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Performance and Immune Performance of Dairy Cows During Heat Stress

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The dairy farming industry is facing massive economic losses as heat stress continues to rise. The purpose of this study was to see how feeding *Saccharomyces cerevisiae* culture (SC) influences productive performance, lactation performance, serum biochemical indexes, hormonal level, antioxidant capacity, and immune function in mid-lactating cows during heat stress. Forty-five healthy mid-lactation dairy cows with comparable milk yield, lactation days, and parity were randomly divided into 3 groups (15 cows in each group). The control group (CON) was fed the

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Effects of *Saccharomyces Cerevisiae* Cultures on

basal diet, while the treatment groups were fed the basal diet + first *Saccharomyces cerevisiae* culture 100 g/d (SC-1) and the basal diet + second *Saccharomyces cerevisiae* culture 30 g/d (SC-2), respectively. The SC-1 and SC-2 groups with SC added in the treatment groups reduced rectal temperature and respiratory rate in heat-stressed cows ($P < 0.05$). The milk yield of SC-1 and SC-2 treatment groups was significantly higher than that of CON ($P < 0.05$). Except for somatic cell count, which was significantly lower in SC-1 and SC-2 than in CON ($P < 0.05$), there were no significant differences in the milk components. The addition of SC: (i) increased serum urea levels ($P < 0.05$), but there was no significant difference in glucose, total cholesterol, alanine transaminase, aspartate aminotransferase, total protein, albumin and alkaline phosphatase levels ($P > 0.05$); (ii) increased serum levels of immunoglobulin-A, immunoglobulin-G, immunoglobulin M, interleukin-4, interleukin-10 and heat shock protein-70 ($P < 0.05$), while decreasing serum levels of interleukin-1 β , interleukin-6, interleukin-2, interferon- γ and tumor necrosis factor- α ($P < 0.05$); (iii) increased total antioxidant capacity, glutathione peroxidase and superoxide dismutase in serum ($P < 0.05$), while decreasing malondialdehyde; (iv) increased serum levels of glucocorticoids, insulin, cortisol and prolactin ($P < 0.05$), while decreasing the serum levels of triiodothyronine and thyroxine ($P < 0.05$). In conclusion, under the current experimental conditions, the addition of SC can reduce rectal temperature and respiratory rate in heat-stressed mid-lactation cows, reduce the number of somatic cells

in milk and improve the mid-lactation cow performance. In addition, SC addition to the diet can raise serum urea levels, regulate serum hormone levels, boost antioxidant capacity in mid-lactation cows, and boost overall immunity.

Keywords: heat stress, *Saccharomyces cerevisiae* culture, mid-lactation dairy cows, milk yield, immunological performance, antioxidant capacity

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INTRODUCTION

Temperatures are rising in every region of the world as a result of global warming (1). According to NASA's Goddard Institute for Space Studies (GISS), global temperatures have risen at a rate of 0.15 to 0.20°C per decade since 1975 (2). As a result, extreme temperature events are becoming more common and severe (3). China has suffered from the effects of global warming in recent decades, which has exacerbated the occurrence of heat stress (4). Some data indicate that the temperature in China rose by an average of 1.4°C between 1951 and 2009 (5). China appears to be one of the regions most affected by high temperatures and one of the most severely heat-stressed (6).

Continued high heat stress can result in significant economic losses for the farming industry, particularly dairy farming (7). The temperature and humidity index (THI) is the sum of the effect of two variables, ambient temperature and humidity, is commonly used to assess the degree of heat stress in cows (8). The Holstein cow, which is known for its high productivity, is the most common breed of dairy cattle raised in China. However, it is extremely sensitive to temperature changes and will suffer heat stress if the ambient temperature exceeds 25°C and the THI exceeds 72 (9). Heat stress can cause a 35–40% reduction in milk production in cows (West, 2003), as well as affect the general health of the cow by reducing normal physiology, metabolism, hormones, antioxidant capacity and immune system (10). Furthermore, the increased incidence of mastitis in dairy cows during the summer poses a significant challenge to dairy farming enterprises (11).

Saccharomyces cerevisiae culture (SC) is a concentrated and dried product obtained by high-density liquid fermentation and deep solid fermentation with *Saccharomyces cerevisiae* as the strain; its composition includes yeast metabolites, yeast cells and denatured medium, which can significantly improve immunity, relieve stress and increase productivity (12). Supplementation of *Saccharomyces cerevisiae* culture in feed has been shown to improve milk production and reduce the inflammatory response of the organism in lactating cows (13). Moreover, we had demonstrated that *Saccharomyces cerevisiae* culture applied to heat-stressed sows could improve their lactation performance and antioxidant capacity (14). We discovered that the majority of previous studies in heat-stressed cows supplemented with *Saccharomyces cerevisiae* culture were concerned with effects on cow performance, reproductive capacity, lactation performance, and rumen fermentation parameters (15, 16). There was consideration given to the cow's immune system, antioxidant capacity, or hormone levels. It was previously unknown how *Saccharomyces cerevisiae* culture affects performance and cow health in mid-lactation cows subjected to heat stress via

antioxidant capacity, immune capacity, and body hormone levels. Therefore, this study systematically investigated the effects of *Saccharomyces cerevisiae* culture on the production performance, serum biochemical indexes, hormone levels, antioxidant capacity, and immune indexes of dairy cows in the middle lactation under heat stress, and provided some data for the development and use of feed additives to mitigate heat stress in dairy cows.

MATERIALS AND METHODS

Animals

The trial was conducted at the cattle farm of Xianghe Dairy Co. in Zaozhuang City, Shandong Province from July 2, 2021 to August 20, 2021. Forty-five Holstein cows in mid-lactation with similar milk yield (29.4 ± 3.7), parity (1.8 ± 0.6), days in lactation (158 ± 14), and good health were selected for the trial and divided into three groups of 15 cows each in a randomized complete block design. The control group (CON) was fed a basal diet, while the treatment groups were fed a basal diet + the first *Saccharomyces cerevisiae* culture at a rate of 100 g/d (SC-1) and a basal diet + the second *Saccharomyces cerevisiae* culture at a rate of 30 g/d (SC-2), respectively. Cows in the treatment groups were fed SC separately at morning feeding. The trial lasted 60 d, with a 10-d adaptation period and a 50-d formal trial period. The base ration is a Total Mixed Ration (TMR) formulated following the Dairy Cattle Feeding Standard (NY/T34-2004). **Table 1** lists the ingredients and nutritional levels. The test cows were kept in loose pens with sprinklers and fans on to keep them during the test period, allowing them to feed and drink freely. The cows were fed three times a day (7:00, 13:00, 18:30) and milked three times a day (5:30, 12:30, 8:30). The Ethical Commission of Shandong Agricultural University reviewed and approved the animal study.

Material

The first *Saccharomyces cerevisiae* culture "Baihuibang" and the second *Saccharomyces cerevisiae* culture "Baihuibang 4C" were provided by Beijing Enhelor International Tech Co., Ltd.

Determination of Temperature and Humidity Index (THI)

An electronic temperature and humidity meter (purchased from Shandong Renke Measurement & Control Technology Co., Ltd. Model: COS-04) was used to record Temperature (°C) and relative humidity (%) at the same height as the cow's head during the experiment. Temperature (°C) and relative humidity (%) data were collected daily in the Dairy barn at 07:00, 14:00, and 22:00,

TABLE 1 | Ration composition and nutrient levels (dry matter basis, %).

Ingredients	Content	Nutrient levels	Contents
Alfalfa hay	11.8	DM	95.9
Corn silage	47.0	CP	15.6
Syrup	3.1	EE	4.2
Mixed concentrates ^a	23.5	NDF	32
Fat powder	0.3	ADF	20.6
Flaked corn	7.8	Ca	0.64
Sugar beet pulp	2.6	P	0.44
Cottonseed	3.1		
Soybean meal	0.8		
Total	100.0		

^aThe mixed concentrate contains self-contained concentrate from Xianghe Dairy Company and lactating cow concentrate supplement [8862], each kg of self-contained ingredients contains 0.55 kg of corn, 0.11 kg of DDGS, 0.07 kg of bran, 0.19 kg of soybean meal, 0.045 kg of Rovimix, 0.027 kg of baking soda, 0.01 kg of magnesium oxide and 0.0027 kg of calcium; The raw material composition of 8,862 includes corn, corn byproduct, wheat by-product, cotton meal, soybean meal, soybean peel, sodium chloride, rock flour, feed grade calcium hydrogen phosphate, vitamin and mineral premix, etc.

and converted to temperature-humidity index (THI) (17). THI is calculated as follows:

$$THI = (0.8 \times T) + [(RH/100) \times (T - 14.3)] + 46.4$$

where T denotes the cowshed temperature (°C); RH denoted the relative humidity of the cowshed (%).

Collection and Determination of Forage Samples

On day 50 of the formal experimental period using the quartering technique, forage samples were collected, dried in an oven at 65°C for 72 h, returned to moisture for 12 h, and then crushed to test the following: dry matter (DM) [GB 6435-86], crude protein (CP) [GB/T 6432-94], crude fat (EE) [GB/T 6433-1994], neutral detergent fiber (NDF), acid detergent fiber (ADF), calcium (Ca) [GB/T 6436-2002], phosphorus (P) [GB/T 6437-2002]. The content of neutral detergent fiber (NDF) and acid detergent fiber (ADF) was determined using the Van Soest method (18).

Determination of Milk Yield and Milk Samples

The milk yield of each test cow was recorded on 2 consecutive days every 10 days during the formal experimental period. Data on milk production data were collected at 05:30, 12:30, and 20:00 each day, and then summarized into daily milk production. On day 50 of the official test period, milk samples were collected from three milking periods, mixed (50 mL) in a 4:3:3 ratio. Next, a potassium dichromate preservative was added, and the milk was sent to the Dairy Research Center of Shandong Academy of

Agricultural Sciences for examination of milk fat percentage, milk protein percentage, somatic cell count, and urea nitrogen.

Measurement of Blood Indicators

On the 50th day of the formal experimental period, blood was drawn from each cow at the caudal root vein before

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the morning feeding. After that, 20 mL of blood was drawn with a disposable syringe, coagulated with a procoagulation tube, and left for 30 min. Next, the blood was centrifuged for 5 min at 3,000 r/min, and the supernatant was divided into 1.5 mL centrifuge tubes and immediately stored in liquid nitrogen. Ten cows in each group were randomly selected to test blood parameters, including T3, T4, GC, IgA, IgG, IgM, PRL, HSP70, INS, COR, IL-1β, IL-6, IL-2, IL-4, IL-10, TNF-α, IFNγ through enzyme-linked immunosorbent assay (ELISA) and ELISA kits with Angle gene products. Malondialdehyde (MDA) was determined using the MDA test kit, purchased from the Angle gene. The superoxide dismutase (SOD) kit, purchased from the Angle gene, was used to measure (SOD). Glutathione peroxidase (GSH-PX) was determined using the GSH-PX test kit, purchased from the Angle gene. Total antioxidant capacity (T-AOC) was determined using an Angle gene total antioxidant capacity (T-AOC) kit. A fully automated biochemical analyzer (Beckman Coulter Model: AU680) was used to determine serum biochemical parameters.

Data Statistics

The test data were compiled using Excel 2010 software; the test data were subjected to Shapiro-Wilk normal distribution test using the shapiro.test() function of R software (version 4.1.1), chi-square test using the bartlett.test() function, and the ANOVA one-way ANOVA using the aov() function analysis of variance (ANOVA). The lsmeans package was used to compute SE values, and Duncan's method from the agricolae package was used for multiple comparisons. 0 < P < 0.001 represents a highly significant difference; 0.001 < P < 0.01 represents a very significant difference; 0.01 < P < 0.05 represents a significant difference; 0.05 < P < 0.1 represents a significant trend of difference. The images in the paper were created with the ggplot2 package of R software.

RESULTS

Changes in Average Temperature and Humidity and Average Temperature and Humidity Index (THI) of the Dairy Barn During the Formal Experimental Period of 50 Days

The THI was >72 for almost all periods during the formal trial period (Figure 1). The average temperature of the Dairy barn during the formal trial period was a minimum of 26.9°C, higher than the temperature threshold of 25.8°C for the appropriate feeding environment for lactating cows (Table 2).

Effect of Different *Saccharomyces Cerevisiae* Cultures on Rectal Temperature and Respiratory Rate in Dairy Cows

Rectal temperature and respiratory rate were significantly lower ($P < 0.05$) in the SC-1 and SC-2 groups of cows fed with *Saccharomyces cerevisiae* cultures than in the CON group; however, there was no significant difference between the SC-1 and SC-2 groups (Table 3).

TABLE 2 | Average temperature and humidity and average temperature and humidity index of the barn during the official test period (50 days).

Time	Average temperature (°C)	Average relative humidity (%)	Average THI
7:00	27.1	82.8	78.47
14:00	33.6	67.2	86.18
22:00	26.9	83.2	78.37

Effect of Different *Saccharomyces Cerevisiae* Cultures on the Productive Performance of Dairy Cows

As shown in Table 4, the milk yield of SC-1 and SC-2 in the

Saccharomyces Cerevisiae Cultures on Serum Biochemical Indices of Dairy Cows

As depicted in Table 5, the urea content was significantly higher in the SC-1 and SC-2 groups than in the CON group ($P < 0.05$), but there was no significant difference in the content of glucose, cholesterol, Alanine transaminase, Aspartate aminotransferase, total protein, albumin, and alkaline phosphatase ($P > 0.05$).

Effect of Different *Saccharomyces Cerevisiae* Cultures on Serum Hormone Indices in Dairy Cows

As shown in Table 6, the levels of glucocorticoids, insulin, cortisol, and prolactin were significantly higher in the SC-1 and SC-2 groups than in the CON group ($P < 0.05$), while the levels of triiodothyronine and thyroxine were significantly lower in the SC-1 and SC-2 groups ($P < 0.05$). The levels of glucocorticoids, insulin, and cortisol were significantly higher in the SC-1 group than in the SC-2 group, while the levels of triiodothyronine and thyroxine content in the SC-1 group were significantly lower.

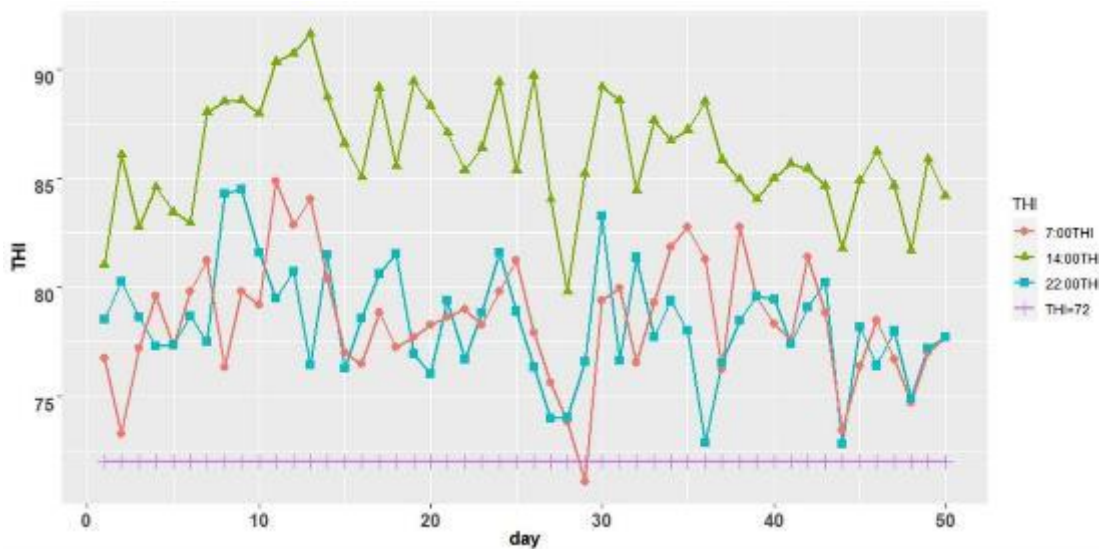


FIGURE 1 | Trend graph of temperature and humidity index (THI) of Dairy barn.

treatment group was significantly higher than that of CON ($P < 0.05$), and there was no significant difference between SC-1

and SC-2 ($P > 0.05$), but SC-1 had a higher milk yield than SC2. The three groups did not differ significantly in terms of milk fat rate, milk protein rate, or urea nitrogen content ($P > 0.05$). The content of somatic cell count (SCC) was significantly lower in SC-1 and SC-2 than in CON ($P < 0.05$).

Effect of Different *Saccharomyces*

Cerevisiae Cultures on Serum Antioxidant Indices in Dairy Cows

Table 7 shows that the total antioxidant capacity, glutathione peroxidase, and superoxide dismutase contents were significantly higher in the SC-1 and SC-2 groups than in the CON group ($P < 0.05$), with the total antioxidant capacity and superoxide dismutase contents in the SC-1 group significantly higher than in the SC-2 group. Malondialdehyde levels were significantly lower in the SC-1 and SC-2 groups than in the CON group ($P < 0.05$), with the SC-1 group having a significantly lower malondialdehyde level than the SC-2 group.

Effect of Different Saccharomyces Cerevisiae Cultures on Serum Immune Indices in Dairy Cows

The levels of IgA, IgG, IgM, IL-1 β , IL-6, IL-2, IL-4, IL-10, TNF α , IFN- γ , and HSP70 in serum were significantly different in the SC-1 and SC-2 groups compared to the CON group ($P < 0.05$),

TABLE 3 | Effect of different Saccharomyces cerevisiae cultures on rectal temperature and respiratory rate in dairy cows.

Items	Groups			SEM	P-value
	CON	SC-1	SC-2		
Rectal temperature/ $^{\circ}\text{C}$	38.89 $\pm$ 0.45 ^a	38.58 $\pm$ 0.48 ^b	38.69 $\pm$ 0.49 ^b	0.06	0.0003
Respiratory rate/(bpm)	65.19 $\pm$ 10.05 ^a	60.58 $\pm$ 13.1 ^b	60.36 $\pm$ 12.44 ^b	1.39	0.0216

Data marked with the same lowercase letter indicate no significant difference ($P > 0.05$), different lowercase letters indicate significant difference ($P < 0.05$). $0 < P < 0.001$ represents a highly significant difference; $0.001 < P < 0.01$ represents a very significant difference; $0.01 < P < 0.05$ represents a significant difference; $0.05 < P < 0.1$ represents a significant trend of difference. The same holds for the tables below.

TABLE 4 | Effect of different Saccharomyces cerevisiae cultures on the productive performance of dairy cows.

Items	Groups			SEM	P-value
	CON	SC-1	SC-2		
Milk yield (kg/d)	26.35 $\pm$ 4.88 ^b	28.87 $\pm$ 4.99 ^a	28.72 $\pm$ 4.79 ^a	0.40	<0.0001
Milk fat percentage/%	3.33 $\pm$ 1.57 ^a	3.23 $\pm$ 1.37 ^a	3.08 $\pm$ 1.41 ^a	0.38	0.8940
Milk protein percentage/%	3.17 $\pm$ 0.15 ^a	3.24 $\pm$ 0.27 ^a	3.15 $\pm$ 0.15 ^a	0.06	0.5000
Milk Urea Nitrogen (ng/dL)	10.9 $\pm$ 3.01 ^a	10.90 $\pm$ 3.40 ^a	10.40 $\pm$ 3.95 ^a	0.99	0.9260
SCC/(10 k/mL)	8.14 $\pm$ 9.40 ^a	2.86 $\pm$ 2.07 ^b	3.29 $\pm$ 3.99 ^b	1.61	0.0461

Different letters (a, b) represent significant differences.

TABLE 5 | Effect of different Saccharomyces cerevisiae cultures on serum biochemical indices of dairy cows.

Items	Groups			SEM	P-value
	CON	SC-1	SC-2		
GLU (mmol/L)	1.99 $\pm$ 0.59 ^a	1.71 $\pm$ 0.48 ^a	1.74 $\pm$ 0.57 ^a	0.18	0.4970
TC (mmol/L)	5.71 $\pm$ 0.75 ^a	6.14 $\pm$ 1.73 ^a	5.39 $\pm$ 0.81 ^a	0.37	0.3850
ALT (U/L)	34.6 $\pm$ 6.35 ^a	39.9 $\pm$ 8.51 ^a	36.6 $\pm$ 7.14 ^a	2.34	0.3740
AST (U/L)	84.6 $\pm$ 21.02 ^a	87.4 $\pm$ 16.39 ^a	91.6 $\pm$ 20.13 ^a	6.40	0.7540
TP (g/L)	83.43 $\pm$ 7.43 ^a	83.94 $\pm$ 6.53 ^a	82.23 $\pm$ 4.78 ^a	2.01	0.8270
ALB (g/L)	25 $\pm$ 1.58 ^a	26.7 $\pm$ 2.51 ^a	26.4 $\pm$ 1.65 ^a	0.62	0.1410
UREA (mmol/L)	5.29 $\pm$ 0.51 ^a	5.46 $\pm$ 0.51 ^b	6.08 $\pm$ 0.84 ^b	0.20	0.0248
AKP (U/L)	53.2 $\pm$ 18.43 ^a	60.9 $\pm$ 14.31 ^a	50.4 $\pm$ 11.84 ^a	4.88	0.3180

Different letters (a, b) represent significant differences.

TABLE 6 | Effect of different Saccharomyces cerevisiae cultures on serum hormone indices in dairy cows.

Items	Groups			SEM	P-value
	CON	TestA	TestB		
GC (ng/mL)	23.91 $\pm$ 0.75 ^c	33.07 $\pm$ 0.89 ^a		06 $\pm$ 0.81 ^b	28.
				0.26	<0.0001

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INS (mU/mL)	8.91 ± 0.43 ^c	12.45 ± 0.53 ^a	82 ± 0.52 ^b	10.
COR (ng/mL)	70.57 ± 3.41 ^c	100.58 ± 5.43 ^a	0.16	<0.0001 85.
T3 (ng/mL)	1.94 ± 0.07 ^a	1.19 ± 0.04 ^c	1.55	<0.0001 52 ± 0.05 ^b 1.
T4 (ng/mL)	90.09 ± 3.49 ^a	66.08 ± 3.74 ^c	0.02	<0.0001 78.
PRL (μIU/mL)	2,684.84 ± 158.45 ^b	2,999.43 ± 153.59 ^a	1.06	<0.0001 2,900

Different letters (a–c) represent significant differences.

as shown in **Table 8**. The contents of IL-10 and HSP70 were significantly higher in SC-1 and SC-2 groups than in the CON group, while the contents of IL-1β, IL-6, IL-2, IFN-γ, and TNFα were significantly lower in the CON group. The contents of

46.70 0.0002
IgA, IgM, IL-10, and HSP70 in the SC-1 group were significantly higher than those in the SC-2 group, while the contents of IL1β, IL-6, IFN-γ, and TNF-α in the SC-2 group were significantly were lower.

TABLE 7 | Effect of different Saccharomyces cerevisiae cultures on serum antioxidant indices in dairy cows.

Items	Groups			SEM	P-value
	CON	SC-1	SC-2		
T-AOC (mmol/L)	0.16 ± 0.01 ^c	0.23 ± 0.01 ^a	0.19 ± 0.01 ^b	0.00	<0.0001
GSH-PX (U/mL)	12.01 ± 1.08 ^b	13.53 ± 0.63 ^a	12.83 ± 0.81 ^a	0.27	0.0020
MDA (nmol/mL)	6.63 ± 0.31 ^a	4.44 ± 0.22 ^c	5.32 ± 0.28 ^b	0.09	<0.0001
SOD (U/mL)	69.38 ± 1.73 ^c	77.84 ± 1.79 ^a	73.89 ± 2.17 ^b	0.60	<0.0001

Different letters (a–c) represent significant differences.

TABLE 8 | Effect of different Saccharomyces cerevisiae cultures on serum immune indices in dairy cows.

Items	Groups			SEM	P-value
	CON	SC-1	SC-2		
IgA (μg/mL)	20.02 ± 0.71 ^c	25.75 ± 0.98 ^a	22.87 ± 0.81 ^b	0.27	<0.0001
IgG (μg/mL)	232.59 ± 13.78 ^b	255.24 ± 11.59 ^a	246.84 ± 12.74 ^a	4.03	0.0028
IgM (μg/mL)	16.09 ± 0.99 ^c	20.06 ± 0.97 ^a	18.24 ± 1.15 ^b	0.33	<0.0001
IL-1β (pg/mL)	191.73 ± 8.16 ^a	151.52 ± 7.87 ^c	171.31 ± 7.14 ^b	2.45	<0.0001
IL-6 (pg/mL)	255.71 ± 7.22 ^a	221.54 ± 6.76 ^c	236.15 ± 6.11 ^b	2.12	<0.0001
IL-2 (pg/mL)	21.11 ± 0.67 ^a	17.04 ± 0.56 ^c	19.08 ± 0.54 ^b	0.19	<0.0001

IL-4 (pg/mL)	412.39 ± 25.17 ^b	446.26 ± 20.40 ^a	433.77 ± 20.21 ^a	6.97	0.0068
IL-10 (pg/mL)	359.46 ± 17.1 ^c	401.21 ± 19.95 ^a	378.06 ± 18.71 ^b	5.89	0.0001
TNF-α (ng/L)	155.67 ± 7.36 ^a	130.11 ± 6.61 ^c	143.11 ± 6.78 ^b	2.19	<0.0001
IFN-γ (pg/mL)	701.96 ± 34.36 ^a	593.17 ± 27.01 ^c	641.29 ± 32.08 ^b	9.90	<0.0001
HSP70 (ng/mL)	7.8 ± 0.37 ^c	10.34 ± 0.45 ^a	9.13 ± 0.51 ^b	0.14	<0.0001

Different letters (a–c) represent significant differences.

DISCUSSION

Effect of Adding Different Types of *Saccharomyces Cerevisiae* Cultures on Rectal Temperature and Respiratory Rate in Heat-Stressed Cows

Heat stress can have a significant impact on the development of our dairy industry. THI=72 has been widely adopted as the threshold value for the occurrence of heat stress in dairy cows over the last decade of heat stress research (19). In this trial, the THI was above 72 for almost all periods, and the times when it was below 72 were due to a brief cooling in the Zaozhuang area of Shandong Province because of the impact of Typhoon No. 6 “Fireworks” in China in 2021. So we believe the cattle were in a heat stress environment. During heat stress, the most common physiological indicators used to assess heat stress in cows are respiratory rate (RR) and rectal temperature (RT) (20). Animals dissipate excess heat from their bodies by increasing their respiratory rate (RR) and allowing it to evaporate in the environment (21). Some researchers, on the other hand, proposed that rectal temperature (RT) is a sensitive indicator of heat stress (22) and that small changes in RT can profoundly influence the function of tissues, organs, and endocrine and that such changes may reduce lactation and causing a variety of growth

60 to 80, with milk production decreasing by 0.13 kg/d for every 1 unit increase in THI (28), and this situation was more prevalent in high yielding cows (29). Additional evidence shows that the number and secretory activity of mammary epithelial cells in cows determine milk production (30). The presence of an intact mammary epithelial barrier, which is an indicator of good mammary gland function, is a major prerequisite for maintaining a high level of milk production (31). According to a study by Collier and colleagues, *in vitro* culture of bovine mammary epithelial cells at high ambient temperatures impaired cell proliferation and induced apoptosis (32), which could reduce milk production. In the present study, the addition of yeast additive improved milk production in cows during heat stress, possibly improving the adaptation of mammary epithelial cells to heat stress. This finding is consistent with the results of many other researchers, including Bruno and the team, who found that adding *Saccharomyces cerevisiae* cultures to the ration improved lactation performance of heat-stressed cows, increasing milk production from 42.2 to 43.4 kg/d. One study found an increase in somatic cell count (SCC) in milk from heat-stressed cows (33). This could be due to the phenomenon that heat stress reduces productive performance and immunity in cows via biochemical and biological pathways, increasing the incidence of intra-mammary infection (IMI), which leads to higher somatic cell counts (SCC) (34). In the present research, the addition of *Saccharomyces cerevisiae* cultures could reduce the level of SCC in milk. Furthermore, SCC is a predictor of IMI and also an indicator of milk quality and assessment of the degree of mastitis (35); as such, adding SC may reduce intra-dairy infection in cows during heat stress, improve milk quality, reduce the prevalence of mastitis, and play a positive role cow health during heat stress.

problems (23). Other studies suggest that as THI increases, so do RT and RR (24). In the present study, the addition of *Saccharomyces cerevisiae* cultures decreased ectal temperature and respiratory rate in cows during heat stress, possibly improving the adaptation of cows to heat stress. Also, we found the results of the same trial in a previous report by Shan and colleagues, where feeding chromium-rich yeast reduced rectal temperature and respiratory rate in lactating cows during heat stress (25).

Effect of Adding Different Types of *Saccharomyces Cerevisiae* Cultures on the Performance of Heat-Stressed Cows

Heat stress causes a decrease in milk production as well as changes in milk composition (26, 27). In a previous study, lactating cows showed a linear decrease in milk production as THI increased from

⁶ Effect of Adding Different Types of *Saccharomyces Cerevisiae* Cultures on Serum Biochemical Indices of Heat-Stressed Cows

Urea is the end product of ammonia and amino acid metabolism in animals; some urea will re-enter the metabolic process if the amount synthesized by the liver exceeds the amount of urea excreted in the urine (36). Cows can suffer from loss of appetite and reduced food intake during heat stress, which potentially results in a negative energy balance (37, 38). Evidence shows that this phenomenon can cause high lipid mobilization from body fat reserves, lipid deposition in the liver, ketogenesis, and hypoglycemia (39). It has been demonstrated that serum urea concentrations are lower in cows with high lipidation and ketogenesis in early lactation when compared to healthy cows,

which may be ascribed to a reduction in dry matter intake in relation to hepatic metabolism (40). In the present trial, the addition of *Saccharomyces cerevisiae* cultures potentially increased urea levels, which may have improved the negative energy balance of cows and increased liver metabolism. There were no significant differences in other serum biochemical indicators. Niacin (NA), an important vitamin in vitamin B that elicits a vasodilatory response, is beneficial for heat-stressed cows. In a study by Khan and colleagues, the addition of NA increased plasma urea concentrations in crossbred cows under heat stress (41), which is consistent with our trials. Previously, it was demonstrated that *Saccharomyces cerevisiae* culture strains are pro-nutrients of all group B vitamins and have high levels of vitamin B (42). Therefore, the elevated urea in the present trial following the addition of *Saccharomyces cerevisiae* cultures may be linked to group B vitamins and positively influence liver metabolism.

Effect of Adding Different Types of *Saccharomyces Cerevisiae* Cultures on Serum Hormones, Antioxidants, and Immune Indices in Heat-Stressed Cows

Heat stress can cause a variety of physiological and hormonal responses via various molecular mechanisms (43). According to one study, cortisol concentrations in cow blood increased after prolonged heat stress exposure (44), which is thought to be one of the main adaptive mechanisms by which the organism is negatively influenced (45). Munksgaard and colleagues also noted that increased cortisol levels ensured nutrient mobilization and availability in response to the stressor (46). In the present trial, cortisol levels were significantly higher in cows fed *Saccharomyces cerevisiae* cultures, which could mobilize nutrients and ensure the supply of substances in the body. Compelling evidence shows that increased milk production necessitates higher levels of glucocorticoids to provide enough energy for galactose production. Cortisol, the main glucocorticoid in galactose production, not only enhances the role of prolactin (PRL) in stimulating mammary epithelial cell differentiation but also increases the expression of milk protein genes during lactation (47). These data imply that prolactin and cortisol can promote increased milk production in cows. Herein, we found that as the concentration of cortisol in the test group increased, so did the level of prolactin, which, when combined with the fact that the combined milk yield increased, suggests that adding *Saccharomyces cerevisiae* cultures can cause cortisol and prolactin to act synergistically in cows to improve their productivity. Heat stress is well known to cause an increase in insulin concentrations (48). Multiple pieces of evidence indicate that insulin action enables the body to respond effectively to heat stress and minimizes heat stress-induced injury (49, 50), and the increased serum insulin concentrations in cows supplemented with SC in our experiment may be because the addition of SC promotes

insulin production in the body, successively reducing heat stress-induced damage in cows.

HSP70 is a key gene in the heat shock protein (HSP) family that has been linked to heat stress response (51). The structure of HSP70 is highly conserved, and it may be involved in protein folding, assembly, and degradation. HSP70 synthesis in cells is low under normal conditions. Under stress, however, they are rapidly synthesized and transferred to the nucleus, nucleolus, and other regions to improve the heat resistance of the organism and play a protective role (52). This heat resistance is positively correlated with the level of HSP70 expression (53). This allows the organism to adapt to heat stress earlier, and this protects tissues and organs from heat stress damage faster and more effectively, as well as improves the organism's resistance to stress (54). Herein, there was a significant increase in HSP70 levels in the treatment group, most likely because the addition of *Saccharomyces cerevisiae* cultures could stimulate the rapid HSP70 synthesis in cows. Notably, Rhoads et al. (50) revealed that HSP70 may improve insulin function and that modulating the insulin-HSP axis during heat stress can improve animal health and productivity, and the treatment group did show a significant increase in insulin metrics as well as a positive correlation between insulin levels and HSP70 levels, which was consistent with previous findings. However, we saw the opposite conclusion in a study where the addition of *Saccharomyces cerevisiae* cultures to periparturient cows under heat stress reduced plasma levels of HSP70 (55), and we speculate that this may be due to the different periods of the herd. Thyroid hormone (T3) is the primary hormone secreted by the thyroid gland, and triiodothyronine (T4) is a deiodination product of T3 and is only biologically active when deiodinated (56). Because the thyroid gland regulates basal metabolic rate in lactating cows, increased release of T4 and T3 increases basal metabolic rate and body heat production (57). Under hot climatic conditions, the decrease in thyroid hormone concentration can be viewed as an adaptive mechanism to reduce metabolic heat production (58). Reduced food intake during heat stress has been linked to thyroid activity suppression, which consequently decreases thyroid hormone levels (59). According to our findings, the addition of yeast additive reduced serum levels of T3 and T4 in heat-stressed cows as well as the metabolic heat production of the organism for better adaptation to the hot environment. The same findings were reported previously (60).

The immune system is one of the mechanisms for resisting stress caused by environmental changes. Heat stress can reduce dry matter intake in cattle, thereby negatively influencing nutrient absorption, and, as a result, the immune system and inflammatory response (61). Serum immunoglobulin is a marker of humoral immunity that promotes monocyte and macrophage phagocytosis and can bind to antigens to produce multiple biological effects. Immunoglobulin-G (IgG) is the major immunoglobulin that promotes the phagocytosis of pathogens and neutralizes bacterial toxins by immune cells. High IgG concentrations in dairy cow blood are thought to boost immunity. Immunoglobulin-A (IgA) is found in low concentrations in serum

but provides significant defense against invading pathogens. Immunoglobulin-M(IgM) lyses bacteria and blood cells and neutralizes viruses, but its retention time is shorter (62, 63). Some studies have demonstrated that heat stress suppresses biological immune functions. One of these effects is a decrease in serum immunoglobulin levels (64). In the present experiment, feeding *Saccharomyces cerevisiae* cultures significantly increased serum levels of IgG, IgA, and IgM in heat-stressed cows, which improved the immunity of the cow organism and reduced heat stress-induced damage to the immune system. As a result, the ability of the organism to resist heat stress is improved.

CD4+ T cells are immune cells that are classified as T helper cells in the lymphocyte classification. CD4+ T cells are classified as either helper T cell 1 (Th1) or helper T cell 2 (Th2) cells. Their presence or activation is thought to have a regulatory effect on immune behavior. Recent studies have shown that the main mechanism for maintaining or restoring homeostasis in the diseased immune system is the relative increase in Th2 cell activity to the relative suppression of Th1 cells (65). Th1 cells mainly secrete IL-2 and IFN- γ , whereas Th2 cells mainly secrete IL-4 and IL-10 (66). Th1 and Th2 cells, respectively, regulate cellular and humoral immunity. Th1 cells promote the inflammatory response, while Th2 cells promote the anti-inflammatory response (67). Researchers have suggested that high temperature is associated with the downregulation

of Th1 cytokines and upregulation of Th2 cytokines, thereby suppressing cellular immunity (68, 69). In our study, we found that the levels of IL-2 and IFN- γ decreased, while the levels of IL-4 and IL-10 increased, which is consistent with previous findings; these data imply that yeast additions potentially reduced inflammation in heat-stressed cows and improved the antiinflammatory response of the organism. Intriguingly, it has been proposed that glucocorticoids may suppress cellular immunity, resulting in a preferential shift toward Th2-mediated humoral immunity (70), which is consistent with our findings. Heat stress has also been shown to increase cortisol concentration in the blood and to inhibit the production of cytokines such as interleukin-4 (IL-4), IL-6, interferon (IFN), and tumor necrosis factor- α (TNF- α) (71). Elsewhere, a study demonstrated that TNF- α and IL-1 β directly promote inflammatory responses in cows, while IL-10 mediates anti-inflammatory responses in cows (72), and that interleukin (IL-6) is a known inflammatory cytokine that promotes inflammation in the organism (73). Herein, elevated serum cortisol concentrations were reported in the treatment group, whereas the levels of inflammatory factors such as TNF- α , IL-6, and IL-1 β decreased. These results suggest that the addition of *Saccharomyces cerevisiae* cultures may regulate the immune system by influencing cortisol levels *in vivo*, and this would result in less inflammation in heat-stressed cows.

Heat stress disrupts the normal regulation of oxidants/antioxidants, causing severe cell damage via enzymatic and non-enzymatic activities (74). High temperatures can promote the occurrence of oxidative stress, which may be attributed to the

excessive production of oxygen radicals by the organism and reduced antioxidant defenses (75). Increased oxygen free radicals result in the formation of malondialdehyde (MDA), which potentially induces cell death by damaging the DNA (76). Superoxide dismutase (SOD) is an enzyme that protects the organism and cells from damage caused by superoxide anion radicals and their active products. Glutathione peroxidase (GSH-Px) is a key enzyme in the antioxidant defense system of organisms, thought to protect various organisms from oxidative stress (77). Total antioxidant capacity (T-AOC), which represents total antioxidants in blood and body fluids, reflects the body's compensatory capacity to resist external stimuli (78). Malondialdehyde, a by-product of lipid peroxidation, is the most common indicator of lipid peroxidation and is regarded as a reliable method for determining the extent of lipid oxidative damage in cell membranes (79). In general, cows with relatively high levels of T-AOC, SOD, and GSH-Px and relatively low MDA values have good antioxidant capacity. In the present investigation, the levels of total antioxidant power, glutathione peroxidase, and superoxide dismutase increased in the experimental group, while malondialdehyde decreased, indicating that brewer's yeast culture can increase the antioxidant capacity of the cow organism under heat stress, reduce cell damage, and decrease the production of oxygen radicals. Harmon and colleagues reported a decrease in plasma antioxidant activity in heat-stressed Holstein cows in mid-lactation, which can indirectly verify our findings (80). In another investigation, researchers reported a 2-fold increase in malondialdehyde in skeletal muscle of acutely heat-stressed broiler chickens (81).

CONCLUSION

The addition of *Saccharomyces cerevisiae* cultures to heatstressed mid-lactation cows increases milk production and reduces the incidence of mastitis, but exerts no significant effect on milk composition. Moreover, the addition of *Saccharomyces cerevisiae* cultures improves the antioxidant capacity of the cows and can reduce inflammatory factors in the body, improve immunity, and synergize hormones in the body to alleviate the negative effects of heat stress. Thus, supplementing mid-lactation cows with *Saccharomyces cerevisiae* cultures can improve the antioxidant capacity and immunity level of the body under heat stress, as well as improve milk yield and cow performance to ensure healthy cow production. The experimental results demonstrated that the effect of *Saccharomyces cerevisiae* cultures in the SC-1 group is superior to that in the SC-2 group.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Commission of Shandong Agricultural University.

AUTHOR CONTRIBUTIONS

DD, ZH, PC, and RZ conceived the project and designed the protocol. DD and WJ performed the experiments. DD, LF, YZ, and WL wrote the manuscript. All authors read and approved the final manuscript.

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culture mitigates heat stress-related damage in dairy cows by multi-omics

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The effect of heat stress on ruminants is an important issue. In recent years, the growth of the Chinese dairy industry has rapidly increased, generating RMB 468,738 million revenue in 2021. A decreased milk yield is the most recognized impact of heat stress on dairy cows and results in significant economic loss to dairy producers. Heat stress also lowers immunity and antioxidant capacity and changes the bacterial composition and metabolites of the rumen. The purpose of this study was to investigate the effect of addition *Saccharomyces cerevisiae* culture on heat-stressed cows. The impact of *S. cerevisiae* culture on microbiota composition, functional profiles, and metabolomics was assessed in heat-stressed cows. A total of 45 Holstein cows in mid-lactation were selected and randomly divided into three groups (15 cows per group). Groups D-C, D-A, and D-B were fed with the basal diet, the basal diet + first *S. cerevisiae* culture 100 g/day, and the basal diet + second *S. cerevisiae* culture 30 g/day, respectively. The trial lasted 60 days. There was an increased abundance of the Phylum Firmicutes in the rumen of heat-stressed dairy cows fed with *S. cerevisiae*, of which four genera had significantly higher abundance, *Ruminococcus_gaurevii_group*, *Butyrivibrio_2*, *Moryella*, and *Ruminiclostridium_6*. At the functional level, ten pathways differed significantly between the three groups ($P < 0.05$), with an increase in fatty acid biosynthesis, fatty acid metabolism, PPAR signaling pathway, ferroptosis, and biotin metabolism in the treatment groups. More differential metabolites were found in the D-C and D-A groups than in the D-C and D-B groups. These results indicate that *S. cerevisiae* cultures can influence the health status of heat-stressed cows by modulating rumen microbial composition, function, and metabolites, thereby improving rumen cellulolytic capacity. This study can provide or offer suggestions or recommendations for the development and utilization of feed additives.

KEYWORDS

heat stress, dairy cows, *Saccharomyces cerevisiae* culture,
metagenomics, metabolomics

Introduction

According to the United Nations Intergovernmental Panel on Climate Change (IPCC) report, the burning of fossil fuels and deforestation (Gray, 2007; Jianbin, 2013) accelerate the pace of global warming, which will be a big challenge for the dairy industry in China (Zhang et al., 2020), impacting the success of its dairy industry. Shandong Province in East China has a temperate monsoon climate with high summer temperatures, and cows in this region are severely affected by heat stress. This has led to a decrease in milk production and feed intake and an increase in the incidence of intramammary bacterial infection (IMI) (West, 2003; Rakib et al., 2020). Heat stress also lowers immunity and antioxidant capacity (Tao et al., 2012; Du et al., 2022) and changes

Effect of *Saccharomyces cerevisiae*

the bacterial composition and metabolites of the rumen (Zhao et al., 2019). This is detrimental to the health of cows and causes serious losses to the dairy industry.

The gastrointestinal tract of cows is a complex ecosystem that has a strong impact on health (Zeineldin et al., 2018a). The rumen, the first stomach of ruminants, has a complex and diverse microbiota that can digest cellulose and provide nutrients to the organism (Bickhart and Weimer, 2018). The rumen microbiota can also stimulate and improve immune responses (Zhang et al., 2021). Recent studies have shown that the negative effects of heat stress on cows can be ameliorated by adding probiotics or other additives to the feed (Liu et al., 2014; Ye et al., 2016; Shah et al., 2020; Li et al., 2021a). These studies have shown varying degrees of improvement in milk production, immunity, and antioxidant capacity, as well as changes in the composition of the rumen microbiota and metabolites following the addition of these products. This may be due to the critical role played by host-rumen-microbiota interactions, and these additives alter the composition of the rumen flora and its microbial metabolites, thereby improving the health of the cow. The same argument is made in a review by Zeineldin et al. (2018b).

Our previous studies showed that *Saccharomyces cerevisiae* cultures could improve the performance, antioxidant capacity, and immunity of heat-stressed cows and reduce corporate losses (Du et al., 2022). Previous studies on heat-stressed cows have centered on rumen microbial diversity and

metabolomics, and this experiment instead focused on rumen microbial diversity, metagenomics, and metabolomics. The objective of this study was to determine the effect of the addition of *S. cerevisiae* cultures on rumen microbial composition, rumen microorganisms' function, and rumen metabolites.

Materials and methods

Animals, diets, and trial design

This study was conducted at the cattle farm of Xianghe Dairy Co., in Zaozhuang City, Shandong Province. A total of 45 Holstein cows in mid-lactation with a similar milk yield (29.4 ± 3.7), parity (1.8 ± 0.6), days in lactation (158 ± 14), and health were selected and divided into three groups of 15 cows each in a randomized complete block design. The control group (D-C) was fed with a basal diet, while the treatment groups were fed with a basal diet + the first *S. cerevisiae* culture at a rate of 100 g/day (D-A) or a basal diet + the second *S. cerevisiae* culture at a rate of 30 g/day (D-B). Both *S. cerevisiae* cultures were provided by Beijing Enhelor International Tech Co., Ltd. Cows in the treatment groups were fed with *S. cerevisiae* culture separately during the morning feeding. The trial lasted for 60 days, with 10-day and 50-day adaptation and formal trial periods, respectively. The average temperature of the dairy barn during the formal trial period was a minimum of 26.9°C. Cows were under heat stress at all times during the trial.

Rumen fluid sampling

Two hours after feeding the cows in the morning of day 50 of the formal trial period, the rumen fluid from 45 cows was sampled using a portable rumen fluid sampling tube. Rumen fluid (100 ml) was collected per cow, filtered through four layers of gauze, packed into 2 ml freezing tubes, and preserved in liquid nitrogen.

DNA extraction and sequencing

DNA was extracted from ruminal fluid samples using the HiPure Stool DNA Kits (Magen, Guangzhou, China) according to the manufacturer's protocols. NanoDrop2000 was used to determine the purity of the extracted DNA. The 16S rDNA V3-V4 region was amplified by PCR using the following primers: 341F, 5-CCTACGGGNGGCWGCAG-3, and 806R 5GGACTACHVGGGTATCTAAT-3. Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions, and quantified using an ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, CA, United States). Purified amplicons were pooled in equimolar ratios and paired-end sequenced on an Illumina platform (PE250) according to standard protocols.

Bioinformatics analysis

Raw reads were further filtered using FASTP to obtain high-quality clean reads (Chen et al., 2018a) (version 0.18.0). Paired-end clean reads were merged as raw tags using FLASH (Magoc and Salzberg, 2011) (version 1.2.11) with a minimum overlap of 10 bp and mismatch error rates of 2%. Noisy raw tag sequences were filtered under specific filtering conditions (Bokulich et al., 2013) (QIIME version 1.9.1) to obtain highquality clean tags. The filtering conditions are as follows: (1) break raw tags from the first low-

quality base site where the number of bases in the continuous low-quality value (the default quality threshold is ≤ 3) reaches the set length (the default length is 3 bp); (2) then, filter tags whose continuous high-quality base length is less than 75% of the tag length. The clean tags were clustered into operational taxonomic units (OTUs) with $h \geq 97\%$ similarity using the UPARSE (Edgar, 2013) (version 9.2.64) pipeline. All chimeric tags were removed using the UCHIME algorithm (Edgar et al., 2011), and effective tags were obtained for further analysis.

The representative OTU sequences were classified into organisms using a naive Bayesian model with the RDP classifier (Wang et al., 2007) (version 2.2) based on the SILVA database (version 132), with a confidence threshold value of 0.8. Chao1, ACE, Shannon, and Simpson were calculated using QIIME (Caporaso et al., 2010) (version 1.9.1) and plotted histograms using the omicsmart online tool.¹ Principal coordinate analysis (PCoA) of unweighted-unifrac distances was generated using the R Vegan package (Oksanen et al., 2012) (version 2.5.3) and plotted using the R ggplot2 package (Wickham, 2011) (version 2.2.1). The stacked bar plot of the phylum and genus composition was visualized using the R ggplot2 package (version 2.2.1). The GraphPad Prism version 8.0.2 software was used to draw horizontal bar charts.

DNA extraction, metagenomic sequencing, and metagenomic data processing

Genomic DNA was extracted using HiPure Bacterial DNA Kits (Magen, Guangzhou, China) according to the manufacturer's instructions. DNA quality was detected using Qubit (Thermo Fisher Scientific, Waltham, MA, United States) and Nanodrop (Thermo Fisher Scientific, Waltham, MA, United States). Qualified genomic DNA was first fragmented by sonication to a size of 350 bp and then end-repaired, A-tailed, and adaptor-ligated using the NEBNext MLtra™ DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, United States) according to the preparation protocol. DNA fragments with a 300–400 bp length were enriched by PCR, PCR products were purified using the AMPure XP system (Beckman Coulter, Brea, CA, United States), and libraries were analyzed for size distribution using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States). Genome sequencing was performed on the Illumina NovaSeq 6000 sequencer using pair-end technology (PE 150).

Raw data from the Illumina platform were filtered using FASTP (version 0.18.0) (Chen et al., 2018b) by removing reads with any of the following: (1) $\geq 10\%$ unidentified nucleotides (N), (2) $\geq 50\%$ bases having Phred quality scores ≤ 20 , or (3) aligned to the barcode adapter. After filtering, clean reads of each sample were assembled individually using MEGAHIT (version 1.1.2) (Li et al., 2014). Genes were predicted based on the final assembly contigs (>500 bp) using MetaGeneMark (version 3.38) (Zhu et al., 2010). The predicted genes ≥ 300 bp in length from all samples were pooled and combined based on $\geq 95\%$ identity and 90% read coverage using CD-HIT (version 4.6) (Fu et al., 2012) to reduce the number of redundant genes for the downstream assembly step. The reads were realigned to the predicted gene using Bowtie (version 2.2.5) (Langmead and Salzberg, 2012) to count the read numbers. The final gene catalog was obtained from non-redundant genes with gene reads > 2 . Quantitative analysis of genes was conducted using Bowtie (version 2.2.5) and PathoScope (version 2.0.7). The unigenes were annotated using DIAMOND (version 0.9.24) (Buchfink et al., 2015) by aligning them with the Kyoto Encyclopedia of Genes and Genomes (KEGG). The histogram of the difference was drawn using the omicsmart online tool (see text footnote 1). The Kruskal-Wallis test was adopted. Meanwhile, Tukey's HSD test was used to understand where the differences originated.

Analysis of the rumen metabolome

The samples (1 mL) were freeze-dried and resuspended in 100 μ L prechilled 80% methanol by well vortex, incubated on ice for 5 min, and centrifuged at 15,000 g at 4°C for 15 min. The supernatants were diluted to a final concentration containing 53% methanol using LCMS-grade water. The samples were subsequently transferred to fresh Eppendorf tubes and centrifuged at 15,000 g at 4°C for 15 min. Finally, the supernatants were injected into the LCMS/MS system for analysis.

UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q Exactive™ HF-X mass spectrometer (Thermo Fisher, Germany) from Gene Denovo Co., Ltd. (Guangzhou, China). Samples were injected onto a Hypersil GOLD column (100 $\times$ 2.1 mm, 1.9 μ m) using a 17-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol), and the eluents for the negative

¹ <https://www.omicsshare.com/>

polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol).

The solvent gradient was set up as follows: 2% B, 1.5 min; 2–100% B, 12.0 min; 100% B, 14.0 min; 100–2% B, 14.1 min; 2% B, 17 min. A Q Exactive™ HF-X mass spectrometer was operated in the positive/negative polarity mode with a spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 40 arb, and aux gas flow rate of 10 arb.

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform the peak alignment, peak picking, and quantitation of each metabolite. The main parameters included are as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; minimum intensity, 100,000. The peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. The peaks were then matched with the mzCloud,² mz Vault and Mass List database, to obtain accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software, namely, R (R version R-3.4.3), Python (Python 2.7.6 version), using CentOS to run this software (CentOS release 6.6). When data were not normally distributed, normal transformations were attempted using the MaxQuant (MaxQuant version 1.6.17.0). The abundance of differential metabolites in the same group was normalized using the z-score,

and the VIP score of OPLS-DA was used to draw the graph. The omicsmart online tool was used to draw differential KEGG enrichment bubble map and VIP score.³

Results 16S rDNA sequencing analysis

16S rDNA in the rumen fluid sample was filtered to obtain clean reads (758,403 ± 3,568 reads in the D-C group, 772,109 ± 4,155 reads in the D-A group, and 760,383 ± 3,892 reads in the D-B group). Our study also obtained 25,312 OTUs.

Bacterial diversity analysis

Alpha diversity indices, including Chao1, ACE, Shannon, and Simpson, showed that microbial community diversity in the rumen fluid samples was higher in the D-A group than in the other two groups (Figures 1A–D); however, this difference was not significant ($P > 0.05$). Beta diversity showed that there was no significant separation between the three groups of microbiota, indicating that there were no significant differences between them (Figure 2). There was also no significant separation at the OTU level using the ANOSIM test with $R^2 = -0.0095$ ($P = 0.549$).

² <https://www.mzcloud.org/>

³ <https://www.omicsmart.com/index.php>

(Supplementary Figure 1).

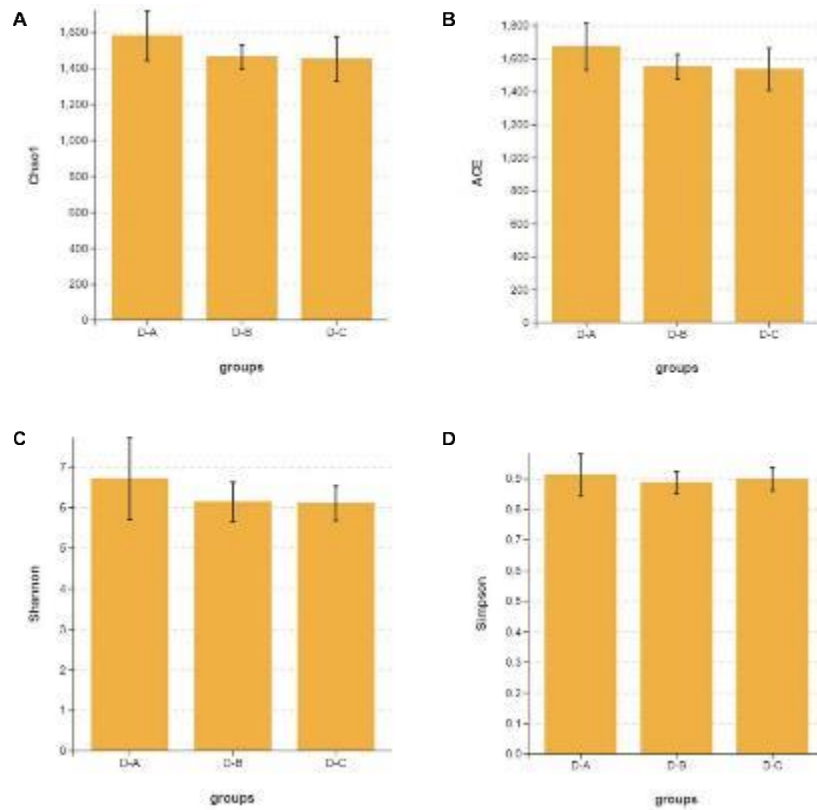


FIGURE 1

The (A) Chao1, (B) ACE, (C) Shannon, and (D) Simpson alpha diversity indices for the rumen microbiota.

Rumen microbial composition at the phylum and genus levels

Figure 3 shows the top ten most abundant microbial species in the three groups. Of these phyla, *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* had the highest abundances of 39.8 ± 2.02 , 29.6 ± 3.7 , and $25.9 \pm 1.7\%$, respectively. The amount of *Firmicutes* was higher in the D-A group than in the other two groups, while the abundance of the *Proteobacteria* was lower in the D-A group, but these differences were not statistically significant ($P > 0.05$). The dominant genera were *Succinivibrionaceae_UCG-001* ($28.9 \pm 3.78\%$), *Prevotella_7* ($18.7 \pm 1.95\%$), *Prevotella_1* ($11.01 \pm 0.95\%$), *Ruminococcaceae_UCG-014* ($3.31 \pm 0.36\%$), *Succiniclasticum* ($3.13 \pm 0.81\%$), *Dialister* ($2.36 \pm 0.35\%$), *Rikenellaceae_RC9_gut_group* ($1.47 \pm 0.21\%$), *Candidatus_Saccharimonas* ($1.16 \pm 0.14\%$), *Shuttleworthia* ($1.049 \pm 0.087\%$), and *Ruminococcus_1* ($1.00 \pm 0.12\%$). Four genera, namely, *Ruminococcus_gauvreauui_group*, *Butyrivibrio_2*, *Moryella*, and *Ruminiclostridium_6*, were significantly different ($P < 0.05$) after filtering species whose abundance was $< 0.1\%$ in all samples. Among the three genera, namely, *Ruminococcus_gauvreauui_group*, *Butyrivibrio_2*, and *Moryella*, the highest abundance was found in the D-A group. In the D-B group,

Ruminiclostridium_6 is the highest abundance (Figure 4).

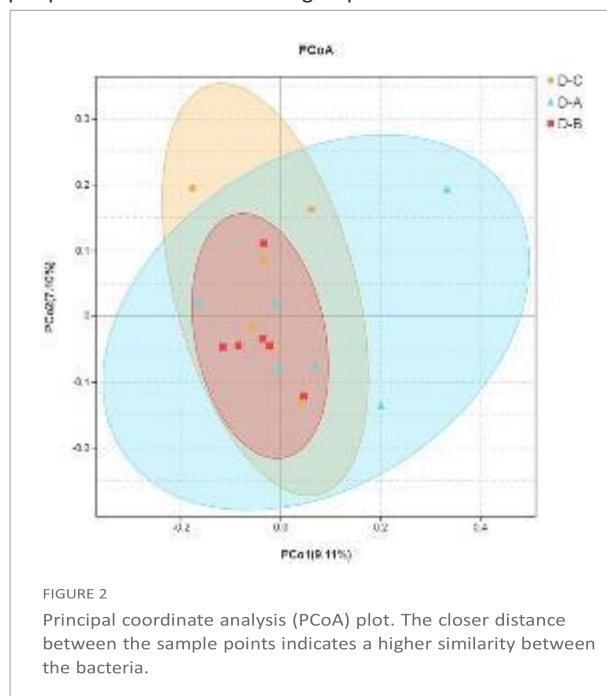
Macrogenome and functional profiles of rumen microbiota

A total of $650,894,398 \pm 4,988,354$ clean reads were sequenced using the Illumina platform after quality control filtering. The sequences were then assembled to obtain 4,417,583 contigs. After gene annotation of the contigs and obtaining non-redundant gene sets, unigenes were compared with the KEGG database and 408 tertiary pathways were assessed. Of these, ten pathways differed significantly among the three phyla ($P < 0.05$), including five metabolic pathways, two cellular processing pathways, two organismal systems, and one genetic information processing pathway (Figure 5).

Metabolomics of the rumen fluid

To further assess the differences in rumen metabolites, the rumen fluid metabolomics were determined. We compared the D-C group with the D-A and D-B groups and identified 41 differential metabolites in the pos mode of the D-C vs. D-A group, 43 in the neg mode, 12 in the D-C vs. D-B pos mode, and 13 in the neg mode ($P < 0.05$, VIP > 1) (Figure 6).

According to the differential metabolites, 50 enriched pathways were identified in the pos pattern of the D-C vs. D-A groups. The



taste transduction pathway was significantly enriched, including upregulation of the four metabolites, D-phenylalanine, maltose, acetylcholine, and serotonin in the D-A group. In the neg model, 52 enriched pathways were identified, of which four, pentose phosphate, primary bile acid biosynthesis, α -linolenic acid metabolism, and carbon metabolism, were significantly enriched. In the pentose phosphate pathway, gluconic acid and D-glucono-1,5-lactone were upregulated in the D-A group and in the primary bile acid biosynthesis pathway, taurine and glycocholic acid were upregulated, sugarcane deoxycholic acid was downregulated in the D-A group and in the α -linolenic acid metabolism pathway, jasmonic acid was upregulated in the D-A group and in carbon

metabolism, and gluconic acid and D-glucono-1,5-lactone were upregulated in the D-A group. In the D-C vs. D-B group, 21 enrichment pathways were found in the pos mode, of which one metabolic pathway, porphyrin, and chlorophyll metabolism were upregulated, and biliverdin and fecal biliverdin were downregulated in the D-B group. Twentyfive enriched pathways were found in the neg model, but none were significantly enriched (Figure 7).

Discussion

To understand the mechanism by which *S. cerevisiae* cultures alleviate heat stress in dairy cows, the experiment investigated the effects of *S. cerevisiae* cultures on the microbial composition, metagenome, and metabolome of the rumen.

Previous studies indicate that the species diversity and abundance of rumen microorganisms play a critical role in

maintaining host physiology (Xu et al., 2021). While Chao1 and

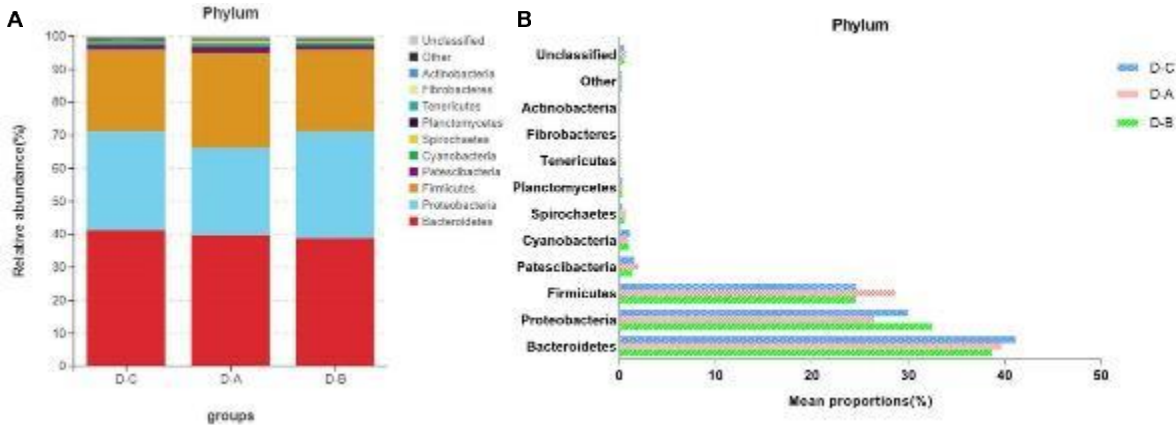
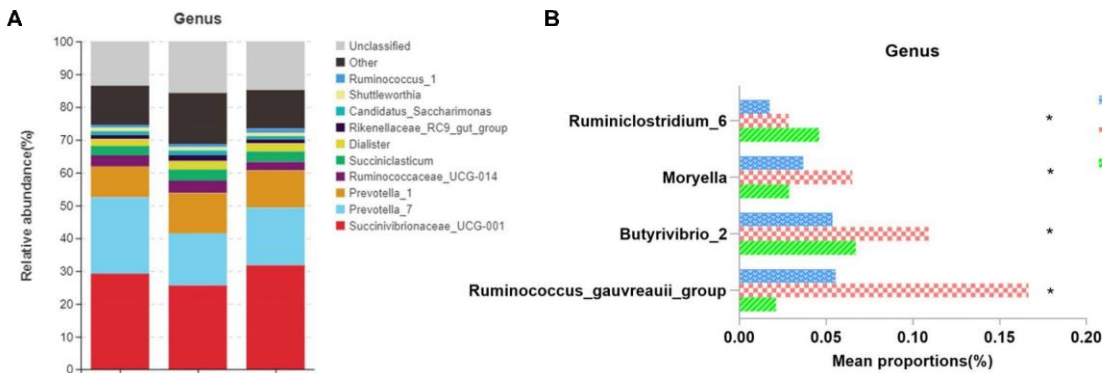
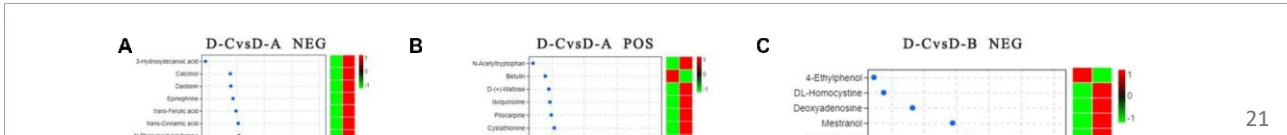
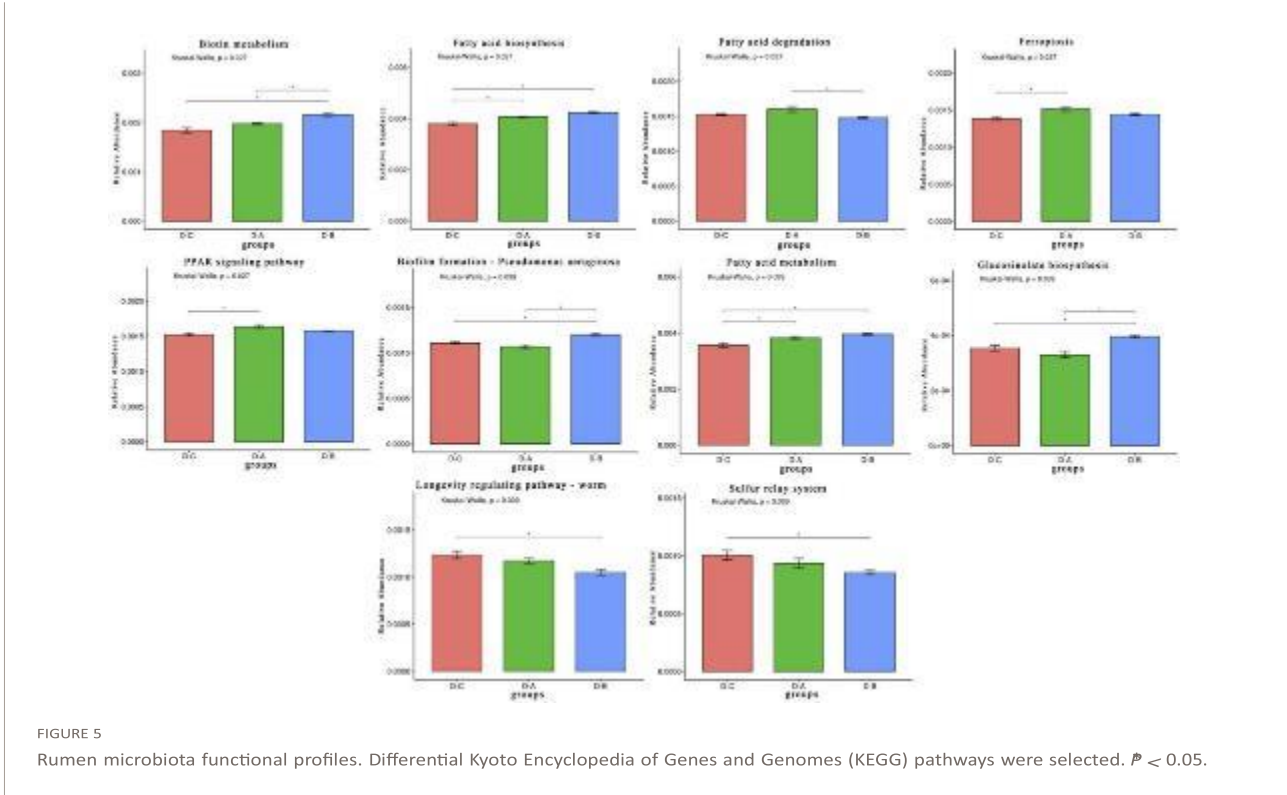


FIGURE 3
Relative rumen microbiota abundance at the(A,B) phylum level in the D-C, D-A, and D-B groups.



ACE indices are primarily used to measure species richness, the Simpson and Shannon indices combine the richness and evenness of a species. Our study found that rumen bacterial abundance and diversity were higher in cows that were fed with the first yeast culture than in the other two groups, although these findings were not statistically significant. This may be due to the increased diversity and abundance of rumen bacteria after the addition of *S. cerevisiae* culture. The environment within the rumen can change to some extent and may affect the bacterial species involved ([Ghazanfar et al., 2017](#)). Our results showed that *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* were the dominant phyla in the rumen of all three groups of dairy cows, which supports the findings of prior studies ([Xue et al., 2019](#)). The abundance of *Firmicutes* was highest in the D-A group, while the abundance of *Proteobacteria* was lowest in the D-A group; however, these differences were not significant.

Firmicutes are associated with the degradation of structural





Pathways with significant enrichment in differential metabolites were selected, and bubble maps were drawn using information about upregulation and downregulation. The X-axis represents the difference between the number of upregulated and downregulated differential metabolites as a percentage of total differential metabolites. The yellow line represents the threshold of $p = 0.05$. The Y-axis represents $-\log_{10}(P\text{-value})$. Different colors represent different categories. (A) Enrichment difference bubble chart of metabolites between D-C and D-A in the neg mode, (B) enrichment difference bubble chart of metabolites between D-C and D-A in the pos mode, (C) enrichment difference bubble chart of metabolites between D-C and D-B in the neg mode, and (D) enrichment difference bubble chart of metabolites between D-C and D-B in the pos mode.

cellulose degradation and reduced inflammation in heat-stressed cows.

The trial assessed the mechanism by which *S. cerevisiae* cultures affect the rumen of heat-stressed cows by metagenome and found ten pathways that differed significantly between the three groups. Fatty acid biosynthesis, fatty acid metabolism, peroxisome proliferator-activated receptor (PPAR) signaling, and ferroptosis and biotin metabolism were all enriched in the treatment groups. Yeast is known as a natural source of B vitamins. According to let's eat right to keep fit by Adelle Davis, yeast is one of the four foods that contain complete vitamin B. *S. cerevisiae* culture is a product obtained from *S. cerevisiae* by fermentation and concentration, it is also rich in high levels of vitamin B, and studies indicate that vitamin B compounds can increase milk production in dairy cows (Chen et al., 2011). Biotin, a water-soluble B vitamin, is an essential nutrient in the rumen of dairy cows and a required cofactor for five carboxylases. These biotin-dependent carboxylases participate in several metabolic pathways, including fatty acid synthesis and fatty acid oxidation, and biotin or carboxylase deficiency can lead to metabolic dysfunction. Saika et al. (2019) and Lauridsen (2020) found that fatty acids are involved in

intestinal mucosal barrier function and oxidative stress and inflammatory responses (Saika et al., 2019; Lauridsen, 2020). By activating fatty acid receptors, they can promote anti-inflammatory and neuronal responses and regulate immune homeostasis (Kimura et al., 2019). The PPAR is also activated by fatty acids and involved in regulating lipid metabolism and inflammation. In addition, PPAR signaling can inactivate NF- κ B, promote antioxidant enzyme expression, and reduce the concentration of reactive oxygen species, thus improving antioxidant capacity (Korbecki et al., 2019). Cellulolytic bacteria in the rumen require biotin for growth, and Abel et al. (2001) showed that biotin improves fiber fermentation in the rumen. Thus, we speculated that the addition of *S. cerevisiae* culture increased B vitamin levels in the rumen, which increased biotin metabolism and production, elevated fatty acid synthesis, activated PPAR signaling, and improved the immune response and antioxidant capacity of the cows. The increase in biotin also elevated the abundance of cellulolytic *Firmicutes* in the rumen and improved the digestibility of the feed.

Ferroptosis is an iron-dependent type of programmed cell death caused by massive lipid peroxidation-mediated membrane damage. This process is distinct from apoptosis,

cellular necrosis, and cellular autophagy (Stockwell et al., 2017; Tang et al., 2019). In most cases, ferroptosis causes an inflammatory response in the body that induces cell death and is harmful to the organism. However, there is evidence that

providing energy to the body (Ohlrogge and Jaworski, 1997). Taurine is a non-essential amino acid that cannot function as a direct energy source (Chesney, 1985) but can provide protection against oxidative stress by increasing superoxide dismutase and glutathione peroxidase activity (Nonaka et al., 2001). Significant enrichment of the pentose phosphate pathway and increased taurine content after the addition of *S. cerevisiae* cultures improved the antioxidant capacity of heat-stressed cows. Glycochenodeoxycholic acid is a hydrophobic bile salt that accumulates in the liver during cholestasis and can induce apoptosis in hepatocytes at certain concentrations. In the D-A group, glycochenodeoxycholic acid production was downregulated, which may have relieved stress on the liver and ensured the health of the organism.

Conclusion

This trial studied the effects of *S. cerevisiae* cultures supplemented diets of heat-stressed cows on rumen microbial composition, metagenomics, and metabolomics and identified a strong relationship between supplementation and vitamin

B. As shown in Figure 8, yeast cultures are rich in vitamin B. Supplementation with *S. cerevisiae* culture enriched the rumen in biotin, a water-soluble vitamin B. Biotin can act as a carboxylase cofactor, participating in the synthesis and metabolism of fatty acids, which in turn can activate PPAR and improve the antioxidant capacity and immunity of the organism. In addition, most cellulolytic bacteria require vitamin B for growth, which may account for the increased abundance of cellulolytic bacteria such as *Ruminococcus* and *Butyrivibrio*. These bacteria stimulate cellulose catabolism, upregulate metabolites in the pentose phosphate pathway, increase primary bile acid biosynthesis and taste transduction pathway signaling, and positively impact the health of cows. Our findings indicate that *S. cerevisiae* cultures enriched with vitamin B provide protection against heat stress and should be considered as an anti-heat stress feed additive in production.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/>, PRJNA834564.

Ethics statement

The animal study was reviewed and approved by Ethical Commission of Shandong Agricultural University.

Author contributions

DD, PC, CW, and ZH conceived the project and designed the protocol. DD and WJ performed the experiments. DD, LF, and YZ wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

PC was employed by Beijing Enhalor International Tech Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.935004/full#supplementary-material>

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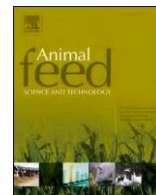
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Effects of *Saccharomyces cerevisiae* fermentation product on the nutrient digestibility and ileal digesta characteristics of cannulated growing pigs fed corn- or barley-sorghum-based diets

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ARTICLE INFO

ABSTRACT

Keywords: This study investigated the effects of *Saccharomyces cerevisiae* fermentation product (SCFP) on Barley-sorghum nutrient digestibility and ileal digesta

characteristics of cannulated growing pigs fed corn- or

Corn ileal digesta characteristics Nutrient digestibility *Saccharomyces cerevisiae* fermentation product barley-sorghum-based diets. Eight ileal-cannulated barrows randomized in a double 4 diet type (corn- or barley-sorghum-based diet), and the second factor was dietary SCFP treat-square design were fed four diets in a 2 × 2 factorial arrangement, in which the first factor was × 4 Latin

Pigs ment (supplemented with or without 5 g/kg SCFP). The cannulated barrows (35.63 ± 0.76 kg) fitted with a T-cannula in the terminal ileum were housed in metabolism cages in an environmentally controlled room. The results show that compared with the corn-based diet, the barley-sorghum-based diet reduced the apparent total tract digestibility (ATTD) and apparent ileal digestibility (AID) of crude protein (CP), the AID of all amino acids, amylase activity, trypsin activity, and pH in the ileal digesta of growing pigs ($P < 0.05$). Dietary 5 g/kg SCFP supplementation increased the digestible energy (DE)/gross energy (GE) ratio ($P < 0.05$), and the metabolizable energy (ME)/GE ratio ($P < 0.10$) of growing pigs in comparison to the non- SCFP-supplemented diet. Additionally, dietary 5 g/kg SCFP supplementation tended to enhance the ATTD of acid detergent fiber (ADF) and the AID of lysine ($P < 0.10$) and elevated the AID of tryptophan in growing pigs ($P < 0.05$). However, the nutrient digestibility and ileal digesta characteristics were unaffected by diet type × SCFP interaction ($P > 0.05$). In conclusion, compared with the corn-based diet, the barley-sorghum-based diet reduced the activities of digestive enzymes and the digestive absorption of CP and amino acids in growing pigs. Dietary 5 k/kg SCFP supplementation had beneficial effects on the energy and ADF digestibility of

Abbreviations: ADF, acid detergent fiber; ADFI, average daily feed intake; ADG, average daily gain; AID, apparent ileal digestibility; ATTD, apparent total tract digestibility; Ca, calcium; CF, crude fiber; CP, crude protein; DE, digestible energy; DM, dry matter; FCR, feed conversion rate; GE, gross energy; ME, metabolizable energy; mPa*s, millipascal-seconds; NDF, neutral detergent fiber; P, Phosphorus; SCFP, *Saccharomyces cerevisiae* fermentation product.

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growing pigs. However, the nutrient digestibility and ileal digesta characteristics of the pigs were unaffected by diet type × SCFP interaction.

1. Introduction

Corn is the primary energy source in the swine feed industry of China, where people consume or produce more than 50 % of the world's pork supply (Chen and Yu, 2018). With soaring of maize prices in recent years in China, increasing attention has been devoted to various alternative energy feeds for swine production, such as barley and sorghum (Piao, 2018). Currently, less is known about the comparative effects of corn-based diets and barley-sorghum-based diets on the nutrient digestibility of pigs.

The use of barley and sorghum in swine feed may face a low digestibility problem because barley and sorghum are less easily digested than corn (Pan et al., 2016, 2017; Thacker et al., 1992). *Saccharomyces cerevisiae* fermentation product (SCFP) is a fermentation product using an unmodified strain of *Saccharomyces cerevisiae*, which includes fermentation products, residual yeast cells, fermentation media, and yeast cell wall components (Shen et al., 2011). Recently, maternal supplementation with SCFP was demonstrated to improve litter weight at weaning (Chen et al., 2020; Zhang et al., 2020) and individual pig weight at weaning (Zhang et al., 2020), which may be explained by the digestibility-enhanced characteristics of SCFP. It has been reported that dietary supplementation of 5 g/kg yeast culture improved the average daily gain (ADG), average daily feed intake (ADFI), and the apparent digestibility of gross energy (GE), dry matter (DM), crude protein (CP), as well as the villus height and villus height to crypt depth ratio in the ileum of weaning pigs (Shen et al., 2009). These references provide support for SCFP in enhancing the nutrient digestibility of pigs fed a barley-sorghum-based diet in place of a corn-based diet.

Therefore, this study was conducted to investigate the impacts of SCFP on nutrient digestibility and ileal digesta characteristics of cannulated growing pigs fed corn- or barley-sorghum-based diets.

Table 1
The composition and nutrient content of basal diets (as-fed basis).

Item	Corn-based diet	Barley-sorghum-based diet
------	-----------------	---------------------------

Ingredients, g/kg Corn		
	706.0	0
Sorghum	0	499.6
Barley	0	240.5
Rice bran meal	0	40.0
Soybean meal	172.0	175.0
Soybean oil	0	12.4
Wheat bran	50.0	0
Wheat flour	40.0	0
Dicalcium phosphate	6.3	5.8
Limestone	7.5	6.0
Salt	3.8	3.8
L-lysine	4.3	5.0
D, L-methionine	1.2	1.4
L-threonine	1.4	1.8
L-tryptophan	0.1	0
Choline chloride	0.8	0.8
Vitamin and mineral premix ^a	1.4	1.4
Cr ₂ O ₃	3.5	3.5
Mold inhibitor	1.0	1.0
Zeolite	0.7	2.5
Total	1000.0	1000.0
Calculated composition, unit Digestible		
energy, MJ/kg	13.51	13.51
Dry matter, g/kg	867.5	871.4
Crude protein, g/kg	155.6	155.6
Crude fat, g/kg	30.3	33.3
Neutral detergent fiber, g/kg	117.9	164.3
Acid detergent fiber, g/kg	45.2	77.5
Ash, g/kg	40.3	48.2
Calcium, g/kg	5.4	5.4
Available phosphorus, g/kg	2.4	2.4
Digestible lysine, g/kg	9.5	9.5
Digestible methionine + cysteine, g/kg Digestible	5.5 5.9	5.5 5.9
threonine, g/kg		
Digestible tryptophan, g/kg	1.6	1.6

^a Vitamin and mineral premix supplied per kilogram of complete diet: 96.46 mg Zn (ZnSO₄·H₂O), 172.70 mg Fe (FeSO₄·H₂O), 35.01 mg Mn (MnSO₄·H₂O), 17.83 mg Cu (CuSO₄·5H₂O), 0.40 mg I (CaI₂O₆), 0.3 mg Se (Na₂SeO₃), 13,000 IU vitamin A, 4000 IU vitamin D₃, 41.08 IU vitamin E, 4 mg K, 0.034 mg vitamin B₁₂, 28.57 mg D-pantothenate, 75.77 mg niacin, 0.30 mg D-biotin, 2.31 mg folic acid.

2. Materials and methods

All animal protocols in this study were approved by the South China Agricultural University (Guangzhou city, Guangdong Province, China) Institutional Animal Care and Use Committee. The animal experiment was carried out at the swine metabolism laboratory at South China Agricultural University (Guangzhou city, Guangdong Province, China). This study was conducted between October 23, 2018 and December 26, 2018.

2.1. Animals, diets, and experimental design

cannula in the distal ileum according to the procedure of Eight growing Duroc × (Landrace × Yorkshire) barrows (initial body weight, 31.06 Stein et al. (1998). On the 14th day after surgery, eight pigs (35.63 ± 0.66 kg) were surgically quipped with a T ± 0.76 kg) were randomly allotted to one of the four experimental diets according to a 4 × 4 Latin square design with 4 dietary treatments and 4 periods. Each period lasted for 10 days.

The 4 diets were as follows: (1) corn-based diet (Table 1), (2) 5 g/kg SCFP supplementation in corn-based diet, (3) barley-sorghum-based diet, and (4) 5 g/kg SCFP supplementation in barley-sorghum-based diet. The SCFP was obtained from Beijing Enhalor Biotechnology Co., Ltd., Beijing, China. The SCFP was manufactured using patent yeast Sa-10 (*Saccharomyces cerevisiae*) fermentation technology that includes liquid and solid anaerobic fermentations, and dried with the media on which it was grown. The analysis of SCFP was not less than 150 g/kg CP, not less than 10 g/kg mannan, not more than 100 g/kg moisture and not more than 80 g/kg ash. All nutrients met or exceeded nutrient requirements of growing pigs recommended by the NRC (2012). All pigs were housed in individual stainless-steel metabolism crates (0.9 × 1.8 × 1.5 m) equipped with a nipple drinker and a feeder during the 40-day experimental period.

2.2. Feeding and sample collection

Pigs had *ad libitum* access to water and were fed a daily amount of feed equivalent to 4% of their body weight, which was divided into two equal meals provided at 0800 and 1700 h. Each 10-day experimental period consisted of 5-days of acclimatization to diets followed by a 3-day collection of feces and urine samples and a 2-day collection of ileal digesta.

2.3. Laboratory analysis

2.3.1. Conventional nutrients in diet, feces, and urine

The energy values of diet, feces, and urine samples were analyzed by the IKA C200 bomb calorimeter (IKA Analysetechnik, Heitersheim, Germany). The DM content was tested according to the standard method (GB6435-1986; People's Republic of China 1986). The CP content was analyzed with an automatic Kjeldahl nitrogen analyzer (Kjeltec® 2300 Analyzer Unit, Foss Tecator AB, Hogan" as, " Sweden). The acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents were analyzed using an A200i automatic cellulose analyzer (Ankom Technology, NY, USA). The calcium and phosphorus contents were analyzed according to standard methods (GB/ T6436-2018 and GB/T6437-2018; Republic of China 2018). The chromium content was measured using a Thermo Scientific ICE3500 (USA) atomic absorption spectrometer according to the standard method (GB/T13088-2006; Republic of China 2016).

2.3.2. Amino acids contents of diets and digesta

The amino acid contents of the diets and digesta were analyzed using an automatic amino acid analyzer using nor-leucine as the internal standard (LP-8900, Hitachi, Tokyo, Japan), which was described in our previous study (Zhang et al., 2017). Before analysis, samples were hydrolyzed using 6 mol/L HCl for 24 h at 110 °C except for tryptophan, methionine, and cystine. Tryptophan was determined after 4 mol/L LiOH hydrolysis for 22 h at 110°C. For methionine and cystine, samples were treated by cold performic acid oxidation overnight and hydrolysis (Zhang et al., 2017).

2.3.3. pH, viscosity, and digestive enzyme activity of digesta

The pH value of digesta was analyzed using a PB-10 digital pH meter (Sartorius, Gottingen, Germany). The viscosity of digesta was " tested using a rheometer (MCR 502, Anton paar, Austria), and readings were expressed in millipascal-seconds (mPa*s). The digestive enzyme activities (trypsin, amylase, and lipase) were analyzed by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. Calculations

The digestible energy (DE), metabolizable energy (ME), the apparent total tract digestibility (ATTD) of nutrients, and the apparent ileal digestibility (AID) of nutrients (including amino acids) were calculated using the following equations:

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$$DE_{kg} = \frac{MJ}{kg} = \frac{\text{Feed intake } (kg) \times \text{Feed gross energy } (MJ/kg) - \text{Feces output } (kg) \times \text{Feces gross energy } (MJ/kg)}{\text{Feed intake } (kg)}$$

Table 2

Effects of fermentation product (SCFP) on the digestible energy and metabolizable energy of cannulated growing pigs fed corn- or barley-sorghum-based diets.

Item	Corn-based diet		Barley-sorghum-based diet		SEM	P-value		
	0 g/kg SCFP ¹	5 g/kg SCFP	0 g/kg SCFP	5 g/kg SCFP		Diet type	SCFP	Diet type × SCFP
N ₂	8	8	8	8				
Average daily feed intake, kg	1.74	1.73	1.72	1.76	0.061	0.976	0.897	0.858
Average daily feces output, g	255.12	245.31	242.74	246.25	8.576	0.752	0.862	0.714
Average daily urine output, L	2.68	2.65	2.00	2.12	0.143	0.032	0.873	0.790
Digestible energy, MJ/kg	13.87	14.05	13.80	13.80	0.069	0.293	0.531	0.558
Metabolizable energy, MJ/kg	13.55	13.66	13.49	13.54	0.076	0.589	0.633	0.826
Digestible energy/Gross energy	0.828	0.856	0.841	0.855	0.004	0.463	0.013	0.391
Metabolizable energy/Gross energy	0.816	0.836	0.812	0.836	0.005	0.869	0.081	0.821

¹ SCFP, *Saccharomyces cerevisiae* fermentation product.

² Pig was the experimental unit for all determined parameters, and the number of replicates per treatment was 8.

Table 3

Effects of *Saccharomyces cerevisiae* fermentation product (SCFP) on apparent total tract digestibility and apparent ileal digestibility of nutrients of cannulated growing pigs fed corn- or barley-sorghum-based diets.

Item	Corn-based diet		Barley-sorghum-based diet		SEM	P-value		
	0 g/kg SCFP	5 g/kg SCFP	0 g/kg SCFP	5 g/kg SCFP		Diet type	SCFP	Diet type × SCFP
N ₂	8	8	8	8				
Apparent total tract digestibility								
Dry matter	0.821	0.847	0.825	0.831	0.0048	0.526	0.113	0.310
Crude protein	0.858	0.876	0.814	0.805	0.0064	<0.001	0.582	0.083
Calcium	0.525	0.542	0.540	0.545	0.0054	0.423	0.307	0.614
Phosphorus	0.425	0.433	0.408	0.415	0.0075	0.266	0.623	0.972
Ash	0.534	0.539	0.507	0.503	0.0094	0.112	0.977	0.817
Crude fiber	0.329	0.340	0.322	0.342	0.0059	0.841	0.209	0.684
Neutral detergent fiber	0.437	0.431	0.423	0.443	0.0149	0.977	0.806	0.683
Acid detergent fiber	0.368	0.386	0.397	0.429	0.0080	0.020	0.098	0.614
Apparent ileal digestibility								
Dry matter	0.767	0.801	0.776	0.760	0.0055	0.131	0.382	0.020
Crude protein	0.848	0.861	0.776	0.780	0.0095	<0.001	0.540	0.751
Calcium	0.497	0.512	0.510	0.511	0.0060	0.606	0.540	0.590
Phosphorus	0.416	0.426	0.407	0.422	0.0073	0.674	0.414	0.877
Ash	0.511	0.528	0.496	0.505	0.0070	0.196	0.372	0.791
Crude fiber	0.284	0.285	0.292	0.299	0.0089	0.573	0.819	0.883
Neutral detergent fiber	0.387	0.390	0.403	0.419	0.0199	0.588	0.816	0.883
Acid detergent fiber	0.332	0.327	0.343	0.338	0.0140	0.713	0.855	0.986

¹ SCFP, *Saccharomyces cerevisiae* fermentation product.

² Pig was the experimental unit for all determined parameters, and the number of replicates per treatment was 8.

$$\text{Feed intake } (kg) \times \text{Feed gross energy } (MJ/kg) - \text{Feces output } (kg) \times \text{Feces gross energy } (MJ/kg)$$

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$$ME_{MJ} = \frac{\text{Urine output (mL)} \times \text{Urine gross energy (mL)} \left(\frac{\text{kg}}{\text{kg}} \right)}{\text{kg}};$$

$$\text{Feed intake (d)}$$

$$\text{Feed intake (kg}_d) \times \text{Nutrient in feed (\%)} - \text{Feces output (kg}_d) \times \text{Nutrient in feces (\%)}$$

$$ATTD = \frac{\text{Feed intake (d)} \times \text{Nutrient in feed (\%)}}{\text{kg}}$$

$$AID = \frac{\text{Nutrient content in digesta (kg DM)} - \text{Chromium content in diet (kg DM)}}{\text{Feed intake (kg}_d) \times \text{Nutrient in feed (\%)}}$$

Table 4

Effects of fermentation (SCFP) product on apparent ileal digestibility of amino acids of cannulated growing pigs fed corn- or barley-sorghum-based diets.

Item	Corn-based diet		Barley-sorghum-based diet		SEM	P-value		
	0 g/kg SCFP	5 g/kg SCFP	0 g/kg SCFP	5 g/kg SCFP		Diet type	SCFP	Diet type × SCFP
N ₂	8	8	8	8				
Indispensable amino acids								
Lysine	0.904	0.916	0.890	0.898	0.0032	0.008	0.099	0.713
Phenylalanine	0.880	0.893	0.826	0.821	0.0067	< 0.001	0.567	0.226
Threonine	0.869	0.885	0.843	0.848	0.0047	< 0.001	0.184	0.436
Isoleucine	0.889	0.899	0.817	0.814	0.0079	< 0.001	0.697	0.382
Leucine	0.883	0.898	0.818	0.800	0.0085	< 0.001	0.803	0.055
Valine	0.859	0.872	0.792	0.790	0.0080	< 0.001	0.510	0.415
Arginine	0.920	0.929	0.882	0.883	0.0044	< 0.001	0.286	0.375
Methionine	0.915	0.920	0.881	0.899	0.0042	< 0.001	0.115	0.330
Histidine	0.879	0.899	0.824	0.820	0.0071	< 0.001	0.285	0.122
Tryptophan	0.879	0.908	0.871	0.881	0.0039	0.011	0.005	0.129
Dispensable amino acids								
Aspartic acid	0.874	0.890	0.822	0.824	0.0064	< 0.001	0.218	0.342
Serine	0.871	0.887	0.817	0.811	0.0069	< 0.001	0.555	0.162
Glutamic acid	0.903	0.914	0.863	0.849	0.0058	< 0.001	0.815	0.082
Glycine	0.809	0.839	0.755	0.756	0.0086	< 0.001	0.208	0.239
Alanine	0.846	0.865	0.787	0.768	0.0088	< 0.001	0.990	0.074
Tyrosine	0.864	0.883	0.825	0.804	0.0066	< 0.001	0.915	0.013
Proline	0.861	0.889	0.804	0.801	0.0096	< 0.001	0.397	0.272
Cystine	0.904	0.910	0.864	0.857	0.0060	< 0.001	0.939	0.466

¹ SCFP, *Saccharomyces cerevisiae* fermentation product.² Pig was the experimental unit for all determined parameters, and the number of replicates per treatment was 8.

2.5. Statistical analysis

Statistical analysis was conducted using the General Linear Model procedure of SPSS 22.0 version (IBM Corp, 2013), arranged as a 2

Saccharomyces cerevisiae

determined parameters. The following model was used: $\times 2$ factorial design with the diet type and dietary SCFP treatment being the main factors. Pig was the experimental unit for all $Y^{ijk} = \mu + AB_j^i + \text{dietary SCFP treatment } (B^j + (AB)^{ij} + e^{ijk})$, in which $j = Y^{ijk}$ yes or no, = dependent variable, AB_{ij} = interaction effect μ = mean, 0.05

A_i = diet type (i = corn-based diet or barley-sorghum-based diet),

were regarded as significant, and probabilities between diet type and dietary SCFP treatment, $e_{ijk} \geq 0.05$ random error. The results are expressed as the mean and SEM. Probabilities ≥ 0.10 were regarded as tendencies among treatments.

3. Results

3.1. The DE and ME

As shown in Table 2, the average daily fecal output, DE, and ME of growing pigs were unaffected by dietary treatments ($P > 0.10$). The average daily urine output of growing pigs was decreased when pigs were fed barley-sorghum vs. corn-based diet ($P < 0.05$). In addition, dietary supplementation with 5 g/kg SCFP increased the DE/GE ratio (P with the non-SCFP-supplemented group. No diet type $\times$ SCFP interaction was found ($P < 0.05$), and the ME/GE ratio ($P > 0.10$). = 0.081) compared

3.2. The ATTD and AID of conventional nutrients

As displayed in Table 3, pigs fed the barley-sorghum-based diet had lower ATTD of CP and higher ATTD of ADF compared to those fed the corn-based diet ($P < 0.05$). Dietary SCFP supplementation tended to increase the ATTD of ADF ($P = 0.098$). The ATTD of CP protein than those fed the corn-based diet (P tended to be affected by diet type $\times$ SCFP interaction ($P < 0.05$). Dietary SCFP supplementation did not affect the AID of conventional nutrients, = 0.083). Pigs fed the barley-sorghum-based diet had lower AID of crude including DM, CP, calcium (Ca), Phosphorus (P), ash, crude fiber (CF), NDF and ADF ($P > 0.10$). The AID of dry matter was affected by diet type $\times$ SCFP interaction ($P < 0.05$).

3.3. The AID of amino acids

As described in Table 4, pigs had decreased AID of all amino acids when they were fed the barley-sorghum-based diet vs. the corn-based diet ($P < 0.05$). Dietary SCFP supplementation increased the AIDs of lysine ($P = 0.099$) and tryptophan ($P < 0.05$). The AIDs of glutamic acid ($P = 0.082$), alanine ($P = 0.074$), and tyrosine ($P < 0.05$) were affected by the diet type $\times$ SCFP interaction.

Table 5

Effects of fermentation product (SCFP) on digestive enzyme activities, viscosity, and pH of ileal digesta of cannulated growing pigs fed corn- or barley-sorghum-based diets.

Item	Corn-based diet		Barley-sorghum-based diet		SEM	P-value		
	0 g/kg SCFP ¹	5 g/kg SCFP	0 g/kg SCFP	5 g/kg SCFP		Diet type	SCFP	Diet type $\times$ SCFP
N ₂	8	8	8	8				
Amylase (U/dL)	69.34	73.35	67.82	67.84	0.896	0.047	0.245	0.249
Lipase (U/L)	276.15	275.60	265.62	274.30	12.556	0.824	0.879	0.862
Trypsin (U/mL)	88.02	90.05	84.57	85.55	1.084	0.073	0.488	0.806
Digesta viscosity (mPa*s) ³	2.23	2.20	2.22	2.22	0.012	0.979	0.651	0.651
Digesta pH	7.27	7.07	6.52	6.57	0.121	< 0.001	0.578	0.341

¹ SCFP, *Saccharomyces cerevisiae* fermentation product.

² Pig was the experimental unit for all determined parameters, and the number of replicates per treatment was 8.

³ mPa*s, millipascal-seconds.

3.4. Digestive enzyme activities, viscosity, and pH of ileal digesta

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As depicted in Table 5, pigs fed the barley-sorghum-based diet had lower amylase activity ($P < 0.05$), trypsin activity ($P = 0.073$), and pH ($P < 0.05$) than those fed the corn-based diet. The digestive enzyme activities, viscosity, and pH of ileal digesta were unaffected by SCFP supplementation or diet type $\times$ SCFP interaction ($P > 0.10$).

4. Discussion

The aim of this study was to evaluate the effects of SCFP on nutrient digestibility and ileal digesta characteristics of cannulated growing pigs fed corn- or barley-sorghum-based diets. It is generally believed that soluble β -glucan and tannin in barley-sorghum-based diets are the main factors affecting the feeding values of barley and sorghum (Pan et al., 2016). β -glucan and tannin are easily combined with proteins, carbohydrates, and minerals to form a complex that is not easily digested by monogastric animals, which reduces the absorption efficiency of nutrients (Pan et al., 2016). β -glucans are the main cell wall component of barley endosperm, and a high content of β -glucan has been reported to decrease the nutritional value of barley (Thacker et al., 1992). Tannins are the primary antinutritional factor affecting the feeding value of sorghum (Pan et al., 2017). Although the chemical contents of sorghum are similar to those of corn, the nutritional value of sorghum is normally considered to be 95 % of the nutritional value in corn (Pan et al., 2017). In the present study, pigs fed the barley-sorghum-based diet had lower amylase activity, trypsin activity, and pH in ileal digesta than those fed the corn-based diet, which indicates that barley-sorghum based diet reduced the nutrient digestive capability of growing pigs. Pigs fed the barley-sorghum-based diet had lower ATTD and AID of CP and higher ATTD of ADF compared to those fed the corn-based diet. Growing pigs have poor digestibility of lignin and cellulose included in the ADF of barley, which was digested in small quantities by intestinal microbes (Fairbairn et al., 1999). Consistent with our results, Pan et al. (2017) found that sorghum in place of corn in growing pigs decreased the feed conversion ratio (FCR), and increased nitrogen output by 27.9 % and the crude protein apparent digestive ratio by 7.2 %. Sorghum partially or totally in place of corn also reduced the FCR and the apparent digestive ratio of weaned pigs (Pan et al., 2017). Consistent with the decreased crude protein digestibility, pigs had decreased AID of all amino acids when pigs were fed barley-sorghum based diet vs. corn-based diet. It has been demonstrated that the amino acid digestibility of sorghum is adversely affected by many factors, such as tannin (Liu et al., 2013) and fiber (Jaworski et al., 2015), which partially explains the decreased AID of amino acids in pigs fed the barley-sorghum based diet in place of the corn-based diet.

In the current study, dietary supplementation with 5 g/kg SCFP did not affect the AID of conventional nutrients. In agreement with our results, Yang et al. (2018) reported that dietary addition with 1 g/kg yeast culture did not influence the ATTD of nitrogen and DM of weaning piglets. However, dietary SCFP supplementation tended to increase the ATTD of ADF in the present study. Consistent with our results, Plata et al. (1994) found that dietary addition with yeast culture (*Saccharomyces cerevisiae*) improved NDF digestibility and ruminal protozoan populations of steers fed oat straw based diets. Jouany et al. (2008) also found that live yeast culture supplementation increased the ADF digestibility of horses fed a high-starch or high-fiber diet. In addition, dietary SCFP supplementation increased the DE/GE ratio, and the ME/GE ratio, as well as the AID of lysine and tryptophan. However, the digestive enzyme activities, viscosity, and pH of ileal digesta were unaffected by SCFP supplementation or diet type $\times$ SCFP interaction, which suggests that the beneficial effects of SCFP on nutrient digestibility may be attributed to microbiota modulation, not physical or chemical digestion. Further research on this topic is warranted and may include a role of the microbiota when feeding SCFP.

5. Conclusions

Compared with corn-based diet, barley-sorghum based diet reduced the activities of digestive enzymes and the digestive absorption of crude protein and amino acids in growing pigs. Dietary supplementation with 5 g/kg *Saccharomyces cerevisiae* fermentation product had beneficial effects on the energy and acid detergent fiber digestibility of growing pigs. However, the nutrient digestibility and ileal digesta characteristics were unaffected by diet type $\times$ *Saccharomyces cerevisiae* fermentation product interaction.

CRediT authorship contribution statement

Jun Chen: Writing - original draft. **Yijiang Wang:** Writing - review & editing. **Jinming You:** Methodology. **Jiaming Chen:** . **Min Tian:** Validation. **Fang Chen:** Methodology. **Shihai Zhang:** Conceptualization. **Wutai Guan:** Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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Effects of yeast culture supplementation from late gestation to weaning on performance of lactating sows and growth of nursing piglets



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Sows

Yeast culture

Dietary yeast culture supplementation can contribute to the performance and health of sows and piglets, but few studies have focused on the relationships between the effects of yeast culture and gut microbiota. This study investigated the effect of yeast culture (*Saccharomyces cerevisiae*) supplementation from late gestation to weaning on the reproductive performance of lactating sows and their faecal microbiota. One hundred and six purebred Landrace sows, of parities two to six were selected and randomly assigned to a control (CON) and yeast culture supplementation (YC) groups based on parity and back fat thickness. The YC sows were individually fed with yeast culture at a dose of 24 g/d from day 90 of gestation to parturition and 40 g/d during lactational period. Blood samples were collected from sows on d 110 of gestation and at weaning at day 21 of lactation for plasma hormone and immunoglobulin analysis. Colostrum and milk on day 20 of lactation were collected for composition analysis. Faecal samples from sows on day 110 of gestation and day 20 of lactation were collected for short-chain fatty acid and faecal microbial analysis. Results showed that the farrowing performance of YC sows did not differ significantly from the CON group ($P > 0.05$). The average daily feed intake by the YC group during the lactation period was significantly increased by 9.98% ($P = 0.004$), the weaning-to-oestrus interval was shortened by 0.96 d ($P = 0.046$) and average daily weight gain of piglets increased by 7.14% ($P = 0.036$) compared with the CON group. Yeast culture supplementation also significantly improved the average daily milk yield in the first week of lactation ($P = 0.035$), lactose content in colostrum ($P = 0.046$), protein ($P = 0.033$) and DM ($P < 0.001$) content of milk. In the YC group, concentrations of plasma ghrelin ($P = 0.02$) and IgG ($P = 0.015$) were increased compared with the CON group, while that of glucagon-like peptide-1 was decreased ($P = 0.006$) on d 110 of gestation. The 16S rRNA gene sequencing showed that faecal microbiota changed at taxonomic levels with yeast culture addition ($P < 0.05$). Dietary yeast culture supplementation from late gestation to lactation improved feed intake, immunity status, milk yield, milk quality and faecal microbiota of sows, resulting in the improved growth performance of piglets.

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Implications

Diet supplemented yeast culture was an effective means to promote the performance and health of sows and piglets, but few studies have focused on its effect on gut microbiota. This study was undertaken to investigate the effects of adding yeast culture to diets on sow performance and its underlying possible mechanisms. The results showed that feed intake, milk quality, faecal microbiota of sows, and maternal health status were altered by

yeast culture supplementation, suggesting *Saccharomyces cerevisiae* yeast culture products may be a promising functional additive to improve the performance of sows and their piglets.

Introduction

Due to genetic selection for prolificacy, modern sows undergo a huge metabolic nutrient demand for milk production to satisfy a large litter size (Zhou et al., 2018), so insufficient nutrient intake will lead to mobilisation of their body reserves during lactation

(Kim et al., 2008). The growth performance of the piglets is determined by the sow's health status and milk yield during lactation (Szyndler-Nećza et al., 2013), which can be altered by maternal

molecule or particle includes activation of cytotoxic macrophages, helper T cells, and NK cells, promotion of T cell differentiation, and activation of the alternative complement pathway (Bohn and Bemiller, 1995). Milk yield and quality not only provide nutrients to support the growth of neonates (Theil and Hurley, 2016) but also ultimately impact the growth performance and gut health of offspring (Leonard et al., 2010; Chu et al., 2016). The milk-derived immunity involves mostly IgA derived from the maternal intestine and directed against various pathogens from the mother's intestinal microbiota (Hanson et al., 1979; Salmon et al., 2009). However, evidence of how yeast culture supplementation affects the gut microbiota of sows is still scarce.

The objective of this study was to supplement sow diets with a novel type of yeast culture *Saccharomyces cerevisiae* (CGMCC No. 6120) and measure the subsequent performance of the sows and their offspring, along with effects on sow milk quality and gut microbiota.

Material and methods

Animals, feeding and management

A total of 106 purebred Landrace multiparous sows of parities two to six were selected and allocated to a control (CON) or a yeast culture group (YC) based on parity (3.50 ± 0.31 and 3.62 ± 0.33) and back fat thickness (15.52 ± 0.55 mm and 15.67 ± 0.56 mm). From d 90 of gestation to parturition, all sows were kept in

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diets (Jang et al., 2013; Hasan et al., 2018), so nutritional manipulations using dietary supplements are direct and effective means to improve sow productivity and litter performance.

Yeast cultures have been widely used as either a probiotic using a small amount of live yeast cells or prebiotic with yeast metabolites or cell wall components as additives to enhance swine performance. Improvements in piglets' numbers weaned (Czech et al., 2010), litter weight at weaning (Czech et al., 2010; Kim et al., 2010; Shen et al., 2011; Zhang et al., 2020) and piglet average daily gain (Shen et al., 2011) have all been noted when sows were fed with yeast products such as dry yeast, yeast cell walls or purified β -glucan and mannan-oligosaccharides from yeast during lactation. This effect may be due to an improvement in the digestibility of DM, gross energy and CP in pigs (Shen et al., 2009). Sow diet supplementation with yeast culture or live yeast has also been demonstrated to have positive effects on the immunological status of their progeny (Shen et al., 2009; Monroy-Salazar et al., 2012; Jang et al., 2013; Zanello et al., 2013). In contrast, no improvements in sows' feed intake during lactation and litter weight at weaning were reported when sows were supplemented with dry yeast (*Saccharomyces cerevisiae*) during gestation and the lactation period (Jurgens et al., 1997). The improvements in immunological state could be due to probiotic components such as β -glucan and mannan-oligosaccharides, which exist in yeast culture, which have shown not only to bind to pathogenic bacteria and facilitate their flushing from the intestinal lumen (Spring et al., 2000) but also to promote the growth of beneficial bacteria (Davis et al., 2004). Immunopotentiality effected by the binding of a (1,3) + glucan

individual stalls and offered 3.0 kg/d of standard gestation diet (Table 1). Sows in the YC group were individually fed a pellet including ten grams of wheat flour, two grams of dextrin and twenty-four grams of *Saccharomyces cerevisiae* yeast culture with moisture 4.87%, CP 18.41%, ash 7.69%, b-glucan and mannan-oligosaccharides 0.82%, and others 69.03% (Enhalor Biotechnology Company, Beijing, China). Sows in the CON group were fed a placebo pellet including ten grams of wheat flour and two grams of dextrin.

Sows were moved into the farrowing house on d 112 of gestation and kept in individual farrowing crates measuring

Table 1

Ingredients and chemical composition of basal diets provided to the sows.

Items	Gestation	Lactation
Ingredients, % Corn		
	56.00	63.40
Wheat bran	10.00	—
Rice bran meal	8.56	—
Soybean meal ¹	9.70	21.20
Extruded-soybean	—	5.30
Flaxseed	—	2.00
Sugar beet pulp	3.00	3.20
Soybean hull	8.50	—
Soybean oil	—	0.50
Limestone	1.00	0.90
Dicalcium phosphate	1.20	1.70
NaCl	0.40	0.40
Sodium bicarbonate	0.20	—
Magnesium oxide	0.18	0.16
L-Lysine sulphate ¹	0.13	0.20
DL-Methionine ¹	0.03	—
L-Threonine	0.11	0.06
L-Tryptophan ¹	0.14	0.13
Choline chloride ¹	0.10	0.10
Mineral premix ²	0.43	0.43
Vitamin premix ³	0.32	0.32
Total	100.00	100.00
Nutritional level ⁴		
DM, %	87.10	87.10
ME, Mcal/kg	2.81	3.20
CP, %	12.5 (12.34)	16.50 (16.52)
Ca, %	0.85 (0.82)	0.90. (0.91)
AP, %	0.32	0.37
Total Lysine, %	0.7 (0.68)	1.08 (1.06)
SID Lysine	0.58	0.96
SID Methionine	0.19	0.25
SID Threonine	0.45	0.61
SID Tryptophan	0.12	0.18

Abbreviations: ME = metabolisable energy; AP = available phosphorus; SID = standardised ileal digestible.

¹ 42% CP content of soybean meal; 70% L-Lysine sulphate content; 99% DLMethionine content; 20% L-Tryptophan content; 60% Choline chloride content. ² Mineral mixture supplied per kilogram of diets: Fe 120 mg; Cu 20 mg; Mn 60 mg; Zn 120 mg; Se 0.3 mg; I 0.5 mg.

³ Vitamin mixture supplied per kilogram of diets: vitamin A 10000 IU; vitamin D₃ 2000 IU; vitamin E 60 IU; vitamin K₃ 5.0 mg; vitamin B₁ 5.0 mg; vitamin B₂ 10.0 mg; vitamin B₆ 6.0 mg; vitamin B₁₂ 50 lg; nicotinic acid 40 mg; d-pantothenic acid 20 mg; folic acid 2.0 mg; biotin 0.2 mg.

⁴ Calculated according to China Feed Database (2018). The measured values are in parentheses, and others are calculated values.

200–80 cm. After farrowing, the feed allowance was 2 kg initially and increased by 0.5 kg/d until the fifth day of lactation. From day six, all sows had free access to standard lactation diet until weaning and similarly, YC sows were individually daily fed a pellet including ten grams of wheat flour, two grams of dextrin and forty-five grams of *Saccharomyces cerevisiae* yeast culture and a placebo pellet including ten grams of wheat flour, two grams of dextrin for the CON group. If needed, cross-fostering was completed within 24 h of parturition to standardise litter size to 10–12 piglets within treatment. The postnatal care of piglets including teeth clipping, tail docking and iron injection was administered within the first 12 h after birth. During lactation, piglets had free access to water via nipple drinkers until weaning on d 21.

Performance measurements and sample collection

Backfat thickness of sows was measured at 6.5 cm to the left side of the dorsal midline at the level of the last rib using an ultrasound device (Renco, Manchester,

MA, USA) on d 90, d 112 of gestation and d 21 of lactation. Piglets were weighed individually at birth and on d 7, 14, and 21 of lactation to calculate weight gain and to estimate milk yield. Colostrum samples were collected

within two hours after the first piglet was born, and milk samples were collected in the morning of d 21 of lactation. During milk sampling, the sow was injected intravenously with 0.3 mL of oxytocin to induce milk let-down, and 20 mL of milk was collected and stored at 20 °C until further analysis. Sow blood samples on d 110 of gestation and d 21 of lactation were drawn by jugular vein puncture two hours after the morning meal and collected in heparinised tubes. Plasma was obtained by centrifuging blood samples at 1 000g at 4 °C for 10 min and then stored at 20 °C until analysis. Fresh faecal samples on d 110 of gestation and d 20 of lactation were individually collected from the rectum of sows using sterile tubes, flash-frozen in liquid nitrogen and stored at 80 °C until analysis.

Milk and plasma sample analysis

Milk composition was analysed in triplicate for DM, fat, protein, lactose, and non-fat solid (SNF) with an Automatic milk analyzer (CombiFoss FT+, Foss, Denmark). Plasma metabolites including neuropeptide Y (NPY), peptide tyrosine-tyrosine (PYY), glucagonlike peptide-1 (GLP-1), Ghrelin, IgG, IgM of sows were determined using respective commercial kits (Jiangsu Meimian Industrial Co., Ltd China) according to the manufacturer's protocols.

Faecal metabolites and microbial analysis

One of the duplicate faecal samples was analysed for shortchain fatty acids (SCFA) including acetate, propionate, butyrate, valerate, isobutyrate and isovalerate using the gas chromatographic method as previously reported by Zhou et al. (2018). Another faecal sample was subjected to microbial analysis. Microbial DNA was extracted using the E.Z.N.A. Stool DNA Kit (D4015, Omega, Inc., Norwalk, CN, USA) according to the manufacturer's protocol. The extracted genomic DNA was measured for purity and integrity before sequencing (LC-Biotechnology Co., Ltd, Hang Zhou, Zhejiang Province, China). The V4 hypervariable region of the 16S rRNA gene was amplified using 515F and 806R primer (5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GGACTACHVGGGTWTC TAAT-3', respectively). The 16sRNA gene sequencing was performed on NovaSeq PE250 platform.

Statistical analysis

This study was a completely randomised design with sow or litter as the experimental unit and yeast culture treatment as the main effect. Milk yield was calculated as litter weight gain and litter size according to Hansen et al (2012). The statistical analysis was performed by the MIXED procedure of SAS (version 9.4) and excluded stillborn piglets, mummified foetuses, piglets' mortality, total born piglets born, live-born piglets, litter size after crossfostering, litter size at weaning and wean-to-oestrus. The percentage of stillborn piglets, mummified foetuses and piglets' mortality during lactation were analysed using the GLIMMIX procedure and fitted with the assumption that the data exhibited a binomial distribution. Total born piglets born, live-born piglets, litter size after cross-fostering, litter size at weaning and wean-to-oestrus interval were analysed using the GLIMMIX procedure with the Poisson distribution.

For analysis of intestinal microbiota, data of relative abundance at phylum and genus levels were log-transformed before statistical analysis. Difference in α -diversity was presented by Shannon index, and differential abundance between the dietary groups of specific genus or phylum were evaluated by the non-parametric Wilcoxon sum rank test. Differences were considered significant when $P < 0.05$ and $0.05 < P < 0.1$ were considered as showing a trend.

Results

Sow and litter performance

Two sows from the CON and the YC group were removed from the study due to low consumption of feed during the experimental period, and two sows from the YC group were excluded due to postpartum paralysis. Supplementation of yeast culture from late gestation to lactation did not change the farrowing performance of YC sows compared with the CON group ($P > 0.05$) as seen in Table 2. The YC sows had higher weekly average daily feed intake (ADFI, $P < 0.05$) as seen in Fig. 1A, whole-lactation ADFI at 5.95 compared with 5.41 kg

for the CON group ($P = 0.004$) and a tendency to lose less back fat as (0.06 compared with Table 3. Yeast culture sup-1.15 mm

$P = 0.083$) during lactation as shown in plementation improved the weaning-to-oestrus interval of YC sows compared to CON sows at 5.16 d compared with 6.12 d ($P = 0.046$), and piglet average daily gain (ADG) was improved at 1.65 compared with 1.54 kg ($P = 0.036$) as seen in Table 3.

Milk yield and milk composition

As shown in Table 3, milk yield during the whole lactation was not changed by yeast culture supplementation ($P > 0.05$), except that the average daily milk yield (ADMY) in the first week of lactation was markedly increased compared with the CON group ($P = 0.035$), as seen in Fig. 1B. The contents of lactose ($P = 0.046$), protein ($P < 0.001$), DM ($P = 0.033$) and urea N contents in milk ($P = 0.017$) were significantly improved in the YC group compared with the CON group as seen in Table 4.

Concentration of faecal short-chain fatty acids, appetite-related hormone and immunological parameters in plasma

As shown in Table 5, consumption of yeast culture did not change faecal SCFA concentrations. However, a significant reduction of isovalerate, a branched-chain fatty acid (BCFA) in the faeces of YC sows, was detected on d 21 of lactation compared with the CON group ($P = 0.043$). Yeast culture supplementation significantly increased concentration of plasma ghrelin ($P = 0.02$) and decreased GLP-1 ($P = 0.006$) on d 110 of gestation and tended to enhance concentration of NPY on d 21 of lactation ($P = 0.074$) as seen in Fig. 2. The plasma IgG level of YC sows was greater than that of CON sows at weaning ($P = 0.015$).

Changes of gut microbial composition

Yeast culture supplementation did not change faecal microbial α - or β -diversity of sows as seen in Fig. 3A and B. The relative abundance of Bacteroidetes was lower ($P = 0.047$), while the Firmicutes/ Bacteroidetes ratio was higher ($P = 0.046$) for YC sows compared to those of the CON sows, as shown in Fig. 3E. During gestation, yeast culture addition remarkably increased the abundances of Ruminococcaceae_UCG-005, Ruminococcus_1, Prevotellaceae_UCG001, Family_XIII_AD3011, Acetivomaculum and Lachnospiraceae_NK4B4 ($P < 0.05$) while decreasing the abundance of Bacteroidales and Lachnospiraceae_UCG-010 at genus level ($P < 0.05$) as shown in Fig. 3C. For the lactation period, the relative abundance of genera Eggerthella and Alistipes were significantly higher ($P < 0.05$), while the relative abundances of genera Anaerostipes, Saccharimonadaceae, Oscillospira and Eubacterium eligens were significantly lower in the YC group than those in the CON group ($P < 0.05$) as shown in Fig. 3D.

Table 2

Effect of yeast culture supplementation from late gestation to lactation on farrowing performance of sows.

Items	CON	YC	SEM	P-value
No. of Sows	53	53		
Litter size				
Total born piglets, n	11.96	12.33	0.752	0.538
Live-born piglets, n	11.30	11.25	0.447	0.934
Stillborn piglets, %	3.34	5.13	0.013	0.103
Mummified foetuses, %	1.20	1.67	0.010	0.372
Piglet weight				
Average piglet weight at birth, kg	1.46	1.41	0.033	0.244
Litter weight at birth, kg	16.39	15.53	0.561	0.275
CV of piglet birth weight, %	20.96	23.53	2.356	0.173

Abbreviations: CON = control group; YC = yeast culture group.

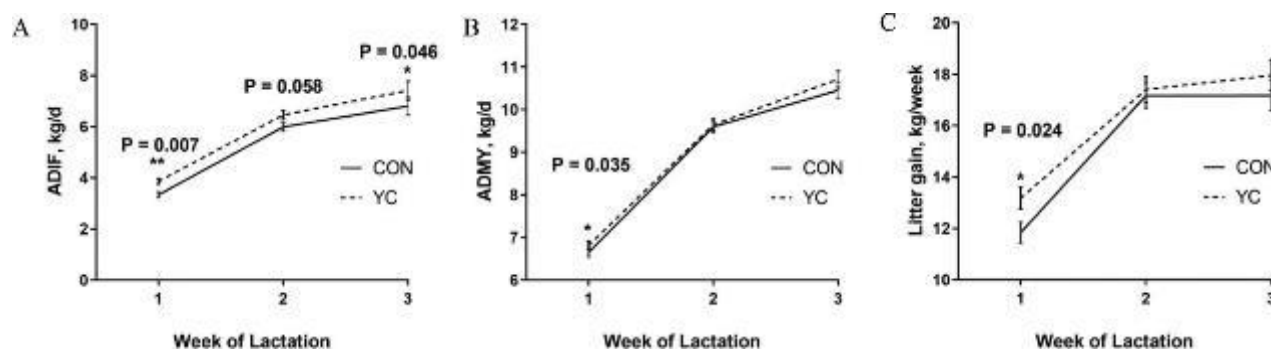


Fig. 1. The weekly ADFI (A), ADMY (B) and litter gain (C) of sows from control and yeast culture group during lactation. * $P < 0.05$, ** $P < 0.01$. Abbreviations: CON = control group; YC = yeast culture group; ADFI = average daily feed intake; ADMY = average daily milk yield.

Table 3

Effects of yeast culture supplementation from late gestation to lactation on lactational performance of sows.

Items	CON	YC	SEM	P-value
No. of sows	52	50		
Sow back fat thickness change, mm G 90–G 112 d	2.51	1.52	0.658	0.14
G112–L21 d	1.15	0.66	0.224 0.206	0.083
Weaning-to-oestrus interval, d	6.12	5.16		0.046
Litter size after cross-fostering, n	10.17	10.24	0.114	0.68
Litter size at weaning, n	9.83	10	0.12	0.329
Piglet mortality, %	2.65	2.93	0.267	0.782
Litter weight after cross-fostering, kg	14.79	14.75	0.321	0.923
Piglets weight after cross-fostering, kg	1.46	1.45	0.031	0.825
Average daily feed intake (0–21 d), kg/d	5.41 ^b	5.95 ^a	0.159	0.004
Milk yield, kg/d	8.88	9.08	0.116	0.19
Litter weight gain per week, kg	15.31	16.54	0.498	0.063
Piglet average daily gain per week, kg	1.54 ^b	1.65 ^a	0.039	0.036

Abbreviations: CON = control group; YC = yeast culture group; G 90–G 112 d = from day 90 of gestation to day 112 of gestation; G112–L21 d = from day 112 of gestation to day 21 of lactation. ^{a,b}Values within a row with different superscripts differ significantly at $P < 0.05$.

Discussion

To meet the continuous need for nutrients for foetal and mammary gland development, sows undergo metabolic and physiological changes during late gestation (Luan et al., 2014). These changes can cause insulin resistance (Barbour et al., 2007) and elevated systemic pro-inflammatory cytokines and oxidative stress (Saltiel and Olefsky, 2017). This study found that dietary yeast culture supplementation from late gestation to weaning appeared beneficial for sows and piglet performance, which could be due to improved sow feed intake, milk quality, maternal immune status and altered intestinal microbiotal homeostasis.

In this study, supplementing sow diets with 24 g/d yeast culture from late gestation did not affect gestational backfat thickness change and farrowing

performance. These results are in agreement with Kim et al. (2008) and Shen et al. (2011) who supplemented sows with *Saccharomyces cerevisiae* fermentation products throughout the whole gestation period. Czech et al. (2010) reported increased live-born piglets and piglet birth weight when sows' diet was supplemented with yeast cell wall product or manna-oligosaccharides beginning on d 28 before farrowing. Similarly, a recent study found that the number of live-born piglets was increased and IUGR percentage was decreased with increasing inclusion of *Pichia guilliermondii* in sow diet throughout gestation (Bass et al., 2019). The inconsistent results suggested that improved farrowing performance may be associated with breed of sow, duration and dose of yeast supplementation, as well as diets that varied in ingredients.

Table 4

Effects of yeast culture supplementation from late gestation to lactation on colostrum and milk composition of experimental sows.

Items	CON	YC	SEM	P-value
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Colostrum Fat, %	4.52	3.71	0.428	0.162
Protein, %	14.59	14.66	0.658	0.912
Lactose, %	2.82 ^b	3.13 ^a	0.145	0.046
DM, %	25.15	24.85	0.717	0.768
SNF, %	21.53	21.01	0.482	4.457
Urea N, mmol/L	66.66	58.76	2.993	0.075
Milk on day 21 of lactatio Fat, %	7.76	9.6	0.992	0.066
Protein, %	4.86 ^b	5.44 ^a	0.212	0.009
Lactose, %	5.7	5.74	0.28	0.922
DM, %	21.06 ^b	24.00 ^a	1.182	0.033
SNF, %	14.02	14.95	0.342	0.066
Urea N, mmol/L	64.27 ^a	51.77 ^b	4.344	0.017

Abbreviations: CON = control group; YC = yeast culture group; SNF = solid of not-fat in milk. ^{a-b}Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 5

Effects of yeast culture supplementation from late gestation to lactation on faecal SCFA concentration of sows.

Items	CON	YC	SEM	P-value
Day 110 of gestation, l mol/g				
Total SCFA	100.41	104.66	9.059	0.744
Acetate	69.81	74.12	6.095	0.622
Propionate	18.62	18.41	2.161	0.945
Butyrate	9.71	9.89	1.144	0.911
Valerate	2.27	2.24	0.164	0.896
Total BCFA	8.32	7.79	0.8	0.952
Isobutyrate	3.24	3.08	0.326	0.671
Isovalerate	5.07	4.71	0.479	0.486
BCFA/SCFA	0.082	0.076	0.001	0.355
Day 21 of lactation, l mol/g				
Total SCFA	96.28	98.4	7.587	0.845
Acetate	67.25	68.18	5.046	0.897
Propionate	17.27	18.58	1.502	0.545
Butyrate	9.64	9.53	1.168	0.948
Valerate	2.13	2.12	0.146	0.964
Total BCFA	8.09	7.1	0.415	0.107
Isobutyrate	3.07	2.92	0.161	0.529
Isovalerate	5.03 ^a	4.18 ^b	0.276	0.043
BCFA/SCFA	0.086	0.075	0.005	0.097

Abbreviations: CON = control group; YC = yeast culture group; SCFA = short-chain fatty acid; BCFA = branched-chain fatty acid. ^{a-b}Values within a row with different superscripts differ significantly at $P < 0.05$.

Feed intake is regarded as a major limiting factor for nutrients available for milk production in lactating sows (Strathe et al., 2017), which may be due to the hormonal regulation of appetite (Zhou et al., 2018). The underlying reason for the increased lactational feed intake of YC sows could be explained by altered plasma gut hormones with reduced concentration of GLP-1, which suppresses feeding desire by relaying meal-related information on nutritional status to the brain (van Bloemendaal et al., 2014) and increased concentration of ghrelin and NPY. Neuropeptide Y is one of the most active stimulating agents for food intake, and ghrelin released from the stomach is the only known hormone which transmits hunger signals to the brain (Nogueiras et al., 2010; Zhang et al., 2012). Despite it, the hormone was changed by supplementation of yeast culture in late gestation and lactation compared to the CON group which had a higher ghrelin (G_{110}), a lower GLP-1(G_{110}) concentration and a trend to increased NPY concentration (L_{21}) in the plasma of sows. Although the mechanisms of yeast

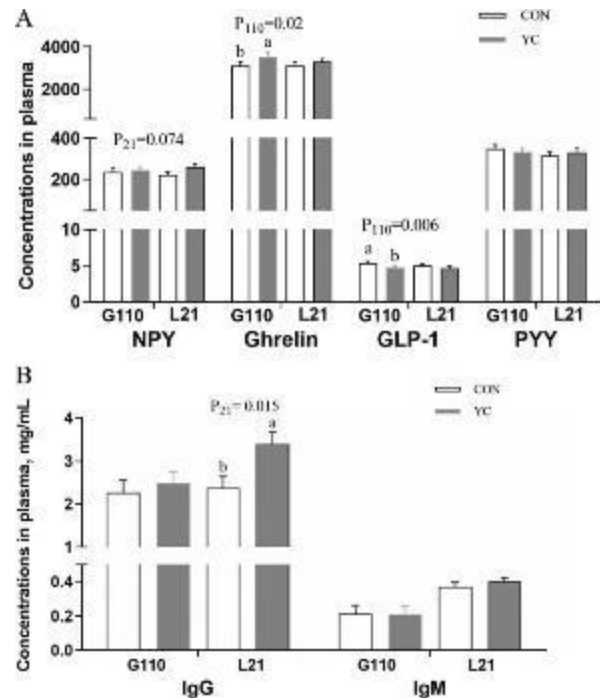


Fig. 2. Concentration of plasma hormones and immunoglobulin in sows. (A) Concentration of plasma NPY (ng/L), Ghrelin (ng/L), GLP-1 (pmol/L) and PYY (pg/ml). (B) Concentration of plasma IgG and IgM. (a-b) Values with different superscripts differ significantly at $P < 0.05$. Abbreviations: CON = control group; YC = yeast culture group; G 110 = day 110 of gestation; L21 = day 21 of lactation; NPY = neuropeptide Y; GLP-1 = glucagon-like peptide-1; PYY = peptide tyrosinetyrosine; IgG = immunoglobulin G; IgM = immunoglobulin M.

culture supplementation are still unclear, this result might be due to improved sow health condition by the positive actions of yeast culture in their intestinal tract. Some slowly fermentable probiotic components such as β -glucan and mannan-oligosaccharides exist in yeast culture, which may not only have the potential to modulate the secretion of GLP-1 (Qin et al., 2021) but could also be associated with enriched beneficial bacteria. The lactation process is generally accompanied by extreme loss of body fat and minor protein catabolism, resulting in a decline in sow BW (Hoving et al., 2012). Excessive loss of BW can increase the weaning-to-oestrus interval and cause reduced ovulation rate and embryonic survival, which will reduce the subsequent reproductive performance of affected sows (Van den Brand et al., 2000; Thaker and Bilkei, 2005; Vinsky et al., 2006). The tendency of decline in backfat loss and weaning-to-oestrus interval in YC sows was closely related to the higher feed intake due to yeast culture supplementation and the results are in agreement with those of Jang et al. (2013). The probiotic components such as β -glucan and mannanoligosaccharides exist in yeast culture, which could reduce the interval from weaning to next oestrus (Pettigrew et al., 2005), but the mechanisms by which yeast culture reduces weaning-to-oestrus interval have not been fully determined, but will be at least in part to better sow health resulting from the positive actions of yeast culture in their gastrointestinal tract.

The improvements in piglet performance in YC sows are similar to results obtained by Kim et al. (2008) and Song et al. (2017). It is well established that yield and composition of milk are major determinants for piglet growth (Lewis et al., 1978), so the higher milk yield in the first week of lactation, as well as improved colostrum, and milk composition in YC sows are likely to be the main reasons for improved individual piglet ADG.

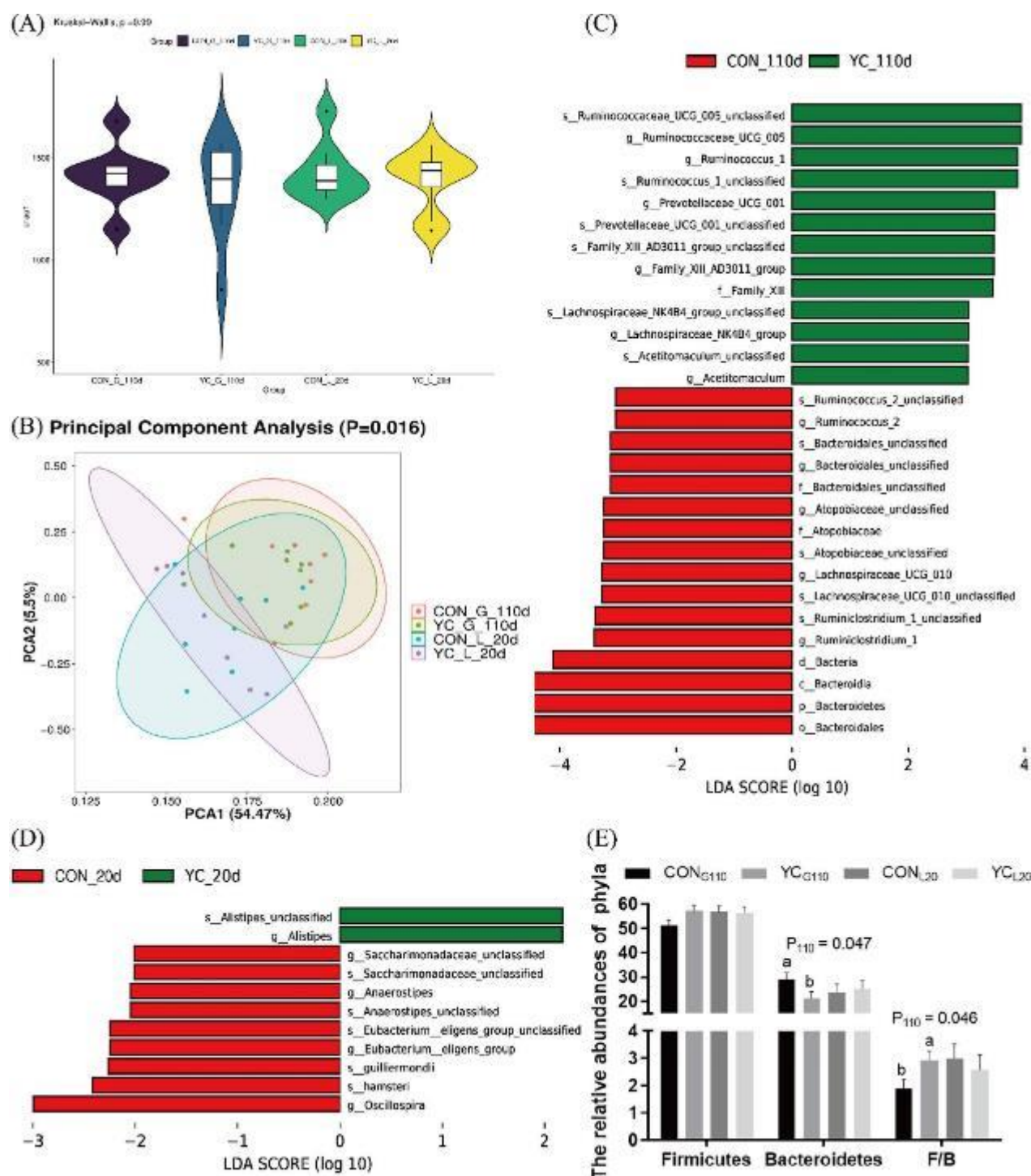


Fig. 3. Effects of yeast culture supplementation from late gestation to lactation on sows' faecal microbial diversity and relative abundance at different taxonomic levels. (A) aDiversity (Shannon index) of the faecal microbial community; (B) b-diversity (Principal Component Analysis) of faecal microbial community; (C) LDA score of significantly differential bacterial taxa on d 110 of gestation; (D) LDA score of significantly differential bacterial taxa on d 20 of lactation; (E) The relative abundance of phyla Firmicutes and Bacteroidetes and Firmicutes/Bacteroidetes ratio (F/B). (a–b) Values with different superscripts differ significantly at $P < 0.05$. Abbreviations: CON = control group; YC = yeast culture group; G 110 = day 110 of gestation; PCA = principal component analysis; L20 = day 20 of lactation; LDA = linear discriminant analysis.

The reduction of specific BCFA concentration detected on d 21 of lactation may be indicative of a lower colonic protein fermentation (Windey et al., 2012; Korpela, 2018) and a higher protein digestion and absorption in the foregut (Shen et al., 2009).

Supplementation with yeast culture to the gestation and lactation diet did elevate sow serum IgG concentration at weaning, suggesting a better maternal

immunological state. Similarly, Czech et al. (2010) reported that serum IgG on d 110 of gestation and IgA on d 21 of lactation were increased when sows consumed yeast cell walls from d 84 of gestation to d 28 of lactation. The improvements in maternal immunological state could be due to probiotic components such as b-glucan and mannan-oligosaccharides that exist in yeast culture, which were not only proven to bind to pathogenic bacteria and flush

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them away from the intestinal lumen (Spring et al., 2000) but also to promote the growth of beneficial bacteria (Davis et al., 2004).

Although yeast culture supplementation showed no effect on faecal bacteria diversity in the present study, a higher Firmicutes/ Bacteroidetes ratio and more abundant beneficial and fermentative bacteria, such as Prevotellaceae_UCG-001, Ruminococcaceae_UCG005, Ruminococcus_1, were observed on d110 of gestation. A high Firmicutes/Bacteroidetes ratio has been directly related with promoting obesity (Ley et al., 2006; Zou et al., 2018) and according to Jumpertz et al. (2011), an increase in Firmicutes and a corresponding decrease in Bacteroidetes was associated with an increased energy harvest. The results indicated some degree of increased energy harvest with yeast culture, which may be associated with increased milk yield and quality in the first week of lactation. Yue et al. (2019) reported that Prevotellaceae_UCG-001 was associated positively with faecal moisture but negatively with gastrointestinal transition time, and related to glycolipid metabolism disorders (Song et al., 2019). Genus Ruminococcaceae_UCG-005 and Ruminococcus_1, both belonging to family Ruminococcaceae, have been demonstrated to participate in degradation of diverse polysaccharides (Shang et al., 2018) with a negative correlation with lipopolysaccharide biosynthesis, and a lower abundance had been reported to lead to inflammatory response (Kang et al., 2017; Shao et al., 2020). All of those mentioned above indicate that yeast culture treatment contributed to improving energy utilisation for milk composition and increased storage in body reserves, as well as improved systemic inflammatory status. Differences of Firmicutes/Bacteroidetes ratio with yeast culture supplementation vanished in lactation and genera Eggerthella and Alistipes were significantly enriched. Decreased body fat content leads to a lower Firmicutes/Bacteroidetes ratio (Turnbaugh et al., 2006; Zou et al., 2018). Lower Firmicutes/Bacteroidetes ratio indicated some degree of increased energy expenditure, as well as a catabolic metabolic state during lactation. Eggerthella and Alistipes can degrade dietary fibres, produce polyphenols and suppress microbial pathogenicity to barrier function (Li et al., 2009; Whisner and Aktipis, 2019).

Conclusion

The present study showed that dietary yeast culture supplementation from late gestation to weaning improved ADG of piglets and weaning-to-oestrus interval, which could be due to improved sow feed intake, milk yield and composition, maternal health status as well as gut microbiota. Saccharomyces cerevisiae yeast culture products may be a promising functional additive to improve the performance of sows and their offspring.

Ethics approval

The animal study protocol was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University under ethic approval number 20 180 082. Animal study was conducted on a commercial pig farm in southwest of China (TIE QI LI SHI, Mian Yang) from October 2018 to January 2019 and was in accordance with the Guidelines on Ethical Treatment of Experimental Animals (2006) No. 398 of the Ministry of Science and Technology of China.

Data and model availability statement

The sequencing data have been uploaded to NCBI, which can be accessed with NO. PRJNA815319.

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Declaration of interest

All authors have read, approved the final manuscript, and declared no competing interests existed.

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Article

Effects of Yeast Culture Supplementation in Wheat–Rice-Based Diet on Growth Performance, Meat Quality, and Gut Microbiota of Growing–Finishing Pigs

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Simple Summary: In recent years, the soaring price of corn–soybean meal has led to an increase in the price of feed, which has brought significant challenges to the pig industry. The addition of more grains to pig diets will help develop new feed resources and improve profits. In this study, we focused on the effects of yeast culture (YC) supplementation on growth performance, meat quality, and gut microbiota of growing–finishing pigs. Three experimental diets were compared: a corn–soybean-based diet (CON), a wheat–rice-based diet (GRA), and a wheat–rice-based diet supplemented with YC. The growth performance of pigs on the wheat–rice-based diet was similar to that of pigs on the corn–soybean-based diet, but the feed cost reduced by 6.7% compared with CON. The dressing percentages of pigs in GRA and YC also increased by 1.43% and 2.15%, respectively. Meat color and antioxidant capacity improved after YC supplementation. YC supplementation promotes the proliferation of probiotics, inhibits the growth of harmful bacteria, and improves intestinal health. Application of a cereal-based diet and YC supplementation in growing pigs appears to be a very promising strategy for cost saving and economic benefits for the swine industry.

Abstract: The aim of this study was to investigate the effects of yeast culture (*Saccharomyces cerevisiae*) supplementation on the growth performance, meat quality, gut health, and microbiota community of growing–finishing pigs. A total of 45 growing–finishing pigs were randomly allocated to three treatments: a corn–soybean-based diet (CON, $n = 15$), a wheat–rice-based diet (GRA, $n = 15$), and GRA supplemented with 500 mg/kg yeast culture (YC, $n = 15$). The results show that compared to the CON group, the GRA group exhibited no significant differences in feed intake, daily gain, or feed conversion ratio, but had significantly reduced feed cost per kilogram BW gain of the finishing pigs ($p < 0.05$). Compared to that of the CON group, the GRA and YC groups showed an increase in the dressing percentage ($p < 0.1$). The meat color redness of the YC group increased ($p < 0.1$), whereas the b^* value at 24 h decreased ($p < 0.1$). Meanwhile, the addition of YC significantly increased total superoxide dismutase activity on day 30 and catalase activity on day 60 ($p < 0.05$), and decreased serum urea nitrogen content on day 60 ($p < 0.05$). Furthermore, YC supplementation increased the gene expression of the duodenal anti-inflammatory factor IL-10 ($p < 0.05$), while it significantly decreased the gene expression of the ileal pro-inflammatory factor IL-8 ($p < 0.05$). The intestinal microbial identification results show that compared to the CON group, the YC group showed an increase in the relative abundances of *Lactobacillus*, *Streptococcus*, and *Clostridium* in the colon, and a decrease in the relative abundances of *Bacteroides*, *Clostridia*, and *Prevotella* in the cecum.

In conclusion, the growth performance of pigs on a wheat–rice-based diet was similar to that of pigs on a corn–soybean-based diet. Supplementation of 0.5% YC in the wheat–rice-based diet could improve the dressing percentage and meat color of growing–finishing pigs, which might be due to

the increase in nitrogen utility and antioxidant capacity, and the improvement of the immune system and changes in microbiota communities.

Keywords: growing–finishing pigs; meat quality; yeast culture

1. Introduction

Yeast and yeast products, as high-quality protein sources, have been shown to improve growth performance, modulate the gut microbiome, and reduce post-weaning diarrhea in weaned piglets [1,2]. However, growing–finishing pigs have mature intestines, and the application of and research into yeast in growing–finishing pigs is less than that in piglets. Recent research has shown that yeast products have a positive effect on meat quality, as an economically important trait. Namted et al. [3] found that autolyzed yeast supplementation in pigs showed an increase in the protein percentage and a^* value (redness) in the meat but reduced feed intake. Active dry yeast (ADY) and yeast cultures (YC) improve beef tenderness, while ADY improves the growth performance and carcass traits of bulls [4]. However, Zhang et al. [5] reported that although growing–finishing pigs fed a brewer's yeast hydrolysate diet showed linear improvement in average daily body gain and feed conversion ratio, there was no effect on the carcass weight, backfat, or lean muscle percentage of pigs. Similarly, yeast culture supplementation in broiler chickens has no effect on meat quality, nutrient utilization, or bacterial count [6]. Possible reasons for these differences are related to the composition and functional properties of the different yeast products.

Wheat, rice, and corn are widely available, and wheat-based and corn-based diets are commonly used in commercial swine farms. The nutrient composition and non-starch polysaccharides of wheat and maize are relatively different. Research has shown that there were no significant differences in growth performance and pork quality in growing–finishing pigs between pigs fed a wheat-based diet and those fed a corn–soybean meal diet [7]. However, there are few studies on the effects of yeast culture supplementation on pig growth and pork quality in wheat–rice-based diets. Furthermore, supplementation with YC in growing–finishing pigs improved intestinal development and morphology and increased the relative abundance of probiotics [8]. Dietary YC supplementation in sows increased the milk lactose, protein and DM content, and changed the fecal microbiota of sows, resulting in improved growth performance of piglets [9,10]. Sun et al. [11] reported that mixed yeast culture supplementation increased body weight gain, digestibility of dry matter, and population of *Lactobacillus* in broilers. These results suggest that yeast and yeast products have important effects on intestinal health and microbial flora. Therefore, this study was conducted to test the hypothesis that YC can improve pork quality by improving intestinal health and microbial composition. Thus, the aim of this study was to determine the effects of corn and wheat–rice-based diet, and YC (*Saccharomyces cerevisiae* (*S. cerevisiae*), CGMCC No. 6120) supplementation on growth performance, antioxidant capacity, intestinal development and flora in growing–finishing pigs fed wheat–ricebased diets.

2. Materials and Methods

The experimental protocol was approved by the Sichuan Agricultural University Animal Care and Use Committee (SAU-ANI-2021-106).

2.1. Animals and Experimental Design

A total of 45 crossbred ((Yorkshire × Landrace) × Yorkshire) pigs (50.75 ± 1.319 kg body weight (BW)) were randomly allotted to one of three groups. The temperature of room was maintained around 23–27 °C. The pen was a slatted concrete floor facility equipped with a feeder and a water nipple to provide feed and water *ad libitum*. Pigs were fed daily at 800, 1400, and 2000 h. Pigs had *ad libitum* access to food and water during 60 days trial. BW was measured every 30 days, and feed intake was recorded daily.

2.2. Diets and Yeast Culture

Saccharomyces cerevisiae yeast culture with moisture 4.87%, CP 18.41%, ash 7.69%, β glucan and mannan oligosaccharides 0.82%, and others 69.03% (Enhelor Biotechnology Company, Beijing, China). The diets were control (CON; corn–soybean meal diets), wheat–rice (unhusked)-based diets (GRA), and GRA + 0.5% YC-supplemented diet (YC). Experimental diets were formulated to meet or exceed the predicted requirements for growing–finishing pigs. All pigs were fed a mash diet according to the three-phase feeding program (Table 1).

Table 1. Ingredient and nutrient values of the experimental diet (%).

Ingredients, %	50–75 kg			75–100 kg			100–110 kg		
	CON	GRA	YC	CON	GRA	YC	CON	GRA	YC
Corn	74.59	10.00	10.00	74.90	0.00	0.00	79.90	0.00	0.00
Wheat	0.00	55.87	55.37	0.00	55.00	54.50	0.00	59.20	58.70
Unhusked rice	0.00	16.40	16.45	0.00	27.50	27.50	0.00	29.03	29.07
Soybean oil	1.01	2.00	2.00	1.00	2.00	2.00	1.00	2.00	2.00
Soybean meal	21.24	12.60	12.55	21.40	12.87	12.87	16.62	7.41	7.37
Yeast culture	0.00	0.00	0.50	0.00	0.00	0.50	0.00	0.00	0.50
L-Lysine HCl	0.30	0.48	0.48	0.12	0.30	0.30	0.10	0.30	0.30
DL-methionine	0.03	0.05	0.05	0.03	0.05	0.05	0.00	0.00	0.00
L-Threonine	0.07	0.17	0.17	0.02	0.10	0.10	0.00	0.10	0.10
L-Tryptophan	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Choline 50%	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Limestone	0.64	0.80	0.80	0.60	0.80	0.80	0.50	0.78	0.78
Dicalcium phosphate	1.18	0.70	0.70	1.00	0.45	0.45	0.95	0.25	0.25
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin premix1	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix1	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Calculated Values									
DE, Mcal/kg	3.37	3.29	3.29	3.38	3.23	3.22	3.39	3.23	3.22
CP, %	15.75	15.77	15.77	15.63	15.62	15.63	13.87	13.86	13.86
Total Lysine	0.98	0.98	0.98	0.85	0.85	0.85	0.72	0.73	0.73
Total methionine	0.28	0.29	0.29	0.28	0.29	0.29	0.23	0.22	0.22
Total Threonine	0.65	0.67	0.67	0.60	0.60	0.60	0.52	0.52	0.53
Total Tryptophan	0.18	0.18	0.18	0.17	0.18	0.18	0.14	0.16	0.16
Ca, %	0.59	0.59	0.59	0.53	0.54	0.54	0.47	0.47	0.47
TP, %	0.55	0.52	0.52	0.52	0.49	0.49	0.49	0.44	0.44

Notes: CON, corn–soybean-based diet; GRA, wheat–rice-based diet; YC, GRA supplemented with yeast culture.

2.3. Sample Collection and Chemical Analyses

Fasting blood samples were collected from the anterior vena cava on days 30 and 60 in disposable culture tubes and centrifuged for 10 min at 3000 rpm at 4 °C. The supernatant was stored at –20 °C. Serum glucose (Glu), albumin (ALB), total protein (TP), and blood urea nitrogen (BUN) levels were detected using an automatic biochemical analyzer (Hitachi 7600-110R, Hitachi Co., Japan). Total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD) and catalase (CAT) activities, and malondialdehyde (MDA) level were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

Six female pigs per group were selected and slaughtered by electronarcosis and exsanguination, based on standard commercial procedures. Carcass weight and tare weight were measured and used to calculate the dressing percentage. Average backfat thickness was calculated as the average of the first rib, the last rib, and the last lumbar

vertebra. Eye muscle area (EMA) was measured using Vernier calipers [12]. Carcass length was measured using dressmaker tape with an accuracy of 1 mm. The heart, liver, spleen, kidney, lung, and abdominal fat were immediately removed, properly cleaned, and individually weighed (g). The organ index = (organ weight in grams)/(live weight, in grams) $\times$ 100.

The longissimus dorsi muscle from the 10th rib was used to determine meat color, pH, marbling score, dripping loss, and cooking loss, according to the method described by Chen [12]. Muscle pH_{45min} and pH_{24h} were calculated using a pH meter (pH-STAR, SFKTechnology, Denmark), which was calibrated with pH 4.6 and pH 7.0 buffers. Meat color (L* lightness, a* redness, and b* yellowness) was recorded 45 min and 24 h after slaughter using a portable chromameter (CR-410, Kinica Minolta Sensing Inc., Minolta, Japan). The marbling score at 24 h was measured using the National Pork Producer Council standards (NPPC; Des Moines, IA, USA).

2.4. Intestinal Morphology

Intestines fixed in 4% paraformaldehyde were prepared based on the conventional paraffin-embedding techniques. In brief, cross-sections of the segments were cut into 5 μ m-thick slices, then stained with hematoxylin and eosin (H&E) (Servicebio Technology Co., Ltd., Wuhan, China). In each sample, 15 intact, well-oriented crypt villus units were chosen. Villus height and crypt depth (CD) were measured using Image-Pro Plus 6.0 (Media Cybernetics Inc., Bethesda, MD, USA).

2.5. Bacterial Community Analysis

To determine the microbial composition, 16S rDNA sequencing was used. Thirty-six fresh content samples of colon and cecum collected from six pigs in each group were used to evaluate the microflora communities. The total DNA was extracted from the samples using the E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions, and the concentration of the total DNA was determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, NC, USA). Extracted DNA samples were sent to Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) for amplicon pyrosequencing using the Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA). The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the 338F and 806R primers (5'-ACTCCTACGGGAGGCAGCAG-3' and 5'-GGACTACHVGGGTWTCTAAT-3', respectively) using an ABI GeneAmp 9700 PCR thermocycler (ABI, CA, USA). Operational taxonomic units (OTUs) with a 97% similarity cutoff were clustered using UPARSE version 7.1 [13]. The taxonomy of the representative sequence of each OTU was analyzed using RDP Classifier version 2.2 [14] against the 16S rRNA database (SILVA v138) with a 70% confidence threshold.

2.6. Real-Time Quantitative PCR

Total RNA was isolated and purified using a total RNA extraction kit (TaKaRa, China). Reverse transcription to synthesize cDNA was performed using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, China). Table 2 contains the probes and primers used for each gene. Fluorescence-based quantitative polymerase chain reaction (PCR) assays were conducted using a 7900HT Real-Time PCR system (Applied Biosystem, USA) with the following cycle profile: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, and 60 °C for 30 s. Each sample was analyzed in triplicate. Relative expression was expressed as the ratio of the target gene to β -actin mRNA according to the 2^{- $\Delta\Delta$ CT} method [15].

Table 2. Primer sequences used in this study.

Gene	Primers	Accession No	Product Length (bp)
<i>IL-10</i>	F: CACTGCTCTATTGCCTGATCTTCC	NM_214041.1	136
	R: AAACCTCTTCACTGGGCCGAAG		
<i>TNF-α</i>	F: TGGCCCCCTTGAGCATCA	NM_214022.1	68
	R: CGGGCTTATCTGAGGTTTGAGA		

<i>IL-1β</i>	F: TACCCTCTCCAGCCAGTCTTCA R: AGGTCCAGGTTTTGGGTGCAG	NM_214055.1	167
<i>IL-6</i>	F: AGGGAAATGTCGAGGCTGTGC R: CCGGCATTTGTGGTGGGGTT	NM_214399.1	112
<i>IL-8</i>	F: AGGACCAGAGCCAGGAAGAGAC R: CACAGAGAGCTGCAGAAAGCAG	NM_213867.1	108
<i>ZO-1</i>	F: CAGCCCCCGTACATGGAGA R: GCGCAGACGGTGTTCATAGTT	XM_021098896.1	114
<i>Occludin</i>	F: ATGCCTCCTCCCTTTTCGGA R: CGCCCGTCGTGTAGTCTGTC	NM_001163647.2	295
<i>Claudin-1</i>	F: GATCGGCTCCA TCGTCAGCA R: CATTGACTGGGGTCATGGGGTC	NM_001244539.1	416
<i>β-actin</i>	F: TCTGGCACACACCTTCT TGATCTGGGTTCATCTTCTCAC	XM_021086047.1 R:	114

2.7. Statistical Analysis

Data were analyzed using the one-way ANOVA procedure of the Statistical Product and Service Solutions (SAS) statistical software (V9.4; SAS Institute Inc., Cary, NC, USA), followed by a generalized linear model (GLM) analysis and Tukey's tests. Values are expressed as mean $\pm$ SEM, with differences being considered statistically significant at $p < 0.05$ and a value of $0.05 \leq p < 0.10$ being indicative of a 'tendency.'

3. Results

3.1. Growth Performance and Cost

The effects of the diet types and YC supplementation on growth performance are shown in Table 3. No differences were observed in BW, average daily gain (ADG), or average daily feed intake (ADFI) among the groups. When the price of wheat was 85–88% of that of corn, the weight gain cost per kilogram of GRA was significantly lower than that of CON during 0–31 days and 0–61 days ($p < 0.05$).

Table 3. Effect of dietary yeast culture supplementation on growth performance of finishing pigs.

Item	CON	GRA	YC	<i>p</i> Value
BW (kg)				
0 d	45.61 $\pm$ 0.98	45.76 $\pm$ 1.02	46.82 $\pm$ 0.91	0.642
30 d	79.04 $\pm$ 1.06	79.01 $\pm$ 1.31	80.21 $\pm$ 1.56	0.771
60 d	111.55 $\pm$ 0.70	111.40 $\pm$ 2.42	112.46 $\pm$ 2.36	0.921
ADG (g)				
0–30 d, g	1078.28 $\pm$ 20.56	1072.69 $\pm$ 14.73	1076.99 $\pm$ 22.69	0.978
31–60 d, g	1083.78 $\pm$ 31.27	1079.56 $\pm$ 38.19	1075.11 $\pm$ 38.19	0.987
0–60 d, g	1080.98 $\pm$ 18.56	1076.07 $\pm$ 24.19	1076.07 $\pm$ 26.13	0.985
ADFI (g)				
0–30 d	2960.69 $\pm$ 47.00	2860.75 $\pm$ 84.15	2926.50 $\pm$ 77.37	0.615
31–60 d	3340.67 $\pm$ 137.53	3389.89 $\pm$ 173.17	3269.53 $\pm$ 155.70	0.862
0–60 d	3147.57 $\pm$ 89.82	3120.98 $\pm$ 117.20	3095.20 $\pm$ 113.70	0.943
F:G ratio				
0–30 d	2.75 $\pm$ 0.05	2.73 $\pm$ 0.02	2.72 $\pm$ 0.03	0.851
31–60 d	3.09 $\pm$ 0.12	3.14 $\pm$ 0.10	3.04 $\pm$ 0.10	0.828
0–60 d	2.91 $\pm$ 0.06	2.90 $\pm$ 0.08	2.88 $\pm$ 0.05	0.928
Cost/kg of gain (US\$)				
0–30 d	1.52 $\pm$ 0.03 _a	1.42 $\pm$ 0.01 _b	1.45 $\pm$ 0.02 _{ab}	0.040
31–60 d	1.78 $\pm$ 0.07	1.66 $\pm$ 0.05	1.55 $\pm$ 0.05	0.130

0–60 d	1.65 ± 0.07 ^A	1.54 ± 0.06 ^B	1.58 ± 0.06 ^{AB}	0.080
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Notes: ADG, average daily gain; ADFI, average daily feed intake; F:G, feed-to-gain ratio. Results are presented as the mean ± SEM. Small letters within the same line indicate $p < 0.05$ and capital letters indicate $0.05 \leq p < 0.10$.

3.2. Carcass Characteristics and Organ Development

Compared to that of the CON group, the YC group tended to exhibit an increase in the dressing percentage by 2.9% (Table 4, $p < 0.1$), while there was no significant difference between the GRA and the YC groups ($p > 0.05$). YC supplementation of the GRA decreased the kidney index ($p < 0.05$) and had no effect on the development of other organs ($p > 0.05$).

Table 4. Effect of yeast culture supplementation on carcass characteristics and organ development of finishing pigs.

Item	CON	GRA	YC	<i>p</i> Value
Eye muscle area, cm ²	53.63 ± 2.66	54.39 ± 2.61	49.32 ± 1.73	0.301
Backfat thickness, mm	17.03 ± 0.47	18.96 ± 1.89	19.07 ± 1.38	0.627
Dressing percentage, %	74.15 ± 0.23 ^A	75.58 ± 0.52 ^{AB}	76.30 ± 0.31 ^B	0.060
Carcass length/cm	83.70 ± 1.93	84.36 ± 0.86	85.68 ± 0.63	0.612
Abdominal fat index, %	1.05 ± 0.07	1.06 ± 0.09	1.15 ± 0.15	0.789
Heart index, %	0.36 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	0.474
Liver index, %	1.46 ± 0.04	1.45 ± 0.05	1.43 ± 0.03	0.878
Spleen index, %	0.14 ± 0.01	0.13 ± 0.00	0.15 ± 0.01	0.370
Lung index, %	0.65 ± 0.07	0.62 ± 0.03	0.62 ± 0.07	0.912
Kidney index, %	0.31 ± 0.01 ^a	0.28 ± 0.01 ^{ab}	0.27 ± 0.01 ^b	0.011

Notes: Results are presented as the mean ± SEM (n = 6). Small letters within the same line indicate $p < 0.05$ and capital letters indicate $0.05 \leq p < 0.10$.

Abdominal fat index (%) = Abdominal fat weight/live weight × 100.

Heart index (%) = Heart weight/live weight × 100.

Liver index (%) = Liver weight/live weight × 100.

Spleen index (%) = Spleen weight/live weight × 100.

Lung index (%) = Lung weight/live weight × 100.

Kidney index (%) = Kidney weight/live weight × 100.

3.3. Meat Quality

As shown in Table 5, diet type and YC supplementation had no effect on meat quality, except for the Hunter values. Compared to CON, YC improved the meat quality of finishing pigs, increased the muscle a* value, and decreased the muscle b* value at 24 h ($p < 0.10$).

pH	6.34 ± 0.09	6.08 ± 0.16	6.16 ± 0.18	0.475
pH _{24h}	5.43 ± 0.03	5.42 ± 0.03	5.41 ± 0.02	0.888
pH _{48h}	5.45 ± 0.02	5.42 ± 0.03	5.44 ± 0.02	0.727
Drip loss, %	2.33 ± 0.41	2.95 ± 0.30	3.05 ± 0.24	0.303
Cooking loss, %	34.87 ± 1.07	34.49 ± 0.82	34.67 ± 1.00	0.965
Marbling score	3.75 ± 0.22	3.25 ± 0.31	3.20 ± 0.15	0.189
45 min				
L*(lightness)	42.19 ± 0.47 ^A	44.80 ± 0.50 ^B	44.35 ± 0.37 ^B	0.063
a* (redness)	8.27 ± 0.40	7.66 ± 0.39	7.39 ± 0.40	0.330
b* (yellowness)	6.77 ± 0.33	7.29 ± 0.19	7.18 ± 0.12	0.421
24 h				

L*(lightness)	51.41 ± 0.37	52.65 ± 0.30	52.06 ± 0.25	0.129
a* (redness)	11.88 ± 0.57 _A	12.54 ± 0.31 _{AB}	12.82 ± 0.26 _B	0.067
b* (yellowness)	9.53 ± 0.15 _B	9.96 ± 0.12 _B	8.74 ± 0.27 _A	0.052
Items	CON	GRA	YC	p Value

45min

Table 5. Effect of yeast culture supplementation on meat quality of finishing pigs.

Notes: Results are presented as the mean ± SEM (*n* = 6). Capital letters within the same line indicate 0.05 ≤ *p* < 0.10.

3.4. Blood Metabolites

As shown in Table 6, diet type and YC supplementation had no significant effects on the serum parameters on day 30. However, compared to CON, dietary YC supplementation significantly decreased TP, ALB, and urea nitrogen levels on day 60 (*p* < 0.05).

Table 6. Effect of dietary yeast culture supplementation on blood metabolites of growing–finishing pigs.

Item	CON	GRA	YC	p Value
D 30				
Total protein, g/L	70.43 ± 3.45	68.33 ± 5.46	68.45 ± 3.63	0.927
Albumin, g/L	38.58 ± 1.81	35.90 ± 2.17	37.55 ± 0.73	0.539
GLU, mmol/L	4.23 ± 0.39	3.78 ± 0.35	5.13 ± 0.62	0.151
UN, mmol/L	6.95 ± 0.78	5.95 ± 0.69	6.70 ± 0.54	0.693
D 60 Total				
protein, g/L	67.87 ± 3.02 _a	61.35 ± 2.81 _{ab}	55.00 ± 1.66 _b	0.048
Albumin, g/L	39.25 ± 2.47 _a	32.67 ± 2.23 _{ab}	28.28 ± 2.61 _b	0.041
GLU, mmol /L	4.25 ± 0.51 _B	3.14 ± 0.26 _A	3.04 ± 0.36 _A	0.085
UN, mmol L	7.92 ± 0.73 _a	6.35 ± 0.73 _{ab}	4.84 ± 0.57 _b	0.038

Notes: GLU, glucose; UN, urea nitrogen. Results are presented as the mean ± SEM (*n* = 6). Small letters within the same line indicate *p* < 0.05 and capital letters indicate 0.05 ≤ *p* < 0.10.

3.5. Serum Antioxidant Capacity

As shown in Table 7, compared to the corn–soybean meal diet, the wheat–rice-based diet increased the T-SOD activity on day 30 in serum, while dietary YC supplementation not only increased CAT activity in serum, but also decreased the MDA level in serum (*p* < 0.05).

Table 7. Effect of dietary yeast culture supplementation on antioxidant capacity of finishing pigs.

Item	CON	GRA	YC	p Value
D 30				
CAT, U/mL	1.93 ± 0.29	1.68 ± 0.27	1.48 ± 0.28	0.557
T-AOC, U/mL	4.70 ± 0.29	5.05 ± 0.43	4.57 ± 0.37	0.643
T-SOD, U/mL	73.69 ± 1.52 _a	80.13 ± 1.86 _b	82.13 ± 0.49 _b	0.002
MDA, nmol/mL	3.08 ± 0.27	2.92 ± 0.19	3.02 ± 0.43	0.936
D 60				
CAT, U/mL	± 0.16 _a	± 0.43 _a	3.50 ± 0.36 _b	0.048
T-AOC, U/mL	4.68 ± 0.28	4.53 ± 0.23	4.04 ± 0.34	0.279
T-SOD, U/mL	82.79 ± 1.47	82.69 ± 1.99	79.13 ± 1.50	0.243
MDA, nmol/mL	2.50 ± 0.21 _A	2.74 ± 0.17 _{AB}	2.26 ± 0.19 _B	0.050

Notes: T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; CAT, catalase; MDA, malonaldehyde. Results are presented as the mean ± SEM (*n* = 6). Small letters within the same line indicate *p* < 0.05 and capital letters indicate 0.05 ≤ *p* < 0.10.

3.6. Intestinal Morphology

Intestinal morphology results are presented in Figure 1 and Table 8. Compared to that of the CON group, the ratio of the villous height to crypt depth of jejunum in the GRA and YC groups increased by 15% and 23%, respectively, although there was no significant difference ($p > 0.05$). However, the VH and CD were not significantly altered in the ileum ($p > 0.05$).

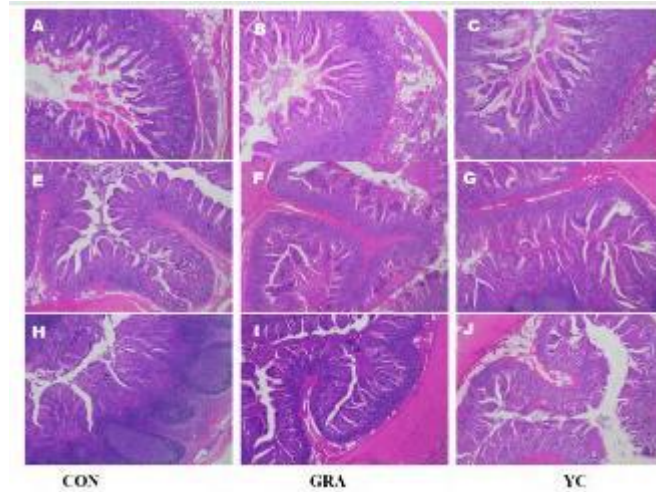


Figure 1. Effect of yeast culture supplementation on intestinal histology of finishing pigs. (A–C) Duodenum; (E–G) jejunum; (H–J) ileum. Figure presented at 200-fold field of vision, $n = 6$ per group.

Table 8. Effect of dietary yeast culture supplementation on intestinal morphology of finishing pigs.

Items	CON	GRA	YC	<i>p</i> Value
Jejunum VH,				
μm	359.65 ± 17.10	346.78 ± 19.98	351.95 ± 24.60	0.908
CD, μm	108.94 ± 6.13	104.44 ± 10.54	91.32 ± 4.32	0.286
VH/CD	2.98 ± 0.24	3.42 ± 0.34	3.68 ± 0.05	0.104
Ileum VH,				
μm	370.16 ± 10.16	377.86 ± 13.79	330.29 ± 26.14	0.178
CD, μm	115.70 ± 7.71	117.15 ± 5.65	105.15 ± 8.84	0.489
VH/CD	3.26 ± 0.22	3.27 ± 0.25	3.24 ± 0.40	0.997

Notes: VH, villous height; CD, crypt depth. Results are presented as the mean $\pm$ SEM ($n = 6$).

3.7. Gene Expression

As shown in Figure 2, compared to that of the CON group, the expression of IL-1 β in the duodenum was downregulated in the YC group ($p < 0.01$). Moreover, the expression of IL-8 and IL-10 was significantly upregulated in the GRA and YC groups ($p < 0.05$), and the relative expression of IL-10 mRNA in the YC group tended to increase ($p < 0.05$). However, there was no effect on the expression of IL-6, TNF- α , occludin, claudin, or zonula occludens-1 (ZO-1) among the groups ($p > 0.05$).

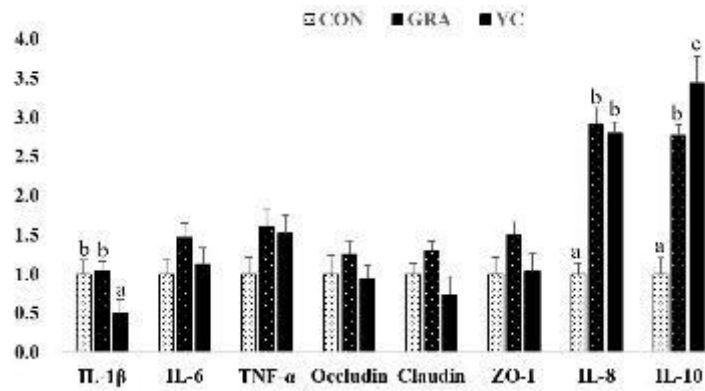


Figure 2. Effect of yeast culture supplementation on the mRNA levels of immunity-related genes in duodena of finishing pigs. IL-10, interleukin 10; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin1 β ; IL-6, interleukin-6; IL-8, interleukin-8; ZO-1, zonula occludens-1. Values are means and SEMs, $n = 6$ per group.

As shown in Figure 3, compared to that of the CON group, the expression of IL-10 in the jejunum was upregulated in the GRA and YC groups ($p < 0.05$). Furthermore, the expression of IL-10 in the YC group was higher than that in the GRA group, whereas the expression of IL-1 β was significantly downregulated in the YC group ($p < 0.05$).

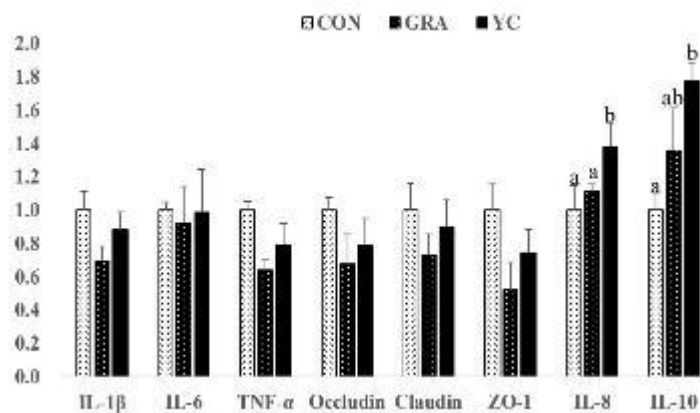


Figure 3. Effect of yeast culture supplementation on the mRNA levels of immunity-related genes in jejunum of finishing pigs. IL-10, interleukin 10; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-8, interleukin-8; ZO-1, zonula occludens-1. Values are means and SEMs, $n = 6$ per group.

As shown in Figure 4, compared to that of the CON group, the expression of IL-1 β in the ileum was downregulated in the GRA group ($p < 0.05$), and the expression of IL-8 was also significantly downregulated in the GRA and YC groups ($p < 0.05$). The relative mRNA expression of claudin and IL-10 in the YC group was significantly upregulated ($p < 0.05$).

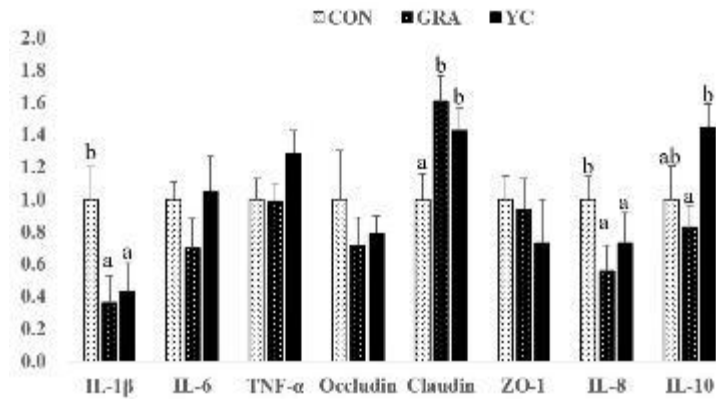


Figure 4. Effect of yeast culture supplementation on the mRNA levels of immunity-related genes in ilea of finishing pigs. IL-10, interleukin 10; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL6, interleukin-6; IL-8, interleukin-8; ZO-1, zonula occludens-1. Values are means and SEMs, n = 6 per group.

3.8. Overview of 16S rRNA Sequencing Data

To explore the effect of dietary yeast culture on gut microbiota, chyme samples were collected for 16S rRNA sequencing. In total, 1423816 sequences and 594334820 bases were obtained. The average length of the sequence was 417 bp.

Furthermore, the microbial composition of the colonic chyme was analyzed to observe alterations in the bacterial community composition. In brief, at the phylum level (Figure 5A), the dominant phyla were Firmicutes and Bacteroidetes, and the abundance of Firmicutes in the GRA (83.98%) and YC (84.33%) groups was higher than that in the CON group (72.11%). At the order level (Figure 5B), the abundance of Lactobacillales in the GRA (50.51%) and YC groups (41.88%) was higher than that in the CON group (24.44%) ($p < 0.05$), whereas the abundance of Bacteroidetes (22.20%) in the CON group was higher than that in the GRA (11.51%) and YC (11.60%) ($p < 0.01$) groups. At the family level (Figure 5C), the abundances of *Prevotellaceae*, *Selenomonadaceae*, *Christensenellaceae*, *Butyrivibrionaceae*, and *UCG-010* in the GRA and YC groups were higher than those in the CON group ($p < 0.05$). At the genus level (Figure 5D), the abundance of *Prevotella* was higher in the CON and GRA groups than in the YC group ($p < 0.05$). Moreover, the abundances of *Prevotellaceae_NK3B31_group*, *Christensenellaceae_R-7_group*, *Anaerovibrio*, and *norank_f_UCG-010* were higher in the CON group than in the GRA and YC groups.

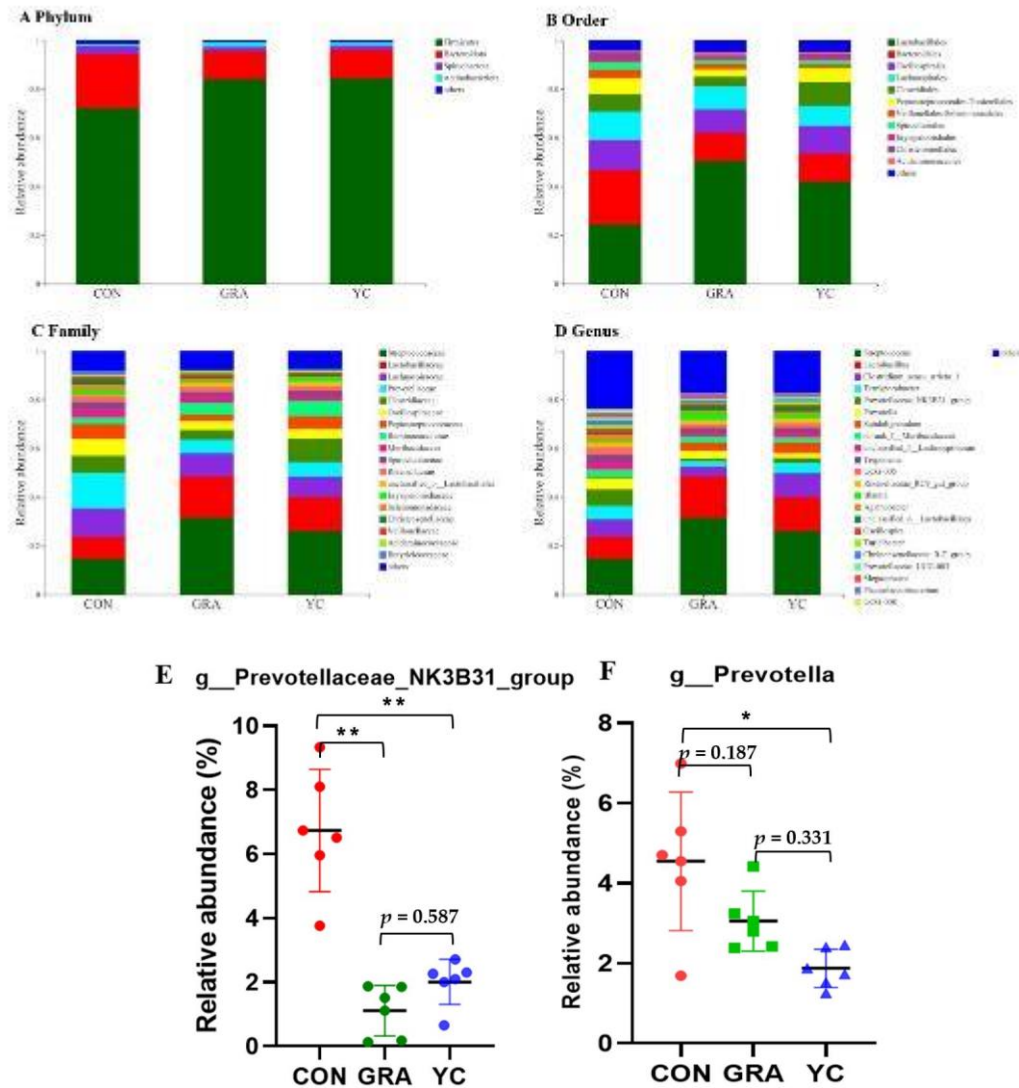


Figure 5. Effects of yeast culture on colon chyme microbial composition. (A) The relative amounts of chyme microbiota at the phylum level. (B) The relative amounts of chyme microbiota at the order level. (C) The relative amounts of chyme microbiota at the family level. (D–F) The relative amounts of chyme microbiota at the genus level. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$.

Dietary YC supplementation changed the microbial composition of cecal content. Figure 6A showed that Firmicutes and Bacteroidetes were the dominant phyla in all groups, and the abundance of Firmicutes in the GRA (76.99%) and YC (80.75%) groups was higher than in the CON group (71.11%). At the order level (Figure 6B), the abundance of RF39 in the CON and YC groups was higher than in the GRA group ($p < 0.05$), whereas the abundance of Peptococcales in the GRA group was higher than in the CON group ($p < 0.05$). At the family level (Figure 6C), the abundances of *Selenomonadaceae* and *Butyrivibrionaceae* in the CON group were higher than in the YC group ($p < 0.05$). The abundance of *norank_o_Coriobacteriales* in the YC group was higher than in the CON group ($p < 0.05$), whereas the abundances of *norank_o_RF39* in the CON and YC groups were higher than in the GRA group ($p < 0.05$). At the genus level (Figure 6D), the abundances of *norank_f_norank_o_Coriobacteriales* in the YC group were higher than in the CON group ($p < 0.05$), whereas the abundances of *Blautia* and *Peptococcus* in the GRA group were higher than in the CON group ($p < 0.05$), and the abundances of *Prevotellaceae_NK3B31_group*, *Turicibacter*, and *Lachnospira* in the CON group were higher than in the GRA group ($p < 0.05$).

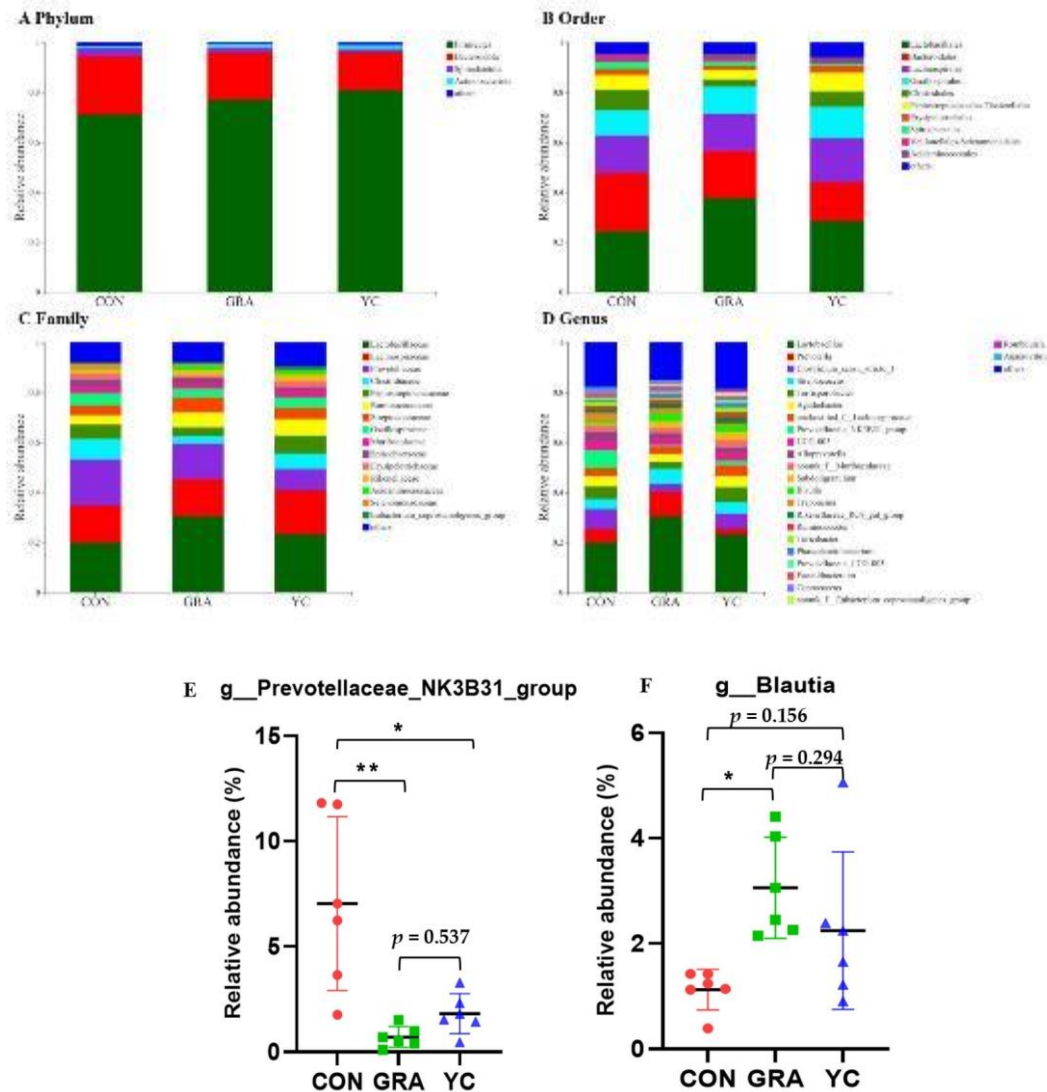


Figure 6. Effects of yeast culture on cecum chyme microbial composition. (A) The relative amounts of chyme microbiota at the phylum level. (B) The relative amounts of chyme microbiota at the order level. (C) The relative amounts of chyme microbiota at the family level. (D–F) The relative amounts of chyme microbiota at the genus level. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$.

4. Discussion

These findings are useful for the rational utilization of feed resources in the swine industry. There are significant differences in nutritional composition between wheat–rice-based and corn-based diets. In our study, we found that the type of diet and YC supplementation had no significant effect on the growth performance of growing pigs in the isoenergetic and isonitrogen models. A previous study reported that diet type did not influence ADG, but growing pigs fed corn–soybean meal diets from days 0 to 64 had decreased ADFI and increased G:F compared to the pigs fed 30% dried distillers grains with solubles and 19% wheat middling diets during this period [16]. The apparent ileal digestibilities of dry matter, neutral detergent fiber, and gross energy (GE) of growing pigs fed a corn-based diet were similar to those of pigs fed a wheat-based diet [17]. Weaned pigs that were fed maize-based diets had a 60 g higher ADG than pigs that were fed wheat–barley-based diets, even though the daily feed intake was similar because of higher N retention [18]. Corn-based and wheat-based diets had no effect on ADG, ADFI, or final BW over the entire 4-week study period or final BW in weaned piglets [19]. Navarro et al. [20] found that the apparent total tract digestibility of GE in growing pigs was higher in pigs fed wheat-based diets than in pigs on DDGS diets, but was similar to that of the pigs fed corn-based diets. Dietary administration of 3 g/kg yeast hydrolysate improved the growth

performance of growing–finishing pigs [21]. Although supplementation with YC had no effect on growth performance, it resulted in better intestinal development and morphology. Mayorga et al. [22] reported that dietary live yeast did not affect growth performance; however, it tended to improve G:F. Furthermore, when the price of wheat and rice was 13% lower than that of corn, the weight gain cost per kilogram was reduced by 6–7%.

Different carbohydrate sources in the diet may have different effects on meat quality because of the differences in starch composition and structure. Interestingly, the dressing percentage of pigs fed GRA and YC increased by 1.93% and 2.90%, respectively, compared to that of the CON group. This may be related to an improvement in nitrogen utilization. In this study, we found that dietary YC supplementation significantly decreased the TP, ALB, and urea nitrogen levels on day 60. Meanwhile, Namted et al. [3] reported that BUN decreased with the addition of 0.5% autolyzed yeast. Pigs fed diets containing mycotoxins and yeast cell wall extract showed increased apparent ileal digestibility of crude protein, and the GE of nursery piglets [23]. Dietary live yeast supplementation during gestation and lactation, and during the nursery phase, increased nutrient digestibility in the offspring [24]. Similarly, yeast hydrolysate supplementation in broiler chickens increased the eviscerated yield rate and chest muscle yield [25]. In addition to the role of yeast, diet type may also affect the dressing percentage. Although there are few studies on the effects of dietary types on pork carcass quality, Li et al. [26] reported that compared to a waxy maize starch diet, a pea starch diet increased the loin eye area and pH_{45min} value of finishing pigs. Ran et al. [27] reported that lambs fed a sorghum-based diet had lower dressing percentage and meat quality than those fed a corn-based diet. This implies that the different effects of carbohydrate sources on pork quality may be related to starch structure and composition. More in-depth and detailed research is required in the future.

Furthermore, the GRA-based diet and YC tended to improve the meat quality of finishing pigs. Namted et al. [3] reported that 0.5% autolyzed yeast in the diet of pigs increased their springiness and tended to increase the redness and pH of the meat at 6 h postmortem. The improvement in beef tenderness following active dry yeast supplementation of finishing bulls is related to an increase in the level of CAT in the serum and glutathione reductase in the meat [4]. Furthermore, dietary live yeast supplementation in broilers alleviates transport-stress-impaired meat quality [8]. Antioxidant capacity and inflammatory responses have an important impact on meat quality. In this study, the T-SOD activities in the GRA and YC groups were higher than that in the CON group, while dietary YC supplementation increased antioxidant capacity in pigs. Yeast hydrolysate supplementation in growing–finishing pigs tended to increase T-AOC activities and enhanced serum IL-10 levels [21]. Yeast hydrolysate increased SOD and GSH-Px activities in the serum and meat quality of broiler chickens [25], and enhanced the antioxidant status of juvenile Nile tilapia [28].

The intestine plays an important role in immunity function. In this study, YC supplementation changed the expression levels of tight-junction-related genes, improving intestinal barrier and immunity. Fu et al. [21] showed that supplementing weaned piglets with yeast hydrolysate upregulated the mRNA expression of ZO-1. The supplementation of yeast culture increased the mRNA levels of ZO-1, claudin, and occludin in juvenile largemouth bass [29]. Furthermore, a wheat–rice-based diet upregulated IL-8 and IL-10 gene expression in the intestine, while YC supplementation decreased the expression of the pro-inflammatory cytokine IL-1 β and upregulated the expression of the anti-inflammatory cytokines IL-8 and IL-10 in the duodenum, jejunum, and claudin of the ileum, respectively, which implies that dietary type and YC have positive effects on the intestinal development and immunity. In fact, research has shown that dietary superfine yeast powder supplementation increased lysozyme levels at day 7 and jejunum mucosal sIgA secretion, while dietary live yeast supplementation increased jejunum mucosal sIgA secretion in piglets [30]. Broiler chickens fed a live yeast culture diet show decreased serum TNF- α , IL-1 β , and IL-6 levels [31]. Oral treatment with yeast β -glucan in non-obese diabetic mice has positive changes to immune functions by increasing IL-10, IL-17, and IL-21 levels and decreasing TNF- α level, although levels of some pro-inflammatory cytokines (IL-1b and IFN- γ) were increased [32]. Meanwhile, β -glucan from *S. cerevisiae* ameliorated inflammation in a DSS-induced mouse model of colitis by regulating the levels of

cytokines (IL-1 β , IL-6, TNF- α , and IFN- γ), increasing the expression levels of tight junction proteins [33], implying that yeast culture has favorable immunomodulatory activity. Gut microbial communities play a considerable role in metabolism and immunity [34]. However, there is minimal research on gut microbes and meat quality. Research has shown that an 80% feed restriction in bearded chickens could improve the meat quality and flavor by changing the lipid metabolism and the structure of the cecal microbial community [35]. Black pepper extract supplementation in finishing pigs linearly increased fecal *Lactobacillus* counts and backfat thickness at week 10 [36]. Zhu et al. [37] found that there is a significant positive correlation between the abundance of the ileal genera *Streptococcus* and L* value at 24 and 48 h, and a significant negative correlation between the abundance of unidentified *Ruminococcaceae* in both the ileum and colon and L* value at 24 h. Furthermore, the gut microbiota of obese Jinhua pigs intrinsically promotes intramuscular fat accumulation and can be transferred to mouse recipients by transplanting fecal microbiota [38]. The aforementioned research shows that the microbial composition has a significant impact on pork quality. In this study, we found that yeast culture supplementation tended to increase the relative abundance of probiotics, such as *Lactobacillus*, *Streptococcus*, and *Clostridium*, decreasing the relative abundance of pathogenic microorganisms, such as *Bacteroides* and *Prevotella* in the cecum, thereby increasing the meat quality. In fact, in vitro research has shown that a live yeast probiotic (*S. cerevisiae*) can improve microflora composition in a colonic simulation model of pigs [39]. *Saccharomyces cerevisiae* yeast-supplemented horses showed increased abundances of *Lactobacilli* and lactic acid utilizers in the cecum but remained similar in the colon, and the abundance of *Lactobacilli* for the high-starch diet was higher than that in the for high-fiber diet in the cecum and colon [40]. Furthermore, hydrolyzed yeast supplementation in weaned piglets increased the *Lactobacillus* spp. count and acetate and propionate production [1]. The supplementation with 1.0% brewer's yeast hydrolysate can improve ADFI and G:F, which may be related to the fecal *Escherichia* microbial counts and diarrhea in weanling pigs [41]. Supplementation with the live yeast *S. cerevisiae* significantly affected microbial diversity in cecal contents and short-chain fatty-acid-producing bacteria in suckling piglets [42]. The supplementation with yeast culture increased the abundance of probiotics (such as *Lactobacillus*, *Bacillus*, and *Bifidobacterium*) and decreased the abundance of intestinal potential pathogenic bacteria (*Plesiomonas*) in juvenile largemouth bass [29]. Supplementation with live yeast cultures increased the abundance of the phyla Firmicutes and genera *Lactobacillus*, *Prevotella*, and *Enterococcus* in broiler chickens [31]. In addition, studies on humans and pigs have shown that diet type significantly affects the intestinal microflora. Lower vegetable intake mediates differences in the abundances of *Lachnospira* and *Ruminococcus-1*, and higher red meat intake mediates differences in the abundances of *Lachnospira* and *Ruminococcus-1* [43]. Pea starch supplementation in growing barrows increased the relative abundance of amylolytic bacteria, such as *Lactobacillus* spp. and *Streptococcus* spp., and decreased the relative abundance of some inflammatory bacteria, such as *Tyzzzeria*, *Porphyromonas*, and *Tannerella* in the cecum of pigs. Thus, a high ratio of amylose to amylopectin in diets may be beneficial for the intestinal health of pigs [44].

5. Conclusions

Compared with the corn-soybean meal diet, the wheat-rice diet had a similar effect on the growth performance of growing pigs. If the price of wheat-rice is 90% lower than of the price of corn, the application of wheat-rice-type diet will greatly save the feed cost. Meanwhile, yeast culture supplementation in wheat-rice-type diet positively increased the dressing percentage and meat color by increasing nitrogen utility, antioxidant capacity, and modulating the immune system and microbiota communities of growing-finishing pigs.

Author Contributions: D.W. and Y.L. conceived and designed this study. Y.L., C.Y., Z.M., L.C., Z.F. and S.X. performed the experiments and analyzed the data. B.F., J.L., C.Y., Y.Z. and M.Y. lab analysis. Y.L. and J.Z. writing the draft. Y.L. and P.C. funded this study. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data for 16S rRNA gene sequencing and RNA-seq are available in the National Center for Biotechnology Information under the accession numbers PRJNA814434. Data available on reasonable request from the corresponding author. **Conflicts of Interest:** The authors declare no conflict of interest.

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Article

Effect of Yeast Cultures on Reproductive Performance, Gut Microbiota, and Milk Composition in Primiparous Sows

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Simple Summary: Compared with multiparous sows, primiparous sows have a poor reproductive performance and insufficient nutritional reserves, resulting in a decreased growth performance in their offspring, which affects the efficiency of pig production. Yeast culture (YC) is rich in nutrients, and the supplementation of yeast culture in gestation and lactation diets is beneficial to improving the nutritional level of primiparous sows. Therefore, the objective of this experiment was mainly to investigate the effects of supplementing 0.5% and 0.8% yeast cultures into diets during gestation and lactation on the reproductive performance and intestinal health of primiparous sows, and the nutrient content of their colostrum and milk. The results showed that the dietary yeast cultures improved reproductive performance, improved the intestinal environment and energy metabolism, and increased the nutrient content of colostrum from primiparous sows. This study provides a theoretical basis for improving the performance of primiparous sows, and also provides a reference for the application of yeast cultures in sow production.

Abstract: The objective of this study was to evaluate the effects of yeast culture (YC) on the reproductive performance, gut microbiota, and milk composition in primiparous sows. A total of 60 primiparous sows were randomly assigned to control (CON) treatment and YC treatment (0.5% YC during gestation and 0.8% YC during lactation) groups consisting of 30 replicates, with one sow in each. The results showed that dietary YC supplementation increased the piglet birth weight and backfat thickness at 28 d of lactation ($p < 0.05$). Dietary YC supplementation increased the apparent total tract digestibility (ATTD) of gross energy and calcium during lactation, the content

4. Introduction

The increasing demand for pig production requires modern sows to have a higher reproductive performance and nutritional status [1]. However, the widespread use of

of acetic acid and propionic acid at 110 d of gestation, and the content of acetic acid and butyric acid at 28 d of lactation in feces ($p < 0.05$). Furthermore, dietary YC supplementation decreased the abundance of Firmicutes, *Lachnospiraceae_XPB1014_group*, and *Terrisporobacter* ($p < 0.05$), and increased the

abundance of *Prevotellaceae_NK3B31_group* and *Rikenellaceae_RC9_gut_group* ($p < 0.05$). Compared to the control group, dietary YC supplementation increased the fat and lactose content of the colostrum ($p < 0.05$). Metabolomics analysis showed that YC increased 26 different metabolites in the colostrum. Among them were mainly pantothenic acid, proline, isoleucine, phenylalanine, acylcarnitine, and other metabolites. In conclusion, these results suggested that dietary YC supplementation improves reproductive performance and gut health and increases the nutrient content in the colostrum of primiparous sows.

Keywords: primiparous sows; prebiotics; reproductive efficiency; gut microbiota; milk

lean-type pig breeds has led to a negative nutritional balance in sows before and after production, affecting their health status. Compared to multiparous sows, primiparous sows have a lower feed intake during gestation and lactation, and need to mobilize more body reserves to feed piglets [2]. The nutritional intake of primiparous sows during lactation cannot meet the needs of lactation, growth, and development, which will lead to the body being in a catabolic state, and affect the reproductive life, and the growth performance of piglets [1,3]. Previous studies have shown that the loss of body reserves during lactation in primiparous sows affects reproductive performance and piglet production performance [4,5]. In addition, due to the high replacement rate of sows and the large proportion of primiparous sows, an insufficient nutritional intake of primiparous sows will cause significant economic losses to the pig industry [6]. Therefore, it is necessary to increase the nutritional intake of primiparous sows through nutritional regulation to improve the production performance of primiparous sows and the growth performance of the next generation of piglets.

Yeast culture (YC), as a prebiotic, consists of a small number of residual yeast cells, yeast metabolites, cell-wall components, and part of the culture medium [7]. YC contains rich nutrients, including oligosaccharides, proteins, vitamins and other nutrients, as well as unknown “growth factors”, which are beneficial in improving the nutrition level and maintaining the health of animals [8]. In studies in ruminants, YC has been used to improve milk production in dairy cows [9], as well as beef tenderness [10]. With the continuous research on the function of YC, there is an increasing interest in the effects of adding YC to pig diets, especially for sows. Zhao et al. [11] found that the average daily feed intake (ADFI), the milk yield in the first week of lactation, and the dry matter content in the milk of lactating sows were significantly increased through adding YC to the diets of sows in late gestation and lactation. Kim et al. [3] also reported that the supplementation of YC into the diet during gestation and lactation improved the performance of sows. Although numerous studies have been conducted on the role of YC in ruminants and multiparous sows, there are few reports on the effects of YC on primiparous sows. Therefore, this experiment aimed to supplement YC into the diet of primiparous sows during gestation and lactation, to investigate the effects of YC on the reproductive performance, gut microbiota, and milk composition of primiparous sows.

2. Materials and Methods

2.1. Experiment Design

A total of 60 primiparous sows with a similar backfat thickness (14.26 ± 0.22 mm) at 30 d of gestation from Heilongjiang Great Northeast Animal Husbandry Co., Ltd. (Harbin, China) were used in this experiment. All sows were randomly divided into a

control (CON) group (basal diet) and YC group (basal diet with 0.5% YC during gestation and 0.8% YC during lactation) with 30 replicates in each. The YC is a fermentation product of *Saccharomyces cerevisiae* with a crude protein content $\geq 15\%$ and a mannan content $\geq 1\%$. The basal diets (Table 1) were formulated to meet or exceed the nutritional requirements of gestating and lactating sows, as recommended by the National Research Council (NRC 2012) [12]. The diet was fed twice a day (8:00 a.m. and 2:00 p.m.) at 2.5 kg from day 30 to day 86 of gestation, and at 3 kg from day 86 to day 110 of gestation. All sows were reared in a single pen, and the temperature in the gestating house was controlled at 18 °C–20 °C. At 110 d of gestation, the sows were transferred to the farrowing room and started on a lactation diet, followed by daily feeding restriction until farrowing according to the sow's actual condition. The birth weight, stillbirth, deformities, and mummification of piglets were recorded at the time of delivery. Within 24 h of birth, piglets within the same treatment group were cross-fostered, and litter sizes were adjusted to 9–12 piglets. Piglets were routinely treated (ear-notching, tail-docking, castration, and iron injection) within 3 days after farrowing. During this period, sows were fed 4 times a day (5:00 a.m., 10:00 a.m., 4:00 p.m., and 8:00 p.m.). All sows were fed 1 kg of the lactation diet on the day of farrowing, which was

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thereafter increased by 1 kg daily, until ad libitum feeding. In addition, sows and piglets could drink freely throughout the experimental period. The temperature in the delivery room was maintained between 20 °C and 23 °C. After 28 d weaning, the sows were transferred to the gestation room. Two estrus detections were performed in the presence of mature boars (9:00 a.m. and 4:00 p.m.), and the interval between weaning and estrus was calculated.

Table 1. The composition and nutrient levels of basal diets (as-fed basis).

Items	Gestation	Lactation
Ingredients, %		
Corn	55.81	61.24
Wheat bran	9.23	5.00
Soybean meal	16.38	21.70
Expanded soybean	14.63	–
Fish meal	–	2.50
Soybean oil	–	5.00
L-lysine-HCL	0.04	0.27
DL-methionine	–	0.12
Chloride choline	0.15	0.15
NaCL	0.40	0.40
Calcium carbonate	0.16	0.17
Dicalcium phosphate	1.20	1.45
Vitamin and mineral premix ¹	2.00	2.00
Total	100.00	100.00
Nutrient levels ²		
Net energy, MJ/kg	9.88	9.91
Crude protein	16.89	16.65
Calcium	0.86	0.92
Phosphorus	0.65	0.63
Lysine	0.73	1.11
Methionine	0.66	0.61

¹ The vitamin and trace minerals supplied per kilogram of complete diet: 13,000 IU vitamin A; 3630 IU vitamin D₃; 26.4 IU vitamin E; 240 mg vitamin K; 21.4 µg vitamin B₁₂; 4.2 mg riboflavin; 12.5 mg d-pantothenic acid; 16.5 mg niacin; 20 mg Cu; 40 mg Mn; 160 mg Fe; 160 mg Zn; 0.4 mg I; 0.4 mg Cr; 0.4 mg Se. ² The net energy, lysine, and methionine were calculated; the other values were measured.

2.2. Sample Collection and Measurement

The backfat thickness at point P2 (6.5 cm from the midline over the last rib) of primiparous sows was measured via B-mode ultrasound (Renco Lean Meater type 7, Minneapolis, MN, USA) at day 29 of gestation, day 1 and day 28 of lactation, to calculate the backfat loss during lactation. The sows' weights were measured on the day of farrowing and weaning, and the weight loss during lactation was calculated. In addition, the daily feed intake during lactation was recorded, and the ADFI was calculated. After production, the number born, live number, stillbirth number, deformity number, and weak litter number of the sows were calculated. The piglets were weighed for their birth weight and weaning weight. After farrowing, 6 sows in each group were selected via hand-milking to collect colostrum samples (20 mL) after the sows were injected with 1 mL of oxytocin. Part of the colostrum was stored at -20 °C for assaying the colostrum composition, and the other part was stored at -80 °C for metabolomics analysis. Likewise, a 20 mL milk sample per sow was collected on day 14 of lactation. All colostrum and milk samples were taken from the sow's anterior, middle, and posterior teats and then pooled. Fecal samples were collected from 12 sows selected per treatment group from day 14 to 16 of lactation. The fecal

samples from each sow on these days were mixed, and homogenized with 10 mL of 10% hydrochloric acid per 100 g fecal sample, and then stored at -20 °C until chemical analysis. Twelve sows per group were randomly selected at 110 d of gestation and 28 d of lactation (the day of weaning). Fresh fecal samples were collected and stored at -20 °C for subsequent short-chain fatty acid (SCFA) analysis. A portion of the fecal samples collected at day 28 of lactation were stored at -80 °C for subsequent microbial analysis.

2.3. Chemical Analysis of Feed and Feces

All fecal samples were thawed and dried at 60 °C to a constant weight. Feed and fecal samples were ground and passed through a 1 mm screen, and then analyzed for crude protein (method 984.13; AOAC, 2006) [13], crude fiber (Van Soest method) [14], calcium (method 935.13; AOAC, 2006), and phosphorus (method ; AOAC, 2006). The gross energy was measured via a bomb calorimeter (Parr 6300 bomb calorimeter; Parr Instrument Company, Moline, IL, USA). The apparent total tract digestibility (ATTD, %) of nutrients was calculated according to the methods of Zhe et al. [15].

2.4. SCFA Analysis

Two grams of fecal sample was weighed into a 10 mL centrifuge tube, and 5 mL of deionized water was added. The samples were swirled for 30 s, left at 4 °C for 30 min, and then centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through a 0.22 µm filter, and this was repeated three times. The final supernatant was added to 25% (w/v) metaphosphoric acid in a 5:1 ratio for SCFA assay via gas chromatography (Shimadzu GC-2010, Japan) [16].

2.5. Microbial Analysis

The MO BIO-power stool DNA isolation kit was used to extract the total bacterial DNA from the fecal samples of the CON group and YC group ($n = 12$) sows at 28 d of lactation, according to the manufacturer's protocols. Before sequencing, the integrity of the extracted genomic DNA was determined via 1% (w/v) agarose gel electrophoresis. The

extracted fecal DNA samples were sent to Applied Protein Technology (Shanghai, China) for amplified pyrosequencing on the Illumina HiSeq PE250 platform. The V4 hypervariable region of the 16S rRNA gene was amplified with the primers 515F and 806R (5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GGACTACHVGGGTWTCTAAT-3', respectively).

The valid label was mapped to the OTU using UParse V7.0.1001, with a 97% similarity. Selected a representative sequence for each OTU. The ribosome database project (RDP) classifier version 2.2 was used to assign a classification level to each representative sequence. The relative abundance of each OTU was detected at different taxonomic levels.

2.6. Metabolite Extraction and Analysis

The collected colostrum and milk were thawed on ice, and extracted with 50% methanol buffer. Briefly, the sample was extracted in 20 µL using 120 µL of 50% methanol, vortexed for 1 min, and incubated for 10 min at room temperature. Extracts were stored overnight at -20 °C. After centrifugation at 4000× g for 20 min, the supernatant was transferred to a new 96-well plate. The samples were stored at -80 °C before LC-MS analysis. In addition, 10 µL of each extract was taken and mixed to prepare QC samples. All samples were collected via the LC-MS system, according to machine commands. The liquid phase conditions and mass spectrometer Q-Exactive Triple TOF 5600plus (SCIEX, Cheshire, UK) parameters were as described by Li et al. [17].

The XCMS software was used to pre-process the acquired mass spectrometry data. The LC-MS raw data were converted to the mzXML format using MSConvert software, and then processed through the XCMS, CAMERA, and metaX toolboxes in the R software.

The combined retention time (RT) and m/z data were used to characterize each ion. Further screening of differential metabolites in the samples was conducted using metaX.

2.7. Statistical Analysis

One sow, due to miscarriage (YC group), was excluded from the experiment. For the analysis of reproductive performance, sows and their litters were used as the experimental units. For the analysis of milk composition, sows were used as the experimental unit. Data were analyzed via an independent samples t-test, using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). The test results were expressed as means ± standard deviation (SD), and $p < 0.05$ was considered to be significant.

The relative abundance data of the gut microbiota were analyzed using the GLIMMIX program of SPSS 25. The alpha diversity within the community was calculated, and the taxonomic community was evaluated, using QIIME software (Version 1.7.0). Student's t -test was performed to calculate the differences in metabolite concentrations of the colostrum and milk, and the p -values for multiple tests were adjusted using FDR (Benjamini-Hochberg). To differentiate variables between groups, principal component analysis (PCA) and supervised partial least-squares-discriminant analysis (PLS-DA) were performed via metaX. We combined the conditions of ratio ≥ 2 or ratio ≤ 0.5 , p -value < 0.05 and variable importance in the projection (VIP) ≥ 1.5 to screen for important features.

3. Results

3.1. Production Performance of Primiparous Sows

The results of the reproductive performance of primiparous sows are shown in Table 2. Dietary YC supplementation increased the piglet birth weight and backfat thickness at 28 d of lactation ($p < 0.05$). However, no significant difference was observed on the litter size at birth, number of piglets born alive, litter birth weight, piglet birth weight, stillborn piglets, number of low-body-weight piglets, number of weaned piglets, litter weaning weight, piglet weaning weight, ADFI during lactation, body weight at the beginning and end of lactation, loss of body weight during lactation, backfat thickness at the beginning

and end of lactation, loss of backfat (BF) during lactation, and days to return to estrus ($p > 0.05$).

Table 2. The effect of dietary YC supplementation during gestation and lactation on the reproductive performance of primiparous sows ¹.

Items	CON	YC	<i>p</i> -Value
Litter size at birth, heads	12.23 ± 2.16	12.45 ± 2.37	0.717
Number of piglets born alive, heads	11.93 ± 2.18	12.34 ± 2.33	0.487
Litter birth weight, kg	16.97 ± 3.29	18.47 ± 3.39	0.271
Piglet birth weight, kg	1.42 ± 0.04	1.49 ± 0.07	0.046
Stillborn piglets, heads	0.30 ± 0.53	0.10 ± 0.31	0.091
Piglets < 1.0 kg, heads	0.90 ± 0.96	0.52 ± 0.57	0.069
Number of weaned piglets, heads	11.03 ± 2.31	11.86 ± 2.13	0.158
Litter weaning weight, kg	87.18 ± 4.26	89.68 ± 6.58	0.648
Piglet weaning weight, kg	7.39 ± 0.19	7.53 ± 0.19	0.155
ADFI during lactation ² , kg/d	5.12 ± 0.23	5.10 ± 0.47	0.302
Body weight at 1 d of lactation, kg	138.4 ± 10.52	140.2 ± 9.44	0.776
Body weight at 28 d of lactation, kg	132.8 ± 9.08	134.8 ± 9.69	0.142
Loss of body weight during lactation, kg	6.30 ± 0.66	5.48 ± 0.60	0.755
Backfat thickness at 1 d of lactation, mm	18.12 ± 1.43	18.18 ± 1.22	0.395
Backfat thickness at 28 d of lactation, mm	17.84 ± 1.28	18.93 ± 1.04	0.031
Loss of BF during lactation ³ , mm	0.30 ± 0.02	0.27 ± 0.03	0.255
Days to return to estrus, d	5.41 ± 0.91	4.90 ± 0.72	0.862

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¹ CON = control group; YC = yeast culture group. All values are expressed as means ± standard deviation ($n = 30$ in the control group and $n = 29$ in the YC group). ² ADFI = Average daily feed intake. ³ BF = Backfat.

3.2. The ATTD of Primiparous Sows during Lactation

As shown in Table 3, the dietary supplementation with YC increased the ATTD of gross energy and calcium in primiparous sows ($p < 0.05$). However, the ATTD of crude ash, crude fiber, crude protein, and phosphorus was not affected by dietary YC supplementation ($p > 0.05$).

Table 3. The effect of dietary YC supplementation on the nutrient apparent total tract digestibility in lactation primiparous sows ¹.

Items (%)	CON	YC	<i>p</i> -Value
Crude ash	76.24 ± 0.44	75.22 ± 0.59	0.388
Crude fiber	13.36 ± 0.63	12.51 ± 0.47	0.361
Gross energy	83.10 ± 0.22	84.20 ± 0.26	0.049
Crude protein	81.60 ± 0.22	82.31 ± 0.42	0.302
Phosphorus	40.53 ± 0.44	40.84 ± 0.60	0.054
Calcium	70.12 ± 0.55	73.44 ± 0.72	0.032

¹ CON = control group; YC = yeast culture group. All values are expressed as means ± standard deviation ($n = 12$).

3.3. The SCFA Concentration in Fecal Samples of Primiparous Sows

The SCFA concentrations in the feces are shown in Figure 1. Dietary YC supplementation increased the concentrations of acetic acid and propionic acid in the feces of 110day-gestation sows, and increased the concentrations of acetic acid and butyric acid in the feces of 28-day-lactation sows ($p < 0.05$).

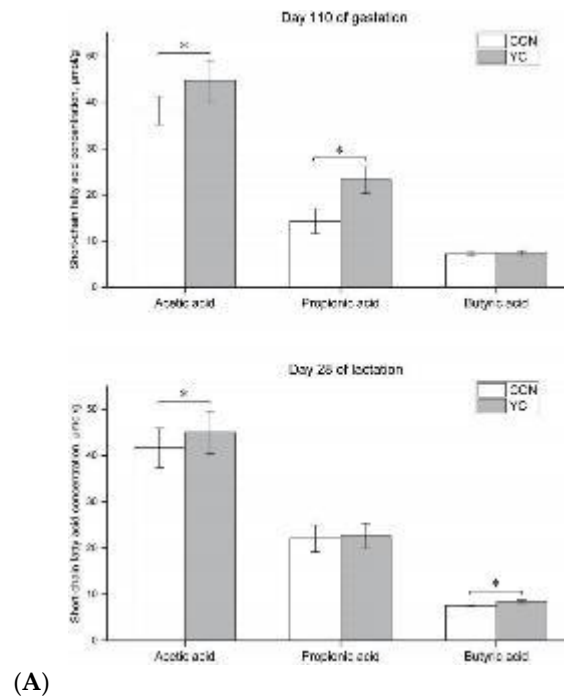


Figure 1. Short-chain fatty acid (SCFA) concentrations in the feces of primiparous sows during gestation and lactation. (A) SCFA concentration during gestation. (B) SCFA concentration during lactation. CON, control group; YC, yeast culture group. The data are expressed as mean $\pm$ standard deviation ($n = 12$).

3.4. Fecal Microbiota Analysis of Primiparous Sows

The effect of dietary YC supplementation on the fecal microbiota of primiparous sows is shown in Figure 2. Compared to the CON group, dietary YC supplementation significantly decreased the good coverage index of fecal microbiota, and significantly increased the Shannon, Simpson, and Sobs indexes ($p < 0.05$) (Figure 2A–D). At the phylum level, the fecal microbiota of primiparous sows consisted mainly of phyla such as Firmicutes and Bacteroidetes (Figure 2E). However, dietary YC supplementation significantly decreased the abundance of Firmicutes ($p < 0.05$). At the genus level, the fecal microbiota of primiparous sows was mainly composed of the genera *Prevotellaceae_NK3B31_group*, *Ruminococcaceae_UCG-005*, and *Lachnospiraceae_XPB1014_group*. Among them, dietary YC supplementation significantly increased the abundance of *Prevotellaceae_NK3B31_group* and

Rikenellaceae_RC9_gut_group ($p < 0.05$) and decreased the abundance of *Lachnospiraceae_XPB1014_group* and *Terrisporobacter* ($p < 0.05$).

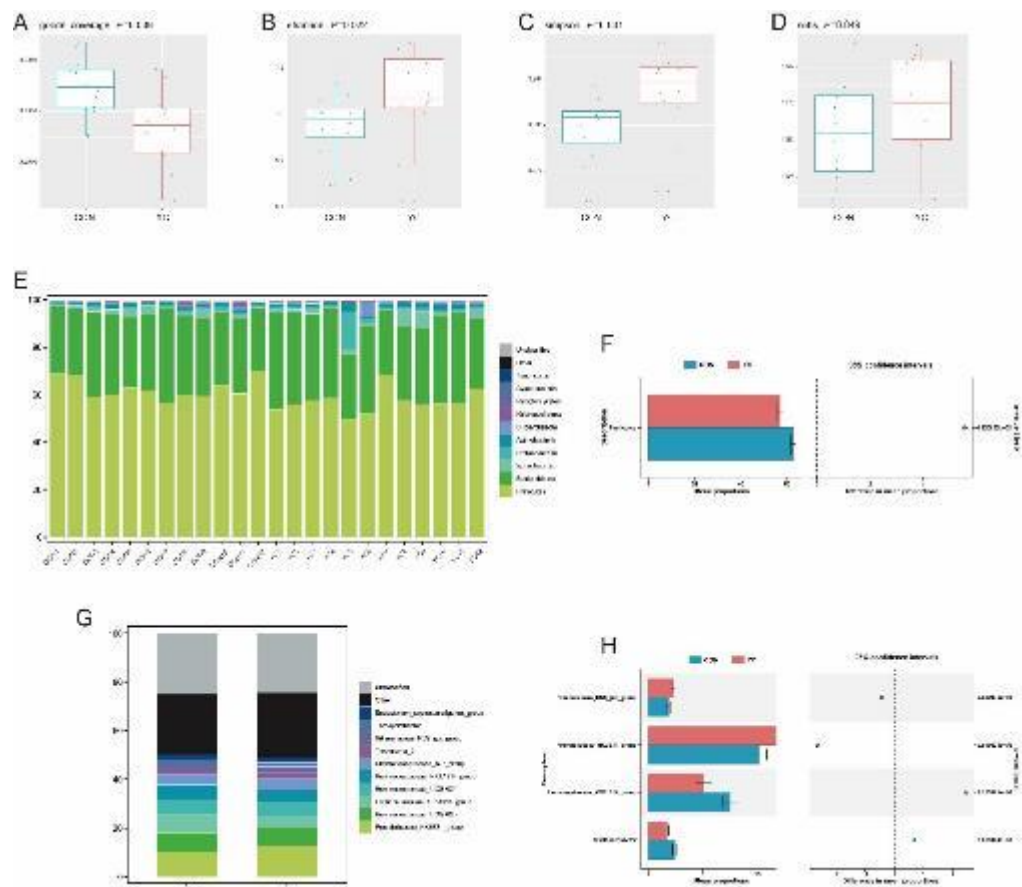


Figure 2. The microbiota analysis of feces during lactation in primiparous sows. (A–D) The good coverage index, Shannon index, Simpson index, and Sobs index. (E) The microbiota composition at the phylum level. (F) Differential microbiota at the phylum level. (G) The microbiota composition at the genus level. (H) Differential microbiota at the genus level. CON, control group; YC, yeast culture group. The data are expressed as mean ± standard deviation ($n = 12$).

3.5. Colostrum and Milk Composition

As shown in Table 4, dietary YC supplementation increased the fat and lactose content in the colostrum ($p < 0.05$).

Table 4. The effect of YC on the colostrum and milk composition from primiparous sows 1.

Items	CON	YC	<i>p</i> -Value
Colostrum			
Fat, %	5.03 ± 0.16	5.19 ± 0.10	0.046
Protein, %	15.75 ± 0.33	15.92 ± 0.53	0.345
Lactose, %	3.47 ± 0.67	3.94 ± 0.77	0.027
Dry matter, %	28.04 ± 0.40	28.11 ± 0.77	0.804
Milk			
Fat, %	5.02 ± 0.11	5.12 ± 0.63	0.055
Protein, %	5.72 ± 0.37	5.81 ± 0.25	0.444

Lactose, %	5.21 ± 0.71	5.39 ± 0.11	0.060
Dry matter, %	19.67 ± 0.51	20.04 ± 0.73	0.163

¹ CON = control group; YC = yeast culture group. All values are expressed as means ± standard deviation ($n = 6$).

3.6. Metabolomic Analysis of Colostrum

A total of 418 effective metabolites, mainly including lipids and lipid-like molecules, organic acids and derivatives, and organoheterocyclic compounds, were analyzed via LCMS from the CON and YC groups (Table S1). PCA and PLS-DA showed that the CON and YC groups were independently distributed, with no overlap (Figure 3A,B). The first principal component (PC1) explained 55.06% and 55.42% of the PCA and OPLS-DA, respectively. The second principal component (PC2) explained 13.03% and 12.73% of the PCA and OPLS-DA, respectively. This indicates that the metabolites in the CON and YC groups differed significantly with the supplementation of YC in the diets. In addition, the PLS-DA model parameters $R_2 = 0.7528$ and $Q_2 = -0.8014$ indicate that the PLS-DA model is valid and reliable (Figure S1).

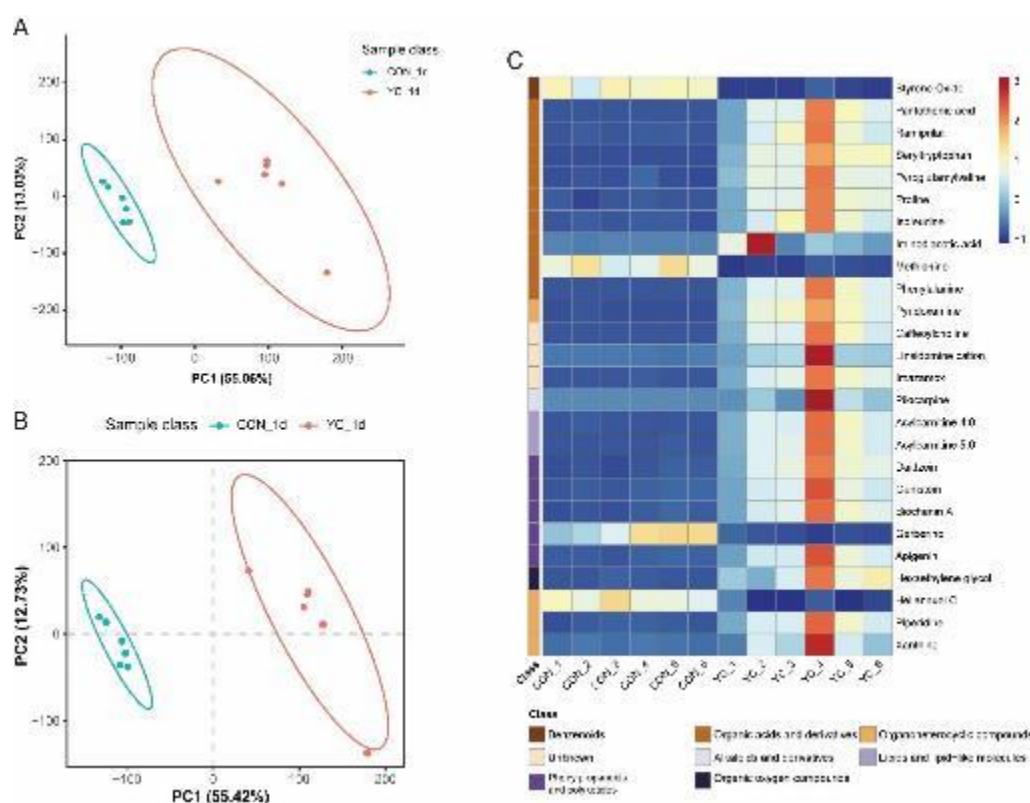


Figure 3. The metabolomic analysis of colostrum from primiparous sows. (A) Principal component analysis (PCA) of colostrum samples. (B) Partial least-squares–discriminant analysis (PLS-DA) of

The detected metabolites were further screened through combining the screening conditions of ratio ≥ 2 or ratio ≤ 0.5 , p -value < 0.05 and VIP ≥ 1.5 , and a total of 26 differential metabolites were significantly influenced by YC treatment (Table S2). The differential metabolites mainly belonged to organic acids and derivatives, phenylpropanoids and polyketides, and organoheterocyclic compounds (Table 5). Among them, compared with the CON group, the YC group showed significantly decreased contents of styrene oxide, methionine, gerberinol, and heliannuol C, whereas the contents of 22 differential metabolites, including linsidomine cation, pilocarpine, and xanthine, were significantly increased (Figure 3C).

Table 5. Differential metabolites in colostrum from primiparous sows.

Metabolite	Class	Ratio ¹	p -Value	VIP ²	MZ ³	RT ⁴
Styrene oxide	Benzenoids	0.05	7.70×10^{-5}	1.96	121.08	1.41
Pantothenic acid	Organic acids and derivatives	60.50	1.03×10^{-6}	2.33	220.13	3.18
Ramiprilat	Organic acids and derivatives	24.57	1.14×10^{-6}	2.04	387.19	3.25
Seryltryptophan	Organic acids and derivatives	49.14	1.93×10^{-7}	2.25	290.11	3.36
Pyroglutamylvaline	Organic acids and derivatives	14.82	6.94×10^{-7}	1.90	227.10	3.34
Proline	Organic acids and derivatives	15.99	6.05×10^{-3}	2.14	116.07	3.31
Isoleucine	Organic acids and derivatives	17.67	3.17×10^{-6}	1.91	132.10	3.23
Iminodiacetic acid	Organic acids and derivatives	31.52	3.65×10^{-3}	1.77	134.04	3.74
Methionine	Organic acids and derivatives	0.15	2.98×10^{-7}	1.68	150.06	1.23
Phenylalanine	Organic acids and derivatives	8.55	4.39×10^{-5}	1.69	166.09	3.54
Pyridoxamine	Organoheterocyclic compounds	6.63	2.36×10^{-5}	1.61	169.10	3.15
Caffeoylcholine	Unknown	112.25	1.01×10^{-8}	2.50	266.16	3.41
Linsidomine cation	Unknown	22.39	6.94×10^{-4}	2.14	189.12	2.31
Imazamox	Unknown	57.85	4.80×10^{-6}	2.32	304.15	3.19
Pilocarpine	Alkaloids and derivatives	102.54	1.52×10^{-3}	2.34	226.15	1.86
Acylcarnitine 4:0	Lipids and lipid-like molecules	100.27	2.84×10^{-8}	2.52	232.17	3.33
Acylcarnitine 5:0	Lipids and lipid-like molecules	76.79	2.16×10^{-8}	2.46	246.19	3.45
Daidzein	Phenylpropanoids and polyketides	6.59	1.06×10^{-5}	1.65	255.06	3.97
Genistein	Phenylpropanoids and polyketides	9.36	1.15×10^{-5}	1.80	271.06	4.17
Biochanin A	Phenylpropanoids and polyketides	7.04	2.42×10^{-5}	1.65	285.07	4.01

Gerberinol	Phenylpropanoids and polyketides	0.12	9.03×10^{-6}	1.84	365.10	3.32
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colostrum samples. (C) Heat map of differential metabolites. CON, control group; YC, yeast culture group, $n = 6$.

Apigenin	Phenylpropanoids and polyketides	9.24	3.32×10^{-5}	1.78	269.04	4.18
Hexaethylene glycol	Organic oxygen compounds	11.15	1.42×10^{-5}	1.56	300.19	3.31
Heliannuol C	Organoheterocyclic compounds	0.22	1.80×10^{-4}	1.56	307.15	0.69
Piperidine	Organoheterocyclic compounds	9.14	2.04×10^{-5}	1.61	86.10	1.76
Xanthine	Organoheterocyclic compounds	11.37	1.36×10^{-3}	1.75	151.02	1.35

¹ ratio = the average value of the YC group/average value of the CON group. ² VIP = variable importance in the projection. ³ MZ = charge-to-mass ratio. ⁴ RT = retention time.

4. Discussion

In this study, the piglet weight at birth and the backfat thickness at 28 d of lactation of primiparous sows were improved via dietary supplementation with YC. The level of nutrition provided by sows to their offspring depends on the nutrition in their diet and the body's nutritional reserves [18]. As we failed to observe an increase in ADFI in primiparous sows with YC supplementation, it is hypothesized that the increase in piglet weight at birth is attributed to the supplementation of YC in the diet to improve the nutritional reserves of primiparous sows. However, Kim et al. [3] reported that dietary YC supplementation did not influence the piglet birth weight. This may be due to Kim et al. supplementing 12 g/d of YC into sows' gestation diets, whereas the present study supplemented 0.5% of YC into sows' gestation diets. In addition, in the present study, on day 86 of gestation, sows were fed an increased amount (from 2.5 kg/d to 3 kg/d) due to fetal development, compared to 2 kg/d in Kim et al. Therefore, this may be one of the reasons for the inconsistent results of different studies. In addition, it has been shown that the supplementation of YC into the diet improves backfat thickness in lambs [19]. It is hypothesized that the increase in backfat thickness in different animals may be related to the nutrients, such as protein, vitamins, and oligosaccharides, in YC [8].

As an important indicator to measure the efficiency of digestion and absorption in animals, ATTD is important to animal health and development [20]. In the present study, we observed that dietary YC supplementation increased the ATTD of gross energy and calcium during lactation. Energy digestibility is the total energy digestibility of protein, carbohydrates, and lipids in the diet [21]. An increased energy digestibility contributes to offspring growth in primiparous sows [15]. In addition, calcium in the sow's diet during gestation and lactation is also critical for offspring bone development [22]. Previous studies have shown that the addition of mannan, one of the yeast cell-wall components, to the diet improves the gross energy digestibility [23,24]. In addition, it is reported that each kilogram of YC contains 1400 units of phytase [25]. This may be the reason why dietary YC supplementation improves the ATTD of calcium. However, Veum et al. [26] reported that dietary YC supplementation did not affect the apparent digestibility in sows. As there are few reports on the effect of YC on ATTD in sows, the exact reasons need to be further studied.

SCFAs are metabolites produced by gut microbes through the fermentation of fiber and resistant starch, and mainly include acetate, propionate, and butyrate [27]. In this study, dietary YC supplementation increased acetic acid and propionic acid concentrations at 110 d of gestation, as well as acetic acid and butyric acid

concentrations at 28 d of lactation, in primiparous sows. It has been reported that the SCFA is associated with maternal hepatic lipid metabolism during gestation [28]. Increased concentrations of acetic and propionic acids contribute to the regulation of lipid metabolism, anti-inflammatory, and feeding behaviors [29]. In addition, the SCFA has a direct relationship with the milk composition. By infusing different SCFAs into cows, Rook et al. [30] observed that acetic

acid could increase the milk yield and the fat content in milk, and butyric acid could increase the fat content in milk. Therefore, it was hypothesized that supplementation with YC during gestation and lactation increased different SCFA concentrations in primiparous sows, which may be beneficial in improving lipid metabolism and nutrient composition in the milk of primiparous sows. In addition, a previous study suggested that one possible reason for the enhancement of volatile fatty acids by the *Saccharomyces cerevisiae* fermentation product was the increased lactic acid utilization, which improves the microbial environment, and increases the abundance of cellulolytic flora [31]. In this study, we observed that YC increased the abundance of *Prevotellaceae_NK3B31_group* by analyzing the gut microbiota and hypothesized that this could be one of the reasons for the increased SCFA concentration [32].

In this study, we observed a significant increase in Shannon, Simpson, and Sobs indexes in the YC group compared to the CON group. It is suggested that dietary YC supplementation helps to increase the richness and diversity of the gut microbiota, which improves the gut health of primiparous sows. As YC contains nutrients such as β -1, 3glucan and mannan, this may account for the increased gut microbial richness and diversity [33,34]. In the mammalian gut, Firmicutes and Bacteroidetes are generally the two most abundant phyla [35]. Consistent with this finding, our study also observed that Firmicutes and Bacteroidetes are the most abundant phyla. In addition, by analyzing the microbial composition at the phylum level, we observed that dietary YC supplementation significantly decreased the abundance of Firmicutes. This is similar to the results of Liu et al. [36], who found that the abundance of Firmicutes decreased from 90.76% to 77.64% with the supplementation of YC in late-laying hens. As a dominant phylum in the gut, Firmicutes regulates host carbohydrate metabolism and lipid metabolism, and plays an important role in the maintenance of internal homeostasis [37]. The decrease in the abundance of Firmicutes in this study may imply that YC has a regulatory effect on glycolipid metabolism in lactating sows. It is hypothesized that YC may have expanded the competition among the gut microbiota, leading to a decrease in the abundance of *Lachnospiraceae_XPB1014_group*, belonging to the Firmicutes, and an increase in the abundance of *Rikenellaceae_RC9_gut_group* and *Prevotellaceae_NK3B31_group*, belonging to Bacteroidetes. Through further analyzing the species composition at the genus level, we found that dietary YC supplementation increased the abundance of *Rikenellaceae_RC9_gut_group* and *Prevotellaceae_NK3B31_group* and decreased the abundance of *Lachnospiraceae_XPB1014_group* and *Terrisporobacter*.

Rikenellaceae, as a common bacterium in the gut, regulates nitrogen and protein metabolism, and promotes butyric acid synthesis, as well as negatively correlating with host obesity [38]. *Prevotella* is associated with a diet rich in carbohydrates and fiber, and makes a significant contribution to the production of SCFA (acetic, propionic, and butyric acids) [39]. *Lachnospiraceae* are involved in the host's carbohydrate metabolism and are, likewise, one of the major SCFA-producing microbiotas [40]. In addition, by feeding silage to finishing pigs, Niu et al. [41] observed that *Terrisporobacter* and *Lachnospiraceae* were strongly associated with SCFA content. In the present study, all four of the genera that changed significantly with the supplementation of YC in the diet were correlated with SCFA concentrations, although the changes in the abundance of different microbes

varied. It is hypothesized that YC may be involved in regulating energy metabolism in primiparous sows.

As the nutritional status of primiparous sows was improved by the supplementation of YC, we analyzed the components in the colostrum and milk. In this study, dietary supplementation with YC increased the fat and lactose contents in the colostrum compared to the CON group. A previous study showed that dietary YC supplementation increased the lactose content of colostrum from sows [11]. In addition, Dias et al. [42] observed that the supplementation of YC into the diet increased the fat and lactose content of milk from dairy cows. The uptake and oxidation of lactose and fat in colostrum provide energy for piglets and influences their survival and constant body temperature [43]. However, sow colostrum is characterized by a high immunoglobulin content and a low lactose and fat

content [44]. In this study, dietary YC supplementation improved the low lactose and fat content of colostrum in sows. In addition, the yield and quality of milk affect the proper development of piglets, which, in turn, is related to the profitability of pork production [45]. Therefore, dietary YC supplementation resulted in a higher nutrient content in colostrum and milk from primiparous sows, which is significant for piglet development and pork production.

The quality of sow colostrum directly affects piglet mortality and growth performance [46]. We further performed a metabolomic analysis of the components in colostrum from primiparous sows. Dietary YC supplementation mainly increased organic acids and derivatives, and lipids and lipid-like molecules, mainly including pantothenic acid and acylcarnitine. Pantothenic acid, also known as vitamin B5, is phosphorylated by pantothenic kinase after entering the cell, and is converted to coenzyme A through a variety of intermediates in all tissues to function [47]. A previous study showed that piglets deficient in pantothenic acid developed severe diarrhea within 2–4 weeks, and uncoordinated locomotion at 4–7 weeks [48]. Additionally, the levels of pantothenic acid in sows' blood and milk were influenced by the levels of pantothenic acid in the diet [46,49]. Therefore, dietary YC supplementation for primiparous sows is beneficial to piglet supplementation with pantothenic acid and improves piglet health. In addition, we observed that dietary YC supplementation to primiparous sows increased the levels of several amino acids, including proline, isoleucine, and phenylalanine. Proline is synthesized from glutamate in a series of steps, through enzymes such as γ -glutamyl phosphate and γ -glutamyl phosphate reductase, and is considered to be an important amino acid in the metabolic processes of cellular redox control, bioenergetics, and apoptosis and cancer [50,51]. As one of the branched-chain amino acids, isoleucine is involved in physiological functions such as protein metabolism, fatty acid metabolism, and glucose transport, and is conducive to promoting the body's immune function [52]. Dietary phenylalanine enters the liver through the blood, and is eventually absorbed by the tissues to synthesize proteins, and the excess is hydroxylated by the liver enzyme phenylalanine-4-hydroxylase to form tyrosine, then hydroxyphenylpyruvate and, finally, metabolize to carbon dioxide [53]. In this study, dietary YC supplementation increased the levels of various amino acids, and provided more nutrients for the production of primiparous sows and the development of piglets. In addition, in the present study, we observed an up-regulation of acylcarnitine content. In the cytoplasm, fatty acids are converted to the form of acylcarnitines, which are transported by the acylcarnitine transporter into the mitochondrial matrix to generate energy through β -oxidation [54]. Increased acylcarnitine in the plasma of lactating sows has been reported to be beneficial for improving sow performance and the growth of weaned piglets [55]. Therefore, we hypothesized that dietary YC supplementation also promoted energy metabolism in

primiparous sows, and that the increased acylcarnitine content of colostrum was beneficial in enhancing piglet growth.

There are several limitations to this study. First of all, the present study did not study piglets excessively, including their feed intake, detailed body weights at different stages, diarrhea, or intestinal microbiota. Additionally, considering that YC is thought to have an immunity-enhancing effect [11,56], we did not study the immunity indexes of primiparous sows or the immunity indexes of piglets. Therefore, we could not know whether the decrease in sow body weight loss and the increase in the weaning weight of piglets were related to the immunity-enhancing effect of YC. Additionally, whether the supplementation of YC had an effect on subsequent litter size and sow longevity in primiparous sows was limited by the experimental conditions and remained unobserved in this study. However, multiple beneficial effects of YC on the performance of primiparous sows were found in this study, and this study was conducted in a commercial swine farm setting, which is more in line with the actual production environment. Therefore, the supplementation of YC in the diets of primiparous sows is beneficial to the performance of sows and piglets, and to improvements in the efficiency of the pig farming industry.

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5. Conclusions

This study suggested that dietary YC supplementation during gestation and lactation improved the reproductive performance of primiparous sows, increased energy metabolism as well as adjusted the microbial structure in the gut. In addition, dietary YC supplementation was able to increase a variety of nutrients in colostrum. Our results suggested that dietary YC supplementation is beneficial to the health of primiparous sows and the performance of their offspring and that YC is a suitable prebiotic for primiparous sows.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: partial least-squares–discriminant analysis (PLS-DA) model validation; Table S1: Effective metabolites detected between the CON and YC groups; Table S2: Differential metabolites detected between the CON and YC groups (ratio ≥ 2 or ratio ≤ 0.5 , p -value < 0.05 and VIP ≥ 1.5).

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Informed Consent Statement:

Data Availability Statement: The data presented in this study are available in the present article, and is shared with consent and in accordance with all authors.

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Conflicts of Interest: Peng Chen is an employee of Beijing Enhalar International Tech Co., Ltd. (Beijing, China). The authors declare no conflicts of interest.

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 [®]
BIOYEASTAR 4C Postbiotics


Yeast culture promotes the production of aged laying hens by improving intestinal digestive enzyme activities and the intestinal health status

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Key words: yeast culture, aged laying hen, performance, egg quality, intestinal health

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ABSTRACT Yeast culture (YC) positively affects the

performance of laying hens. The purpose of the present study was to explore the underlying mechanism for the YC-mediated performance improvement. Sixty 67-week-old Hy-Line Brown laying hens were randomly allocated into 2 experimental groups with 5 replicates of 6 birds each. One group was fed a control diet, whereas the other received the control diet supplemented with YC at 3.0 g/kg; treatment lasted for 8 wk. The results showed that dietary YC supplementation increased ($P < 0.05$) the total egg weight (11.2–13.6%) and egg-laying rate (13.0–13.5%) but decreased ($P < 0.05$) the feed/egg ratio by 9.3 to 11.0% during weeks 5 to 6 and 7 to 8 compared with the control. However, egg quality, including eggshell strength, eggshell thickness, egg weight, albumen height, egg yolk

INTRODUCTION

Older laying hens present several problems for the poultry industry, including decreased performance and egg quality (Bar et al., 1999). The number of broken eggs from aged laying hens increases by 7.5%, an amount that leads to great economic losses (Roland, 1988). A previous study showed that reduced intestinal health in aged laying hens, which causes digestion, absorption, and immune problems, is one of the major reasons for altered laying performance (Jing et al., 2014). Therefore, poultry producers are

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substances, including yeast cells, vitamins, peptides, amino acids, proteins, peptides, organic acids, and oligosaccharides (Jensena et al., 2008). YC improves the performance of monogastric and ruminant animals (Bontempo et al., 2006; Desnoyers et al., 2009; Ozsoy et al., 2018). However, few studies have focused on the effects of YC on the performance of laying hens.

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Previous studies reported that yeast cell wall and yeast autolysate supplied to laying hens increase egg production and egg weight and improve feed efficiency (Yalcin et al., 2010; Hashim et al., 2013). These findings are consistent with our previous large-scale production study with 20,400 laying hens, which showed that dietary YC supplementation increases the performance of aged laying hens (Li et al., 2016). Specifically, dietary supplementation of 0.2% YC decreases the feed/egg ratio (1.8%) and mortality rate (20.6%), while increasing eggshell strength (1.2%), egg weight (3.4%), yolk weight (4.0%), albumen height (7.4%), and Haugh unit (4.5%; Li et al., 2016). However, the mechanism behind the positive effects of YC on laying hens' performance remains unclear.

Numerous studies showed that YC affects intestinal mucosal morphology and ileal villus development in broilers (Santin et al., 2001; Zhang et al., 2005). These studies evidenced the positive influences of YC on the intestinal health of chickens. Intestinal health problems are a major reason for reduced laying performance in aged

color, and Haugh unit, was not affected ($P > 0.05$) by YC supplementation. Furthermore, dietary YC supplementation increased ($P < 0.05$) chymotrypsin and α -amylase activities by 54.8 to 62.5% in the duodenal chyme and reduced ($P < 0.05$) plasma endotoxin by 44.1%. YC dietary supplementation also upregulated ($P < 0.05$) the mRNA levels of intestinal barrier-related genes (occludin and claudin 1) and antimicrobial peptides genes (b-defensin 1 and 7 and cathelicidin 1 and 3) in the duodenum or jejunum compared with the control. In conclusion, dietary YC supplementation improved the performance of aged laying hens, potentially through the upregulation of intestinal digestive enzyme activities and intestinal health-related gene expression.

constantly pursuing solutions to enhance intestinal health, improve laying performance, and extend the laying period in aged laying hens. Feeding poultry various microorganisms to enhance intestinal health and thus improve performance is one strategy that has attracted prominent attention (Shamsi et al., 2015; Forte et al., 2018; Ozsoy et al., 2018; Adetunji and Adejumo, 2019).

Yeast culture (YC) is a natural yeast fermentation product that comprises a variety of biologically active

laying hens, and thus, we hypothesized that YC may augment the performance of aged laying hens because of improved intestinal health. Intestinal barrier function and digestive capacity play key roles in intestinal health (Katherine et al., 2009). In general, intestinal permeability-related genes, including tight junction protein, that is, occludin (OCLN), claudin (CLDN), and zonula occludens (ZO; Shin et al.,

2018), and antimicrobial peptides, that is, avian b-defensins (AvBD1-14; Lynn et al., 2007) and cathelicidins (CAHT1-3, B1; Achanta et al., 2012), play important roles in the intestinal barrier function. Furthermore, enzymes related to

the digestive capacity, including amylase, chymotrypsin, and lipase, play pivotal roles in feed digestion. However, it is still unclear whether YC improves the performance of laying hens by regulating these genes. Therefore, we selected aged laying hens to determine whether dietary YC supplementation could alleviate the reduction in performance and egg quality; and YC improved performance of aged laying hens by regulating these intestinal barrier function and digestive capacity genes.

MATERIALS AND METHODS Birds, Treatment, Growth Performance, and

Sample Collection

The Institutional Animal Care and Use Committee of Huazhong Agricultural University, China, supervised and approved the experimental protocol of this study. In total, sixty 67-week-old Hy-Line Brown laying hens were randomly divided into 2 groups with 5 replicates of 6 birds each. The control group received a basal diet (BD, Table 1)

with nutrients that met the recommendations provided by the National Research Council (NRC, 1994). The dietary treatment group was prepared by supplementing the same BD with 3.0 g/kg YC as a dry powder (1.8×10^{18} cfu/kg *Saccharomyces cerevisiae*;

after collection of the chyme. The intestinal and chyme samples were divided into aliquots, snap-frozen in liquid nitrogen, and stored at 280C until further analysis.

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Table 2. Effects of dietary YC supplementation on laying performance of laying hens.¹

Item	Weeks 1 to 2		Weeks 3 to 4		Weeks 5 to 6		Weeks 7 to 8	
	Control	YC	Control	YC	Control	YC	Control	YC
Total feed intake, kg/hen	1.38 6 0.06	1.34 6 0.07	1.61 6 0.07	1.59 6 0.11	1.66 6 0.07	1.67 6 0.10	1.75 6 0.04	1.77 6 0.04
Total egg weight, kg/hen	0.56 6 0.05	0.56 6 0.08	0.60 6 0.08	0.64 6 0.12	0.59 6 0.04 ^a	0.67 6 0.02 ^b	0.60 6 0.03 ^a	0.67 6 0.05 ^b
Feed/egg ratio, g/g	2.49 6 0.25	2.48 6 0.50	2.71 6 0.37	2.52 6 0.31	2.81 6 0.07 ^b	2.50 6 0.12 ^a	2.91 6 0.11 ^b	2.64 6 0.15 ^a
Egg-laying rate, %	66.7 6 7.7	66.7 6 15.6	69.3 6 10.6	75.9 6 6.2	66.9 6 5.6 ^a	75.9 6 2.0 ^b	66.9 6 4.4 ^a	75.6 6 5.4 ^b

^{a,b}Means within a row with different superscripts differ significantly (P, 0.05).

Table 1. Composition and nutrient content of basal diet.

Ingredients	Contents (%)
Corn	53.5
Soybean meal	23.5
Wheat bran	6.5
Soybean oil	5.0
Limestone	8.5
Salt	0.3
DL-methionine	0.11
Dicalcium phosphate	1.59
Premix ¹	1.0
Total	100
Nutrient levels ²	
Metabolizable energy MJ/kg ⁻¹	14.59
Crude protein	15.2
Calcium	3.42
Total phosphorus	0.62
Available phosphorus	0.39
Lysine	0.91
Methionine	0.42

¹Contained the following per kilogram of diet: vitamin A, 12,000 IU; vitamin D₃, 4000 IU; vitamin E, 35 IU; vitamin K, 5 mg; thiamine, 2 mg; riboflavin, 8 mg; vitamin B₆, 5 mg; vitamin B₁₂, 50 mg; D-biotin, 200 mg; pantothenic acid, 15 mg; nicotinic acid, 50 mg; choline, 500 mg; folic acid, 1.5mg; Mn, (MnSO₄·H₂O), 120mg; Zn (ZnO), 80mg; Fe (FeSO₄·H₂O), 120 mg; Cu (CuSO₄·5H₂O), 15 mg; I (KI), 1 mg and Se (Na₂SeO₃), 0.3 mg. ²Nutrient Levels were a calculated value.

Beijing Enhalar Biotechnology Co. Ltd., Beijing, China). The YC was produced by fermentation of a substrate that contained corn germ meal, wheat bran, sugar cane molasses, yeast extract, and inorganic salts by *S. cerevisiae*. The nutrient composition of the YC powder is listed in the Supplemental Table 1 (Zhang et al., 2018). All birds were allowed ad libitum access to the mash diets and distilled water for 8 wk. Egg weight, feed intake, and mortality were recorded daily (Hashim et al., 2013). The feed/egg ratio and egg production rate were calculated biweekly. Interior egg and eggshell quality were measured from the eggs laid on the last day of weeks 4 and 8. At the end of the feeding trial, 10 hens (2 birds/cage) from each group were slaughtered to collect blood samples, duodenum, jejunum, and the chyme from the duodenum and jejunum. The mesentery was cut to uncoil the intestine, and then the entire duodenum and jejunum were removed. The chyme was collected from one end of the intestine by massaging the other end. A small segment of duodenum and jejunum tissue was collected

Plasma Immunoglobulin and Endotoxin Levels and Chyme Digestive Enzyme Activity Analysis

The plasma samples were prepared by centrifuging the collected blood samples at 1000 ! g at 4C for 10 min (Sun et al., 2016). The concentrations of immunoglobulin (Ig) A, IgG, and IgM and endotoxin in plasma were measured with ELISA kits (A40199-S, A04022-S, A02721-S, A049165-S; Shanghai Jining Shiye Co. Ltd., Shanghai, China), according to the manufacturer's

¹Results are reported as the mean 6 SD, n 5 5; YC 5 yeast culture.

instructions. The activities of α -amylase, lipase, and chymotrypsin in chyme from the duodenum and jejunum were determined by a colorimetric method using specific assay kits (C016-1, A054-1, A080-1; Nanjing Jiancheng Bioengineering Institute of China, Nanjing, China).

Real-Time Quantitative Polymerase Chain Reaction Analyses

Total RNA was isolated from the duodenum and jejunum (20 mg tissue; n 5 10 hens per group). The RNA sample preparation, q-PCR procedure, and relative RNA abundance quantification were conducted as previously described (Luo et al., 2019; Zhao et al., 2019). Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The RNA quality and concentration were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) at 260 and 280 nm. Reverse transcription to synthesize the complementary DNA library was performed using the PrimeScript™ RT reagent kit (TaKaRa, Kusatsu, Japan). The mRNA levels of the genes were analyzed using a q-PCR machine (CFX384; Bio-Rad, Hercules, CA) with SYBR Green Dye (Bio-Rad), following the manufacturer's instructions. Primers for the intestinal barrier-related genes, including OCLN, CLDN1, and ZO-1, antimicrobial peptide genes, including AvBD1, 4, and 7 and CAHT1 and 3, and

the reference gene *bactin* were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) and are presented in [Supplemental Table 2](#). The primer quality was verified by amplification plots and dissociation curves. The 2^{ddCt} method was used for the quantification with *bactin* as a reference gene, and the relative abundance was normalized to the control group.

Statistical Analysis

Statistical analysis was performed with SPSS Statistics 20 (SPSS Inc., IBM, Chicago, IL). Data are presented as mean $\pm$ SD. Dietary effects were determined by one-way ANOVA with a significance level of $P < 0.05$. The Tukey–Kramer method was used for multiple mean comparisons.

RESULTS Laying Performance and Egg Quality

Laying performance results are presented in [Table 2](#). There were no differences ($P > 0.05$) in the initial egg production rate between the 2 groups (data not shown). Dietary YC supplementation did not affect ($P > 0.05$) the

total egg weight, feed/egg ratio, and laying rate during weeks 1 to 2 and 3 to 4 and total feed intake throughout the entire experimental period. However, it increased ($P < 0.05$) total egg weight (11.7–13.6%) and egg-laying rate (13.0–13.5%) but decreased ($P < 0.05$) the feed/egg ratio (9.3–11.0%) compared with the control during weeks 5 to 6 and 7 to 8. Egg quality results are presented in [Table 3](#). Eggshell strength, eggshell thickness, egg weight, albumen height, egg yolk color, and Haugh unit were not affected ($P > 0.05$) by dietary YC supplementation at weeks 4 and 8.

Plasma Immunoglobulin and Endotoxin Levels and Chyme Digestive Enzyme Activity

Plasma immunoglobulin and endotoxin concentration results are presented in [Table 4](#). After 8 wk of experimental treatment, dietary YC supplementation decreased ($P < 0.05$) plasma endotoxin by 44.1%

Table 3. Effects of dietary YC supplementation on egg quality¹.

Item	Week 4		Week 8	
	Control	YC	Control	YC
Eggshell strength, N	28.9 $\pm$ 5.6	32.1 $\pm$ 6.4	30.8 $\pm$ 5.1	31.2 $\pm$ 6.5
Eggshell thickness, mm	0.353 $\pm$ 0.022	0.349 $\pm$ 0.036	0.351 $\pm$ 0.029	0.372 $\pm$ 0.031
Egg weight, g	62.4 $\pm$ 2.4	60.2 $\pm$ 5.6	64.0 $\pm$ 3.3	63.6 $\pm$ 2.1
Albumen height, mm	8.42 $\pm$ 0.78	8.68 $\pm$ 0.88	8.76 $\pm$ 1.07	8.94 $\pm$ 0.87
Egg yolk color	6.05 $\pm$ 0.82	6.01 $\pm$ 0.57	6.02 $\pm$ 0.91	5.83 $\pm$ 0.64
Haugh unit	91.3 $\pm$ 3.7	92.7 $\pm$ 5.4	92.1 $\pm$ 5.4	94.0 $\pm$ 4.2

¹Results are reported as the mean $\pm$ SD, $n = 5$; YC 5 yeast culture.

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Expression of Intestinal Barrier-Related and Antimicrobial Peptide Genes

The mRNA levels of the examined gene results are presented in [Figure 1](#). In the duodenum, dietary YC supplementation enhanced ($P < 0.05$) OCLN and CLDN1 (intestinal barrier-related genes) and AvBD1, CATH1, and CATH3 (antimicrobial peptide genes) mRNA levels at week 8 compared with the control ([Figure 1A](#)). Dietary YC supplementation had a minimal effect in the jejunum; it only increased ($P < 0.05$) the AvBD7 mRNA level compared with the control ([Figure 1B](#)).

DISCUSSION

The current study showed that dietary supplementation of 0.3% YC improved the performance of aged laying hens. Specifically, dietary YC supplementation reduced the feed/egg ratio and increased the total egg weight and egg-laying rate during weeks 5 to 8. These outcomes were similar to previous studies that revealed YC supplementation for 1 to 4 wk is necessary to detect positive effects on the performance of livestock and poultry ([Mathew et al., 1998](#); [Lesmeister et al., 2004](#); [Gao et al., 2008](#)). However, in the present study, interior egg and eggshell quality were not affected by dietary YC

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Table 4. Effects of YC supplied in diets on concentration of immunoglobulin and endotoxin in plasma¹.

Item	Control	YC
IgM, mg/mL	327.5 $\pm$ 134.9	318.3 $\pm$ 97.1
IgA, mg/mL	184.9 $\pm$ 74.0	174.1 $\pm$ 37.7
IgG, mg/mL	1,192.8 $\pm$ 443.5	1,149.1 $\pm$ 405.5
Endotoxin, mg/mL	53.6 $\pm$ 5.5 ^a	29.9 $\pm$ 10.6 ^b

^{a,b}Means within a row with different superscripts differ significantly ($P < 0.05$).

Abbreviation: Ig, immunoglobulin.

¹Results are reported as the mean $\pm$ SD, $n = 5$; YC 5 yeast culture.

compared with the control. However, the concentrations of plasma immunoglobulins, including IgA, IgG, and IgM, were not affected ($P > 0.05$) by YC supplementation. Chyme digestive enzyme activity results are presented in [Table 5](#). Although dietary YC supplementation did not affect ($P > 0.05$) α -amylase and chymotrypsin in the jejunum or lipase in the duodenum and jejunum, it increased ($P < 0.05$) α -amylase and chymotrypsin activities in the duodenum by 54.8 and 62.5%, respectively, compared with the control.

supplementation, data that are consistent with previous studies (Yalcin et al., 2008; Li et al., 2016). This divergence may be because of the differences in the domestic animal age, duration, doses, and varieties of yeast.

Activities of intestinal digestive enzymes, including lipase, chymotrypsin, and α -amylase, play pivotal roles in nutrient digestion and have been described as valuable parameters for feed utilization efficiency and performance of domestic animals (Yi et al., 2013). Interestingly, dietary YC supplementation significantly increased the activities of chymotrypsin and α -amylase in duodenal chyme. These findings are similar to a previous study that showed yeast products—autolyzed whole yeast and yeast cell wall—can improve pancreatic function and increase digestive enzyme synthesis of broilers (Ahiwe et al., 2019). These results indicated that YC supplementation improved the digestibility of the protein and starch components of the feed. Furthermore, these data explain the improved feed conversion efficiency observed in hens supplemented with YC. These outcomes were consistent with previous studies, which provided evidence that dietary YC supplementation can improve nutrient digestibility in dairy cows, sheep, and lambs (Chademana and Offer, 1990; Haddad and Goussous, 2005; Dias et al., 2017).

YC-mediated enhancement of intestinal health and immune function are recognized as pivotal factors for improved performance of domestic animals (Gao et al., 2008; Shen et al., 2009; Lee et al., 2018). Endotoxin, a component of the cell wall of gram-negative bacteria, is released by cell lysis. Once released, it exerts toxic effects on the host that cause severe intestinal damage (Hutcheson et al., 1990). Plasma endotoxin concentration has been well documented as a valuable parameter of intestinal permeability and health (Liu et al., 2018). In the current study, dietary YC supplementation reduced plasma endotoxin in laying hens, concomitant with improved intestinal barrier function. OCLN and CLDN1, which code tight junction proteins that play pivotal roles in maintaining intestinal barrier function (Pinton et al., 2009; Zhao et al., 2011), were upregulated by YC in the duodenum. These novel findings might explain the lower plasma endotoxin observed in the YC treated laying hens. Moreover, the YC-mediated improvement in gut integrity may be associated with several mechanisms: (1) increased abundance of *Lactobacillus* (Wu et al., 2018), which can improve intestinal barrier

Table 5. Effects of dietary YC supplementation on activities of digestive enzymes in chyme from duodenum and jejunum of aged laying hens¹.

Item	Chyme in duodenum		Chyme in jejunum	
	Control	YC	Control	YC
Lipase, U/mgprot	44.3 6 21.8	39.0 6 16.8	101.3 6 45.6	101.5 6 86.7
Chymotrypsin, U/mgprot	1.04 6 0.42 ^b	1.61 6 0.21 ^a	4.71 6 2.12	4.01 6 1.06
α -amylase, U/mgprot	6 0.06 ^b	6 0.07 ^a	6 0.23	0.56 6 0.17

^{a,b}Means within a row with different superscripts differ significantly (P, 0.05). ¹Results are reported as the mean 6 SD, n 5 5; YC 5 yeast culture.

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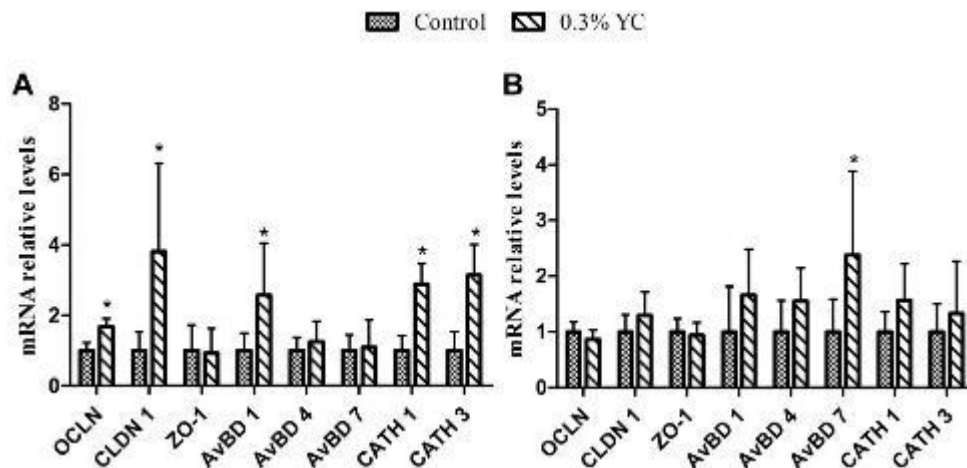


Figure 1. Effect of dietary YC supplementation on mRNA abundances of intestinal barrier-related genes and antimicrobial peptides genes relative to the control (set at 1.0) in the duodenum (A) and jejunum (B) of hens. Values are means 6 SD, n 5 5. Means with * are different from the control, P, 0.05. YC, yeast culture.

function by modulating goblet cells and increase the tight junction-related genes expression in intestine (Anderson et al., 2010); (2) decrease in *Escherichia coli* and *Salmonella* colonization in the intestine (Shanmugasundaram et al.,

2013), which can produce endotoxin and induce intestinal barrier dysfunction (Garber et al., 2012; Ren et al., 2017). Meanwhile, AvBD1 and AvBD7 code β -defense peptides that exhibit stronger activity against gram-negative bacterial strains (Derache et al., 2009); CATH1 and CATH3 code

antimicrobial peptides that can kill a broad range of gram-negative and gram-positive bacteria (Xiao et al., 2006). Interestingly, these genes were upregulated by dietary YC supplementation, a novel finding in the current study. Upregulation of these b-defense and antimicrobial peptide genes in the duodenum and jejunum of YC-treated laying

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hens could mediate the improved intestinal immune function. However, dietary YC supplementation did not affect the plasma IgA, IgG, and IgM levels, data that are inconsistent with a previous study (Fathi et al., 2012). This discrepancy may be because of the differences in the domestic animal species, age, and yeast varieties (Gao et al., 2008; Zhang et al., 2018).

In conclusion, the present study successfully confirmed that dietary supplementation of 0.3% YC improved the performance of aged laying hens, including reducing the feed/egg ratio and improving total egg weight and egg-laying rate. Furthermore, the positive effects of YC on the performance of laying hens were associated with enhanced intestinal digestive enzyme activities and intestinal health. Moreover, the improvement in the intestinal health by dietary YC supplementation was related to the upregulation of intestinal barrier-related genes and antimicrobial peptides genes. Overall, these findings provide a potential explanation for the mechanisms that mediate the positive effects of YC on laying hens. Thus, these findings will be beneficial for the nutritional management of aged laying hens.

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Conflict of Interest: All authors have read and approved the final manuscript and declared that no competing interests exist.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2019.11.017>.

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- Zhang, P., S. Cao, T. Zou, D. Han, H. Liu, J. Jin, Y. Yang, X. Zhu, S. Xie, and W. Zhou. 2018. Effects of dietary yeast culture on growth performance, immune response and disease resistance of gibel carp



Article

Effects of Yeast Culture Supplementation on Growth Performance, Nutrient Digestibility, Blood Metabolites, and Immune Response in Geese

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Simple Summary: Antibiotics benefit animal growth performance and health. However, an overuse of antibiotics can lead to antibiotic residue and the development of drug-resistant bacteria. Considering the possible serious consequences of antibiotic overuse, non-antibiotic alternatives, such as yeast and yeast products, are widely used in animal diets to improve growth and productive performance. Geese

of drug-resistant bacteria, and the inhibition of the animal's immune system [1–3]. Therefore, novel dietary supplements, such as probiotics, prebiotics, and other feed additives, have been used to replace antibiotics to improve animal health and maintain production efficiency [4–6]. Yeast cultures (YCs) are widely used as excellent feed additive candidates in animals [7]. YCs are

limited. On this basis, the effects of dietary yeast culture supplementation on growth performance, nutrient digestibility, blood metabolites, and immune functions of geese were studied. The results showed that the dietary addition of yeast culture could improve growth performance and nutrient digestibility and can modulate the immune response of geese. This dietary strategy based on feed additives is an effective method for improving the growth efficiency of the geese.

Abstract: The present study was conducted to investigate the effects of dietary yeast culture (YC) supplementation on growth performance, nutrient digestibility, blood metabolites, and immune functions in geese. One-day-old Sichuan white geese ($n = 300$) were randomly divided into five groups containing 0 (control), 0.5%, 1.0%, 2.0%, and 4.0% of YC in the diet for 70 days. In general, the dietary supplementation of YC significantly increased the average daily gain and feed conversion ratio ($p < 0.05$) in which the 1.0% or 2.0% levels were better and significantly reduced the average daily feed intake at the 2.0% level ($p < 0.05$). YC supplementation increased digestibility of P (quadratic, $p = 0.01$) and gross energy (quadratic, $p = 0.04$) from days 23 to 27 and crude protein from days 23 to 27 and days 64 to 68 (quadratic, $p \leq 0.05$), with the 2.0% level being the most effective. Serum metabolites were significantly affected by dietary YC ($p < 0.05$). Supplemental YC increased IL-2 on day 28 (linear, $p = 0.01$; quadratic, $p = 0.04$) and lysozyme on day 70 (quadratic, $p = 0.04$) and decreased complement C4 on day 70 (linear, $p = 0.05$). Interferon- γ , interleukin-2, and tumor necrosis factor- α genes were mostly up-regulated after YC supplementation, and interferon- γ and interleukin-2 gene expression levels were significantly increased at the 2.0% level ($p < 0.05$). Taken together, dietary YC supplementation improved growth performance and affected nutrient digestibility, serum metabolites, and immune function in geese, which was optimized at the 2% YC level in the present study.

Keywords: yeast culture; growth performance; digestibility; immune function; geese

1. Introduction

With the rapid development of large-scale and intensive animal husbandry, animal diseases have become more

complex and diverse, and veterinary drugs and antibiotic feed additives are widely and increasingly being used. However, frequent and excessive antibiotic use is an increasing concern because of possible antibiotic residues, the development

microecological products formed by yeast after sufficient anaerobic fermentation on specific media and are mainly composed of yeast metabolites, fermented medium, and a few yeast cells [3,8]. YCs are rich in vitamins, saccharides, minerals, enzymes, growth-promoting factors, and amino acids, which may benefit animal growth, metabolism, and health [8,9].

Many studies have reported the application of YCs in animals because of the potential benefits to animal production yields. For example, supplementing dairy cow diets with YCs improved lactation performance [10–12] and increased feed efficiency [13]. Supplemental YCs increased the carcass weights of calf-fed Holstein steers throughout the growingfinishing period [14]. Supplementing buffaloes with YC increased milk production by increasing the feed, energy, and nitrogen conversion efficiency [15]. Adding YCs significantly affected the ruminal pH in rams [16] and helped prevent and treat acute ruminal lactic acidosis in sheep [17]. In horses, dietary YC supplementation increased variability in *Streptococcus bovis* [18] and aided in fiber digestion [19]. In pigs, the dietary supplementation of YC improved growth performance and may be a good alternative to antibiotics [3,8]. YC feeding benefitted the gut microbiota, growth, and biochemical parameters in grass carp [7] and promoted feed intake, weight gain, and disease prevention in Nile tilapia [20]. YC supplementation improved growth performance and affected immune functions, Ca and P digestibility, and intestinal mucosal morphology in broilers [4] and increased egg weight and decreased egg yolk cholesterol without affecting performance or egg traits [9].

In summary, YCs have been extensively studied in livestock, especially ruminants, and several studies have been conducted on aquatic animals and poultry. However, no study has reported dietary YC supplementation in geese. Geese are economically important herbivorous waterfowl in central Europe and Asia, especially in China (90% of the world's production), and are characterized by rapid growth, high fecundity, and strong disease resistance [21]. Geese provide nutritious meat and eggs worldwide, are a valuable protein source for humans, and provide high-quality liver fat and feathers [22]. Because of its many benefits, we hypothesized that YC supplementation would influence performance in geese by improving digestion and immune functions. Therefore, this study was conducted to evaluate the effects of YC dietary supplementation on growth performance, nutrient digestibility, blood biochemical parameters, and immune functions in geese.

2. Materials and Methods

2.1. Animals, Diets, and Experimental Design

In total, three hundred healthy Sichuan white goose (mixed sex) at 1-day-old age having an average body weight (BW) of 95.57 ± 2.42 g were used and randomly divided into five groups containing 0 (control), 0.5%, 1.0%, 2.0%, and 4.0% of a commercial YC (Baihuibang, Beijing Enhalar Biotechnology Co., Ltd., Beijing, China) product in the diet. YC is formed by *Saccharomyces cerevisiae* (Sa-10) through a two-step continuous fermentation of liquid and solid. In the process of aerobic liquid fermentation, *Saccharomyces cerevisiae* cells fully proliferate. Then, the fermentation broth is injected into a medium composed of a variety of high-quality grains for anaerobic solid fermentation. The ingredient analysis shows that YC contains ~15.0% CP, ~3.5% crude fat, ~8.7% crude fiber, ~14.2% amino acid, ~3.3% mannan, ~14.0% β -glucan and rich in deformed medium, yeast cell walls, cell contents, and vitamins. The basal diets with corn-soybean meal in recommendations of the NRC (1994) [23] for starter (days 1–28) and for grower (days 29–70) periods were formulated (Table 1). Geese were housed from April to June in pens (3.5 m $\times$ 3.0 m) and reared in a windowed poultry house. Each group consisted of 6 replicates with 10 geese per pen. Geese were allowed access to feed (in pellet form) and water *ad libitum* throughout the entire experimental period. Feed was provided four times daily at 7:30, 12:30, 17:00, and 21:00 h. The house average temperature during the experiment period was $(21.32 \pm 2.39) ^\circ\text{C}$, and relative humidity was $(84.03 \pm 5.15)\%$. All geese management was consistent with recommendations of goose feeding management technical procedures. **Table 1.** Composition and nutrient content of basal diets (dry basis).

Ingredients (%)	Starter (Days 1–28)	Grower (Days 29–70)
Corn	63.80	53.60
Wheat bran	2.99	14.50
Soybean meal	20.00	11.50
Rapeseed meal	4.00	/
Rice bran	/	13.40

Silkworm chrysalis	4.30	1.79
CaHPO ₄	1.59	0.90
Limestone	0.87	0.75
L-Lysine (98.5%)	0.15	0.18
DL-Methionine	0.05	0.07
Salt (NaCl)	0.20	0.20
Choline chloride	0.05	0.12
Premix ¹	2.00	2.00
Sand	/	1.00
Total	100	100
Nutrient content		
Crude protein (%)	11.97	11.21
Metabolizable energy (MJ·kg ⁻¹) ²	20.43	14.81
Crude fiber (%)	4.12	8.04
Calcium (%)	0.87	0.80
Available P (%) ²	0.43	0.40
Lysine (%)	1.14	0.85
Methionine (%)	0.36	0.30

¹ The premix provides the following per kilogram of diet: VA 40,000 IU, VD₃ 2000 IU, VE 60 mg, VK₃ 2 mg, VB₁ 4 mg, VB₂ 24 mg, VB₆ 4 mg, VB₁₂ 50 µg, nicotinic acid 12 mg, pantothenic acid 36 mg, folic acid 4 mg, biotin 0.4 mg, Fe 120 mg, Cu 4 mg, Mn 300 mg, Zn 160 mg, I 0.2 mg, and Se 0.2 mg. ² Calculated values.

2.2. Sampling and Analyses

2.2.1. Growth Performance

Every morning the feed consumption before the feeding was recorded and BW was measured on day 1, 28 and 70 with empty stomach. Then calculated the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) for starter, grower and overall periods. Mortality was recorded when occurred during the experiment, including mortality date, BW, mortality reason, and feed consumed.

2.2.2. Nutrient Digestibility

The determination of nutrient digestibility was conducted by collecting total fecal once daily for 5 consecutive days from day 23 and again from day 64, as previously described [4] with minor modifications. In brief, total excreta collection was made from 3 geese in metabolic cages that were randomly selected in each pen. Metabolic cages were used only during excreta collection periods, and they were removed afterward. Excreta from each pen were collected, mixed, and weighed, and a 10% aliquot was sampled and frozen in -20 °C. Daily aliquots of excreta were combined per pen within each period, and a 10% aliquot was taken and dried in 70 °C. Dried excreta samples were crushed by a pulverizer and passed through a 40-mesh sieve and stored in a glass vial. Excreta samples were analyzed for Ca, P, crude protein (CP), and gross energy (GE).

2.2.3. Serum Metabolites

Blood samples of 18 fasted geese randomly selected from each dietary treatment (3 geese per pen) were collected from vena brachialis under the wing at day 28 and 70 for a biochemical parameter assay. Serum samples were collected by centrifugation at aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), 3500× g for 10 min and stored at -80 °C. Serum samples were analyzed for aspartate total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), and low-density lipoprotein (LDL) using a CL-8000 clinical chemical analyzer (Shimadzu, Kyoto, Japan) via standard enzymatic procedures.

2.2.4. Immune Factors

Serum lysozyme activity was measured by the method of turbidimetry using micrococcus lysodeikticus cells as substrate. Serum immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), complement C3, and complement C4 were measured by immunoturbidimetry using monoclonal antibodies. Serum interleukin-2 (IL-2) level was determined by antigen competition method of ELISA. All above tests were carried out using commercial kits (Solarbio, Beijing, China).

2.2.5. Immune Genes

On day 70, 18 geese from each treatment (3 geese per pen) were killed by cervical dislocation for measurement of spleen interferon- γ (*IFN- γ*), interleukin-2 (*IL-2*), and tumor necrosis factor- α (*TNF- α*) genes expression using quantitative PCR (q-PCR). Total RNA was extracted from spleen using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified using an RNeasy column (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using the oligo (dT) 6-mer primers provided in the PrimeScript RT Master Mix kit (TaKaRa, Dalian, China). q-PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa) on a CFX96 Real-Time PCR detection system (Bio-Rad). The house-keeping gene (β -actin) used as the endogenous control and target gene primer sequences used for q-PCR are shown in Table 2. All measurements were performed in triplicate, and negative controls (no cDNA template) were always included. The relative amounts of objective genes were calculated using the $\Delta\Delta C_t$ method.

Table 2. Primer sequences used for quantitative PCR.

Gene Name ¹	Primer Sequences (5'→3')	Product Size (bp)	GenBank No.
<i>IFN-γ</i>	F: ACATCAAAACCTGTCTGAGCAGC R: AGGTTTGACAGGTCCACGAGG	94	AF087134
<i>IL-2</i>	F: AATACTAGCACAGAGACAACCAG R: TTACTGAAATTTATTAATATCATCTA	768	AF294323
<i>TNF-α</i>	F: GAATGAACCTCCTCCG R: ATCTGGTTACAGGAAGG	139	EU375296
β -actin	F: CAACGAGCGGTTCAAGGTGT R: TGGAGTTGAAGGTGGTCTCGT	92	M26111

¹ *IFN- γ* : interferon- γ ; *IL-2*: interleukin-2; *TNF- α* : tumor necrosis factor- α .

2.3. Statistical Analysis

All data were analyzed using one-way ANOVA of SPSS 22.0 software package for Windows (IBM Corporation, 2014) with LSD multiple comparison tests. The effect of supplemental levels of YC was determined using orthogonal polynomials for linear and quadratic effects. Variability in data is expressed as the SE of means, and data were expressed to be statistically significant when a probability level of $p \leq 0.05$ was attained.

3. Results

3.1. Growth Performance

As shown in Table 3, dietary YC supplementation significantly affected the performance of BW, ADG, ADFI, and FCR of geese ($p < 0.01$), with the exception of ADFI during the starter period (days 1–28, $p > 0.05$). Compared with the control group, the YC-treated group showed improved ADG and FCR, whereas at the 2.0% level, YC significantly reduced ADFI during the grower (days 29–70) and overall (days 1–70) periods ($p < 0.05$). No differences in ADG and FCR were found between the YC-treated group at 1.0% and 2.0% ($p > 0.05$), specifically not at the 0.5% and 4.0% levels, which were more effective ($p < 0.05$) during the starter period. ADG was greater and ADFI and FCR were lower at the 2.0% level than at the 0.5%, 1.0%, and 4.0% levels during the grower and overall periods ($p < 0.05$), whereas the 0.5%, 1.0%, and 4.0% levels did not differ ($p > 0.05$). As the dietary YC level increased, BW, ADG, and FCR tended to be improved during the starter period (quadratic, $p = 0.06$), with the 1.0% and 2.0% levels being more effective. Mortality during the experimental period was 1.0% ($n = 3$) and was unaffected by the dietary treatments ($p > 0.05$).

Table 3. Effect of supplemental yeast cultures in geese diets on growth performance.

	Yeast Culture Supplementation (% of Diet)						<i>p</i> -Value	
	Control	0.5%	1.0%	2.0%	4.0%		L	Q
Mortality (goose)	0	1	1	0	1	0.26	/	/

BW (g/goose) d								
1	94.50	96.33	94.42	95.63	96.67	1.60	0.24	0.57
d 28	1001 ^a	1090 ^b	1184 ^c	1172 ^d	1000 ^a	2.32	0.78	0.06
d 70	2868 ^a	3025 ^b	3027 ^b	3474 ^c	3063 ^b	46.82	0.52	0.21
ADG (g/goose) d 1								
to 28	32.39 ^a	35.48 ^b	38.93 ^c	38.43 ^c	32.27 ^a	0.11	0.77	0.06
d 29 to 70	44.44 ^a	46.07 ^{ab}	45.86 ^a	54.82 ^c	49.12 ^b	1.14	0.33	0.29
d 1 to 70	39.62 ^a	41.83 ^b	41.89 ^b	48.26 ^c	42.38 ^b	0.66	0.52	0.21
ADFI (g/goose) d 1								
to 28	52.10	51.51	51.92	51.72	51.60	0.58	0.39	0.72
d 29 to 70	227.4 ^a	226.0 ^a	226.2 ^a	221.2 ^b	227.5 ^a	1.14	0.94	0.27
d 1 to 70	157.3 ^a	156.2 ^a	156.5 ^a	148.0 ^b	157.2 ^a	0.87	0.83	0.37
FCR d 1 to								
28	1.61 ^a	1.45 ^b	1.33 ^c	1.35 ^c	1.60 ^a	0.02	0.78	0.06
d 29 to 70	5.12 ^a	4.92 ^{ab}	4.93 ^{ab}	3.87 ^c	4.63 ^b	0.12	0.39	0.29
d 1 to 70	3.97 ^a	3.74 ^b	3.74 ^b	3.07 ^c	3.71 ^b	0.07	0.56	0.22
Item	SEM							

Note: Means within the same row with different superscript letters differ significantly ($p < 0.05$). Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

3.2. Nutrient Digestibility

The digestibilities of P and GE from days 23 to 27 and Ca and CP from days 64 to 68 were significantly affected ($p < 0.05$) by dietary YC supplementation (Table 4). Compared with the control group, Ca digestibility from days 64 to 68 and GE digestibility from days 23 to 27 were significantly decreased ($p < 0.05$) at the 4.0% level; at the 1.0% and 2.0% levels, YC significantly increased ($p < 0.05$) P and GE digestibility from days 23 to 27 and CP digestibility from days 64 to 68. With the increase in dietary YC, P and GE digestibility from days 23 to 27 and CP digestibility from days 23 to 27 and days 64 to 68 exhibited quadratic responses ($p \leq 0.05$), with the 2.0% level being the most effective.

3.3. Serum Metabolites

YC dietary treatments significantly affected ($p < 0.01$) the serum metabolite parameters on days 28 and 70 (Table 5). The concentration of ALT, ALP, and TG in the YC-treated groups significantly increased ($p < 0.05$) compared with those of the control group, whereas TC and HDL significantly decreased ($p < 0.05$) in general. Interestingly, the YC-treated groups had lower AST and LDL concentrations than did the control group on day 28 but greater AST concentration on day 70. Furthermore, the inclusion of YC tended to increase ALT (linear, $p = 0.05$), AST (linear, $p = 0.07$), and TG (linear, $p = 0.06$) concentrations on day 70.

Table 4. Effect of yeast culture supplementation in goose diets on nutrient digestibility.

Yeast Culture Supplementation (% of Diet)

p-Value

Note: Means within the same row with different superscript letters differ significantly ($p < 0.05$). Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

Table 5. Effect of yeast culture supplementation in goose diets on serum parameters.

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HDL (mmol/L) d								
28	4.65 ^a	4.09 ^b	5.15 ^c	4.26 ^b	4.58 ^a	0.08	0.97	1.0
d 70	3.24 ^a	3.61 ^b	2.91 ^c	2.66 ^d	2.64 ^d	0.01	0.13	0.32
LDL (mmol/L) d								
28	1.98 ^a	1.70 ^b	2.39 ^c	1.91 ^d	1.87 ^e	0.01	0.82	0.9
d 70	1.61 ^a	1.75 ^b	1.42 ^c	1.64 ^a	1.61 ^a	0.01	0.95	0.96

Parameter

SEM

Note: Means within the same row with different superscript letters differ significantly ($p < 0.05$). Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

3.4. Immune Factors

Adding YC to the geese diets significantly influenced ($p < 0.05$) the response of lysozyme, IL-2, IgM, complement C3, and complement C4 on days 28 and 70 (Table 6); however, the level of IgA was unaffected ($p > 0.05$). Dietary YC significantly affected IgG on day 70 ($p < 0.01$), but this response was not observed on day 28 ($p > 0.05$). Compared with the control group, YC supplementation significantly increased IL-2 and IgM at the 0.5% level; the lysozyme, IL-2, and IgM at the 2.0% level; and IgM at the 4.0% level on day 28 and on day 70 ($p < 0.05$). Moreover, 1.0% level also significantly increased IL-2 on day 28 and the lysozyme and complement C4 on day 70 ($p < 0.05$). However, 1.0% YC level significantly decreased the lysozyme on day 28 and IgG on day 70 ($p < 0.05$), and complement C4 on days 28 and 70 significantly decreased at the 2.0% and 4.0% levels ($p < 0.05$), respectively. As dietary YC increased, IL-2 increased on day 28 (linear, $p = 0.01$; quadratic, $p = 0.04$), and complement C4 decreased on day 70 (linear, $p = 0.05$). Lysozyme exhibited quadratic responses ($p = 0.04$) on days 70, with the 2.0% level being the highest.

Table 6. Effect of yeast culture supplementation in goose diets on immune factors.

	Yeast Culture Supplementation (% of Diet)					<i>p</i> -Value		
	Control	0.5%	1.0%	2.0%	4.0%	L	Q	
Lysozyme (μg/mL) d								
28	0.79 ^b	0.65 ^a	0.78 ^b	1.01 ^c	0.91 ^{bc}	0.06	0.25	0.47
d 70	0.40 ^a	0.73 ^b	0.79 ^b	1.09 ^c	0.48 ^a	0.04	0.99	0.04
IL-2 (ng/mL) d								
28	46.12 ^a	50.65 ^b	64.93 ^c	68.81 ^d	83.81 ^e	0.28	0.01	0.04
d 70	17.62 ^a	20.02 ^b	18.97 ^{ab}	19.72 ^b	17.50 ^a	0.63	0.61	0.32

IgA (g/L) d								
28	0.32	0.33	0.33	0.33	0.33	0.01	0.36	0.35
d 70	0.33	0.34	0.33	0.34	0.34	0.004	0.31	0.63
IgG (g/L) d								
28	2.34	2.54	2.48	2.49	2.39	0.15	0.83	0.46
d 70	2.44 ^a	1.65 ^b	2.23 ^a	2.50 ^a	2.31 ^a	0.11	0.65	0.92
IgM (g/L) d								
28	0.15 ^a	0.30 ^b	0.18 ^a	0.28 ^b	0.30 ^b	0.02	0.28	0.59
d 70	0.20 ^a	0.33 ^b	0.15 ^c	0.32 ^b	0.31 ^b	0.02	0.45	0.8
Complement C3 (g/L) d								
28	0.13 ^a	0.19 ^b	0.15 ^{ab}	0.15 ^{ab}	0.13 ^a	0.01	0.52	0.72
d 70	0.20 ^a	0.18 ^a	0.24 ^b	0.18 ^a	0.27 ^b	0.01	0.22	0.45
Complement C4 (g/L) d								
28	0.08 ^a	0.06 ^{ab}	0.08 ^a	0.04 ^b	0.06 ^{ab}	0.01	0.42	0.51
d 70	0.07 ^a	0.08 ^a	0.07 ^a	0.07 ^a	0.04 ^b	0.01	0.05	0.06
Item	SEM							

Note: Means within the same row with different superscript letters differ significantly ($p < 0.05$). Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

3.5. Immune Genes

Quantitative evaluation indicated that dietary YC supplementation significantly affected ($p < 0.05$) immune gene expression levels (Figure 1). Compared with the control group, *IFN- γ* gene expression levels significantly increased at 1.0% and 2.0% ($p < 0.05$) and decreased at 4.0% ($p < 0.05$). *IL-2* gene expression levels increased significantly at 0.5%, 1.0%, and 2.0% ($p < 0.05$), and *TNF- α* gene expression levels were greater at 0.5%, 2.0%, and 4.0% ($p < 0.05$). *IFN- γ* and *IL-2* gene expression levels were the highest at 2.0%, moderate at 0.5% and 1.0%, and the lowest at 4.0%. The *TNF- α* gene expression level tended to increase linearly ($p = 0.06$) with increasing levels of YC.

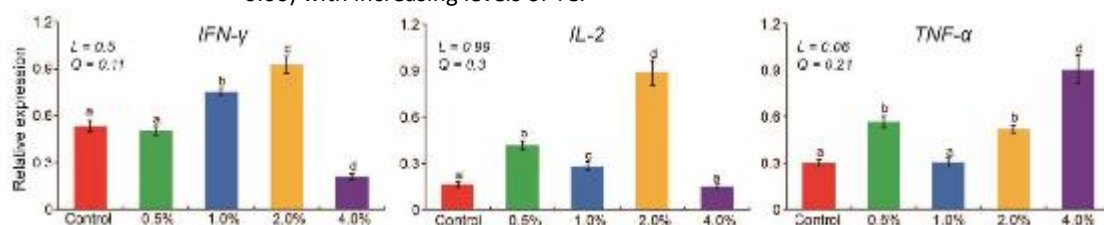


Figure 1. Effect of yeast culture supplementation in goose diets on immune gene expression. Means with different letters differ significantly ($p < 0.05$). Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

4. Discussion

In this study, YC supplementation did not affect goose mortality ($p > 0.05$), which is consistent with previous studies on laying hens [9], broilers [4], quails [24], and calf-fed Holstein steers [14]. Conversely, Tangendjaja [25] reported that YC supplementation in laying hens significantly reduced mortality ($p < 0.05$). Similarly, Fathi et al. [26] found that broilers fed a diet supplemented with 1.25 g/kg of YC had lower mortality rates throughout the experiment ($p < 0.05$). Barens et al. [27] indicated that mortality was significantly reduced in rainbow trout during the first 4 weeks of rearing with YC-supplemented diets ($p < 0.05$). The specific mechanisms of YC on health and/or viability remained unclear because the results are affected by multiple factors such as species, environment, and feed.

In the current study, supplemental YC improved body weight gain (BWG) and FCR in geese. In poultry, YC research mainly focused on chickens, and the results suggest that YC benefits BWG and FCR [2,4,9,26]. At appropriate YC levels, BWG was unattributed to increased feed consumption, and even Liu et al. [28] indicated that 0.2% dietary YC supplementation decreased feed intake in laying hens. Studies with livestock on cattle [14,29,30], sheep [31], and pigs [3,8,32] also demonstrated that dietary YC supplementation increased ADG. These benefits may be because YC supplementation influences gastrointestinal microbiota and intestinal morphology to improve nutrient digestion and retention [3,10,33], and it increases the relative abundances of cellulolytic, amylolytic and lactate-utilizing bacteria [34]. Additionally, YCs contain peptides, organic acids, oligosaccharides, amino acids, and other factors that promote animal growth [4]. In the present study, BWG and FCR tended to be best at the 2.0% level, possibly because YC interacted with the gastrointestinal system by triggering proper levels. Similarly, low YC levels would more effectively improve immune response performance because demand for the immune response is minimal under low disease challenge or stress conditions [4]. However, other studies reported that YC supplementation did not increase BWG [15,35,36]. Different responses may be related to different dietary nutritional densities, durations of supplementation, and yeast product formulations, making comparisons among studies difficult [4,15].

In the present study, YC supplementation improved Ca, P, CP, and GE digestibilities, with better responses at the 2.0% level for P, CP, and GE. Several studies have demonstrated that probiotics, including YCs, are one approach to improving nutrient digestibility. Shen et al. [8] indicated that dietary YC supplementation improved GE and CP digestibility in nursing pigs. Gao et al. [4] and Bradley et al. [37] showed that supplemental YC improved the utilization of digested P and Ca in poultry. This improvement in nutrient utilization may have been due to YC constituents, including phytase [4], oligosaccharides [38], and growth factors [39]. Previous studies reported that increased villus/crypt ratios [40] and feed retention times [41] exert positive effects on nutrient digestibility. The effect of YC on the villus/crypt ratio and feed retention times for geese was not measured in our experiment and, thus, warrants further research. However, other studies showed that YC did not affect energy or protein digestibility [4,36]. Dias et al. [13] found that YC supplementation did not affect the total-tract apparent digestibility of nutrients in dairy cows.

Blood metabolite values are frequently used to assess growth and general metabolic health status in animals [15,42]. For example, serum ALT and AST are used to indicate liver damage; TC, TG, HDL, and LDL are used to indicate fat mobilization, and ALP is used to indicate growth [15]. The ALT and AST results in the present study indicated that YC supplementation negatively affected liver health, possibly because the liver had not adapted to the rapid growth, which was confirmed by ALP. Studies on laying hens [9], buffaloes [15], lactating ewes [43], dairy cows [44], goats [45], and rams [16] reported that YC supplementation did not affect serum ALT or AST. Nursoy and Baytok [46] found increased TG in YC-fed dairy cows, but Chen et al. [2] indicated that TG was unaffected in YC-fed broiler chickens. Research on rabbits [47] and broiler chickens [48,49] showed that probiotic supplementation reduced TC and LDL, which was consistent with the results of the present study. However, other studies reported no difference in TC or LDL levels between probiotic-supplemented and control groups [2,50]. Evidence has shown that probiotics and their components regulate host lipid metabolism via active substances that deconjugate bile salts in the intestines, thereby preventing bile salts from acting as precursors in cholesterol synthesis [51,52]. However, other studies reported inconsistent results, likely because of differing doses, ration compositions, animal species, animal ages, and supplementation strains.

Studies have reported that yeast supplementation modulated immune responses in humans [53], chicks [4], pigs [8], dairy cows [54], and fish [20] to maintain health and improve growth performance by reducing pathogenic bacteria and improving gut health. Similarly, cellular studies also support the role of YC in innate immune functions [55]. In this study, dietary YC increased the serum lysozyme, IL2, IgM, and complement C3 contents, particularly at the 0.5% and 2.0% levels, which is consistent with the findings of Gao et al. [4] and Fathi et al. [26] who reported increased lysozyme and IgM in YC-fed birds. Studies have shown that probiotics (e.g., YCs) stimulate animal immunity via the following methods: (i) flora from the probiotics migrate throughout the gut wall and multiply to a limited extent; (ii) dead organisms release antigens that are absorbed and stimulate the immune system [51]; (iii) the luminal and mucosal gut microbiotas are modulated and protected against inflammation [53]; (iv) yeast cell wall polysaccharides can positively affect immune functions [54]; (v) harmful and pathogenic microorganisms are competitively excluded [56]; (vi) probiotics benefit the intestinal environment and improve intestinal morphology [3,57]; (vii) probiotics help maintain a physiological balance of immunopotent cells, thus providing a healthy environment for the immune system; (viii) oligosaccharides in yeast cell walls bind to viruses and function as vaccine adjuvants to increase antibody titers [26]. Surprisingly, YC did not affect IgA and even reduced IgG in the present study. The reason for this is unknown, and further studies are necessary to interpret these results.

Dong and Wang [58] indicated that the dietary prebiotic significantly increased immune gene expressions in red swamp crayfish, suggesting that YC potentially affects immune genes. In the present study, YC supplementation upregulated three immune genes, *IFN-γ*, *IL-2*, and *TNF-α*, in the spleen, especially at the 2% level, with *IFN-γ* and *IL-2* exhibiting the highest expressions. This result is supported by the protein study of

Shen et al. [8], who reported that YC increased cytokine IFN-γ production by triggering a Th-1 response in the gut. IFN-γ and IL-2 activate macrophages, while TNF-α is produced by macrophages. Therefore, the results may be explained in that YC improves animal immunity by activating macrophages [4,8] that can rapidly and efficiently phagocytize the bacteria.

Based on the response of the measured indicators to different levels of YC, the results showed that the optimal YC level was 2% on the whole, and 4.0% YC was not more effective in the present study, which may be due to the high levels of YC causing geese to develop immune tolerance, leading to the wastage of energy and nutrients and suppressing growth performance [59].

5. Conclusions

Dietary YC supplementation improves growth performance and affects nutrient digestibility, serum metabolites, and immune function of geese. Growth performance was the best at the 2% YC supplementation level under the experimental conditions of this study. These results may be attributed to YC modulating the animals' immune status.

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Poultry

Effect of yeast culture supplementation on blood characteristics, body development, intestinal morphology, and enzyme activities in geese

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Abstract

The objective of this experiment is to evaluate the effects of yeast culture (YC) supplementation on blood characteristics, body size, carcass characteristics, organ weights, intestinal morphology, and enzyme activities. Five groups of geese were randomly assigned to five dietary treatments: the basal diet (control) and basal diets plus 0.5%, 1.0%, 2.0%, or 4.0% YC. Compared with the controls, YC supplementation at 0.5% and 1.0% increased the serum total protein (TP), albumin (ALB), and globulin (GLO) and decreased the uric acid and creatine kinase (CK) contents ($p < 0.05$). YC supplementation at 2.0% and 4.0% increased the CK, growth hormone, catalase and glutathione reductase contents, and relative proventriculus weights, and decreased the TP, ALB, and GLO contents, relative liver, gizzard, jejunum, ileum, and thymus weights ($p < 0.05$). YC supplementation at 2.0% improved fossil bone length, breast muscle percentage, jejunal villus height, ileal and jejunal villus height/crypt depth ratios, pepsin, lipase, amylase and pancreatic trypsin activities, and decreased abdominal fat percentage ($p < 0.05$). Furthermore, YC inclusion increased the body slope length (linear, $p = 0.002$; quadratic, $p = 0.02$), breast width (quadratic, $p = 0.02$), ileal (linear, $p = 0.04$; quadratic, $p = 0.01$) and duodenal villus height (cubic, $p = 0.04$), and decreased the relative gizzard (quadratic, $p = 0.04$) and thymus (linear, $p = 0.002$; quadratic, $p = 0.02$; cubic, $p = 0.02$) weights, liver (linear, $p = 0.002$; quadratic, $p = 0.02$), and serum (linear, $p = 0.006$; quadratic, $p = 0.03$) malondialdehyde contents, and jejunal crypt depth (quadratic, $p = 0.03$). The findings indicated that the YC supplementation had a positive effect on the growth and development of geese, with 2% YC being the most effective.

KEYWORDS

blood characteristics, body development, enzyme activity, geese, intestinal morphology, yeast culture

Jie Zhang and Yancong Yuan contributed equally to this study.

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1 | INTRODUCTION

Yeast cultures (YCs) are micro-ecological products formed by yeast after sufficient anaerobic fermentation on specific media and are mainly composed of yeast metabolites, fermented medium, and a few yeast cells (van der Peet-Schwering et al., 2007; Shen et al., 2009). YCs are rich in vitamins, saccharides, minerals, enzymes, growth-promoting factors, and amino acids (Shen et al., 2009; Yalçın et al., 2008), which have been used to replace antibiotics to improve animal growth, metabolism, health, and maintain production efficiency (Bach, 2001; Gao et al., 2008; Smith et al., 2002). In dairy cows, supplementing diets with YCs improved lactation performance (Dias et al., 2017a, 2017b; Salvati et al., 2015) and increased feed efficiency (J. Dias et al., 2018). In sheep, adding YCs can be beneficial in the prevention and treatment of acute ruminal lactic acidosis (Reis et al., 2018). In pigs, dietary supplementation of YC improved growth performance and modulated gut immune response (van der Peet-Schwering et al., 2007; Shen et al., 2009). In aquatic animals, YC feeding benefitted the gut microbiota and promoted feed intake, weight gain, and disease prevention (Berto et al., 2016; Liu et al., 2018). Currently, studies about YC in poultry are mainly focused on chickens. For example, YC supplementation improved growth performance, immune functions, and carcass yield in broilers (Fathi et al., 2012; Gao et al., 2008). YC supplementation to laying hen diets increased egg weight and decreased egg yolk cholesterol without affecting performance and egg traits (Yalçın et al., 2008). In addition, YC also provides other benefits such as improved organ weights (Adejumo et al., 2006), enzyme activity (Jin et al., 2000; Tagang et al., 2013), meat quality, and antioxidant capacity (J. Zhang et al., 2021) in poultry. However, research on the application of dietary YC supplementation to geese is limited. Therefore, the objective of this study was to evaluate the effects of dietary YC supplementation in geese on blood characteristics, body development, intestinal morphology, and enzyme activities.

2 | MATERIALS AND METHODS

2.1 | Experimental design and goose husbandry

Three hundred 1-day-old healthy mixed-sex Sichuan white geese, with average body weights of 95.57 ± 2.42 g, were used in a completely randomised study and divided into five groups fed either 0% (control), 0.5%, 1.0%, 2.0%, or 4.0% commercial YC product (Beijing Enhelor Biotechnology). The YC was a fermented product composed of inactivated *Saccharomyces cerevisiae* grown on a medium. The ingredient analysis shows that YC contains ~15.0% crude protein, ~3.5% crude fat, ~8.7% crude fibre, ~14.2% amino acid, ~3.3% mannan, ~14.0% β -glucan, and other microcomponents. The corn-soybean meal basal diets were formulated (Table 1) to meet the recommendations of the National Research Council (1994) during the starter (Days 1–28) and grower (Days 29–70) periods. Geese were housed in pens (3.5 m $\times$ 3.0 m) and net-reared in a windowed poultry house from April to June 2019. Each group consisted of 6 replicate pens with 10 geese per pen. Geese were injected with *Astragalus* polysaccharides (Yongjian Biological) and administered multidimensional electrolytes in their water on Days 1 and 7. Geese were allowed access to feed (in pellet form) and water ad libitum

throughout the experimental period. Feed was provided four times daily at 7:30, 12:30, 17:00, and 21:00 h. The average house temperature during the experimental period was $21.32 \pm 2.39^\circ\text{C}$, and the relative humidity was $84.03 \pm 5.15\%$.

TABLE 1 Composition and nutrient content of starter (Days 1–28) and grower (Days 29–70) basal diets (dry basis)

Items	Starter	Grower
Ingredients (%)		
Corn	63.80	53.60
Wheat bran	2.99	14.50
Soybean meal	20.00	11.50
Rapeseed meal	4.00	–
Rice bran	–	13.40
Silkworm chrysalis	4.30	1.79
CaHPO ₄	1.59	0.90
Limestone	0.87	0.75
L-lysine (98.5%)	0.15	0.18
DL-methionine	0.05	0.07
Salt (NaCl)	0.20	0.20
Choline chloride	0.05	0.12
Premix ^a	2.00	2.00
Sand	0	1.00
Total	100	100
Nutrient content		
Metabolisable energy (MJ kg ⁻¹) ^b	11.97	11.21
Crude protein (%)	20.43	14.81
Crude fibre (%)	4.12	8.04
Calcium (%)	0.87	0.80
Available P (%) ^b	0.43	0.40
Lysine (%)	1.14	0.85
Methionine (%)	0.36	0.30

^aThe premix provides the following per kilogram of diet: VA 2000 IU, VD₃ 1000 IU, VE 3000 mg, VK₃ 200 mg, VB₁ 100 mg, VB₂ 1200 mg, VB₆ 200 mg, VB₁₂ 2.5 mg, nicotinic acid 600 mg, pantothenic acid 1800 mg, folic acid 200 mg, biotin 20 mg, Fe 6 g, Cu 0.2 g, Mn 15 g, Zn 8 g, I 10 mg, and Se 30 mg. ^bCalculated according to Chinese Feed Database News Web Center (2005).

2.2 | Experimental procedures

2.2.1 | Blood characteristics

Blood samples of 18 fasted geese randomly selected from each group (3 geese per pen) were collected from vena brachialis under the wing at Day 70. Samples were collected without anticoagulant, let stand for 10 min, and centrifuged at 3500 g for 10 min at room temperature, and then the resultant sera were stored at -80°C . Serum samples were analysed for total protein (TP), albumin (ALB), globulin (GLO), uric acid (UA), and creatine kinase (CK) using CL-8000 clinical chemical analyzer (Shimadzu) via standard enzymatic procedures.

2.2.2 | Body development

Body size

The body size was determined with reference to the national standard NY/T 823-2020 (MOAPRC, 2020). The body slope length, fossil bone length, breast depth, breast width, shank length, shank circumference, pelvis width, neck length, and half-diving depth were measured with a caliper or tape.

Carcass characteristics

After blood collection, the same geese were weighed individually and sacrificed by cervical dislocation and exsanguinated to evaluate the carcasses. The feathers, foot cuticle, toe shell, and beak shell were manually removed after scalding at 60°C for approximately 2 min, then the carcass was weighed. Next, the half eviscerated, eviscerated, leg muscle, breast muscle and abdominal fat were weighed, and each portion was expressed as a percentage of the live body weight.

Organ weights

The heart, liver, spleen, gizzard, proventriculus, thymus, pancreas, bursa of Fabricius, duodenal, jejunum, and ileum were removed and weighed. Organ weight was expressed as a percentage of live body weight.

2.2.3 | Intestinal morphology

Three centimeters of the duodenum, jejunum, and ileum were removed, rinsed in Tris-buffered saline, and fixed in 4% paraformaldehyde. Fixed intestinal segments were routinely processed, embedded in paraffin, sliced into 5- μm -thick sections, and stained with hematoxylin and eosin for light microscopy measurements of the villus height and crypt depth at $\times 22$ magnification. All villi and crypts were measured in triplicate for each tissue.

2.2.4 | Enzyme activities

Immediately following organ weighing, aliquots of the liver, proventriculus, pancreas, and duodenal samples (approximately 5 g each) were removed from each goose, cryogenically frozen in liquid nitrogen, and stored in a -80°C freezer until use. The activities of glutathione (GSH), catalase (CAT),

glutathione reductase (GR), malondialdehyde (MDA), growth hormone (GH), pepsin, trypsin, lipase, and amylase were measured using commercial analytical kits according to the manufacturer's instructions (Solarbio).

2.3 | Statistical analysis

Data were analysed using one-way analysis of variance of the SPSS 22.0 software package for Windows (IBM Corporation, 2014) with least significant difference multiple comparison tests. The effect of supplemental YC levels was determined using orthogonal polynomials for linear, quadratic, and cubic effects. Variability in the data is expressed as the standard error of means and a probability level of $p \leq 0.05$ was considered to be statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Blood characteristics

Dietary YC significantly affected the serum TP, ALB, GLO, UA, CK, and GH contents ($p < 0.05$; Table 2). Compared with the controls, the serum TP, ALB, and GLO contents increased at 0.5% and 1.0% YC supplementation and decreased at 2.0% and 4.0% YC supplementation; 0.5% supplementation showed a higher increase than did 1.0% supplementation ($p < 0.05$). No differences occurred at 2.0% or 4.0% supplementation. Supplemental YC at 2.0% and 4.0% increased the CK contents, whereas 0.5% and 1.0% decreased the UA and CK contents compared with those of the controls ($p < 0.05$). CK mainly exists in the cytoplasm and mitochondria and is directly related to intracellular energy operation, muscle contraction, and ATP regeneration. The increase in CK contents in the 2% and 4% may be due to accelerated muscle contraction and energy operation caused by the goose struggling vigorously during sampling, which, in turn, resulted in elevated contents in the blood. GH content did not differ among the 0.5%, 1.0% and control groups but improved at 2.0% and 4.0% ($p < 0.05$). The 2.0% level showed the best improvement, which is consistent with previous findings that 2% YC yielded the maximum growth rate. Dietary YC supplementation affected the blood metabolism because yeast products are rich in vitamins, saccharides, minerals, enzymes, growth-promoting factors, and amino acids, which improve digestibility, immune status, and growth (Gao et al., 2008; Salinaschavira et al., 2017). Abouelnour (1998), Doto et al. (2011), and Xiong et al. (2015) reported similar results. However, Stanley et al. (2004) and Chen et al. (2013) indicated that dietary YC did not affect the TP, ALB, or GLO contents. The results also showed that TP and GH varied inconsistently as the YC levels increased, suggesting that YC efficiency exerts different effects on protein absorption and utilisation (Doto et al., 2011).

TABLE 2 Effects of yeast culture supplementation in goose diets on blood biochemical parameters

Items	Yeast culture supplementation (% of diet)					P L		Q C	
	(Control)	0.5	1.0	2.0	4.0				
TP (g/L)	37.60 ^a	44.10 ^b	39.17 ^c	35.43 ^d	35.33 ^d	0.15	0.27	0.62	0.58
ALB (g/L)	15.30 ^a	18.33 ^b	16.13 ^c	14.17 ^d	14.10 ^d	0.06	0.25	0.59	0.53
GLO (g/L)	22.30 ^a	25.83 ^b	23.00 ^c	21.17 ^d	21.23 ^d	0.18	0.29	0.65	0.62
UA (μmol/L)	221.00 ^a	134.00 ^b	166.00 ^c	227.00 ^d	224.67 ^d	5.43	0.42	0.75	0.40
CK (U/L)	515.00 ^a	486.00 ^b	399.00 ^c	717.33 ^d	660.67 ^d	17.16	0.22	0.54	0.39
GH (ng/ml)	0.94 ^a	0.93 ^b	0.99 ^b	1.88 ^c	1.33 ^b	0.05	0.42	0.39	0.53

Note: ^{a-c}Means within a row with no common superscripts differ significantly ($p < 0.05$).

Orthogonal contrasts: L = linear, Q = quadratic, and C = cubic effect of yeast culture supplementation.

Abbreviations: ALB, albumin; CK, creatine kinase; GH, growth hormone; GLO, globulin; TP, total protein; UA, uric acid.

3.2 | Body development

3.2.1 | Body size

As shown in Table 3, YC supplementation at 2.0% significantly increased the fossil bone length compared with that of the controls ($p < 0.05$). The body slope length (linear, $p = 0.002$; quadratic, $p = 0.02$) and breast width (quadratic, $p = 0.02$) increased as dietary YC increased. Body size is an important trait that directly or indirectly affects animal growth performance (Munim et al., 2013). The results of this study confirmed that the body size is consistent with the positive effect of YC supplementation on body weight (Gao et al., 2008; Yalçın et al., 2008). This may be because YC improves intestinal morphology and increases the absorption rates of Ca and P required for body size growth (Gao et al., 2008). Furthermore, it is also confirmed from the results of intestinal morphology in this article.

3.2.2 | Carcass characteristics

Compared with that of the controls, 2.0% YC supplementation significantly increased breast muscle percentages ($p < 0.05$) and decreased abdominal fat percentages ($p < 0.05$). Fathi et al. (2012) observed significantly increased breast muscle percentages in broilers fed 1.5 g/kg of YC. Similarly, broilers fed a diet containing 1% yeast extract showed significantly improved breast yields. Lactobacillus supplementation in broiler diets effectively reduced abdominal fat deposition after 28 days of age (Kalavathy et al., 2003). Onifade et al. (1999) also showed that broilers fed yeast (*S. cerevisiae*) had lower abdominal fat depositions. Also, Li et al. (2019) reported an increased carcass and breast yield, gizzard weight, and decreased abdominal fat by supplementing yeast up to 0.3% in diets of broiler. The higher carcass yield of geese-fed diets supplemented with YC in the present study may be attributed to better digestive function as it provides more available nutrients including vitamins and minerals, required for muscle growth (Cheng et al., 2017).

3.2.3 | Organ weights

Dietary YC did not affect the relative heart weights. YC supplementation at 2.0% and 4.0% increased the relative weights of the proventriculus, decreased those of the liver, gizzard, jejunum, ileum, and thymus (all $p < 0.05$); and did not affect those of the pancreas, spleen, and bursa of Fabricius compared with the controls. YC inclusion also decreased the relative gizzard (quadratic, $p = 0.04$) and thymus (linear, $p = 0.002$; quadratic, $p = 0.02$; cubic, $p = 0.02$) weights, and these decreases were minimal at the 2.0% and 4.0% levels. Similarly, Alcicek et al. (2004) and Bozkurt et al. (2011) reported that dietary supplementation with prebiotics and probiotics decreased the relative liver and small intestinal weights in broilers. Engberg et al. (2000) found that antimicrobial activity in the gut lumen thinned the intestinal wall and reduced the intestinal weight. However, Adejumo et al. (2006), Tagang et al. (2013), and Jin et al. (1998) reported inconsistent results for broilers, and Chen et al. (2013) reported inconsistent results for geese.

3.3 | Intestinal morphology

YC supplementation and sampling sites variably affected the intestinal villus heights and villus height/crypt depth ratios (VCRs) of the geese (Table 4). Dietary YC supplementation significantly increased the jejunal villus height ($p < 0.05$), which was significantly higher after 2.0% YC supplementation than after 0.5%, 1.0%, or 4.0% YC supplementation ($p < 0.05$). YC supplementation increased the ileal (linear, $p = 0.04$; quadratic, $p = 0.01$) and duodenal (cubic, $p = 0.04$) villus heights. Increasing the YC supplementation rate did not affect the intestinal crypt depth and decreased the jejunal crypt depth (quadratic, $p = 0.03$). Similarly, Gao et al. (2008) found that YC supplementation increased the duodenal villus height and reduced jejunal crypt depth in 42-day-old broilers. Shen et al. (2009) observed increased jejunal villus heights, while crypt depth was unaffected in pigs supplemented with YC, and van der PeetSchwering et al. (2007) reported that dietary YC did not affect crypt

TABLE 3 Effects of yeast culture supplementation in geese diets on goose body development

Yeast culture supplementation (% of diet)							p		
Items	0 (Control)	0.5	1.0	2.0	4.0	SEM	L	Q	C
Body size (cm)									
Body slope length	26.17 ^a	26.31 ^a	26.36 ^a	27.17 ^{ab}	28.33 ^b	0.42	0.002	0.02	0.10
Fossil bone length	12.50 ^a	14.67 ^{b,c}	13.83 ^{a,b,c}	15.33 ^c	13.33 ^{a,b}	0.54	0.94	0.59	0.81
Breast depth	6.54	6.69	6.48	6.64	6.27	0.13	0.17	0.28	0.61
Breast width	5.65	5.95	6.06	6.24	5.99	0.14	0.41	0.02	0.13
Shank length	7.78	8.44	7.97	8.19	8.36	0.29	0.38	0.72	0.87
Pelvis width	6.29	6.44	5.68	5.86	5.97	0.22	0.46	0.48	0.80
Half-diving depth	64.83	66.33	61.67	61.33	61.00	1.64	0.15	0.30	0.65
Carcass characteristics (%)									
Carcass	89.53	91.38	91.53	89.84	91.73	1.17	0.47	0.81	0.06
Half eviscerated	81.04	83.14	83.25	82.63	82.64	1.08	0.65	0.57	0.25
Eviscerated	72.13	75.04	74.70	74.24	73.80	1.22	0.82	0.57	0.43
Leg muscle	10.92	11.38	11.74	11.27	11.94	0.42	0.16	0.45	0.23
Breast muscle	4.45 ^a	4.49 ^a	4.66 ^a	5.42 ^b	4.27 ^a	0.18	0.89	0.36	0.16
Abdominal fat	3.36 ^a	2.49 ^{b,c}	2.95 ^{a,b}	2.18 ^c	2.93 ^{a,b}	0.16	0.74	0.37	0.72
Organ weights (%)									
Heart	0.88	0.85	0.85	0.88	0.82	0.04	0.25	0.52	0.19
Liver	2.25 ^a	1.83 ^b	1.82 ^b	1.90 ^b	1.77 ^b	0.08	0.28	0.46	0.27
Gizzard	3.16 ^a	2.89 ^b	2.59 ^c	2.50 ^c	2.70 ^{b,c}	0.08	0.33	0.04	0.16
Proventriculus	0.35 ^a	0.27 ^c	0.34 ^a	0.39 ^b	0.39 ^b	0.01	0.23	0.56	0.57
Pancreas	0.35 ^{a,b}	0.33 ^{b,c}	0.32 ^{b,c}	0.38 ^a	0.31 ^c	0.01	0.64	0.69	0.22
Duodenal	0.26 ^a	0.20 ^c	0.25 ^{a,b}	0.22 ^{b,c}	0.25 ^{a,b}	0.01	0.84	0.77	0.94
Jejunum	0.72 ^a	0.53 ^d	0.66 ^b	0.71 ^{a,b}	0.59 ^c	0.02	0.73	0.91	0.73
Ileum	0.60 ^a	0.50 ^b	0.52 ^b	0.53 ^b	0.51 ^b	0.02	0.41	0.60	0.49
Spleen	0.07 ^{ab}	0.06 ^b	0.08 ^a	0.07 ^{a,b}	0.08 ^a	0.01	0.32	0.68	0.91
Bursa of Fabricius	0.29 ^{a,b,c}	0.26 ^c	0.27 ^{b,c}	0.32 ^a	0.30 ^{a,b}	0.01	0.36	0.68	0.12
Thymus	0.16 ^a	0.14 ^b	0.13 ^{b,c}	0.12 ^c	0.07 ^d	0.01	0.002	0.02	0.02

Note: ^{a-c}Means within a row with no common superscripts differ significantly ($p < 0.05$).

Orthogonal contrasts: L = linear, Q = quadratic, and C = cubic effect of yeast culture supplementation.

depth. Taller villi, by providing an increased surface area, have the capability of greater absorption of available nutrients (Khalid et al., 2021).

Compared with the controls, dietary YC supplementation at 1.0%, 2.0%, and 4.0% significantly increased the ileal and jejunal VCRs ($p < 0.05$), with a maximal increase at 2.0%. Gao et al. (2008) reported increased intestinal VCRs and that broilers fed 2.5 g/kg showed enhanced performance after YC supplementation. Shen et al. (2009) also observed increased jejunal VCRs in pigs supplemented with YC. This may be due to the mannan oligosaccharides (MOS) and β -glucan in YC promoting intestinal health by enhancing intestinal immunity. VCRs reflect the development of the intestinal mucosa.

The better the development and the more perfect the structure, the more the number of goblet cells, which can secrete more mucin to improve the immune protection of the mucosa, thus ensuring the smooth immunity of the intestinal mucosa (Liao, 2009). It also has been reported that MOS can bind to the receptors on the surface of intestinal mucosal immune cells of piglets to activate the intestinal mucosal immune response and promote the production of intestinal mucosal secretory immunoglobulin A (Halas & Nochta, 2012). Moreover, the improvement in intestinal health by dietary YC supplementation was related to the upregulation of intestinal barrier-related genes and antimicrobial peptides genes (J.-C. Zhang et al., 2020).

TABLE 4 Effect of dietary yeast culture supplementation on intestinal morphology of geese

Yeast culture supplementation (% of diet)							p			
Items	(Control)	0.5	1.0	2.0	4.0	SEM	Q	C		
Villus height (μm)										
Ileum		787.67 ^a	813.67 ^{a,b}	857.67 ^{b,c}	895.67 ^c	908.67 ^c	16.44	0.04	0.01	0.12
Jejunum		1363.00 ^a	1325.67 ^a	1438.00 ^b	1670.33 ^c	1555.67 ^d	22.80	0.18	0.23	0.13
Duodenum		969.00 ^a	986.33 ^a	1052.67 ^b	1194.67 ^c	1105.67 ^b	16.94	0.21	0.12	0.04
Crypt depth (μm)										
Ileum		206.00	206.33	192.67	197.67	220.00	9.38	0.32	0.12	0.43
Jejunum		236.33	228.33	214.00	208.00	214.67	8.49	0.22	0.03	0.22
Duodenum		218.33	208.67	195.67	219.33	223.67	11.24	0.40	0.61	0.42
VCR										
Ileum		3.84 ^a	3.96 ^a	4.46 ^b	4.54 ^b	4.14 ^{a,b}	0.15	0.57	0.11	0.41
Jejunum		5.77 ^a	5.82 ^a	6.74 ^b	8.04 ^c	7.27 ^b	0.17	0.18	0.12	0.15
Duodenum		4.46	4.74	5.43	5.46	4.97	0.24	0.54	0.10	0.37

Note: ^{a-d}Means within a row with no common superscripts differ significantly ($p \leq 0.05$).

Orthogonal contrasts: L = linear, Q = quadratic, and C = cubic effect of yeast culture supplementation. Abbreviation: VCR, villus-to-crypt ratio.

TABLE 5 Effects of yeast culture supplementation in goose diets on digestive enzymes

Items	Yeast culture supplementation (% of diet)					SEM	p L		
	0 (control)	0.5	1.0	2.0	4.0			Q	C
Pepsin (U/mg)	9.96 ^a	10.32 ^a	10.18 ^a	12.06 ^b	9.85 ^a	0.50	0.93	0.34	0.32
Duodenum									
Trypsin (U/mg)	3.04	3.11	3.22	3.64	2.79	0.26	0.69	0.16	0.07
Lipase (U/g)	426.06 ^c	473.85 ^b	471.40 ^b	531.47 ^a	428.23 ^c	11.35	0.94	0.11	0.33
Amylase (U/mg)	0.96 ^{b,c}	0.88 ^c	1.06 ^{a,b}	1.12 ^a	1.04 ^{a,b}	0.05	0.36	0.39	0.63
Pancreas									
Trypsin (U/mg)	1128.19 ^a	1439.03 ^b	1411.82 ^b	2135.71 ^c	1747.26 ^d	63.86	0.23	0.16	0.32
Lipase (U/g)	51.17 ^a	50.90 ^b	51.43 ^a	64.77 ^b	55.19 ^a	2.45	0.44	0.41	0.19
Amylase (U/mg)	0.50 ^a	0.53 ^a	0.55 ^{a,b}	0.66 ^b	0.66 ^b	0.04	0.04	0.07	0.11

Note: ^{a-d}Means within a row with no common superscripts differ significantly ($p < 0.05$).

Orthogonal contrasts: L = linear, Q = quadratic, and C = cubic effect of yeast culture supplementation.

3.4 | Enzyme activities

YC supplementation did not affect trypsin activity in the duodenum (Table 5). YC supplementation at 2.0% improved the activities of pepsin, lipase, amylase, and pancreatic trypsin compared with those of the controls ($p < 0.05$). As the dietary YC increased, the pancreatic amylase activity responded linearly ($p = 0.04$), and the duodenal trypsin activity tended to be improved (cubic, $p = 0.07$); these results were maximal at the 2.0% level. Similarly, Jin

et al. (2000) reported that supplementation with *Lactobacillus* cultures increased intestinal amylase activity in chickens. Several studies on probiotics have shown increased activity among digestive enzymes such as pepsin, trypsin, lipase, and amylase (Hunt et al., ; Zacarias-Soto et al., 2011). YCs are rich in enzymes and can modulate intestinal microorganisms that modify the intestinal milieu and deliver enzymes and other beneficial substances to the intestines (Shen et al., 2009). The YC has been shown to improve nutrient

digestibility (Gao et al., 2008), which supports the finding that YC increases digestive enzyme activity in the intestines.

Dietary YC supplementation at 2.0% and 4.0% increased the liver CAT and serum GR contents compared with those of the controls ($p < 0.05$; Table

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

TABLE 6 Effects of yeast culture supplementation in goose diets on antioxidant enzymes

Yeast culture supplementation (% of diet)							p		
Items	0 (Control)	0.5	1.0	2.0	4.0	SEM	L	Q	C
Liver									
GSH (μmol/g)	0.94 ^{a,b}	0.83 ^a	1.06 ^b	1.05 ^b	1.04 ^b	0.05	0.32	0.53	0.82
CAT (U/mg)	124.92 ^a	131.97 ^a	125.55 ^a	175.57 ^b	146.33 ^c	4.14	0.36	0.39	0.37
GR (U/g)	13.41 ^{b,c}	14.00 ^b	10.14 ^d	12.43 ^c	27.52 ^a	0.36	0.08	0.03	0.20
MDA (nmol/mg)	7.30 ^a	6.54 ^b	5.70 ^c	5.31 ^c	2.50 ^d	0.13	0.002	0.02	0.07
Serum									
GSH (μmol/g)	2.88	2.58	2.86	2.76	2.58	0.12	0.33	0.67	0.85
CAT (U/mg)	5.00 ^{b,c}	5.53 ^{a,b}	4.73 ^c	5.78 ^a	4.77 ^c	0.20	0.75	0.69	0.83
GR (U/g)	46.54 ^a	31.20 ^b	46.21 ^a	63.35 ^c	52.82 ^d	1.37	0.35	0.57	0.44
MDA (nmol/mg)	5.72 ^a	5.28 ^a	4.33 ^b	3.95 ^b	2.78 ^c	0.15	0.006	0.03	0.17

Note: ^{a-d}Means within a row with no common superscripts differ significantly ($p < 0.05$).

Orthogonal contrasts: L = linear, Q = quadratic, and C = cubic effect of yeast culture supplementation. Abbreviations: CAT, catalase; GR, glutathione reductase; GSH, glutathione; MDA, malondialdehyde.

6). GSH levels did not differ between the YC-treated and control groups; however, liver GSH increased slightly at 1.0%, 2.0%, and 4.0% supplementation. Inclusion of YC decreased the MDA contents in the liver (linear, $p = 0.002$; quadratic, $p = 0.02$) and serum (linear, $p = 0.006$; quadratic, $p = 0.03$). Similarly, Tagang et al. (2013) reported that yeast probiotic supplementation enhanced serum antioxidant enzyme activities in broilers, but they found no effect on MDA levels. Chen et al. (2013) showed that fermented liquid feed supplemented with *Bacillus subtilis* and *S. cerevisiae* increased the GSH-Px and superoxide dismutase activities and decreased the MDA contents in geese. Nutrition plays a vital role in maintaining the prooxidant/antioxidant balance (Cowey, 1986). Delles et al. (2014) demonstrated that dietary antioxidant supplementation improved oxidative stability, which was associated with enhanced cellular antioxidant enzymatic activity. This may suggest that dietary YC supplementation can significantly improve the health and oxidative statuses of geese.

4 | CONCLUSION

Dietary YC supplementation variously affected blood metabolism parameters and decreased organ weights, and improved body development, intestinal morphology, digestive and antioxidant enzyme activities of geese, with 2% YC supplementation was the most effective under the current experimental conditions.

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DESCRIPTION OF PROBLEM

Antibiotics combat disease in animals and

improve production efficiency and quality, resulting ultimately in economic profits.

Therefore, antibiotics have been widely used as feed

additives for animal husbandry.

However, their overuse has caused many problems, such as presence of antibiotic residue, drug

resistance and others (Baquero et al., 2008).

Therefore, it is imperative to explore antibiotic

anaerobic

Effect of dietary yeast culture supplementation on the cecal microbiota modulation of geese

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SUMMARY

One of the most extensively applied animal feed additives is yeast culture (YC), which can increase production efficiency by altering gastrointestinal tract (GIT) microbiota. Geese are the ideal model to study the interaction between diet and GIT, due to their adaptation to consume different roughage sources. Here, we fed geese with 0 (control), 0.5% (treat1), 1.0% (treat2), 2.0% (treat3), and 4.0% (treat4) of YC for 10 wk. The GIT microbiota was analyzed by 16S rRNA gene V3–V4 region via high-throughput sequencing. The results showed that YC supplementation did not significantly affect α -diversity ($P > 0.05$). However, dietary YC at 2.0% had the highest species richness (quadratic, $P < 0.05$). Principal coordinates analysis showed an obvious separation between control and 4.0% group. The dominant phyla were Firmicutes and Bacteroidetes whereas the predominant genera were Alistipes and Desulfovibrionaceae. The relative abundance of phyla Firmicutes significantly increased in 0.5, 1.0, and 2.0% group and decreased in 4.0% group ($P < 0.05$). YC supplementation increased the relative abundance of Bacteroidetes (linear, $P = 0.04$) and genus Bacteroides (linear, $P = 0.02$; quadratic, $P = 0.05$). 1.0% group contained the highest proportion of the beneficial bacteria, such as Parabacteroides, Enterococcus, Streptococcus, and Pseudomonas. 0.5% group obviously improved the capacity of amino acid and carbohydrate metabolism. Collectively, these findings demonstrate the beneficial effects of YC feeding on gut microbiota for geese, which suggested that dietary strategy based on feed additives is an effective method to maintain the health of geese.

Key words: goose, yeast culture, GIT, microbiota, feed additives

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alternatives, but which are safer and more effective.

Yeast cultures (YCs) are microecological products formed by yeast after sufficient

fermentation on specific media and are mainly composed of yeast metabolites, fermented medium, and a few yeast cells. YCs are rich in vitamins, saccharides, minerals, enzymes, growth-promoting factors, and amino acids, which may benefit animal growth, metabolism, and health. YC is one of the most important feed additives (Denev et al., 2007) as it can improve yield and quality of animal by-products, such as milk (Poppy et al., 2012) and eggs (Ozsoy et al.,

2018). Moreover, YC can enhance animal (i.e., pig, broiler) growth performance, as measured by average daily gain, body weight and average daily intake (van der Peet-Schwering et al., 2007, Shen et al., 2009), and also can strengthen immunity and disease resistance (Davis et al., 2004, Gao et al., 2008, Jensen, et al., 2008). These may act on the gastrointestinal tract (GIT) directly to maintain the balance of the microorganism and promote development of the GIT. For example, YC can significantly affect community structure and composition of gut microbiota of grass carp, increasing the abundance of beneficial bacteria (Liu et al., 2018). Addition of YC in the diet resulted in an increase in beneficial bacteria within GIT, making greater compensatory body weight gains after infection with *Salmonella* than in pigs fed conventional nursery diets (Price et al., 2010). The effect of YC on hindgut microbial communities was evaluated in horses, where it increased the abundance of fiberdegrading bacteria (Jouany et al., 2009).

Geese are important poultry in China (90% of the world's production), and their cecum are more developed compared with other poultry, so it is the ideal model to study the interaction between GIT microbiota and diet (Liu et al., 2018). Supplementation of geese diets with yeast fermented feed regulate cecal microflora that is beneficial of growth performance (Yan et al., 2019). Dietary yeast supplement improve intestinal microflora of goose and lead to better carcass hygiene (Yaman et al., 2006). However, few studies have investigated the effect of YC on the GIT microbiota of geese. Furthermore, the variety in GIT microbiota composition and structure can reflect the effect of diet on the host. Thus, the present study was conducted to investigate the effect of YC supplementation on the GIT microbiota of geese.

MATERIALS AND METHODS

All procedures involving animal care and management were in accordance with and approved by the Animal Use and Care Committee of Southwest University (SWU-20143003).

Animals, Diets, Feeding, and Experimental Design

Sichuan white geese ($n = 300$; 1-day-old; mixed sex) with healthy and similar body weight (BW: 95.57 ± 2.42 g) were randomly divided into 5 groups: 1) basal diet (control), 2) basal diet+0.5% YC (treat1), 3) basal diet +1.0% YC (treat2), 4) basal diet+2.0% YC (treat3), and 5) basal diet+4.0% YC (treat4). The commercial YC (Beijing Enhakor Biotechnology Co., Ltd., Beijing, China) was a fermented product composed of inactivated *Saccharomyces cerevisiae* grown on a medium, which contains »15.0% crude protein, »3.5% crude fat, »8.7% crude fiber, »14.2% amino acid, »3.3% mannan, »14.0% b-glucan and other microcomponents. Two corn-soybean based basal diets were formulated to be fed during starter (0–4 wk) and grower (5–10 wk; Table 1) periods. Each group consisted of 6 replicates pens, and 10 geese were randomly assigned to each pen (3.5 m $\times$ 3.0 m), and each pen is isolated independently, with one pen vacant between each group. Geese were allowed access to feed (in pellet form) and water ad libitum throughout the experimental period. Feed was provided 4 times daily at 7:30, 12:30, 17:00, and 21:00 h. The daily temperature inside the house throughout the experimental period was (21.32 ± 2.39)°C, and the relative humidity was (84.03 ± 5.15)%. Mortality was recorded when occurred during the experiment (0.5%: 1; 1.0%: 1; 4.0%: 1).

Intestinal Content Collection

Three 70-day-old geese were randomly selected from each pen and sacrificed by cervical dislocation and exsanguinated with empty stomach (fasting: 8 h). Nine samples of cecum contents from each group were taken and randomly combined into 3 samples. The cecum was dissected with sterile instruments and

Table 1. The composition of the basal diet of geese.

Items	0–4 wk	5–10 wk
Ingredients (%)		
Corn	63.80	53.60
Wheat bran	2.99	14.50

Soybean meal	20.00	11.50
Rapeseed meal	4.00	/
Rice bran	/	13.40
Silkworm chrysalis	4.30	1.79
Calcium hydrogen phosphate	1.59	0.90
Stone powder	0.87	0.75
Salt	0.20	0.20
L-Lysine (98%)	0.15	0.18
DL-methionine	0.05	0.07
Choline chloride	0.05	0.12
Premix	2.00	2.00
Sand	/	1.00
Total	100	100
Nutrient content		
Metabolizable energy (MJ/kg ¹)	11.97	11.21
Crude protein (%)	20.43	14.81
Crude fiber (%)	4.12	8.04
Calcium (%)	0.87	0.80
Available P (%)	0.43	0.40
Lysine (%)	1.14	0.85
Methionine (%)	0.36	0.30

Note: Premix provides (per kilogram of diet): vitamin A 40,000 IU, vitamin D₃ 2,000 IU, vitamin E 60 mg, vitamin K₃ 2 mg, vitamin B₁ 4 mg, vitamin B₂ 24 mg, vitamin B₆ 4 mg, vitamin B₁₂ 50 mg, nicotinic acid 12 mg, pantothenic acid 36 mg, folic acid 4 mg, biotin 0.4 mg, Fe 120 mg, Cu 4 mg, Mn 300 mg, Zn 160 mg, I 0.2 mg, Se 0.2 mg.

washed in 70% ethanol and sterile water to avoid cross contamination. The content from cecum was squeezed out and mixed thoroughly, then collected into sterilized tubes and immediately stored in liquid nitrogen.

16S rRNA PCR Amplification and Sequencing

Bacterial genomic DNA was extracted from 100 mg of each sample using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The bacterial V3–V4 region of the 16S rRNA was amplified by PCR (95°C for 5 min; followed by 22 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 90 s; and a final extension at 72°C for 8 min) using the following primers: 319F

(5⁰ACTCCTACGGGAGGCAGCAG-3⁰) and 806R (5⁰-GGACTACHVGGGTWTCTAAT-3⁰). The PCR products were purified with Agencourt AMPure XP beads (Beckman, Brea, CA).

The sequencing libraries were generated using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's instructions and barcodes were added. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA) and Bioanalyzer 2100 (Agilent, Germany). The constructed library was mixed in equimolar and paired-end (PE) sequenced (2 × 250 bp) on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA). All control and YC-treated samples were included in the same sequencing run.

Sequences Analysis

According to the overlap relationship between PE reads, the double-end sequences obtained from Hi-seq sequencing were merged into a single sequence tag, to control the quality of reads and filter the effect of merge. This included the following 3 main steps: 1) PE reads merging: used FLASH v1.2.7 to merge the reads of each sample through the overlap, obtaining the original tags data (raw tags); 2) tags filtering: used Trimmomatic v0.33 to filter the raw tags obtained by merging to obtain high quality tags data (clean tags); 3) chimera removal: used UCHIME v4.2 to identify and remove the chimera sequence to obtain the final effective data (effective tags).

Bioinformatics Analysis

Usearch (Edgar, 2013) was used to cluster tags at a similarity level of 97% to identify operational taxonomic units (OTUs). OTUs were annotated based on the bacterial database GreenGenes. Taxonomic assignment was achieved using RDP classifier, clustering the sequences at 97% similarity with a confidence threshold of 0.8. Heat map of the relative abundance of the OTUs at the family level in the 5 groups was performed with MultiExperiment Viewer (MeV).

Microbiota Diversity Analysis

The a- and b-diversity of each sample was evaluated based on the OTU data. a-diversity was evaluated by richness (ACE and Chao1) and diversity (Simpson and Shannon) using Mothur (v1.30). b-diversity was evaluated by weighted and unweighted UniFrac distances of principal coordinate analysis (PCoA) using QIIME. PICRUST (Parks et al., 2014) was used to detect the composition of functional genetic of each group by comparing species information and analyzing differences between groups in KEGG Orthologs (KO) composition including function. This involved metabolism, genetic information processing, human diseases, environmental information processing, cellular processes, and organismal systems. Bugbase was used to detect the potential pathogenic bacteria abundance at the phylum and genus level.

Statistical Analysis

The data analyses were performed with SPSS 22.0 software (IBM Corporation, Armonk, NY) using one-way ANOVA with LSD multiple comparison tests. The effect of supplemental YC levels was determined using orthogonal polynomials for linear and quadratic effects. All data are shown as means $\pm$ SEM unless otherwise indicated. Differences were considered statistically significant when $P < 0.05$ throughout.

RESULTS

Sequencing Data Analysis

A total of 1,195,635 couples of effective reads were obtained from 16S rRNA gene V3–V4 amplicons with an average of 79,709 reads per sample (ranging from 76,541 to 80,376). At a similarity level of 97%, we found a total of 484 OTUs common to all groups (control: 477; 0.5%: 479; 1.0%: 481; 2.0%: 480; 4.0%: 475; [Figure 1A](#)). A heat map with the 50 most abundant bacteria at the family level in the 5 groups ([Figure 1B](#)) suggests that bacterial abundance in the control was different from the YC groups. A total of 462 common OTUs were shared among the 5

groups, and the most abundant taxa of the shared OTUs ([Figure 1C](#)) indicate that Firmicutes, Bacteroidetes, Proteobacteria, Acidobacteria, Verrucomicrobia, and Cyanobacteria form the 6 most dominant phyla. Seven unique OTUs were found in the YC-group compared with the control ([Figure 1D](#)). Among them, 4 OTUs at the genus level, *Butyrivibrio*, *Ruminococcus_1*, DTU014, and *Bacteroides* were found in the YC-group, *Caproiciproducens* and *Clostridiales_vadinBB60_group* were found in the YCgroup except 0.5% group, and [*Eubacterium*] *_coprostanoligenes_group* was found in the 2.0 and 4.0% group.

Variation in Microbiota Diversity

The addition of YC in the diet produced no significant differences ($P > 0.05$) in a-diversity based on the richness (Ace and Chao1) and Shannon's or Simpson's indices ([Table 2](#)). Compared with controls, supplemental YC at 0.5, 1.0, and 2.0% group increased richness and Shannon's indices, and decreased Simpson's index. With the increase of dietary YC, richness exhibited quadratic responses (Ace: $P = 0.009$; Chao1: $P = 0.002$), with the 2.0% feeding level being the highest. Furthermore, the richness and Shannon's indices were lowest in 4.0% group. The b-diversity based on the overall community composition indicated that the similarity between clustered GIT microbiota according to YC treatments ([Figure 2](#)). The PCoA showed that 4.0% group clearly separated from the other groups, using both weighted ([Figure 2A](#)) and unweighted ([Figure 2B](#)) analysis, whereas 0.5 and 2.0% group obviously clustered together. It should also be noted that a large variation was observed in 1.0% group.

Microbial Analysis at the Phylum and Genus Level

At the phylum level, a total of 13 phyla were identified (control: 13; 0.5%: 12; 1.0%: 11; 2.0%: 13; 4.0%: 12). Firmicutes (19.90–54.60%), Bacteroidetes (21.60–59.20%),

Proteobacteria (8.09–23.50%), Actinobacteria (1.77–4.65%), Cyanobacteria (1.01–2.52%), and Verrucomicrobia (0.55–5.46%) constituted the dominant phyla (Figure 3A). At the genus level, a total of 189 genera were identified (control: 186; 0.5%: 188; 1.0%: 189; 2.0%: 187; 4.0%: 184). Alistipes (17.6–49.7%), Desulfovibrionaceae (4.57–18.20%), Lachnospiraceae (1.49–7.64%), CHKCI001 (1.23–14.10%), Blautia (1.42–5.30%), Bacteroides (2.30–8.24%) were predominant (Figure 3B).

Bacteria

YC treatment produced a significant effect on the relative abundance at both phylum and genus levels (Figure 4). Compared with the control, the relative abundance of phyla Bacteroidetes and Firmicutes in 4.0% group significantly increased or decreased, respectively ($P < 0.05$), whereas Firmicutes significantly increased in 0.5, 1.0, and 2.0% group ($P < 0.05$). In 0.5% group, the relative abundance of

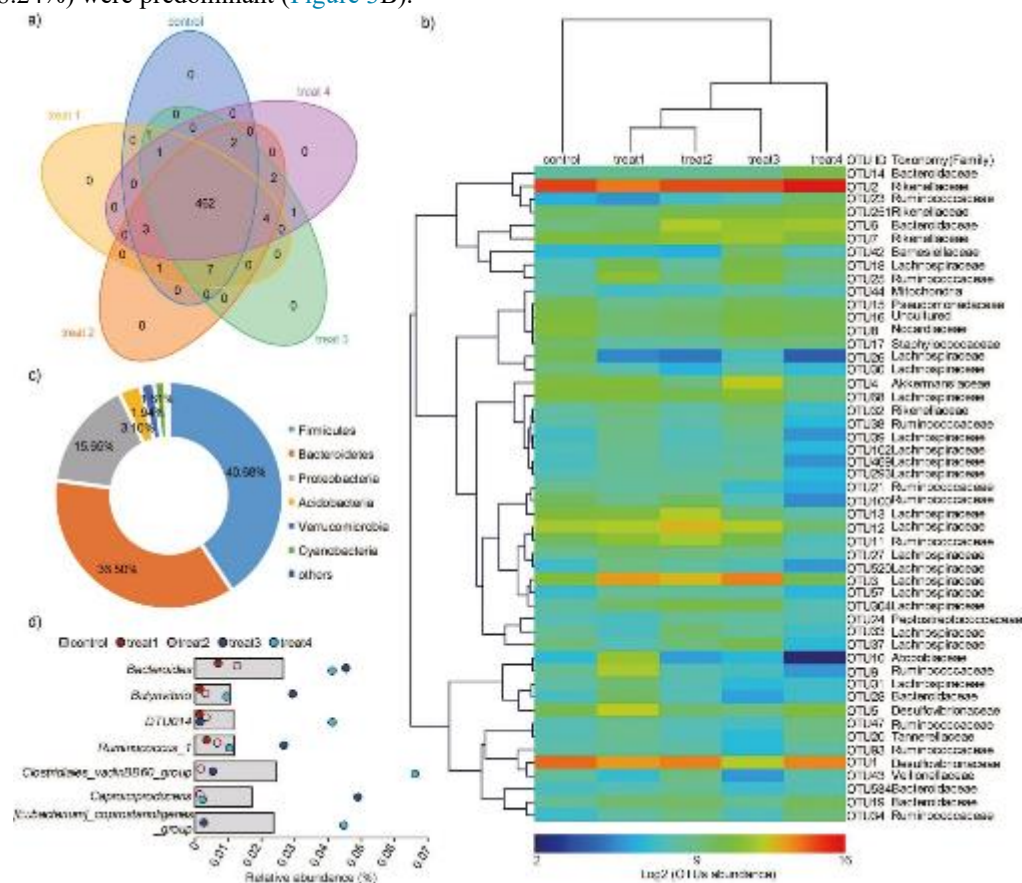


Figure 1. Effect of dietary yeast culture supplementation on bacterial community composition in goose gut. Number of shared OTUs among five groups (A); a heat map of changes in relative abundance of the 50 most abundant OTUs summarized at the family level was performed with MultiExperiment Viewer (MeV) (B); composition of shared gut microbiota in the five groups, at the phylum level (C); relative abundance of gut microbiota that is uniquely present in the YC group, at the genus level (D). Abbreviations: OTUs, operational taxonomic units; YC, yeast culture.

Comparison of the Relative Abundance of

Table 2. Effect of dietary yeast culture supplementation on the gut microbiota a-diversity of geese.

Yeast culture supplementation (% of diet)	P value
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Item	0 (control)	0.5%	1.0%	2.0%	4.0%	L	Q
Shannon	3.48 § 0.29	3.83 § 0.18	3.55 § 0.34	3.56 § 0.35	2.84 § 0.58	0.08	0.11
Simpson						0.29	0.13
Ace	0.144 § 0.013	0.129 § 0.005	0.136 § 0.006	0.130 § 0.021	0.156 § 0.033	0.25	0.009
Chao1	460.85 § 12.71	466.61 § 4.31	467.45 § 2.25	469.30 § 5.53	450.53 § 8.53	0.23	0.002
	463.55 13.23	467.44 6.84	469.58 21.16	470.85 9.17	452.74 7.37		
	§	§	§	§	§		

All data represent means § SEM.

Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

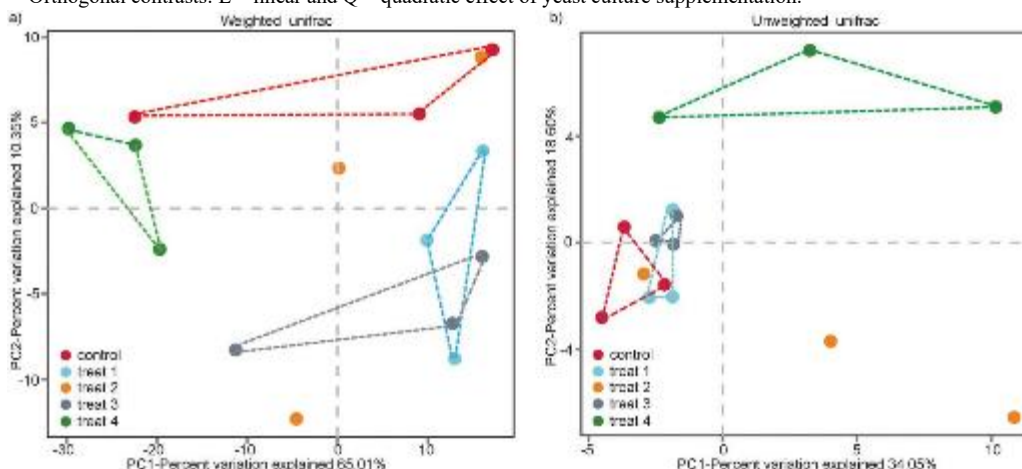


Figure 2. Effect of dietary yeast culture supplementation on microbiota community structure in goose gut. Principal coordinates analysis (PCoA) using weighted (A) or unweighted (B) UniFrac distances.

genus *Shuttleworthia*,
Christensenellaceae_R7_group,
Ruminococcaceae_UCG, *Faecalibacterium*,
Eisenbergiella, *Blautia*, and *Sellimonas*
significantly increased ($P < 0.05$), whereas in
4.0% group, genus *Alistipes* and *Bacteroides*
also increased ($P < 0.05$). Furthermore, inclusion
of YC increased the relative abundance of phyla
Bacteroidetes (linear, $P = 0.04$) and genus
Bacteroides (linear, $P = 0.02$; quadratic, $P =$
 0.05).

Predicted Functions of Microbiota

Based on functionality prediction, we
investigated the KO composition in the 5
groups. We found no significant differences
between control, 1.0, 2.0, and 4.0% group.
However, carbohydrate metabolism, amino acid
metabolism, global, and overview maps achieve
a statistical significant difference ($P < 0.05$)
between control and 0.5% group (Figure 5A). To
determine

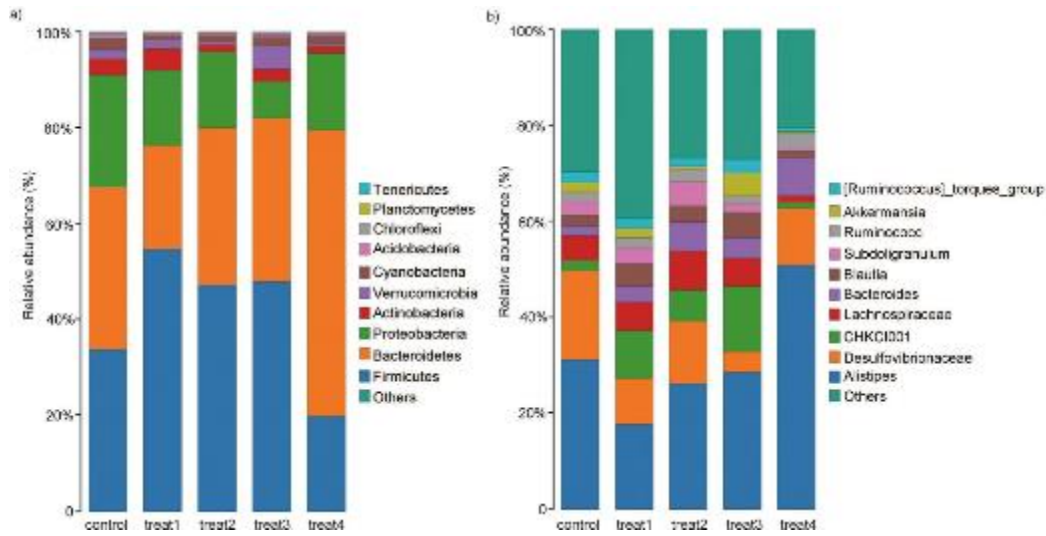


Figure 3. Effect of dietary yeast culture supplementation on the relative abundance of gut microbial population in geese. Relative abundance of bacterial phyla (A) and genera (B).

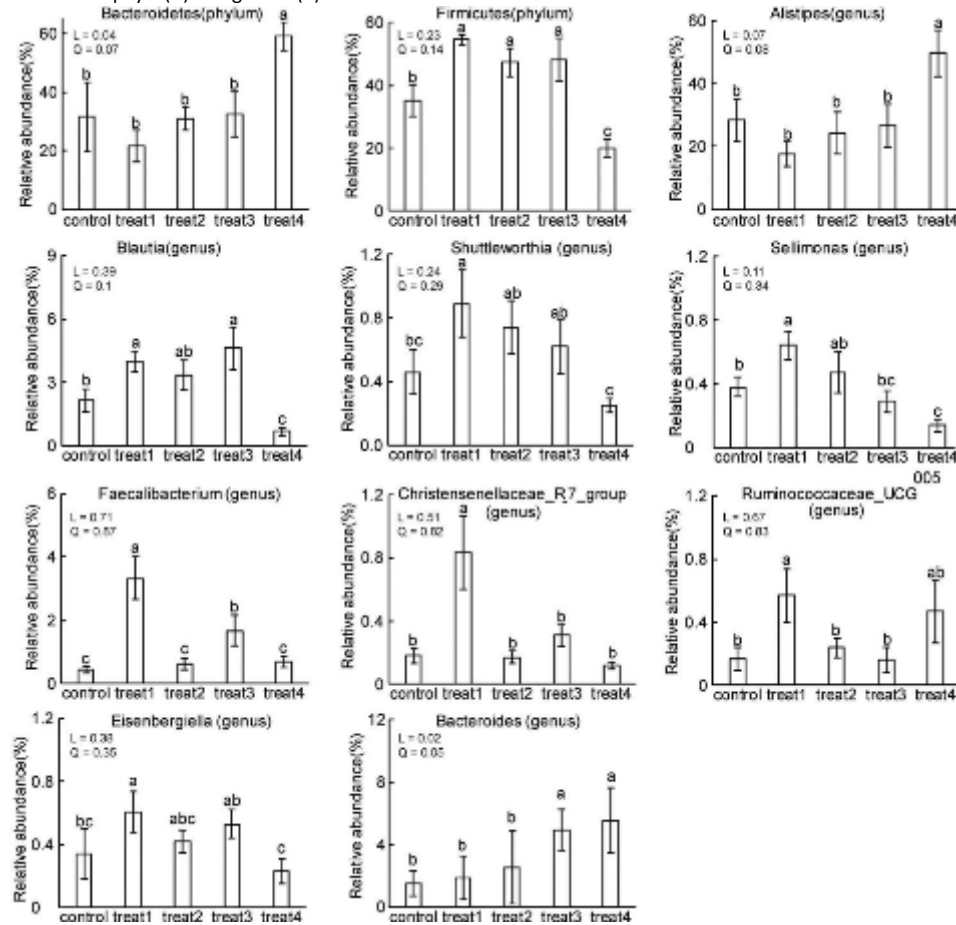


Figure 4. Effect of dietary yeast culture supplementation on relative abundance of gut bacteria in geese which were significantly affected by YC treatment. Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation. Abbreviation: YC, yeast culture.

whether the YC supplementation has a positive or negative effect on the GIT, and what levels are optimal, we predicted what bacteria were pathogenic and beneficial in the 5 groups (Figure 5B). At the genus level, the proportion of potential beneficial bacteria in 1.0% group was higher than in other groups, whereas the proportion of potential pathogenic bacteria in 4.0% group was higher than in other groups. Among these bacteria, the dominant potential pathogenic genera were Rikenellaceae, Bacteroides, and Ruminococcus, whereas the prominent potential beneficial genera were Parabacteroides, Enterococcus, Streptococcus, and Pseudomonas.

DISCUSSION

The GIT microbiota is a community of microorganisms inhabiting the animal GIT, which can be beneficial or harmful. In general, there is a balance between beneficial and harmful microbiota, and this has relevance for the immune system, metabolism (Tremaroli and Backhed, 2012) and diseases (Pennisi, 2013). The composition of the GIT microbiota evolves throughout an individual's lifetime, and the balance may be altered by both endogenous and exogenous factors (Sekirov et al., 2010), such as the diet (Scott et al., 2013). Thus, the GIT microbial composition depends on the interaction between the microorganisms and their host, and with the diet of the host. Therefore, studying the GIT microbiota composition and its structure can be used to better understand how dietary YC affects growth performance of goose at the microbial level. In the present study, we chose 5 commonly used YC diet supplementation levels (0, 0.5, 1.0, 2.0, and 4.0%) to study how YC modulates GIT microbiota. We used high-throughput sequencing of the V3–V4 region of the 16S rRNA gene to explore the diversity and composition of GIT microbiota in geese.

The α -diversity analysis showed that there are no significant differences in species diversity or richness between these treatments, suggesting that the presence or absence of microbial is not easily affected by the rearing environment or changes in diet, although the relative abundance may be affected. This is consistent with a report indicating that there are no significant differences in α -diversity when gilthead sea bream is supplemented with different mannan-oligosaccharide levels (Dimitroglou et al., 2010). However, laying hens with the higher fermentation yeast addition into diets had the lower total bacterial count (Hassanein and Soliman, 2010). The discrepancies between these and the present study may be caused by differences in yeast product used, host species and rearing conditions. In addition, the β -diversity analysis based on PCoA showed a clear separate cluster between the 4.0% and other groups, suggesting that the microbial complexity and enrichments were relatively affected by YC diet in 4.0% group, which adapting to changing and extreme environments (Thomas et al., 2011).

consistent with the richness and diversity was lowest in 4.0% group.

In the present study, Firmicutes and Bacteroidetes were the 2 most dominant phyla in the bacterial communities of the goose. Similar results have been observed in study of chicken (Qu et al., 2008), buffalo (Singh et al., 2012), and goose (Wang et al., 2015). Phylum Firmicutes and Bacteroidetes are beneficial to the host. Firmicutes are tightly related to nutrition and to energy absorption (Jumpertz et al., 2011). In particular, the genera of this phylum degrade fiber and cannot stand extremely acidic environments. In contrast, phylum Bacteroidetes plays a role in maintaining a healthy GIT,

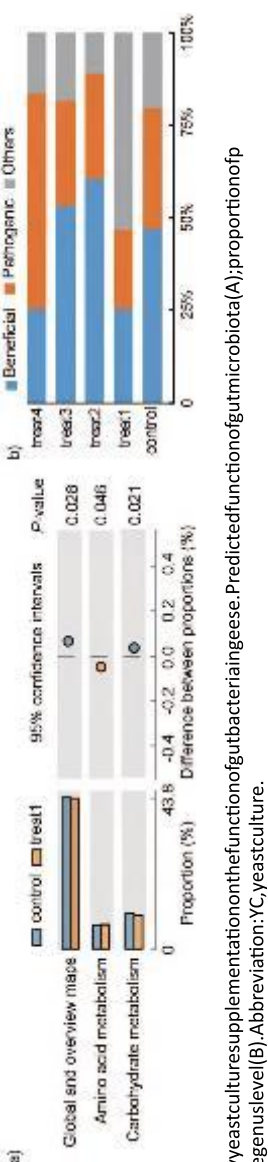


Figure 5. Effect of YC dietary yeast culture supplementation on the function of gut bacteria in geese. Predicted function of gut microbiota (A); proportion of beneficial and pathogenic bacteria at the genus level (B). Abbreviation: YC, yeast culture.

Furthermore, we found that as the amount of YC increased, the relative abundance of the

phyla Firmicutes and Bacteroidetes decreased and increased, respectively, and a similar trend can be found in their respective genera, which consistent with similar studies performed on gut microbiota in humans (David et al., 2014). These results may be explained by YC diet supplementation can enhance gut microbiota fermentation, resulting in an increased in the concentration of volatile fatty acids and a reduced pH of the GIT (Millerwebster et al., 2002).

The dominant bacteria in the GIT may play many important functions essential to the goose (Nicholson et al., 2012). Our study indicates that 0.5% YC can significantly enhance carbohydrate and amino acid metabolism and global and overview maps (Figure 5A). These biochemical processes might promote utilization of energy, absorption of nutrients and growth (Li et al., 2017). Remarkably, the proportion of potential beneficial bacteria after 1.0 and 2.0% YC treatment was higher than in the control (Figure 5B). These results are caused by the presence of oligosaccharides in YC, which can minimize GIT pathogen concentration and stimulate the growth of beneficial bacteria (de Maesschalck et al., 2015), thus alleviating the competition for diet between the host and the pathogenic bacteria. However, the proportion of potential beneficial bacteria in the sample treated with the highest amount of YC (4%) was the lowest (Figure 5B). High concentration of YC may induce the goose to develop immune tolerance, leading to wastage of energy and nutrients, and suppressing the growth of both host and microbiota (Zhen et al., 2019). A similar result was obtained for pigs (Shen et al., 2009) and broilers (Gao et al., 2008), which growth performance did not increase with higher YC supply. Our previous study also showed that the low YC concentration (0.5, 1, and 2%) improved average daily gain and feed conversion rate in goose, whereas high YC concentration (4%) did not (Zhang et al., 2022). Nonetheless, the mechanism of immune tolerance induced by high YC dose requires further investigation.

CONCLUSIONS AND APPLICATIONS

1. In general, dietary YC supplementation had a positive effect on the GIT microbiota in goose, especially 2% feeding level being the highest species richness.
2. The supplementation of YC had no effect on overall microbial structure, but modulated composition and relative abundance of GIT microbiota, such as inclusion of YC increased the relative abundance of phyla Bacteroidetes and genus Bacteroides.
3. The YC could be used as a potential new feed additive for healthy breeding of geese.

DISCLOSURES

The authors declare that there is no conflict of interest for this study.

ACKNOWLEDGMENTS

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Availability of data and materials: The authors declare that the data of this research are not deposited in any official repository. All sequences have been deposited in the NCBI's Sequence Read Archive with the accession number PRJNA616162.

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Effects of dietary yeast culture supplementation on the meat quality and antioxidant capacity of geese¹

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Primary Audience: Nutritionists, Researchers, Farmers

SUMMARY

This study aimed to evaluate the effects of dietary yeast culture (YC) supplementation on the meat quality and antioxidant capacity of geese. Three hundred geese (live weight: 95.57 ± 2.42 g) were fed a basal diet (control), or diets supplemented with 0.5, 1.0, 2.0, or 4.0% YC for 70 d. Compared with the control, dietary YC supplementation increased ($P < 0.05$) the lightness and redness (a^*) readings and catalase and glutathione reductase activities in the goose meat but decreased ($P < 0.05$) the shear force, myofiber diameter, and drip loss. The effects of YC supplementation on the moisture, crude fat, CP, glutathione, and malondialdehyde contents of the meat varied for the different supplementation levels. Meat from the 2.0% YC group had the highest a^* readings and lowest shear force, myofiber diameter, and drip loss values; meat from the 0.5% YC group had the highest crude fat and CP contents and lowest moisture content; and meat from the 1.0% and 2.0% YC groups had the highest glutathione, catalase, and glutathione reductase activities. Supplementing YC had beneficial effects on the meat quality and antioxidant capacity of geese, with different supplementation levels having different effects.

Key words: yeast culture, geese, meat quality, antioxidant

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DESCRIPTION OF PROBLEM

Geese provide nutritious meat and eggs and are therefore a valuable protein source for humans, as well as producing high-quality liver fat and feathers (Yang et al., 2018). With the

¹ Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by Southwest University and Chongqing Three Gorges Vocational College.

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Prebiotics are defined as nondigestive components of feed that improve the health of the host by selectively promoting the metabolism and proliferation of beneficial bacterial species already resident in the digestive tract (Ziemer and Gibson, 1998; Bindels et al., 2015). They have been proven to be effective antibiotic alternatives for improving the health, metabolism, and productive performance of poultry (Gao et al., 2008; Chen et al., 2017; Tavaniello et al., 2018). Yeast culture (YC) is a dried prebiotic product containing yeast and various metabolites of yeast fermentation and is rich in vitamins, saccharides, minerals, enzymes, growth-promoting factors, and amino acids (van der Peet-Schwering et al., 2007; Shen et al., 2009). Several studies have reported that dietary YC supplementation has beneficial effects on meat quality, with Geng et al. (2016) reporting that YC supplementation improved beef quality by increasing fat metabolism. Tavaniello et al. (2018) suggested that prebiotics could have positive effects on the meat quality traits of broiler chickens. In addition, goose feeds fermented liquid feed with probiotics (*Bacillus subtilis* and *Saccharomyces cerevisiae*) were found to increase the superoxide dismutase activity and decrease the malondialdehyde (MDA) content of the breast muscles (Chen et al., 2013). However, studies on the effects of dietary YC supplementation on goose meat quality and antioxidant capacity are still scarce, where previously that improves the growth performance including BW, feed intake, and feed conversion ratio (unpublished results). This study aimed to evaluate the meat quality and antioxidant capacity of geese fed diets containing varying quantities of YC.

rapid increase in demand for goose meat products that are nutritious, healthy, and safe, goose breeding has gradually developed into a largescale industry; however, this increase in intensification has come with a sudden increase in morbidity rates. The excessive use of antibiotics has therefore become common, as producers attempt to reduce the risk of disease. However, geese, especially goslings, are particularly sensitive to drugs, and the imprudent use of antimicrobials can cause casualties owing to drug poisoning, as well as causing environmental pollution and food safety problems.

MATERIALS AND METHODS

All procedures involving animal care and management were in accordance with and approved by the Animal Use and Care Committee of Southwest University.

Experimental Design and Goose Husbandry

Three hundred 1-day-old healthy mixed-sex Sichuan white geese, with average BW of 95.57 ± 2.42 g, were used in a completely randomized study and divided into 5 groups fed either 0% (control), 0.5, 1.0, 2.0, or 4.0% commercial YC product (Beijing Enhelor Biotechnology Co., Ltd., Beijing, China). The YC was a fermented product composed of inactivated *S. cerevisiae* grown on a medium. The ingredient analysis shows that YC contains w15.0% CP, w3.5% crude fat, w8.7% crude fiber, w14.2% amino acid, w3.3% mannan, w14.0% b-glucan, and other microcomponents. The corn-soybean meal basal diets were formulated (Table 1) to meet the recommendations of the NRC (1994) during the starter (Day 1–28) and grower (Day 29–70) periods. Geese were housed in pens (3.5 m × 3.0 m) and netreared in a windowed poultry house from April to June 2019. Each group consisted of 3 replicate pens with 20 geese per pen. Geese were injected with Astragalus polysaccharides

Table 1. Composition and nutrient content of starter (Day 1–28) and grower (Day 29–70) basal diets (dry basis).

Items	Starter	Grower
Ingredients (%)		
Corn	63.80	53.60
Wheat bran	2.99	14.50
Soybean meal	20.00	11.50

Rapeseed meal	4.00	/
Rice bran	/	13.40
Silkworm chrysalis	4.30	1.79
CaHPO ₄	1.59	0.90
Limestone	0.87	0.75
L-Lysine (98.5%)	0.15	0.18
DL-Methionine	0.05	0.07
Salt (NaCl)	0.20	0.20
Choline chloride	0.05	0.12
Premix ⁷	2.00	2.00
Sand	/	1.00
Total	100	100
Nutrient content		
ME (MJ\$kg ⁸¹) ²	11.97	11.21
CP (%)	20.43	14.81
Crude fiber (%)	4.12	8.04
Calcium (%)	0.87	0.80
Available P (%) ²	0.43	0.40
Lysine (%)	1.14	0.85
Methionine (%)	0.36	0.30

(Yongjian Biological, Chongqing, China) and administered multidimensional electrolytes in their water on day 1 and 7. Geese were allowed access to feed (in pellet form) and water ad libitum throughout the experimental period. Feed was provided 4 times daily at 7:30, 12:30, 17:00, and 21:00 h. The average house temperature during the experimental period was 21.32C 6 2.39C and the RH was 84.03 6 5.15%.

Sampling and Measurements

Nine fasted geese randomly selected from each group (3 geese per pen) and sacrificed by cervical dislocation at day 70. Breast muscle samples were collected and divided into 2 parts, one part for measured meat quality and the other part was cryogenically frozen in liquid nitrogen and stored in a -80C freezer for measuring antioxidant enzyme activities. The basic physical characteristics (shear force, pH, drip loss, and color) and chemical composition (moisture, crude fat, CP, and ash) of meat were measured as per the methods of Zhang et al. (2018). The myofiber diameter was measured as described previously (Zhang

et al., 2013). The glutathione, catalase (CAT), glutathione reductase, and MDA content were measured using commercial analytical kits as per the manufacturer's instructions (Solarbio, Beijing, China).

Statistical Analysis

Data were analyzed using 1-way ANOVA of SPSS 22.0 software package for Windows (IBM Corporation, 2014) with least significant difference multiple comparison tests. The effect of supplemental levels of YC was determined using orthogonal polynomials for linear and quadratic effects. Variability in the data is expressed as the SEM, and a probability level of P # 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Meat Quality

Yeast culture supplementation had no effects on the meat pH and yellowness (b*) readings and ash contents (Table 2). In general, compared with the control, the YC supplementation increased (P , 0.05) the lightness (L*) and redness (a*) readings of the meat, while decreasing (P , 0.05) the shear force, myofiber diameter, and drip loss. The highest a* readings and lowest shear force, myofiber diameter, and drip loss values were found for the geese supplemented with 2.0% YC. A lower shear force value indicates tenderer meat, with these results concurring with those of Geng et al. (2016), who reported that YC supplementation improved beef tenderness. However, Pelicano et al. (2005), Gomes et al. (2009), and Geng et al. (2016) reported that meat pH, drip loss, and color (L*, a*, and b*) values were not affected by the supplementation of prebiotics. In contrast, studies on broilers (Maiorano et al., 2012; Tavaniello et al., 2018) and lambs (Rufino et

⁷ The premix provide the following per kilogram of diet: VA, 2,000 IU; VD₃, 1,000 IU; VE, 3,000 mg; VK₃, 200 mg; VB₁, 100 mg; VB₂, 1,200 mg; VB₆, 200 mg; VB₁₂,

2.5 mg; nicotinic acid, 600 mg; pantothenic acid, 1,800 mg; folic acid, 200 mg; biotin, 20 mg; Fe, 6 g; Cu, 0.2 g; Mn, 15 g; Zn, 8 g; I, 10 mg; Se, 30 mg.

⁸ Calculated values.

Table 2. Effects of the dietary supplementation of yeast culture to geese on meat quality.

Items	Yeast culture supplementation (% of diet)					SEM	P-value	
	0	0.5	1.0	2.0	4.0		L	Q
Physical characteristics								
Shear force (N)	33.52 ^a	30.57 ^{b,c}	31.14 ^b	29.93 ^c	32.29 ^a	0.30	0.90	0.21
Myofiber diameter (mm)	16.11 ^a	15.92 ^{a,b}	15.79 ^b	15.14 ^c	15.77 ^b	0.08	0.55	0.12
pH	6.03	5.96	5.93	6.00	6.02	0.04	0.18	0.14
Drip loss (%)	6.43 ^a	5.56 ^b	4.78 ^c	4.58 ^d	5.34 ^c	0.05	0.53	0.15
L *	49.23 ^{a,b}	48.70 ^a	50.70 ^c	50.17 ^{b,c}	50.37 ^c	0.33	0.30	0.50
a*	7.90 ^a	9.07 ^b	8.77 ^b	9.80 ^c	8.23 ^a	0.12	0.95	0.16
b*	11.93	12.10	11.57	11.87	11.33	0.31	0.12	0.37
Chemical composition								
Moisture (%)	75.54 ^a	74.70 ^a	76.67 ^b	75.27 ^a	76.91 ^b	0.25	0.29	0.61
Crude fat (%)	2.06 ^a	2.49 ^b	1.69 ^c	2.65 ^b	1.65 ^c	0.06	0.58	0.68
CP (%)	20.20 ^a	21.13 ^b	19.64 ^a	20.39 ^{a,b}	19.52 ^a	0.29	0.34	0.68
Ash (%)	1.36	1.36	1.50	1.34	1.25	0.08	0.27	0.43

^{a-c}Means in the same row with different superscript letters differ significantly (P, 0.05).

Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

Abbreviations: a*, measure of redness; b*, measure of yellowness; L*, measure of lightness.

al., 2013) indicated that dietary prebiotic supplementation reduced the meat pH and L*, a*, and b* readings. Here, we speculate that it may be related to changes in muscle-fiber types (Zhao et al., 2012), but the specific mechanism is not yet clear.

Yeast culture supplementation at 1.0 and 4.0% significantly increased the moisture content, at 0.5 and 2.0% significantly increased the crude fat content, and at 0.5% significantly increased the CP content (P, 0.05), while 1.0 and 4.0% YC significantly decreased the crude fat content relative to the control (P, 0.05). Yeast preparations have been found to improve animal energy intake and ME, which in turn, affects the growth of protein and fat deposition (Hansen et al., 2017; Purchas et al., 2002). Rufino et al. (2013) showed that inactive dry yeast supplementation increased the meat CP and ash content and decreased the intramuscular fat content in lambs. However, Geng et al. (2016) reported that YC supplementation had no effect on the meat ash, protein, or intramuscular fat content in finishing bulls. Similar results were also reported by Gomes et al. (2009), who observed no effects of YC supplementation on the fat content of the meat from feedlot finished steers. These conflicting results may be linked to differences in the formulations and organisms present in the products supplemented, the duration of the

supplementation and dosages applied, the nutritional densities of the basal diets, and the animal species tested.

Nonetheless, the increase in fat deposition caused by the supplementation of yeast preparations has a positive effect on meat tenderness (Swyers et al., 2014; Geng et al., 2016), and the results for these 2 parameters are consistent in this study.

Antioxidant Enzymes

As shown in Table 3, CAT and glutathione reductase activities were higher in meat samples from the YC-supplemented geese than the controls (P, 0.05), whereas the MDA content was lower in the samples from the 2.0 and 4.0% YC groups (P, 0.05). The glutathione activity was significantly higher in the 1.0 and 2.0% YC groups (P, 0.05) but lower in the 4.0% YC group (P, 0.05) than in the control. In addition, the 1.0%

YC group had higher ($P, 0.05$) CAT and glutathione reductase activities than the 0.5, 2.0,

. 3. The 1 and 2% YC supplementation resulted in the most improvement of the

Table 3. Effects of the dietary supplementation of yeast culture to geese on the antioxidant enzyme activities and MDA content of the meat.

Items	Yeast culture supplementation (% of diet)					SEM	P-value	
	0	0.5	1.0	2.0	4.0		L	Q
GSH (mmol/g)	0.89 ^a	0.85 ^a	1.23 ^b	1.92 ^c	0.55 ^d	0.07	0.90	0.29
CAT (U/mg)	1.59 ^a	2.05 ^b	2.59 ^c	2.26 ^b	2.25 ^b	0.07	0.46	0.36
GR (U/g)	24.37 ^a	25.52 ^a	48.92 ^b	43.49 ^b	33.82 ^c	1.51	0.64	0.32
MDA (nmol/mg)	4.27 ^{a,b}	4.39 ^a	3.92 ^{b,c}	3.69 ^c	3.65 ^c	0.14	0.07	0.15

^{a-c}Means in the same row with different superscript letters differ significantly ($P, 0.05$).

Orthogonal contrasts: L = linear and Q = quadratic effect of yeast culture supplementation.

Abbreviations: CAT, catalase; GR, glutathione reductase; GSH, glutathione; MDA, malondialdehyde.

and 4.0% YC groups did. A similar study on broilers reported that probiotic supplementation increased the SOD activity and decreased the MDA content of the breast muscles of geese (Chen et al., 2013). Tagang et al. (2013) also showed that yeast probiotic supplementation increased the serum glutathione peroxidase and CAT activities in broiler meat. This may be linked to the antioxidant effects of the mannan and β -glucan contained in YC (Kogan et al., 2008; Krizková et al., 2001). However, our results contrasted with the findings of Maiorano et al. (2017), who reported that the MDA content was higher in meat from broilers provided with a prebiotic treatment. These differences may be linked to the higher susceptibility of broiler chickens to oxidative stress, as a result of their intensive genetic selection for improved productive performance (Sihvo et al., 2014). Furthermore, 4.0% YC was not more effective in present study, which may be owing to the high level of YC disrupted the nutritional and energy metabolism balance.

CONCLUSIONS AND APPLICATIONS

1. At appropriate levels, dietary YC supplementation had a positive effect on the meat quality and antioxidant capacity of geese.
2. The supplementation of 2% YC produced the best physical meat properties, and 0.5% YC produced the best chemical meat composition

geese's antioxidant capacity.

4. Further research is needed to determine the most appropriate dietary YC level to optimize both the meat quality and antioxidant capacity of geese.

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DISCLOSURES

The authors declare no conflicts of interest.

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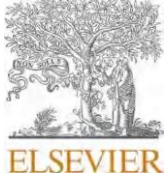
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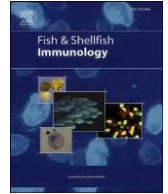
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Yeast culture improved the growth performance, liver function, intestinal barrier and microbiota of juvenile largemouth bass (*Micropterus salmoides*) fed high-starch diet

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ABSTRACT

The present study was conducted to investigate the effects of yeast culture on the growth, health and microflora of the juvenile largemouth bass fed high-starch diet. The experiment set three isonitrogenous and isolipidic diets, control (high-starch diet), HSY₁ (high-starch diet with 1% yeast culture) and HSY₃ (high-starch diet with 3% yeast culture). A feeding trial was conducted in largemouth bass juveniles for 8 weeks. The results indicated fish fed with 3% yeast culture not only could improve specific growth rate (SGR), but also significantly decreased hepatic lipid content, hepatic glycogen content, and hepatopancreas somatic index (HSI) compared with the control group ($p < 0.05$). The total superoxide dismutase (T-SOD) and catalase (CAT) activities of HSY₃ group significantly increased while malondialdehyde (MDA) content significantly reduced in liver compared with the control group ($p < 0.05$). Meanwhile, the mRNA expression levels of hepatic *Sod* and *Cat* were up-regulated ($p < 0.05$), and liver metabolism showed 111 metabolites were significantly changed in HSY₃ group, liver lipid metabolism pathway remarkably changed. Besides, the intestinal anti-inflammatory cytokines were significantly up-regulated, and the pro-inflammatory cytokines were significantly down-regulated as the inclusion of yeast culture ($p < 0.05$). Notably, HSY₃ group diet up-regulated the expression of *Zo-1*, *Claudin* and *Occludin* in intestine compared with the other groups ($p < 0.05$). Serum D-lactate (D-lac), diamine oxidase (DAO) and lipopolysaccharide (LPS) decreased significantly with the inclusion of yeast culture ($p < 0.05$). Furthermore, the abundance of probiotics (such as *Lactobacillus*, *Bacillus* and *Bifidobacterium*) increased significantly, and the abundance of intestinal potential pathogenic bacteria (*Plesiomonas*) decreased in HSY₃ group ($p < 0.05$). The phenotypic analysis showed that gram-negative bacteria significantly decreased while gram-positive bacteria increased in HSY₃ group ($p < 0.05$). All in all, this study revealed that supplementation of 3% yeast culture can improve the growth performance and the health of juvenile largemouth bass, and has the potential to be used as an effective synbiotics for *M. salmoides*.

1. Introduction

It is well known that starch plays a very important role in aquatic animal feed, which act as an economical energy source [1], and has also the function of stabilizers and swelling agent. However, starch utilization is very low in many carnivorous fish [2,3], and high starch diets can negatively affect fish growth [4], induce hepatic lipid accumulation [5], impair immune system [6], and dysregulation of intestinal flora in fish [7]. Therefore, how to solve the adverse effects of high starch in fish is an

urgent problem that we need to explore.

In recent years, many probiotics have shown good efficacy in promoting growth [8], regulating immune system [9], and inhibiting potential pathogens [10]. Yeast culture (YC), as a member of probiotics family is a natural product produced from yeast fermentation, which contains proteins, polysaccharides (mainly β -glucans and mannan-oligosaccharides), lipids, vitamins, peptides, amino acids, nucleotides, organic acids, and antioxidants [11]. Yeast culture supplementation has a range of beneficial effects, including increased feed

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intake [12] and feed digestion [13], enhancement of overall immunity [14], disease resistance [15], modulation of the animal gut microbiota [16] and

consequently improved animal performance [17]. Therefore, yeast culture has attracted extensive attention in aquaculture.

To increase stability of aquafeed, feed processor tend to increase the proportion of starches in the diet [18]. Thus, one of the key goals when feeding largemouth bass is to maintain the optimal starch for feed processing. High-yielding largemouth bass fed a certain amount of starch in their diets can suffer the same problem. However, a high-starch diet can induce oxidative stress, disorders of liver function, suppress innate immunity, consequently inhibit the growth of *M. salmoides* [19]. Most importantly starch diets were inefficiently used as an energy source by *M. salmoides* juveniles [20]. Previous studies have demonstrated that yeast culture could improve growth performance, immune response, intestinal health, antioxidant capacity and disease resistant in aquatic animals [21,22]. To solve the problem, yeast culture is supplemented to the diet of largemouth bass because of its role in health. We hypothesize that yeast culture supplementation still has beneficial effects on the largemouth bass growth. The objective of the study was to examine the effects of supplementing yeast culture to diets containing high proportions of starch in the diet on animal performance, health, and intestinal microbial communities of juvenile largemouth bass.

2. Materials and methods

2.1. Experimental diets

In the present study, three isonitrogenous (47.8% crude protein) and isolipidic (11.8% crude lipid) diets were formulated and their chemical composition is shown in Table 1. The yeast (*Saccharomyces cerevisiae*, microbial preservation registration number: CGMCC NO. 6120 2012.05.18) culture (from Beijing Enhalar International Tech Co., Ltd, Beijing, China)) supplement levels were 0% (control), 1% (HSY₁) and 3% (HSY₃) respectively. The yeast culture is fermented by single colony (including liquid fermentation and solid fermentation). The main protein source of the diet was steam fish meal, domestic chicken meal, soybean meal, soybean protein concentrate, wheat gluten, the main fat source was fish oil, soybean oil (1:2), and the sugar source was cassava starch. The feed materials were crushed and screened by 245 µm, then mixed evenly by step-by-step enlargement method. Pellet feed with a particle size of 3 mm was prepared, which was naturally air-dried and bailed and stored in a refrigerator at 4 °C for later use.

Table 1
Composition and nutrient levels of test diets (air-dry basis).

Items	Groups		
	Control	HSY ₁	HSY ₃
<i>Ingredient (%)</i>			
Fish meal	35.00	35.00	35.00
Domestic chicken powder	10.00	10.00	10.00
Yeast culture	0	1.00	3.00
Soybean meal	9.90	9.90	8.90
Concentrated soy protein	15.00	14.00	13.00
Wheat gluten	3.00	3.00	3.00
Cassava starch	15.00	15.00	15.00
Fish oil	2.00	2.00	2.00
Soy lecithin	1.50	1.50	1.50
Soybean oil	4.00	4.00	4.00
Vitamin premix ^a	1.00	1.00	1.00
Mineral premix ^b	1.50	1.50	1.50
Choline chloride	0.50	0.50	0.50
Ca(H ₂ PO ₄) ₂	1.50	1.50	1.50

2.2. Fish and feeding trial

Largemouth bass juveniles were obtained from a breeding farm in Changshou, Chongqing, China. Prior to the start of the normal feeding experiment, fish was disinfected and placed in a cylinder with a volume of 400L for two weeks. During the period of temporary breeding, commercial feed (Guangzhou Jieda Feed Co. LTD, China) was used for domestication. Next, the largemouth bass with similar size and good health were randomly selected as experimental fish. All fish were fasted for 24 h. Apparently healthy and similar size fish (average initial body weight: 12.17 ± 0.02 g) were randomly distributed into 9 the blue circle cylinder (capacity: 225 L) at a density of 30 fish per tank (each group is assigned 3 tanks). During the growth period, the fish were fed 3 times per day (8:30, 13:30 and 18:30) with two rounds of feeding each time. The water source for aquaculture is fully aerated tap water. During

6.5aquaculture, the water temperature is 23.2 mg/L, ammonia nitrogen < 0.1 mg/L, nitrite -28.5 °C, dissolved oxygen < 0.01 mg/L, and pH ≥ was around 7.5. The photoperiod of culture was 12 h light and 12 h dark, and the illumination time was 08:00–20:00. Water is exchanged every two days and is a quarter the size of the aquarium.

2.3. Collection and preparation of samples

At the end of the breeding experiment, the fish were fasted for 24 h, and then weighed and counted separately after being anesthetized with 0.01% MS-222. The growth characteristics and survival rate of largemouth bass were calculated in each test tank. After feeding for 6 h, twelve fish (4 fish in each tank) were randomly selected from each experimental group. After being anesthetized with 0.01% MS-222, the blood sample was obtained from the caudal vein using the 1 mL syringe. After standing for 12 h, serum was obtained by centrifugation. Body length and weight were measured, viscera, liver and intestines were

Vitamin C	0.05	0.05	0.05
Ethoxyquinoline	0.05	0.05	0.05
<i>Chemical composition (%)</i>			
Crude protein	47.76	47.67	47.69
Crude lipid	11.77	11.77	11.76
Ash	11.48	11.50	11.48

dissected on an ice tray, and weighed. The liver and intestinal tissues were collected and frozen in the 80 °C refrigerator with liquid nitrogen for the determination of tissue physiology and biochemistry. Intestinal fecal tissues of 2 fish were randomly collected from each tank, then put into 1.5 mL sterile test tube with RNA enzyme removed, frozen in liquid nitrogen, and placed in 80 °C refrigerator for microbiome sequencing. Complete livers of 2 fish were randomly collected from each tank and then placed into a 1.5 mL sterile tube with RNA enzyme removed. After freezing in liquid nitrogen, the samples were placed in a 80 °C refrigerator for liver metabolome sequencing. 2.4. Liver antioxidant ability and serum biochemistry

The liver was taken out at 80 °C, 1 g of tissue was accurately weighed, 2 enzyme-free beads and 9 mL double-steamed water were added into a 10 mL enzyme-free tube, homogenized, centrifuged (wuhan Sevicebio technology Co. Wuhan, China) in a frozen centrifuge (4000 RPM) for 15 min, and the supernatant was extracted. The 10% tissue homogenate was obtained. The

activities of total superoxide dismutase (T-SOD), catalase (CAT), malondialdehyde (MDA), and Glutathione peroxidase (GSH-Px) was determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). After the serum was removed from the 80 °C refrigerator and thaw naturally, the activity of D-lactate (D-lac), Diamineoxidase (DAO) and lipopolysaccharide (LPS) in the serum was measured using a commercial ELISA kit (Shanghai Bio-Technology Co., Ltd.). See the instructions of the kit for the operation method. Hepatic lipid was determined by chloroform methanol method. Hepatic glycogen was measured using a commercial kit.

2.5. RNA isolation, reverse transcription and mRNA levels analysis

Total RNA in liver and intestine was extracted using RNAiso Plus reagent (Takara, Japan) according to the manufacturer's instructions. Total RNA concentration and purity were determined using a Nano Drop 2000 ultramicrophotometer (Thermo Fisher, USA). The total RNA concentration was then 1000 µg/L by appropriate dilution. The total RNA extracted was then reverse-transcribed into cDNA using the reverse transcription kit Fastking-RT Supermix (Novoprotein, Shanghai, China). Real-time quantitative PCR (qPCR) system was 20 µL, including 1 µL upstream, 1 µL downstream primer (1 µM), 2 µL first strand cDNA, 10 µL 2 × NovoStart® SYBR qPCR Supermix Plus, and 6 µL free water. The . The quantitate sequences of specific primers used are shown in tve instrument was real-time quantitative PCR (Bio-RAD-CFX96, USA). The procedure was kept at 95 °C for 1 min for 1 cycle, 95 °C for 20 s, 60 °C for 1min and a total of 40 cycles. After that, a fusion curve was performed to verify that there was only one PCR product per PCR reaction. Ct values of cDNA were obtained by qPCR. The relative mRNA expression of target genes was normalized to the *β-actin* mRNA, and determined using the $2^{-\Delta\Delta Ct}$ method [23].

2.6. UHPLC-QTOF-MS non-target metabolomics

Non-target metabolomics data were measured by biotech companies (Biomarker Technologies Co, LTD) and analyzed on the BMKCloud. The main instrument platforms used are high performance liquid chromatography (Waters UPLC Acquity I-Class PLUS), high resolution mass spectrometry (Waters UPLC Xevo G2-XS QToF) and chromatographic columns (Acquity UPLC HSS T3 1.8 µm 2.1*100 mm, Waters). After the metabolites are extracted, the sample can be tested on the machine. In the machine parameters, the positive ion mode includes mobile phase A (0.1% formic acid aqueous solution), mobile phase B (0.1% formic acid acetonitrile anion) and injection volume (1 µL). The flow rate was always maintained at 400 µL/min. The separation was achieved

Table 2

Primer sequences for real-time PCR.

Genes	Primers	Sequence 5'-3'	GenBank
<i>Sod</i>	F ^a R ^b	CCACCAGAGGTCTCACAGCA CCACTGAACCGAAGAAGGACT	XM_038713969.1
<i>Cat</i>	F R	TGGTGTTACGGATGAGATGG GGAGAAGCGGACAGCAATAGG	XM_038704976.1
<i>GSH-Px</i>	F R	ATACCAAGTCTCTTCCCTCTGT CGTCCACCACTTTGCCATT	XM_038715030.1
<i>Zo-1</i>	F R	ATCTCAGCAGGGATTGCGAG CTTTGCGGTGGCGTTGG	XM_038701018.1
<i>Claudin-1</i>	F R	CCAGGGAAGGGAGCAATG GCTCTTTGAACAGTGCAGAC	XM_038713307.1
<i>Occludin</i>	F R	GATATGGTGGCAGCTACGGT TCCTACTGCGGACAGTGTG	XM_038715419.1
<i>IL-10</i>	F R	CGGCACAGAAATCCAGAGC CAGCAGGCTCACAATAAATCATCT	XM_038696252.1

using the following gradient: 0 min, 98% A, 2% B; 0.25 min, 98% A, 2% B; 10.0 min, 2% A, 98% B; 13.0 min, 2% A, 98% B; 13.1 min, 98% A, 2% B; 15.0 min, 98% A, 2% B. Parameters of mass spectrometry were as follows: low collision energy, 2 V; high collision energy range, 10–40 V, sweep frequency, 0.2 s. Parameters of ESI ion source were as follows: capillary voltage, 2000 V (+) and 1500 V (-); cone voltage, 30 V; ion source temperature, 150 °C; degree of desolvation temperature, 500 °C; backblow velocity, 50 L/h; flow rate of desolvation gas, 800 L/h. The original data collected by Masslynx V4.2 were processed by ProGenesis QI software for peak extraction and peak alignment. The identification was carried out based on the online Metlin database of ProGenesis QI software and the self-built database of Biomarker Technologies Co, LTD.

Principal Component Analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) analysis were used to distinguish the differences between the two groups. Cluster analysis can visually show the data characteristics. By mapping the differential metabolites to KEGG database, we can obtain the enrichment metabolic pathways. 2.7. Intestinal microbiota

Intestinal microorganism index entrusts biological company (Biomarker Technologies Co, LTD) to determine, and the data results were analyzed on the BMKCloud. Total bacterial DNA were extracted from samples using the Power Soil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's protocol. The V3 – V4 region of the 16S rRNA was amplified by PCR (forward primer, 5'- ACTCCTACGGGAGGAGCA-3'; reverse primer, 5'- GGACTACHVGGGTWTCTAAT-3') and sequenced using a HiSeq platform (Illumina, USA) at Biomarker Technologies Corporation (Beijing, China). Sequences simi-

larity sequence of OTU can be compared with the microbial reference database ≥97% were assigned to the same OTUs. The representative to obtain the taxonomic information of the corresponding species for each OTU, and then the composition of sample communities can be counted at various levels (Phylum, Class, Order, Family, Genus, Species). Then, the QIIME software was used to generate the species abundance tables at different taxonomic levels, and the VennDiagram R package was used for visualization [24]. Significant differences between groups were analyzed using Metastats [25]. The microbial alpha diversity index [26] was calculated, including Chao1, ACE, Shannon, Simpson and other indicators, and the OTU coverage rate (Sequencing depth) was also calculated. The BugBase tool was used for bacterial phenotypic prediction analysis [27].

<i>TGF-β1</i>	F R	GCTCAAAGAGAGCGAGGATG TCCTCTACCATTCGCAATCC	XM_038693206.1
<i>IL-8</i>	F R	CGTTGAACAGACTGGGAGAGATG AGTGGGATGGCTTCATTATCTTGT	XM_038704088.1
<i>IL-1β</i>	F R	CGTGACTGACAGCAAAAGAGG GATGCCAGAGCCACAGTTC	XM_038696252.1
<i>TNF-α</i>	F R	CTTCGTCTACAGCCAGGCATCG TTTGGCACCCGACCTCACC	XM_038710731.1
<i>β-actin</i>	F R	GGACACGGAAGGATTGACAG CGGAGTCTCGTTCTTATCGG	XM_038695351.1

^a F: forward primer. ^b R: reverse primer.

2.8. Statistical analysis

All data were expressed as the mean value of 3 data + standard error (S.E.M) and analyzed using SPSS25.0 version. One-way ANOVA was used to analyze the differences between the experimental groups, and Tukey's multiple

comparison was performed. The level of significance was set as $P < 0.05$. The graphics were drawn using GraphPad Prism7.0 (GraphPad Software Inc. USA).

3. Results

3.1. Growth performance

The growth performance and feed utilization of *M.salmoides* fed dietary yeast culture supplemental level under high-starch stress were presented in Table 3. In terms of final body weight, WG, and SGR, HSY₃ group was significantly higher than the control group ($p < 0.05$), but there was no statistical difference between HSY₁ group and the other groups ($p > 0.05$).

Hepatic lipid, hepatic glycogen and HSI was significantly decreased with the increase of dietary yeast culture supplemental level ($p < 0.05$, Table 3). In addition, CF was significantly increased with dietary yeast culture supplemental level increasing ($p < 0.05$), but compared with the control group, HSI in HSY₃ was significantly decreased ($p < 0.05$). In terms of body composition, adding yeast culture significantly increased

crude protein and decreased crude ash ($p < 0.05$).

3.2. Hepatic antioxidant capacity

The antioxidant capacity of *M.salmoides* liver was shown in Table 4. The activities of T-SOD and CAT significantly increased and the MDA content reduced as dietary fiber levels increase ($p < 0.05$), and *Sod* and *Cat* gene (Fig. 1) in HYS₃ was significantly up-regulated compared with the control group ($p < 0.05$).

3.3. Liver metabolome

In order to evaluate the metabolites of largemouth bass between the control and HSY₃, principal component analysis (PCA) and orthogonal projections to latent structures - discriminant analysis (OPLS-DA) were used to compare and analyze the samples (Fig. 4A) for PCA and (Fig. 4B) for OPLS-DA. The results showed a significant separation between the two groups.

The Volcano Plot showed that 2836 metabolites were produced in liver (Fig. 4C), of which 5 metabolites were significantly up-regulated and 106 metabolites were significantly down-regulated ("Fold change > 1" and "VIP > 1", $P < 0.05$). Cluster analysis was performed on the 111 differential metabolites (Fig. 4D). Typical metabolites include dide-O-methylsimmondsin, (3 beta, 22 E, 24 R)-ergosta-4, 6, 8 (14), 22-tetraen-3-ol, (PC) (18:0/18:3 (9Z, 12Z, 15Z)), and squamocin L.

The KEGG annotation results of some differential metabolites can be seen in Table 6, which mainly included metabolism, human diseases, organismal systems and genetic information processing. To be specific,

Table 3

Effect of yeast culture on the growth performance and morphological measurements of *M.salmoides* induced by high-starch.

		HSY ₁	HSY ₃
Items	Groups		
	Control		
<i>performance</i>	<i>Growth</i>		
Initial body weight (g)	12.15 ± 0.02 _a	12.19 ± 0.03 _{ab}	12.17 ± 0.04 _b
WG (%)		84.83 ± 1.07 _{ab}	88.40 ± 1.05 _b
Final body weight (g)		595.70 ± 6.95 _{ab}	626.51 ± 10.82 _b
591.601.893.2283.33 ± ± ± 0.030.02±1.178.83 _a		3.23 ± 0.02	3.30 ± 0.02
		1.84 ± 0.05	1.85 ± 0.03
SGR PER		2.82 ± 0.08	2.84 ± 0.05
		1.13 ± 0.03	1.12 ± 0.02
FR ^g _f	2.751.10 ± ± 0.050.02 FCR	100	100
SR ^h (%)	100		
<i>Morphological measurements</i>			
CF ⁱ (g/cm ³)	2.08 ± 0.02 ^a	2.29 ± 0.02 ^b	2.36 ± 0.01 ^c
HSIVSI ^j (%)	9.222.21 ± ± 0.420.03 _b	8.68 ± 0.50 _{ab}	8.371.99 ± ± 0.090.05 _a
j (		2.12 ± 0.06 _b	
Hepatic lipid (%)	3.28 ± 0.28 _b	3.07 ± 0.17 _b	2.21 ± 0.05 _a
Hepatic glycogen (mg/g)	97.65 ± 1.09 _{Body}	97.39 ± 0.62	91.56 ± 0.52
<i>composition</i>			
Moisture (%)	70.86 ± 0.01 _{Crude lipid}	70.99 ± 0.15	71.10 ± 0.11
(%)	6.88 ± 0.44 _a Crude protein (%)	6.76 ± 0.16 _b	6.29 ± 0.14 _c
	17.65 ± 0.01 _a Crude ash (%)	17.82 ± 0.02 _b	18.20 ± 0.07 _a
	4.43 ± 0.02	4.29 ± 0.04	4.07 ± 0.03

^a Values (means ± S.E.M, n = 3) in the same row with different letter superscripts were significantly different ($P < 0.05$), while with the same or no letter superscripts were not significantly different ($P > 0.05$). The same as below. ^b Weight gain rate (WG) =

$100\% \times [\text{final weight (g)} - \text{initial weight (g)}] /$

$\text{initial weight (g)}$. ^c Specific growth rate (SGR) = $100\% \times [\ln (\text{mean final weight}) - \ln (\text{mean initial weight})] / 56 \text{ days}$. ^{def} Feed conversion ratio (FCR) Feeding ratio (FR) Protein efficiency

ratio (PER) = $100\% \times \text{F} / [(\text{Wtotal feed intake in dry basis (g)} / \text{weight gain total weight}$

$\text{gain (g)} / \text{protein intake (g)} \cdot \text{t} + \text{W}_0) / 2 \times \text{t}]$.

(g).

^{ghij} Hepatopancreas somatic index (HSI/) Viscera somatic index (VSI) Survival rate (SR)

Condition factor (CF) = $100 \times \text{final number} / \text{initial number}$. $\text{‰} = 100 \times \text{fish weight} / (\text{body length})\%$ $\text{x} = \text{visceral weight} / \text{fish weight}$. $100\% \times \text{liver weight} / \text{fish weight}$.

³.

Table 4

Effects of yeast culture on hepatic antioxidant capacity of *M.salmoides* induced by high-starch.

Items	Groups		
	Control	HSY ₁	HSY ₃
CAT (U/mgprot)	16.53 ± 0.54		
GSH-Px (U/mgprot)	41.11 ± 2.43	43.74 ± 1.65	44.67 ± 1.91
MDA (nmol/ml)	1.22 ± 0.05 ^b	1.21 ± 0.06 ^{ab}	1.01 ± 0.10 ^a

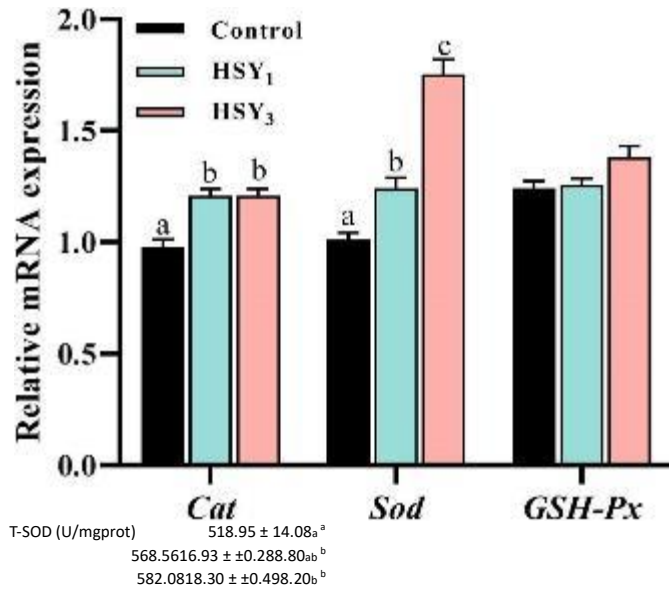


Fig. 1. Liver expression levels of antioxidant-related genes of juvenile largemouth bass fed the experiment diet.

Values (means ± S.E.M, n = 3) in each column with different superscripts have significant differences ($P < 0.05$). The same as below.

the up-regulated differential metabolites were mainly enriched in alpha-linolenic acid metabolism (ko00592), choline metabolism in cancer (ko05231), arachidonic acid metabolism (ko00590), linoleic acid metabolism (ko00591), glycerophospholipid metabolism (ko00564), steroid hormone biosynthesis (ko00140) and cortisol synthesis and secretion (ko04927) pathways. Secondly, the down-regulated differential metabolites were mainly enriched in folate biosynthesis (ko00790), metabolic pathways (ko01100) and tryptophan metabolism (ko00380) pathways. 3.4. Serum biochemical

The serum biochemical parameters of *M.salmoides* are shown in Table 5. The serum levels of D-lac, DAO and LPS in HSY₃ were significantly lower than those in other groups ($p < 0.05$), and there was no significant difference between HSY₁ and the control ($p > 0.05$).

Table 5

Effects of yeast culture on serum biochemical parameter of *M.salmoides* induced by high-starch.

Items	Groups		
	Control	HSY ₁	HSY ₃
D-lactate (umol/L)	528.59 ± 10.59 ^b	513.46 ± 13.10	388.95 ± 8.15
Diamine oxidase (U/L) Lipopolysaccharide (EU/		288.8122.51 ± ±0.428.34 ^b	
	22.48285.22 ± ±0.494.74 ^b	15.27203.41 ± ±2.35 ^a	9.74

Table 6

Annotation results of differentially expressed metabolite KEGG in the control group and 3% yeast culture group (Control vs HSY₃, n=3).

Metabolites	up or down	Log ₂ FC	VIP	KEGG Pathway Annotation
Phosphatidylcholine	up	3.76	1.49	alpha-Linolenic acid metabolism(ko00592) Choline metabolism in cancer(ko05231) Arachidonic acid metabolism(ko00590) Linoleic acid metabolism(ko00591) Retrograde endocannabinoid signaling(ko04723) Glycerophospholipid metabolism(ko00564) Metabolic pathways(ko01100)
Cortisolone	up	2.95	1.38	Metabolic pathways(ko01100) Steroid hormone biosynthesis(ko00140) Cortisol synthesis and secretion(ko04927) Cushing syndrome(ko04934)
Cellobiose	down	-2.82	1.40	Starch and sucrose metabolism(ko00500) Metabolic pathways(ko01100) ABC transporters(ko02010)
5-Hydroxy-L-tryptophan	down	-1.29	1.35	Serotonergic synapse(ko04726) Metabolic pathways(ko01100) Tryptophan metabolism(ko00380)
4-Oxo-1-(3-pyridyl)-1-butanone	down	-1.87	1.45	Metabolism of xenobiotics by cytochrome P450(ko00980) Nicotinate and nicotinamide metabolism(ko00760) Chemical carcinogenesis(ko05204)
10-Formyltetrahydrofolate	down	-2.07	1.40	One carbon pool by folate(ko00670) Aminoacyl-tRNA biosynthesis(ko00970) Metabolic pathways(ko01100) Carbon metabolism(ko01200)
Indole	down	-1.46	1.31	Tryptophan metabolism(ko00380) Metabolic pathways(ko01100) Protein digestion and absorption(ko04974)
Dihydrobiopterin	down	-1.41	1.41	Phenylalanine, tyrosine and tryptophan biosynthesis(ko00400) Folate biosynthesis(ko00790) Metabolic pathways(ko01100)
Oxalic acid	down	-2.00	1.35	Metabolic pathways(ko01100)
ADP-ribose	down	-1.82	1.35	Glyoxylate and dicarboxylate metabolism(ko00630) Purine metabolism(ko00230)

3.5. Intestinal physical and immune barrier

Intestinal expression levels of tight junction related genes are shown in Fig. 2. The results showed that the mRNA levels of *Zo-1*, *Claudin* and *Occludin* increased significantly as the addition of yeast culture ($p<0.05$). In addition, intestinal expression levels of anti-inflammatory cytokines (TGF- β 1) were significantly up-regulated (Fig. 3), and intestinal expression levels of pro-inflammatory cytokines (IL-8, IL-1 β , TNF α) were significantly down-regulated as the addition of yeast culture ($p<0.05$).

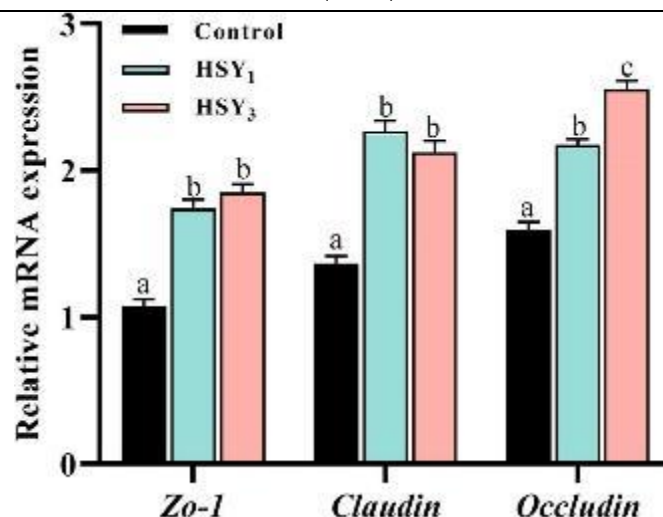
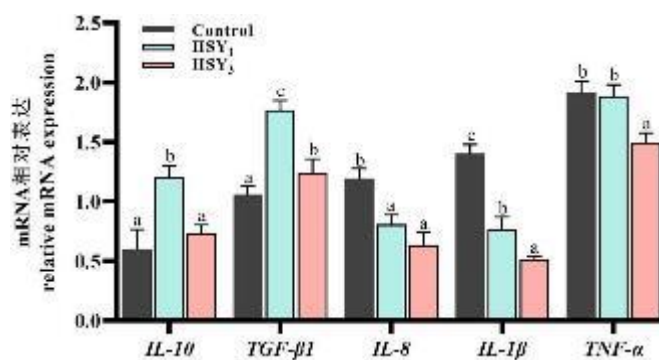


Fig. 2. Intestinal expression levels of tight junction-related genes in juvenile largemouth bass.



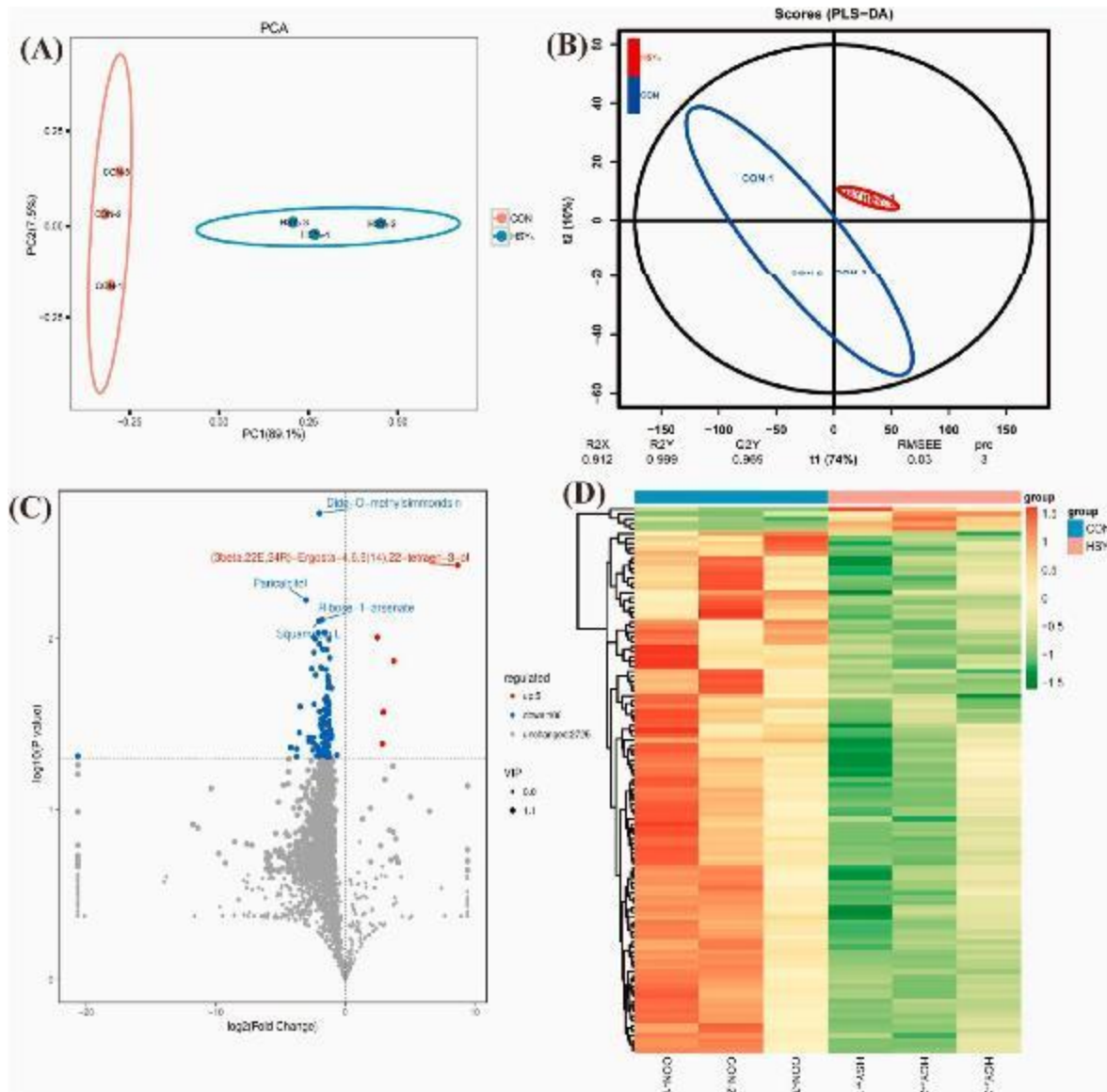


Fig. 4. The degree of difference between the control group and the HSY₃ group was shown in the untargeted metabolic. (A) Principal Component Analysis, The X-axis represents the first principal component, and the Y-axis represents the second principal component. The different colored ellipses represent 95% confidence intervals. (B) Orthogonal feeding Interests-discriminant analysis (OPLS-DA) was used to analyze the results. (C) Volcano Plot looks at the differences between the two groups of metabolites, and each dot represents a metabolite. The abscissa represents the group contrast ratio of each material change (with 2 logs base), and the vertical says the P value (in the logarithm of 10 at the bottom). Scatter represents OPLS - DA model to the size of the VIP value, the greater the scatter VIP value, the greater the screening of differentially expressed metabolites is more reliable. Blue dots represent downregulated and differentially expressed metabolites, red dots represent up-regulated and differentially expressed metabolites, and gray dots represent detected but not significantly different metabolites (D) Clustering analysis of the selected differential metabolites can further excavate the characteristics and obtain the differential metabolic clustering heat map. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. Intestinal expression levels of

inflammation-related genes in juvenile largemouth bass. 3.6. Intestinal microbial

A total of 458,436 pairs of raw Reads were obtained by sequencing of the six samples. A total of 455,335 Clean Reads were generated after quality control and splicing of double-ended Reads, and at least 58,137 Clean Reads were generated from each sample. With the addition of yeast culture, Shannon and Simpson index showed no significant

difference, while OTUs, ACE and Chao1 index increased significantly ($p < 0.05$, Table 7).

The histogram of intestinal microbial species classification is shown in Fig. 5. At the phylum level, *Proteobacteria* and *Firmicutes* were the dominant phylum of largemouth bass, followed by *Actinobacteria* and *Bacteroidetes*. With the increase of yeast culture, the abundance of *Proteobacteria* decreased from 51.90% to 33.37% while the abundance of *Firmicutes* increased from 28.09% to 39.14% (Fig. 5A). At the genus level, *Plesiomonas* (17.34%) and *Stenotrophomonas* (8.39%) were

Table 7

Effects of yeast culture on intestinal microbiota α diversity of *M. salmoides* (means $\pm$ S.E.M, $n = 3$).

Items	Richness estimates	Diversity estimates	Coverage
-------	--------------------	---------------------	----------

	OTUs	ACE	Chao1			
Control	583 ± 112 ^a	681 ± 85.2 ^a	677 ± 92.4 ^a	0.924 ± 0.05	6.16 ± 1.02	99.8 ± 0.00
HSY ₃	988 ± 2.52 ^b	1008 ± 2.66 ^b	1018 ± 2.45 ^b	0.989 ± 0.01	8.09 ± 0.09	99.8 ± 0.00

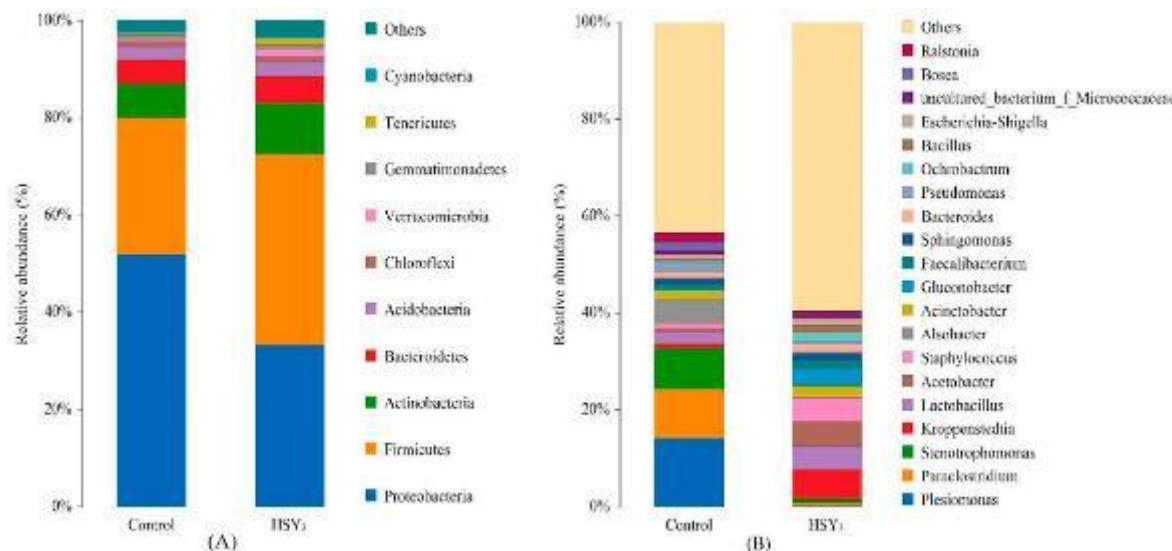


Fig. 5. Histogram of intestinal microbial species classification at phylum (A) and genus (B) levels in control group and HSY₃ group. The phylum level lists the top 10 most abundant (Based on relative abundance) bacterial phyla and the genus level lists the top 20 most abundant bacteria. Other phyla genera were all assigned as 'Others'.

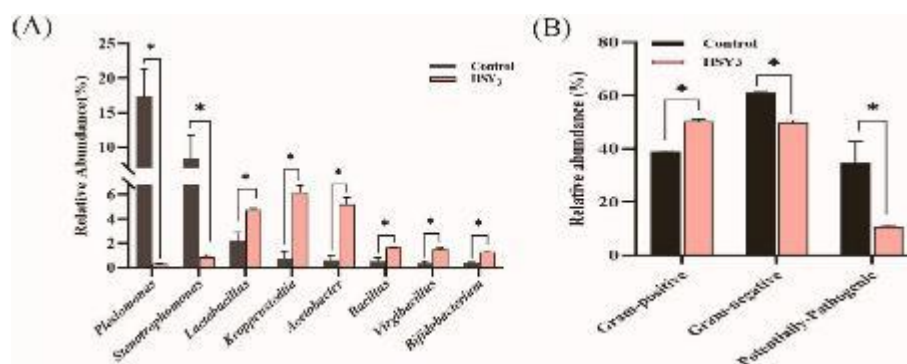


Fig. 6. (A) The differences in microbial community abundance (at genus level) between the two groups were analyzed by Metastats software. (B) BugBase phenotype analysis of control group and HSY₃ group. "*" represents a significant difference between the two groups ($P < 0.05$).

Simpson Shannon (%)

dominant genera in the control group while *Kroppenstedtia* (6.18%) and *Acetobacter* (5.19%) were dominant genera in HSY₃ group (Fig. 5B). Meanwhile, the difference of microbial community abundance (Fig. 6A) between the two groups was analyzed by Metastats software. We found that the abundance of *Plesiomonas* and *Stenotrophomonas* significantly decreased while the abundance of *Lactobacillus*, *Kroppenstedtia*, *Acetobacter*, *Bacillus*, *Virgibacillus* and *Bifidobacterium* significantly increased in HSY₃ group compared to the control group ($P < 0.05$).

The phenotypic prediction (Fig. 6B) showed that the HSY₃ group significantly increased the proportion of Gram-positive bacteria while

decreased the proportion of Gram-negative bacteria, compared with the control group ($P < 0.05$). With the addition of YC, the potential pathogens were significantly reduced ($P < 0.05$).

4. Discussion

The current study demonstrated the benefit of dietary supplementation with yeast culture (YC) on the growth of *M. salmoides*. Similar result was observed in Nile tilapia (*Oreochromis niloticus* L.) [28]. As well, many researchers have reported that YC promotes nutrient absorption and improves the growth performance of weaned piglets [29], broiler [30] and lambs [31]. However, some studies showed that YC has no effect on growth of animals [32]. The different results may be mainly related to fish species, dietary composition and yeast additive form. In addition, growth enhancing effect of YC is dependent on dosage, duration of feeding, the route of administration and species [33]. The results of the present study exhibited that the growth-promoting effect of YC was related to doses of yeast culture, and supplementation of YC at 30.0 g kg⁻¹ was optimal for the growth of *M. salmoides* fed high-starch diets.

Liver is an important metabolic organ that may regulate various metabolism functions, when the hepatic injury may lead to these physiochemical function disorder. Previous studies have confirmed that a high-starch diet leads liver damage of largemouth bass [20]. The present study found that the protective effect of YC against high starch-induced liver damage in largemouth bass, which might be due to the up-regulation of related genes (*Cat* and *Sod*) and resulted in the increase of antioxidant enzymes activities. This is consistent with previous research on Atlantic salmon (*Salmo salar*) [34] and Ussuri catfish (*Pseudobagrus ussuriensis*) [21]. Other studies have also

shown that yeast product can improve the antioxidant status and disease resistance of fish [35]. Some studies have indicated that yeast products are mainly composed of a high content of polysaccharides, which have been reported to play a protective role against oxidative damage preserving the cellular integrity [36,37]. Thus, it can be presumed that the protective effect of YC against high starch-induced liver damage in largemouth bass may be related to the mannose oligosaccharides (MOS), β -glucan and other antioxidants in YC.

In addition, using liver non-target metabolomics techniques, we found that phosphatidylcholine (PC) (18:0/18:3 (9Z, 12Z, 15Z)) was significantly upregulated in HSY₃ group compared to the control group. Studies have shown that decreased hepatic PC content or/and decreased ratio of hepatic PC to phosphatidylethanolamine (PE) have been linked to non-alcoholic fatty liver disease (NAFLD) [38]. The ratio of PC/PE is closely associated with severe fatty liver [39], which generally was regarded as the clinical evidence of liver health [40]. Noteworthy, in the present study, liver PC was upregulated 13.5 times in HSY₃ group, and (PC) (18:0/18:3 (9Z, 12Z, 15Z)) was significantly enriched in the lipid metabolism pathway in KEGG database. This suggested that YC supplementation can could positively influence the hepatic health of largemouth bass. Moreover, it was also observed that 3% YC supplement significantly reduced the liver glycogen and hepatic lipid of largemouth bass. These results suggest that the liver protection mechanism of YC may be related to the change of liver fat metabolism. This is well agreement with the results in broiler chickens, which used yeast to ferment cottonseed meal to improve liver and abdominal fat metabolism [41]. As far as we know, this is the first attempt to explore the effects of YC on a non-target liver metabolome in largemouth bass and needs to be further studied for providing new essential information.

Intestinal barrier represents the first line of defense against environmental pathogens in fish [42]. DAO, D-lac and LPS are three important markers of intestinal mucosal injury [43–45]. We found

that YC reduced the DAO activity and the D-lac and LPS contents in the serum, indicating that it reduced intestinal permeability in largemouth bass. Intestinal permeability is mainly controlled by the tight junction (TJ) proteins between epithelial cells [46,47]. Further studied suggested that YC promoted the expression of intestinal TJ proteins in vivo. When TJ structure is destroyed, the host initiates a series of protective mechanisms to restore the normal state. Using the high starch-treated fish model, we found that YC reduced starch-induced TJ breakdown by enhancing the expression of *Zo-1*, *Claudin* and *Occludin*. This agrees with study by Wang et al. [48] Who found that yeast products (contain yeast culture) significant also increased the expression of *Occludin* and *Zo-1* gene in the small intestinal epithelial. In addition, the efficiency of yeast supplementation for fish varies in relation to the dose of the components used in the diet. The supplementation of a higher dose of YC (3%) was observed to be more beneficial than the dose of 1% supplementation in largemouth bass. These evidences suggest that YC is beneficial to TJ barrier in vitro.

Importantly, the release of proinflammatory cytokines can destroy the integrity of intestinal TJ structure, which exacerbates intestinal permeability [49]. The cytokines in animals reflected systemic immune status to a certain extent. Yeast culture and their glycan components improve mucosal damage and inflammation in intestinal diseases [50]. Previous researches reported that yeast products (contain yeast culture) increased the concentration of anti-inflammatory factor in the jejunum and ileum of weaned piglets [51], and downregulated the expression of proinflammatory cytokines via NF- κ B related pathway in Ussuri catfish [21]. The present study also found that YC significantly up-regulate the expression of anti-inflammatory cytokines (*TGF- β 1*) and down-regulate pro-inflammatory cytokines genes (*IL-8*, *IL-1 β* , *TNF α*) in the intestine. These findings maybe attribute to the immune polysaccharide of yeast cell wall, such as mannan oligosaccharides and β -glucans. Although several studies have shown that *IL-8* dysregulation can influence intestinal inflammation, the contribution of *IL-8* to increase the permeability of TJ remain unclear [52]. Therefore, the yeast culture should be conducted in future investigations to further explore the mechanisms of the anti-inflammatory effects on high starch-induced intestine damage. Intestinal homeostasis is a dynamic process regulated by many factors and can change at any time to achieve a balanced state. Because of the importance of intestinal barrier function, the regulation of TJ proteins involves a complex network that is affected by many factors. Our study provides a reference for YC to participate in the regulation of the integrity of the intestinal barrier in fish.

Probiotics are known to benefit gut healthy by suppressing the number of harmful microorganisms and promoting growth of beneficial bacterium [53]. In this investigation, the dietary YC inclusion altered intestinal microbial community in largemouth bass. We did not find the difference in the diversity of intestinal microbiota in groups of fish pretreated with YC. But administration of YC significantly increased the relative abundance of intestinal microbiota, which suggested that YC could improve the stability of microflora. This observation is in accordance with the findings by Liu et al. [54]. In contrast [55], found that yeast supplementation on Nile Tilapia exerted a significant influence on the alpha-diversity of the autochthonous microbiota. This discrepancy is likely due to the intestinal microbiota variably depending on the environmental factors and the variety of host species. Furthermore, the most abundant two phyla *Firmicutes* and *Proteobacteria* were predominant in the intestine, being over 70% of the total bacteria. When treated with high starch, the abundance of *Proteobacteria* in the intestine increased sharply, while *Firmicutes* decreased, which is consistent with studies on largemouth bass [7,56] and on golden pompano (*Trachinotus ovatus*) [57]. Intriguingly, after the addition of 3% YC, the abundance of *Proteobacteria* in the gut decreased, while the abundance of *Firmicutes* increased. *Proteobacteria* is a microbial signature of inflammation in the gut [58,59]. *Firmicutes* can play a key role in host nutrition and metabolism through the synthesis of short-chain fatty acids (SCFA) [60,61]. This partly suggested that adding 3% YC can improve intestinal flora and reduce the possibility of intestinal inflammation. Besides, the ratio of *Firmicutes/Bacteroidetes* increased from 5.02 to 6.98 in this study. The higher ratio of *Firmicutes* to *Bacteroides* reflect the stronger ability of nutrient

transportation and utilization [61,62]. It revealed that YC might enhance the digestion and absorption of nutrients, which led to the higher growth of largemouth bass.

Noteworthy, significant changes of the gut microbiota in different groups were also found at the genus taxonomic level. Our data show that high-starch stress results in significant increase of *Plesiomonas*, which is known as a potential opportunistic pathogen, positively correlated with gut permeability [63,64]. We hypothesize that *Plesiomonas* bacteria could be related to the disturbance in the TJ proteins expression, observed in control group. Conversely, treatment with YC in high-starch diet (HSY₃) prevented increase of potential pathogenic bacteria and destruction of intestinal barrier. The present results found significant decrease of potential pathogenic bacteria (*Plesiomonas*) and increase of potential beneficial bacteria (*Lactobacillus*, *Bacillus* and *Bifidobacterium*) in microbiota of largemouth bass from HSY₃ group vs the control group. Essential impact of the YC on microbiota during high-starch stress is manifested in elevated number of beneficial bacteria. Beneficial effects of these bacteria were shown in many studies containing in humans and animals. Thus, *Bifidobacterium* are known as a normal component of the gut microbiota and as probiotics for human and animal consumption [65]. *Lactobacillus* are showed to reduce the intestinal permeability and exert a protective effect on intestinal epithelium via inhibiting the regulation of immune cytokines [66]. *Bacillus* could regulate intestinal physiology and maintaining intestinal homeostasis [67,68]. A large number studies have shown that these three strains have the potential function of secreting bile salt hydrolase (BSH) [69–71]. BSH forms free bile acids such as cholic acid (CA), chenodeoxycholic acid (CDCA) and muricholic acid (MCA) through conjugation and increases the rate of bile acid reabsorption into the liver. In addition, our results also showed that the relative abundances of *Kroppenstedtia* and *Acetobact* increased with the supplementation

of YC. Knowledge of the biological functions of these two genera in the intestine reported in the literature so far is too little to explain the altered abundance of the genera in fish with the supplementation of YC and thus warrants further studies. Further phenotypic analysis of BugBase confirmed the decline of potential pathogenic bacteria in fish with the supplementation of YC. Simultaneously, a decrease in the proportion of negative bacteria was also observed, which could explain the decrease of LPS in serum to some extent. It is not surprising that positive bacteria decline under a high-starch diet. Therefore, positive contribution of YC to the change of microbiota was testified in fish model. It is well-known that the intestinal microbial community is associated with animal performance, health, and immunity.

In conclusion, the present results revealed substantial effect of YC synbiotics in prevention of high starch-related complications. Oral treatment of fish with prebiotic in high-starch stress conditions protected disruption of liver and intestine homeostasis, enhanced antioxidant capacity, maintained expression of TJ proteins. We suggest that these effects are associated with beneficial modulation of the gut microbiota by prebiotic. However, the effect of YC depends on its concentration in the high-starch diet of juvenile largemouth bass, and the dose of YC in this study is recommended to be 3%. Our findings may provide new insights into the mechanism by which yeast culture regulates TJ proteins in fish.

Ethics statement

This study was conducted under the standard code of protocol for the Care and Use of Laboratory Animals in China. This research was approved by the Animal Ethics Committee of Southwest University (No. IACUC-20181015-12, 15 October 2018).

CRedit authorship contribution statement

Zhuandong Feng: conducted experiments, Data curation, Writing – original draft, Writing – review & editing. **Yunfei Zhong:** conducted experiments, Data curation, Writing – original draft, Writing – review & editing. **Guanglun He:** Methodology, Project administration. **Hao Sun:** Methodology, Project administration. **Yongjun Chen:** Supervision, Formal analysis. **Wenhao Zhou:** Formal analysis. **Shimei Lin:** idea for the project, experiment design, paper revising.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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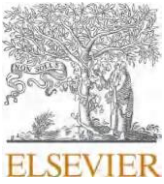
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Partially replacing dietary fish meal by *Saccharomyces cerevisiae* culture improve growth performance, immunity, disease resistance, composition and function of intestinal microbiota in channel catfish (*Ictalurus punctatus*)

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ABSTRACT

The aim of the present study was to investigate the partial replacement of fish meal by *Saccharomyces cerevisiae* culture on growth performance, immunity, composition and function of intestinal microbiota and disease resistance in channel catfish (*Ictalurus punctatus*). Two equal nitrogen and energy diets were prepared including a basal diet (containing 10% fish meal, Control) and an experimental diet (replacing 20% of the fish meal of the basal diet with yeast culture, RFM). Channel catfish were fed with the diets for 12 weeks. The results showed that weight gain and condition factor were significantly increased, and FCR was significantly decreased in RFM group ($P < 0.05$). The gene expression of intestinal *HIF1α* was significantly increased in RFM group ($P < 0.05$), while the expressions of *NF-κB* in the intestine and liver were significantly decreased ($P < 0.05$). The relative abundance of Firmicutes tended to increase, and the *Turicibacter* had an upward trend ($0.05 < P < 0.2$). In addition, the survival rate of channel catfish was significantly increased in RFM group after challenged with *Aeromonas veronii* Hm091 and *Aeromonas hydrophila* NJ-1 ($P < 0.05$). Compared with intestinal microbiota of channel catfish of control group, intestinal microbiota of channel catfish of RFM group significantly increased the expression of *HIF1α*, and decreased the expression of *IL-1β* and *TNF-α* ($P < 0.05$) in germ-free zebrafish. Intestinal microbiota induced by RFM diet also significantly increased disease resistance to *Aeromonas veronii* Hm091 and *Aeromonas hydrophila* NJ-1. In conclusion, replacement of fish meal by the yeast culture improved the growth, immunity and disease resistance of channel catfish, and intestinal microbiota of channel catfish induced by the yeast culture played a critical role in these effects.

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1. Introduction

As an important protein source in feed, fish meal has the characteristics of high protein content, good palatability, less antinutritional factors, balanced composition of essential amino acids, high content of calcium, phosphorus, iodine and selenium, and many kinds of vitamins

[1]. However, the rapid increase of fish meal demand and the decline of

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marine fishery resources have led to the shortage of fish meal resources, the rising price of fish meal and the reduction of profits in the feed industry. It is an important way for the sustainable development of aquaculture to reduce the use of high price protein sources and use appropriate protein sources instead of fish meal [2]. Plant protein sources

freshwater aquaculture species in the United States, accounting for more than 60% of the total fish production in the United States [13,14]. In 1984, China introduced channel catfish from the United States. Since then, the scale of cultivation has gradually expanded. Due to its strong adaptability, fast growth rate and delicious meat, the scale of aquaculture has been increased year by year [15–17]. In 2020, the national aquaculture output of channel catfish reached to 308,488 tonnes, which has become one of the excellent aquaculture species in China (China fishery statistical yearbook, 2021). The yeast culture has high content of crude protein, which can be used as an alternative protein source. However, there were fewer reports on application

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(such as soybean meal, miscellaneous meal, etc.) are important alternatives, but there are also problems such as insufficient resources, nutritional imbalance, and anti-nutritional factors [3]. Thus, there is an emerging need for suitable and sustainable novel feed ingredients for aquaculture. In the past ten years, the research on single cell protein and its application in aquatic feed as a substitute for fish meal have been widely concerned.

Single cell protein refers to the cells of yeast, bacteria, actinomycetes, molds, algae and higher fungi, which are cultivated on a large scale and used as the protein source of human food and animal feed [4–6]. Yeast products are widely used in feed as functional additives for aquatic animals. However, many researchers have gradually begun to pay attention to the possibility of yeast source products as feed raw materials. Yeast is a kind of protein feed with high nutritional value, which contains relatively high protein content (45–55%) and can be produced in large quantities at low cost [7,8]. By adding a certain proportion of yeast products to replace fish meal in feed, the cost of feed raw materials can be reduced and the shortage of feed protein raw materials can be alleviated. At the same time, the yeast products are rich in organic acid, oligosaccharide, flavoring substance and aromatic substance [9], which can enhance the digestion and absorption of aquatic feed, improve growth and metabolism of fish, and improve the survival rate of fish [10–12].

Channel catfish (*Ictalurus punctatus*) is the most important

of fish meal replacing by yeast culture in channel catfish. Therefore, in order to provide theoretical basis for the development of low fish meal artificial feed for channel catfish, yeast culture was used to replace fish meal in this study to evaluate its effects on the growth performance, immune response, intestinal microbiota, and disease-resistance against *Aeromonas veronii* Hm091 and *Aeromonas hydrophila* NJ-1 in channel catfish.

2. Materials and methods

2.1. Bacteria, yeast product and diet preparation

Saccharomyces cerevisiae Sa-10 was preserved in China General Microbiological Culture Collection Center (CGMCC no. 6120). *A. veronii* HM091 was preserved in China General Microbiological Culture Collection Center with its preservation number CGMCC no.22536. *A. hydrophila* NJ-1 was a gift from Dr. Yongjie Liu from Nanjing Agricultural University (Nanjing, China). *Saccharomyces cerevisiae* culture (ESTAQUA®) was provided by Enhalar Biotechnology Company (Beijing, China), and was fermented based on the patent CN107937292A together with technique guidance of Sino-Norway Joint Lab on Fish Gut Microbiota. In brief, when obtaining yeast culture powder, *Saccharomyces cerevisiae* Sa-10 was aerobically fermented in a molasses-based medium (150 g of cane molasses, 10 g of ammonium sulfate, 1.6 g of yeast extract, 1.5 g of magnesium sulfate, 0.08 g of anhydrous calcium chloride, 1.5 g of potassium dihydrogen phosphate, 0.3 g of defoamer in 1 L of sterile water, pH 6.2). The grains were added to the content to form a slurry and undergo anaerobic fermentation to enhance cell wall destruction. Finally, the fermented product was enriched with nucleotides and acetic acid, and this product contained 48.56% more nucleotides and 90.19% more acetic acid than it obtained using the original production method (SI Table 1).

The main source of protein in the basal diet were soybean meal, rapeseed meal and fish meal, and the main source of lipid was soybean

Table 1

Feed formulation and chemical composition of diets for channel catfish.

	Control	RFM
Rice bran	100.00	100.00
Flour	150.00	150.00
Soybean meal	250.00	250.00
Rapeseed meal	150.00	154.00
Fish meal	100.00	80.00
Chicken meal	100.00	100.00
DDGS	50.00	50.00
SCC Sa-10 ^a	0.00	20.00
Bentonite	15.00	10.00
Lys-HCl	2.00	2.00
Methionine	0.50	0.50
Choline Chloride (50%)	2.00	2.00
Ca(H ₂ PO ₄) ₂	20.00	20.00
Soybean oil	40.00	41.00
VC phosphate Premix ^b	0.50	0.50
Total	1000	1000
Chemical composition (g/kg)		
Crude protein	380.4	
376.7 Crude fat		
112.9 116.1 Crude ash		
102.2 98.6		
Moisture		
54.3	66.7	

^a Provided by Enhalar Biotechnology Company, Beijing, China, its composition was as follows: Crude protein, 67.73%; Crude fat, 3.18%. ^b Containing vitamin premix and mineral premix. (1) Vitamin premix con-

taining the following (g/kg): thiamine, 0.438; riboflavin, 0.632; pyridoxine·HCl, 0.908; *D*-pantothenic acid, 1.724; nicotinic acid, 4.583; biotin, 0.211; folic acid, 0.549; vitamin B-12, 0.001; inositol, 21.053; menadione sodium bisulfite, 0.889; retinyl acetate, 0.677; cholecalciferol, 0.116; *DL*- α -tocopherol-acetate, 12.632. (2) Mineral premix containing the following (g/kg): CoCl₂·6H₂O, 0.074; CuSO₄·5H₂O, 2.5; FeSO₄·7H₂O, 73.2; NaCl, 40.0; MgSO₄·7H₂O, 284.0; MnSO₄·H₂O, 6.50; KI, 0.68; Na₂SeO₃, 0.10; ZnSO₄·7H₂O, 131.93; Cellulose, 501.09.

oil. According to previous research results [18,19], in this study the experimental diet was prepared by 20 g/kg *Saccharomyces cerevisiae* culture (SCC) to replace the same amount of fish meal in the basic diet. The feed formulation and approximate analysis were shown in Table 1.

2.2. Fish husbandry and feeding

All experiments and animal care procedures were approved by Institute of Feed Research of the Chinese Academy of Agricultural Sciences by the China Council for

Animal Care (Assurance No. 2019-AF- FRI-CAAS-001). Channel catfish was

Ingredient	g/kg (air dry basis)
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purchased from a fish farm in Hebei, China, and transported to the International Agricultural High-tech Industrial Park of the Chinese Academy of Agricultural Sciences, Langfang, Hebei, China. The channel catfish was domesticated in circulating water for a week before the experiment.

After 24 h of starvation, channel catfish with similar body weight (initial body weight 24.49 ± 0.11 g) were randomly divided into 2 groups. 6 replication tanks were assigned to each treatment, and each tank containing 12 fish. During the trial period, channel catfish were fed 3 times a day (7:00, 12:00 and 18:00). The fish were fed with basic feed and experimental feed for 12 weeks. Initially, they were fed daily at 1.5% of their initial body weight for 2 weeks. From the third week, the feeding amount increased to 2.5% of their initial body weight for 6

weeks. Starting from the ninth week, the feeding amount increased to 4% of their initial body weight for another 3 weeks, and from the twelfth week, the feeding amount increased to 5% of their initial body weight for 1 week. The capacity of the feeding tank was 90 L, the water inflow was 120 L/h, the water temperature was 25.5 °C, the dissolved oxygen

(DO) was ≥ 6 mg/L, the ammonia nitrogen content was 0.06 mg/L. <0.2 mg/L, and the nitrite concentration was <

2.3. Measurement of growth performance and feed utilization efficiency

At the end of the experiment, fish were fasted for 24 h, and then weighed after being anaesthetized with 3-aminobenzoic acid ester methanesulfonate. The weight gain (WG, %), feed coefficient (FCR), survival rate (%), condition factor were calculated according to the method [20].

2.4. Histological observation of intestine and liver

At the end of the experiment, 1 fish was randomly selected from each of the 6 replication tanks for liver and hindgut histological analysis after 24 h fasting ($n = 6$). The samples were dehydrated in 4% paraformaldehyde, and then embedded in paraffin. The sections were stained with hematoxylin and eosin (HE), sealed with neutral gum, and observed with microscope (Leica DMIL-LED, Germany). The K-Viewer image analysis software was used for the image analysis.

2.5. Relative quantification expression of immune genes

Relative quantification analysis was used to evaluate the effect of feeding RFM diet on the immunity of channel catfish. Total RNA was extracted from liver and intestine of channel catfish using the TRIzol Reagent extraction method ($n = 6$). According to the instructions of the reverse transcription kit (TianGen, Beijing, China), 2 μ g RNA was used as a template to reverse transcribe into cDNA, and then the cDNA was used as a template for Quantitative real-time PCR reaction (TianGen, Beijing, China). The primers are shown in Table 2.

2.6. Western blot analysis of immune protein expression

The intestine and liver of channel catfish were treated with Hank's Balanced Salt (14065056, Gibco™, Grand Island, N.Y.) containing protease/phosphatase inhibitor cocktail (5872, Cell Signaling Technology). The equivalent proteins were loaded in 12% SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) for immunoblotting. The membrane incubated with antibody GAPDH (1:3000, SAB2701826, Sigma- Aldrich), NF- κ B-p65 (1:1000, LS-C31758, LifeSpan BioSciences, Inc.) at 4 °C overnight. Secondary peroxidase conjugated antibodies were used (anti-mouse: 1:5000, A21010, Abbkine, Wuhan, China;

or anti-rabbit: 1:1000, CW0103, CWBIO, CoWin Biotech Co. Ltd., Beijing, China). The blots were developed by chemiluminescence using an ECL kit (WBKLS0500, Merck). The optical density of the bands was determined by the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Analysis of the quantity and structure of intestinal microbiota

At the end of the 12-week feeding experiment, the intestinal contents of channel catfish were collected and mixed into a duplicate at 4 h after the last feeding. The 16S rRNA gene sequencing technique was used to analyze the intestinal microbiota of channel catfish. The 16S V3–V4 region was amplified by using the primers 338F_806R-F (5'-ACTCCTACGGGAGG-CAGCAG-3') and 338F_806R-R (5'-GGACTACHVGGGTWCTAAT-3'). The

Table 2
16S rRNA gene sequencing was performed on the Illumina platform in Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China).

Gene	Forward primer (5–3)	Reverse primer (5–3)
18sRNA	GAGAAACGGCTACCATCC	GATACGCTCATTCCGATTACAG
<i>HIF1α</i>	CCAGCAGTCCAGCAGATTCTTC	GCAGCAGGTGGAGAGGAGAGG
<i>NF-κB</i>	GACGAGGTGGACGAGGGCTAC	CTGAGGCGGAAGCATTGATGAG
<i>IL-1β</i>	GGCTGTGTGTTGGGAATCT	TGATAAACCAACCGGGACA
<i>TNF-α</i>	AAGGAGAGTTGCCTTTACCG	ATTGCCCTGGGTCTTATGG
<i>IL-10</i>	TCATGTCATGAACGAGATCC	CCTCTGCATTCCACCATATCC

The total number of bacteria was quantified by *q*-PCR, using Universal bacteria primers (forward 5'-ACTCCTACGGGAGGAGCAGCAGT-3'; reverse 5'-ATTACCGGGGCTGTGCGC-3') [21]. DNA was extracted from

The primer sequences of immune gene of channel catfish for *q*PCR quantitative analysis.

each sample by using a bacterial genomic DNA extraction kit (Tiangen, Beijing, China). According to the principle of homologous recombination, the bacterial 16S rDNA was cloned into the pLB vector (Tiangen, Beijing, China) as the target gene to form a recombinant plasmid. For each standard plasmid, the copy number was calculated based on the length of the PCR product and the average quality of a DNA base pair. The results were expressed as the number of Log10 copies of bacterial 16S rDNA per milligram of intestinal contents.

2.8. Bacterial disease resistance

At the end of the 12-week feeding experiment, the channel catfish was subjected to a bacterial challenge experiment. The pathogenic *A. veronii* Hm091 and *A. hydrophila* NJ-1 were obtained from the Sino-Norway Joint Lab on Fish Gut Microbiota. Channel catfish were injected intraperitoneally with *A. veronii* Hm091 and *A. hydrophila* NJ-1. The

8.5dose for the challenge was Hm091 of 1.3×10^7 CFU per fish [22]. The

cumulative survival rate of groups were $\times 10^7$ CFU per fish and NJ-1 of observed

for 48 h after bacteria challenge and fish were not fed at the challenge period.

2.9. Functions of intestinal microbiota tested by germ-free zebrafish

In order to evaluate roles of the intestinal microbiota of channel catfish induced by RFM diet on immune response. Germ-free (GF) zebrafish were prepared following established protocols as described previously [23]. Intestinal contents of channel catfish were collected and resuspended in PBS. 30 GF zebrafish and 30 ml of gnotobiotic zebrafish medium (GZM) were included in each tank. Then 300 μ L suspension (10^6 CFU/ml) was added to each tank of GF zebrafish at 4 dpf. One group of GF zebrafish was not treated, and the other two groups were transplanted with intestinal microbiota of control or RFM-treated fish. 9 replicates were assigned to each treatment. After 3 days of colonization, some the larvae were sampled to evaluate the expression of immune regulatory genes, and the primers are shown in Table 2. The other larvae were challenged with mixture of *A. veronii* Hm091 and in this study. The zebrafish mortality was observed for 20 h after *A. hydrophila* NJ-1. The dose of

Hm091 and NJ-1

were 1.5×10^7 CFU/ml infection.

2.10. Statistical analysis

Graph Prism 8 software (GraphPad Software Inc. San Diego, CA, USA) was used to analyze the experimental data. The results were presented as mean with standard error mean (mean $\pm$ SEM). Student's *t*-test was used to make the difference between the two groups of data. Difference was considered as significant when $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), P between 0.05

and 0.2 was considered there was a trend of difference between groups.

3. Results

3.1. Growth performance and survival of channel catfish

After feeding experimental diets to channel catfish for 12 weeks, the results showed that fish of RFM group exhibited notably improved growth performance than the control fish. The weight gain (Fig. 1A and B, $P < 0.05$) and the condition factor (Fig. 1D, $P < 0.05$) in the RFM group were significantly higher than the control group, and the FCR in the RFM group was significantly lower than the control group (Fig. 1C, $P < 0.05$). There was not significantly different in the survival rate (Fig. 1E, $P > 0.05$) between RFM groups and control group.

3.2. Effect of RFM diet on the liver health in channel catfish

Histological changes of liver were observed, the result showed that RFM diet reduced microvesicular fat lesions and infiltration of inflammatory cell (Fig. 2). Moreover, compared with the control group, the expression of pro-

inflammatory mediator NF- κ B significantly reduced in the RFM group both at the transcriptional and protein level ($P < 0.05$, Fig. 3A and B).

3.3. Effect of RFM diet on the intestinal health in channel catfish

As shown in Fig. 4, the intestinal structural integrity of channel catfish was improved and infiltration of inflammatory cells was reduced in RFM group compared with the control group. Meanwhile, compared with the control group, the intestinal expression of *HIF1 α* was significantly increased ($P < 0.05$, Fig. 5A), and the intestinal expression of NF- κ B was

significantly reduced in RFM diet ($P < 0.001$, Fig. 5B).

3.4. RFM diet protected channel catfish against bacterial infection

To investigate the disease resistance of the channel catfish in control group and RFM group, channel catfish were injected intraperitoneally with *A. veronii* Hm091 and *A. hydrophila* NJ-1. At 28 h post challenge, the mortality rate of the control group was 100%, while the mortality rate of the RFM group was 66.7% ($P < 0.05$, Fig. 6). The results showed that RFM diet significantly increased the resistance of channel catfish against Hm091 and NJ-1 compared with control group.

3.5. Effect of RFM diet on intestinal microbiota of channel catfish

Compared with the control group, the total number of bacteria in the

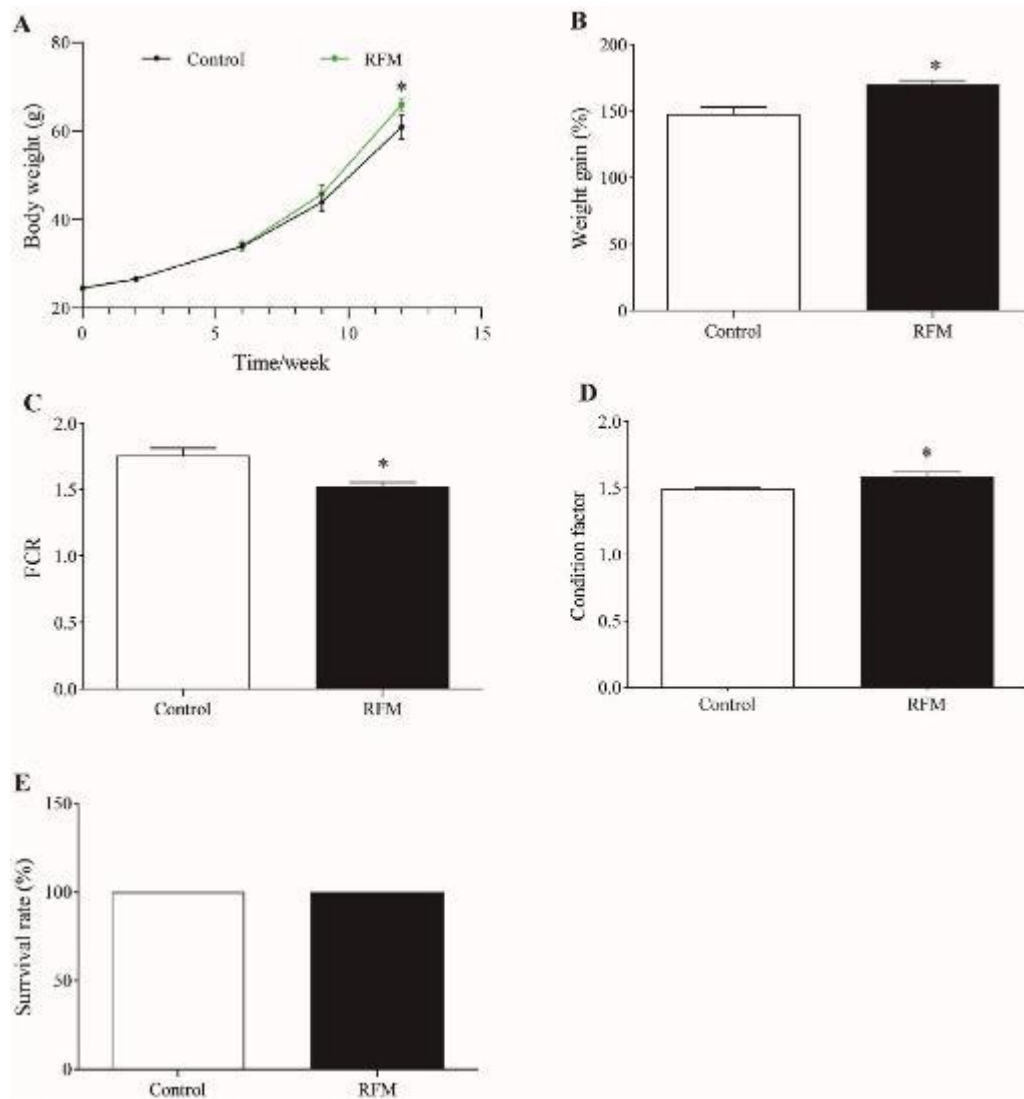


Fig. 1. Effects of RFM diet on the body weight, weight gain, FCR, survival rate and condition factor of channel catfish. (A) Body weight, (B) Weight gain (%), (C) FCR, (D) Condition factor, (E) Survival rate. Data are presented as the means $\pm$ SEM ($n = 6$). Differences are considered significant at $P < 0.05$ (*).

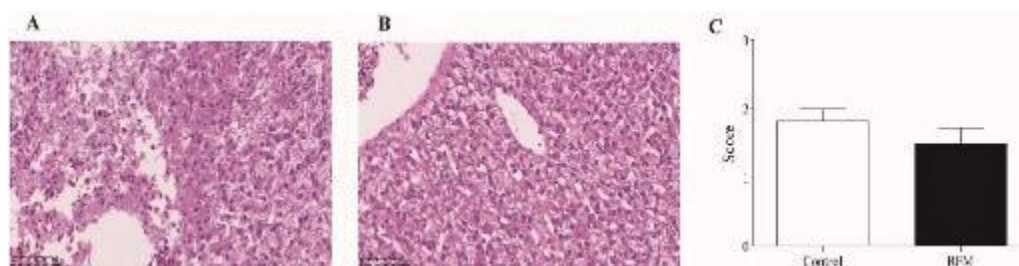


Fig. 2. Effects of RFM diet on the histology (H&E staining) of liver of channel catfish. (A) Control, (B) RFM. (C) Score of liver inflammation. Data are presented as the means $\pm$ SEM (n = 6).

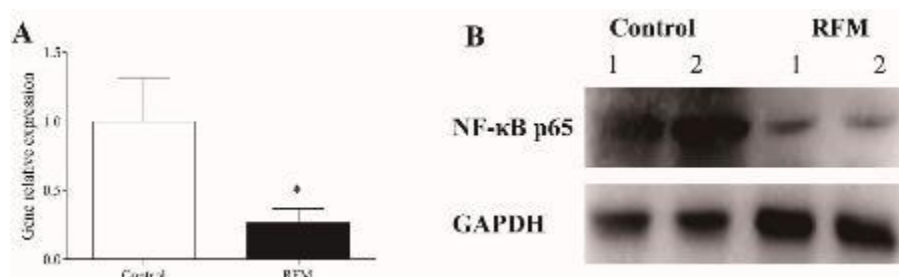


Fig. 3. Effect of RFM diet on the expression of NF- κ B gene and protein in liver of channel catfish. (A) qPCR showing the gene expression of *NF- κ B* in the liver, (B) Western blot showing the protein expression of NF- κ B in the liver. Data are presented as the means $\pm$ SEM (n = 6). Differences are considered significant at $P < 0.05$ (*).

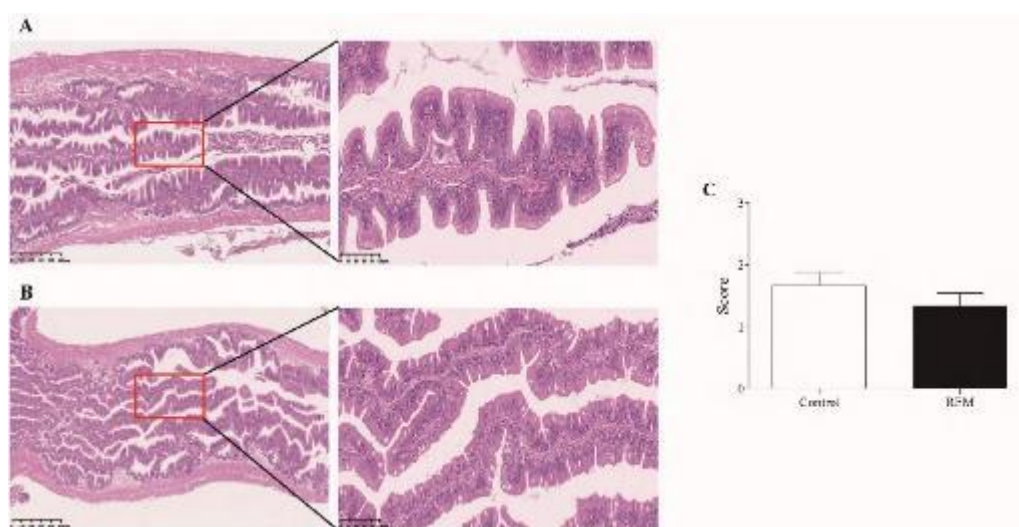


Fig. 4. Effects of RFM diet on the histology (H&E staining) of intestine of channel catfish. (A) Control, (B) RFM. (C) Score of intestinal histopathology. Data represent the means $\pm$ SEM (n = 6).

RFM group significantly increased (Fig. 7A). The 16s rRNA gene sequencing technique was used to analyze the intestinal microbiota of channel catfish. The results showed that Firmicutes, Proteobacteria and Bacteroidetes were the dominant phyla. Compared with control group, the relative abundance of Firmicutes had an upward trend (Fig. 7B; Table 3). The dominant genera were *Romboutsia*, *Turicibacter* and *Brvibacillus*, and the *Turicibacter* had an upward trend in RFM treatment ($0.05 < P < 0.2$, Fig. 7C; Table 4). The results of PcoA analysis showed that the structure of intestinal microbiota was altered by the RFM diet (Fig. 7D).

3.6. Effects of intestinal microbiota of channel catfish induced by RFM diet on the expression of genes associated with host health in the GF zebrafish model

Compared with GF zebrafish colonized microbiota from the control group, the expression of *HIF1 α* in GF zebrafish colonized microbiota from RFM group

was significantly increased ($P < 0.01$, Fig. 8A), while the expression of *NF- κ B* was reduced ($P > 0.05$, Fig. 8B). Meanwhile, we found that the treatment group transplanted from RFM diet significantly decreased the expression of pro-inflammatory genes (*IL-1 β* , *TNF- α*) ($P < 0.01$, Fig. 8C and D) and increased the expression of anti-inflammatory genes (*IL-10*) ($P > 0.05$, Fig. 8E).

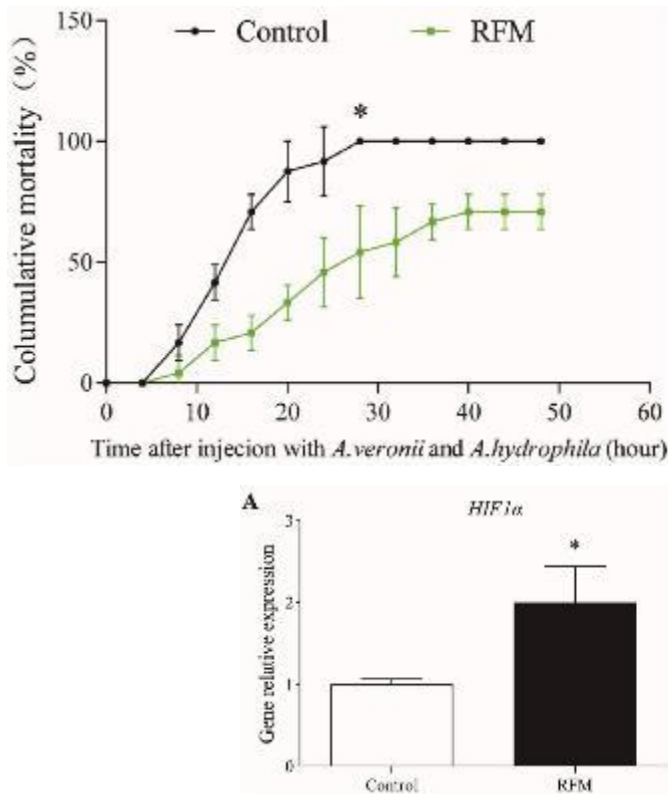


Fig. 5. Effect of RFM diet on the intestinal expression of *HIF1α* and *NF-κB* in channel catfish. (A) *HIF1α*, (B) *NF-κB*. Data are presented as the means $\pm$ SEM ($n = 6$). Differences are considered significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Fig. 6. Protection ability of RFM diet in channel catfish. Data are presented as the means $\pm$ SEM ($n = 3$). Differences are considered significant at $P < 0.05$ (*).

3.7. Effects of intestinal microbiota of channel catfish induced by RFM diet on the resistance of GF zebrafish against *Aeromonas*

To further investigate the effects of intestinal microbiota of channel catfish fed the RFM diet or the control diet, GF zebrafish colonized with either intestinal microbiota of channel catfish were challenged with mixture of *A. veronii* Hm091 and *A. hydrophila* NJ-1. Mortality was recorded in every 2 h. The results showed that compared with the control group, the treatment group colonized with intestinal microbiota of channel catfish of RFM diet significantly decreased the mortality rate against the *A. veronii* Hm091 and *A. hydrophila* NJ-1 ($P < 0.001$, Fig. 9). **4. Discussions**

In recent years, the replacement of fish meal with single cell protein has become a research hotspot of animal nutritionists, and yeast is the most used single cell protein in aquatic feed. Many studies have shown that it is feasible to use feed yeast instead of fish meal in aquatic animals, but it is related to many factors, such as culture species, fish feeding habits and so on. Indexes such as growth rate and feed utilization rate are usually used as the criteria for evaluating the feeding effect [24].

The present study showed that partially replacing fish meal by yeast culture could significantly improve the growth status and feed conversion utilization rate of channel catfish, which was more suitable for the growth of channel catfish. In previous studies, when using yeast culture instead of fish meal to feed white shrimp (*Litopenaeus vannamei*), FBW and WGR increased, FCR and HSI decreased [18]. The body length, body weight and survival time of tilapia (*Oreochromis mossambicus*) larvae were greatly improved when the larvae Q. Hao et al.

were fed with single cell protein diet [25]. This may be due to feed yeast can produce or release various digestive enzymes after cell lysis in the digestive tract of aquatic animals. These enzymes can effectively promote the absorption of nutrients in the digestive tract of animals, so as to improve the weight gain and reduce the feed coefficient [26,27].

Most of the previous researches on fish meal replacement in aquaculture have focused on the effects on the growth performance of fish, but gut health cannot be ignored either. The gut of animals plays a major role in nutrition digestion and absorption and in providing immune barriers against disease infection [28]. It is well known that morphological and histological assessment of the gut is crucial, which may be affected by dietary nutrient absorption, and also shows immune blockade against pathogen infection. Previous studies have shown that mannan oligosaccharides, as a component of yeast culture, can improve the intestinal tract of white shrimp and European lobster (*Homarus gammarus* L.), which is consistent with our results [29,30]. In this study, compared with the control group, RFM diet improved the intestinal structural integrity and reduced inflammatory cell infiltration. Intestinal tract

is a highly hypoxic tissue. *HIF1α* contributes to maintaining hypoxic environment, intestinal barrier, nutrient absorption and immune regulation [31]. Studies have shown that *HIF-1α* can upregulate claudins and mucins to enhance the integrity of the epithelial barrier, thereby promoting microbial colonization. And it can also downregulate *NF-κB* signaling and the production of anti-inflammatory cytokines, thereby reducing intestinal inflammation [32]. *NF-κB* is a first responder to harmful cellular stimuli and plays an important role in many biological processes including innate and adaptive immunity [33]. In this study, compared with the control group, the expression of *HIF1α* in the intestine of RFM group was significantly increased, and the expression of *NF-κB* in the intestine of RFM group was significantly decreased, which indicated that the positive effect of *Saccharomyces cerevisiae* culture on the expression of *HIF1α* and *NF-κB* may contribute to its impact on intestinal health.

Intestinal microbiota plays a key role in nutrition, immunity and resistance against invasive pathogens [34]. Generally speaking, the most common phyla in intestinal microbiota of fish are Firmicutes and Proteobacteria [35], which is also consistent with our research. Our study showed that the abundance of Firmicutes in the RFM group was higher than the control group. Among them, *Turicibacter* is one of the most genus of Firmicutes, and its abundance is also higher than the RFM group. The majority of bacteria in the phylum Firmicutes play an important role in the fermentation of undigested carbohydrates and amino acids into short-chain fatty acids [36]. As described earlier, *Turicibacter* could produce short-chain fatty acids, and it was positively correlated with the content of butyric acid [37], which is a functional fatty acid that inhibits enteritis and repairs the intestine, and *Turicibacter* deficiency could lead to intestinal diseases in animals [38]. Combined with the results of intestinal microbiota transplant in the GF zebrafish model, we also found that the treatment group colonized with intestinal

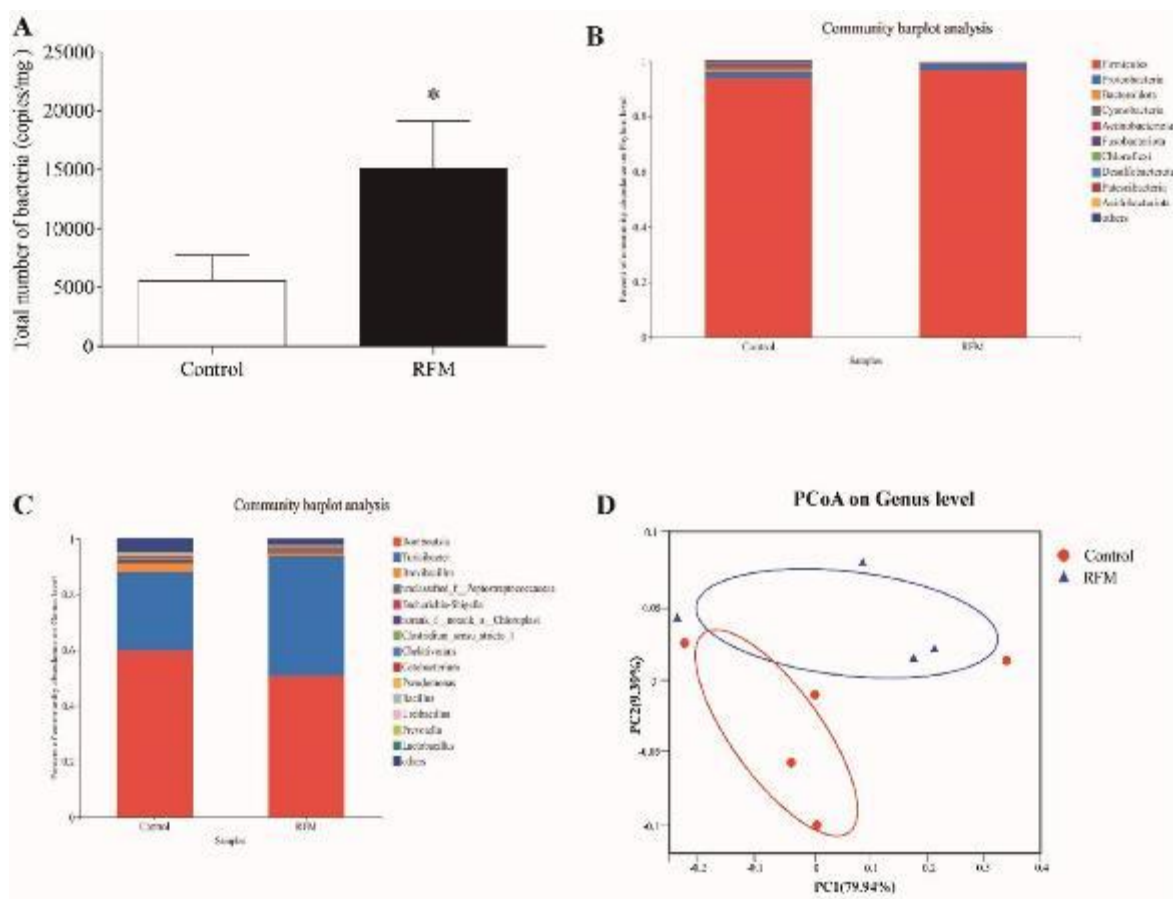


Fig. 7. Effects of RFM diet on the intestinal microbiota of channel catfish. (A) The total number of bacteria in channel catfish fed with control and RFM diet. The relative phylum (B) and genus (C) abundance of the microbiota. PCoA of the intestinal microbiota (D) of the intestinal microbiota (n = 4).

microbiota of channel catfish of RFM diet had effect to improve the

diet and RFM diet. **Table 3** Intestinal microbiota at main phylum level of channel catfish fed with control speculate that the RFM diet can improve intestinal health which mainly mediated by intestinal microbiota. immune system and disease resistance. Based on the above evidence, we

Bacteria Phylum (%)	Control	RFM	P Value
Firmicutes Proteobacteria	93.97 ± 4.63	96.42 ± 6.49	0.24
Bacteroidetes	2.51 ± 2.10	2.55 ± 4.62	0.76
Cyanobacteria	0.82 ± 0.60	0.40 ± 0.77	0.90
Actinobacteria Fusobacteria	0.92 ± 1.79	0.10 ± 0.17	0.84
Chloroflexi	0.64 ± 0.46	0.31 ± 0.54	0.76
Desulfobacterota Acidobacteria	0.56 ± 0.58	0.01 ± 0.01	0.98
Patescibacteria	0.16 ± 0.14	0.03 ± 0.06	0.94
	0.07 ± 0.10	0.02 ± 0.04	0.90
	0.06 ± 0.06	0.02 ± 0.04	0.97
	0.07 ± 0.07	0.01 ± 0.01	

The outbreak of infectious diseases is the bottleneck of the development of aquaculture. *Aeromonas* can cause motile aeromonad septicemia, which can lead to a large number of deaths of intensive high-density cultured freshwater fish and cause serious economic losses to farmers [22,39]. In aquatic animals, the survival rate after bacterial attack is usually used to determine the immune response to disease [40]. The non-specific immune system of channel catfish plays an important role in protecting it from bacterial infection. In this study, the survival rate of channel catfish fed with RFM was increased after challenged with a mixture of *A. veronii* Hm091 and *A. hydrophila* NJ-1, which was consistent with the results of shrimp fed with 2% yeast culture [18]. The increased resistance to *Vibrio harveyi* indicated that yeast culture could stimulate and prolong the immune response of rockfish to pathogenic infection. A large number of these chemicals, such as β -Glucan, chitin and mannan oligosaccharide showed immune stimulation after pathogen attack [41,42], so as to improve survival rate. Therefore, the effect of yeast culture on bacterial resistance indicated that feeding yeast culture could effectively improve the resistance of channel catfish to *Aeromonas* infection.

Table 4 and

RFM diet.

1 genus level of channel

Bacteria Genus (%)	Control	RFM	P Value
<i>Romboutsia Turicibacter</i>	59.83 ± 3.91	51.52 ± 12.93	0.90
<i>Brevibacillus Escherichia-Shigella</i>	28.47 ± 11.05	41.88 ± 21.41	0.16
<i>unclassified_f__Peptostreptococcaceae</i>	2.96 ± 4.05	0.91 ± 1.67	0.94
<i>norank_f__norank_o__Chloroplast</i>	0.21 ± 0.17	1.03 ± 2.04	0.90
<i>Chelativorans</i>	0.33 ± 0.62	0.77 ± 0.52	0.33
<i>Clostridium_sensu_stricto_1</i>	0.90 ± 1.77	0.10 ± 0.17	0.72
<i>Cetobacterium</i>	0.48 ± 0.62	0.26 ± 0.47	0.94
<i>Pseudomonas</i>	0.21 ± 0.20	0.48 ± 0.43	0.24
	0.56 ± 0.58	0.01 ± 0.01	0.98
	0.31 ± 0.26	0.16 ± 0.27	0.94

5. Conclusion

In conclusion, yeast culture can be used as a substitute for fish meal in aquafeed of channel catfish. In this study, the results showed that partially replacing dietary fish meal by *Saccharomyces cerevisiae* culture

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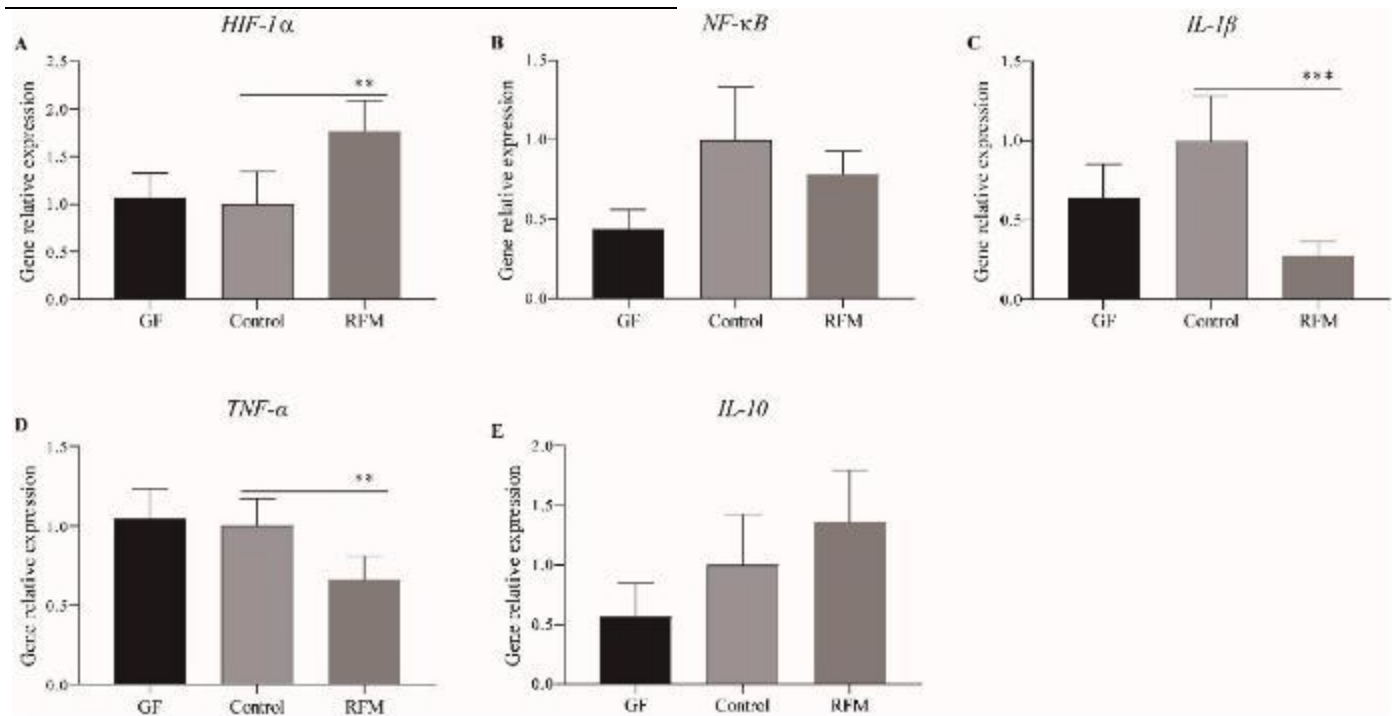


Fig. 8. The effect of RFM induced intestinal microbiota of channel catfish on genes associated with host health in the GF zebrafish. (A) *HIF1α*, (B) *NF-κB*, (C) *IL-1β*, (D) *TNF-α*, (E) *IL-10*. Data are presented as the means ± SEM (n = 6). Differences are considered significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

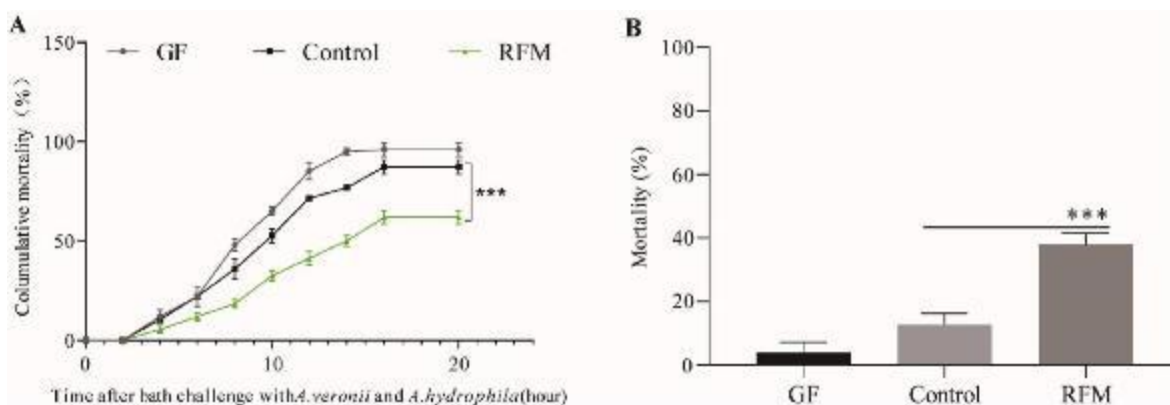


Fig. 9. Effects of RFM induced intestinal microbiota of channel catfish on cumulative mortality in the GF zebrafish against pathogenic *Aeromonas*. Data are presented as the means $\pm$ SEM (n = 3). Differences are considered significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

improve the growth performance and feed utilization rate of channel catfish, effectively improve the immune function of channel catfish, maintain intestinal health and improve the ability of channel catfish to resist *Aeromonas* infection, and further showed the intestinal microbiota induced by the *Saccharomyces cerevisiae* culture played a critical role in probiotic effects of *Saccharomyces cerevisiae* by using the germ-free zebrafish model.

CRedit authorship contribution statement

Qiang Hao: Formal analysis, Writing – original draft, Writing – review & editing, conducted the experiments and biochemical analyses, analyzed the data, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Rui Xia:** Formal analysis, Writing – original draft, Writing – review & editing, conducted the experiments and biochemical analyses, analyzed the data, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Qingshuang Zhang:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Yadong Xie:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Chao Ran:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Yalin Yang:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Wenhao Zhou:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Fuwei Chu:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Xiaomei Zhang:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Yu Wang:** Methodology, Writing – original draft, Writing – review & editing, developed the methodology. All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Zhen Zhang:** Resources, Formal analysis, Writing – original draft, Writing – review & editing, designed study and provided the laboratory resources, analyzed the data, All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript. **Zhigang Zhou:** Resources, Writing – original draft, Writing – review & editing, designed study and provided the laboratory resources, All authors

participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2022.05.014>.

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Effects of dietary *Saccharomyces cerevisiae* on growth, intestinal and liver health, intestinal microbiota and disease resistance of channel catfish (*Ictalurus punctatus*)

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Intestinal microbiota Disease resistance

ABSTRACT

Probiotics are an eco-friendly alternative to antibiotics and play an important role in ensuring the sustainability of aquaculture. The aim of the present study was to investigate the effects of *Saccharomyces cerevisiae* on the growth, intestinal and liver health, intestinal microbiota, and disease resistance of channel catfish (*Ictalurus punctatus*). Channel catfish were randomly allotted into two treatments, control group (basal diet) and SCC group (basal diet with 2% *Saccharomyces cerevisiae* culture), and fed for 12 weeks. The results showed that the weight gain of SCC group was significantly increased ($P < 0.05$) and FCR was significantly decreased ($P < 0.05$) when compared with the control group. SCC diet significantly improved the intestinal structural integrity of channel catfish and increased the number of intestinal goblet cell. The expression of intestinal gene *HIF1α* was increased in SCC group, while the expressions of *NF-κB* in the intestine and liver were significantly decreased ($P < 0.05$). The relative abundance of Firmicutes tended to increase, while Proteobacteria had a tendency to decrease ($0.20 > P > 0.05$). In addition, the survival rate of channel catfish was significantly increased in SCC group after being challenged with mixture of *A. veronii* HM091 and *A. hydrophila* NJ-1. In conclusion, the supplementation of *Saccharomyces cerevisiae* culture improved the growth, intestinal and liver health and disease resistance of channel catfish.

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1. Introduction

China has the world's largest aquaculture industry, accounting for more than 60% of global production. In 2018, the national fish production has reached about 179 million tons (FAO. The State of World Fisheries and Aquaculture 2020). However, at the same time, disease problems have become increasingly prominent under the intensive and industrialized farming systems (Gui, Tang, Li, Liu, and De Silva, 2018), and bacterial diseases accounted for the highest proportion, leading to the overuse of antibiotics. Improper use of antibiotics will destroy intestinal microbiota in the aquaculture environment, and threaten food safety and human health (Chen et al., 2020). People pay more attentions to restrict the uses of antibiotics in aquaculture, and probiotics may be

Wang, and Zhou, 2018; Subuntith Nimrat, Suksawat, Boonthai, and Vuthiphandchai, 2012).

Nowadays, yeast has been widely used as probiotics in aquaculture. Yeast products could reduce the number of harmful bacteria in the intestine of fish, and promote the reproduction of beneficial bacteria. In addition, yeast products could improve intestinal function and maintain intestinal integrity, thereby improving growth performance and immunity of fish (Islam, Rohani, and Shahjahan, 2021; Jahan, Islam, Rohani, Hossain, and Shahjahan, 2021; Rohani et al., 2021). Yeast culture is a complex yeast fermentation product, including yeast, its metabolites, and culture medium (Barnes, Durben, Reeves, and Sanders, 2006; Sanderson and Jolly, 1994). The first generation of yeast culture is DVAQUA® of Diamond V Mills Inc., IA. It is yeast culture of *Saccharomyces cerevisiae*, and contains "unknown growth factors", which could adjust the microecological balance of the intestinal tract of aquatic animals, so as to improve the production performance of animals (Barnes et al., 2006; Sanderson and Jolly, 1994). The experiments of hybrid DVAQUAtilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂) showed that could affected the autochthonous gut bacteria community and improved non-specific immunity (He et al., 2009, 2011a; Zhou et al., 2009, 2011). The second generation of yeast culture is Saccharoculture of Veterinary Pharmaceuticals, Korea. It not only contains yeast culture of *Saccharomyces cerevisiae*, but also

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one of the most promising approaches (Castex, Daniels, and Chim, 2014). Probiotics have many positive impacts, such as activating the host's immunity, inhibiting the growth of pathogenic microorganisms, balancing the host's intestinal microbiota and improving the host's metabolism (Hoseinifar, Sun,

has *Bacillus amyloliquefaciens*, with a live bacterial content of more than 4

Feed formulation and chemical composition of diets for channel catfish.

billion/g (He et al., 2011b). Saccharoculture is also rich in a large number of β -Glucan, amino acids, antimicrobial peptide and other metabolites. The experiments of crucian carp (*Carassius auratus*) and juvenile common carp (*Cyprinus carpio*) both showed that Saccharoculture could improve weight gain, special growth rate and survival rate after *A. hydrophila* infection of fish (He et al., 2011b; Huang et al., 2015).

Studies have shown that nucleotides and short-chain fatty acids have a very positive significance for improving disease resistance of the host. Nucleotides, as essential substances for cell synthesis, have physiological functions such as enhancing growth, immune responses, and disease resistance in various fish species (Guo et al., 2017, 2019; Ran et al., 2021; Xu et al., 2015). Short-chain fatty acids could significantly promote the weight gain and daily feeding rate of fish, improve intestinal digestive enzyme activities, energy metabolism, and inhibit the reproduction of harmful bacteria (Liu et al., 2014; Wang et al., 2021; Zhang et al., 2020). Because short-chain fatty acids (especially acetic acid, Guo et al., 2017, 2019; Ran et al., 2021) and nucleotides (especially AMP, Wang et al., 2021; Zhang et al., 2020) have these prominent probiotic effects, the third generation of yeast culture was developed in China, which is characterized by high acetic acid and high AMP.

Channel catfish (*Ictalurus punctatus*) is an important freshwater aquaculture fish. Due to its wide adaptability, miscellaneous food habits, strong disease resistance, fast growth, high yield, and tender meat, the scale of aquaculture has expanded rapidly and the degree of intensification has been continuously improved. Channel catfish was used as the research subjects in this study, and we evaluated the effects of *Saccharomyces cerevisiae* culture (ESTAQUA®) on the growth, intestinal and hepatic health, intestinal microbiota and disease resistance of channel catfish.

2. Material and methods

2.1. Bacteria and culture conditions

Aeromonas veronii HM091 was preserved in China General Microbiological Culture Collection Center with its preservation number CGMCC no.22536. *Aeromonas hydrophila* NJ-1 was a gift from Dr. Yongjie Liu from Nanjing Agricultural University (Nanjing, China). *Saccharomyces cerevisiae* Sa-10 was preserved in China General Microbiological Culture Collection Center with its preservation number CGMCC no.6120. *Saccharomyces cerevisiae* culture (ESTAQUA®) was provided by Enhalar Biotechnology Company (Beijing, China), and was fermented based on the patent CN107937292A together with technique guidance of Sino- Norway Joint Lab on Fish Gut Microbiota. The fermented product was enriched with nucleotides and acetic acid by adding the processes of liquid low-temperature anaerobic fermentation and solid-state enzymatic aerobic fermentation, and this product contained 48.56% more nucleotides and 90.19% more acetic acid than it obtained using the original production method (SI Table 1). The cell wall of *Saccharomyces cerevisiae* Sa-10 was destroyed by high temperature, which allowed its contents to exert their potential functional roles.

Table 1

Ingredient	g/kg (air dry basis)	Control	SCC
Rice bran		100.00	100.00
Flour		150.00	150.00
Soybean meal		250.00	230.00
Rapeseed meal		150.00	144.00
Fish meal		100.00	100.00
Chicken meal		100.00	100.00
DDGS		50.00	50.00
SCC Sa-10 ^a		0.00	20.00
Bentonite		15.00	20.85
Lys-HCl ^b		2.00	2.40
Methionine ^b		0.50	0.55
Choline Chloride (50%)		2.00	2.00
Ca(H ₂ PO ₄) ₂		20.00	20.00
Soybean oil		40.00	39.70
VC phosphate Premix ^c		0.50	0.50
		20.00	20.00
Total		1000	1000
Chemical composition (g/kg)			
Crude protein		380.4	380.5
Crude fat		112.9	114.1
Crude ash		102.2	107.7
Moisture		54.3	54.8

^a Provided by Enhalar Biotechnology Company, Beijing, China, its composition was as follows: Crude protein, 67.73%; Crude fat, 3.18%. ^b The additive quantities of Lys-HCl and methionine in the two feeds were adjusted according to the differences between SCC and SBM (China Feed- database Information Network Centre). ^c Containing vitamin premix and mineral premix. (1) Vitamin premix containing the following (g/kg): thiamine, 0.438; riboflavin, 0.632; pyridoxine-HCl, 0.908; *d*-pantothenic acid, 1.724; nicotinic acid, 4.583; biotin, 0.211; folic acid, 0.549; vitamin B-12, 0.001; inositol, 21.053; menadione sodium bisulfite, 0.889; retinyl acetate, 0.677; cholecalciferol, 0.116; *d*- α -tocopherol-acetate, 12.632. (2) Mineral premix containing the following (g/kg): CoCl₂·6 H₂O, 0.074; CuSO₄·5 H₂O, 2.5; FeSO₄·7 H₂O, 73.2; NaCl, 40.0; MgSO₄·7 H₂O, 284.0; MnSO₄·H₂O, 6.50; KI, 0.68; Na₂SeO₃, 0.10; ZnSO₄·7 H₂O, 131.93; Cellulose, 501.09.

2.2. Fish husbandry and feeding

All experiments and animal care procedures were approved by Institute of Feed Research of the Chinese Academy of Agricultural Sciences by the China Council for Animal Care (Assurance No. 2018-AF- FRI-CAAS-001). The experiment was conducted in the International Agricultural High-tech Industrial Park of the Chinese Academy of Agricultural Sciences, Langfang, Hebei, China. Channel catfish was purchased from a fish farm in Hebei and domesticated in a circulating water system for a week before the experiment.

After 24 h of starvation, channel catfish with similar body weight (initial body weight 24.49 $\pm$ 0.11 g) were randomly divided into 2 groups with 6 repetitive per group at a density of 12 fish per 90 L tank. The basal diet was formulated with fish meal, soybean meal, rapeseed meal as the main protein source, and soybean oil as the main fat source. Based on previous findings, supplementation of 2% yeast culture could improve the growth performance, immunity and disease resistance of juvenile largemouth bass, hybrid grouper and shrimp (Ayiku et al., 2020; Feng et al., 2021; Wang et al., 2022), and could relieve liver damage and inflammatory response induced by CCl₄ (Tao et al., 2021), so in this study the experimental diet was prepared by 20 g/kg *Saccharomyces cerevisiae* culture (SCC, ESTAQUA®) to replace the same amount of soybean meal (SBM) in the basic diet. The feed formulation and approximate analysis were shown in Table 1.

During the trial period, channel catfish were fed to apparent satiation by hand three times (7:00, 12:00 and 18:00) daily for 12 weeks. The fish were fed daily at 1.5% of their initial body weight for 2 weeks. From the third week, the feeding amount increased to 2.5% of their initial body weight for 6 weeks. Starting from the ninth week, the feeding amount increased to 4% of their initial body weight for another 3 weeks, and from the twelfth week, the feeding

amount increased to 5% of their initial body weight. The water inflow was 120 L/h, the water temperature was 25.5 °C, the dissolved oxygen (DO) was ≥ 6 mg/L, the ammonia nitrogen content was < 0.2 mg/L, and the nitrite concentration was < 0.06 mg/L.

2.3. Measurement of growth performance and feed utilization efficiency

To evaluate the effect of SCC on performance, the fish were weighed at 2 week, 6 week, 9 week, and 12 week. Weight gain (WG, %) = 100%

$\times (g)$, feed conversion ratio (FCR) [(final body weight (g) - initial body weight (g))/initial body weight $\times$ food intake (g)/weight gain of fish

(g), survival rate (SR, %) = (number of fish at the end of the experiment/ 100%,

and condition number of fish at the start of the experiment) $\times 3$. factor = 100

$\times$ body weight (g)/ [body length]

2.4. Histological observation of intestine and liver

After 24 h fasting, the channel catfish was anesthetized with 3-aminophenoic acid ester methanesulfonate. 1 fish was randomly selected from each of the 6 replication tanks for liver and hindgut histological analysis (n = 6). The liver and hindgut of channel catfish were collected, dehydrated in 4% paraformaldehyde, and then embedded in paraffin. The sections were stained with hematoxylin and eosin (HE), sealed with neutral gum and observed with microscope (Leica DMIL-LED, Germany). The images were analyzed with K-Viewer image analysis software.

2.5. Relative quantification expressions of intestine and liver health related genes

The liver and hindgut tissues of channel catfish were taken for RNA extraction (n = 6), and the total RNA was extracted using the TRIzol Reagent extraction method (Invitrogen, Carlsbad, CA, USA). According to the instructions of the reverse transcription kit (TianGen, Beijing, China), 2 μ g RNA was used as a template to reverse transcribe into cDNA, and the cDNA was used as a template for Quantitative real-time PCR reaction (TianGen, Beijing, China). The primers were shown in Table 2.

2.6. Western blot analysis of NF- κ B-p65

The intestine and liver of channel catfish were treated with Hank's Balanced Salt (14065056, Gibco™, Grand Island, N.Y.) containing protease/phosphatase inhibitor cocktail (5872, Cell Signaling Technology). The same amounts of total protein were loaded in 12% SDS-PAGE for electrophoresis, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). After the PVDF membrane was blocked non-specific binding with TBST containing 5% skimmed milk powder, and incubated with primary antibodies, i.e., antibodies against GAPDH (1:3000, SAB2701826, Sigma-Aldrich), NF- κ B-p65 (1:1000, LS- C31758, LifeSpan BioSciences, Inc.). Secondary peroxidase conjugated antibodies were used (anti-mouse: 1:5000, A21010, Abbkine, Wuhan, China; or anti-rabbit: 1:1000, CW0103, CWBIO, CoWin Biotech Co. Ltd., Beijing, China). The blots were developed by chemiluminescence using an ECL kit (WBKLS0500, Merck) and the ChemiDoc XRS+ System (BIO-

determined by the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Table 3.

2.7. Analysis of the quantity and structure of intestinal microbiota

At the end of the 12-week feeding experiment, the intestinal contents of channel catfish were collected at 4–6 h after the last feeding. 12 fish from each group were randomly selected for intestinal contents sampling, and intestinal content samples from 3 fish were mixed into a replicate sample (n = 4). The 16 s rRNA gene sequencing technique was used to analyze the intestinal microbiota of channel catfish. According to the manufacturer's instructions, DNA was extracted from each sample using a bacterial genomic DNA extraction kit (Tiangen, Beijing, China).

For the DNA copy of the total bacteria, the obtained DNA was used as a template to amplify 16 S rRNA, using Universal bacteria primers (forward 5'-ACTCTACGGGAGGCAGCAGT-3'; reverse 5'-ATTACCGCGGCTGCTGGC-3') with specific barcodes for each samples (Zhang et al., 2019).

2.8. Bacterial disease resistance

In order to evaluate the effect of supplementary SCC on the disease resistance of channel catfish, the fish were challenged with mixture of highly pathogenic *A. veronii* HM091 and *A. hydrophila* NJ-1. *A. veronii* HM091 and *A. hydrophila* NJ-1 were placed in a 250 ml flask containing LB culture medium and cultured in an incubator at 37 °C and 180 r/min and 4.25×10^8 CFU/ml before challenge test, respectively. After 12×10^7 CFU/ml for 20 h. The concentration of Hm091 and NJ-1 were 6.5 weeks of feeding trial, each fish was injected with pathogenic bacteria.

The size of the challenge tank was $50 \times$ CFU/g fish body weight, and the $50 \times$

40 cm, the final challenge concentration of Hm091 was 2.1×10^{10} CFU/g fish

body weight. There were two groups: group A was the control group; group B

final challenge concentration of NJ-1 was $1.3 \times$

was SCC group, each treatment group had 3 replicates, and each replicate had 8 fish. The survival rates of the two groups were calculated and fish were not fed in the challenge period.

2.9. Statistical analysis

Graph Prism 8 software (GraphPad Software Inc. San Diego, CA, USA) was used to analyze the experimental data. The results were presented as mean $\pm$ SEM. Paired t-tests were used to make the difference between the two groups of data. Difference was considered as significant when $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)

Table 3
Growth performance and morphological parameters of channel catfish fed experimental diets at 12 weeks.

	Control	SCC

Table 2

The primer sequences of immune gene of channel catfish for qPCR quantitative analysis.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
18sRNA	GAGAAACGGCTACCATCTCC	GATACGCTCATTCCGATTACAG
HIF1 α	CCAGCAGTCCAGCAGATTACTTCC	GCAGCAGGTGGAGAGGAGAGG
NF- κ B	GACGAGGTGGACGAGGGCTAC	CTGAGCGCGAAGGCATTGATGAG

RAD). The optical density of the bands was

Initial weight (g/fish)	24.57 ± 0.11	24.48 ± 0.12
Final weight (g/fish)	60.92 ± 2.68	65.09 ± 2.18 *
WG (%) FCR		
SR (%)	147.95 ± 10.06	165.91 ± 8.84 *
CF (g/cm3)	1.76 ± 0.11 100 ±	1.56 ± 0.09 *
	0	100 ± 0
	1.49 ± 0.05	1.54 ± 0.07

3. Results

3.1. Growth performance and survival of channel catfish

During the experimental period, no mortality of channel catfish in each treatment was observed. At 12 weeks, the weight gain of fish in the SCC group was significantly higher than the control group ($P < 0.05$, Fig. 1A, B; Table 1), and the FCR was significantly lower than the control group ($P < 0.05$, Fig. 1C;

Table 1). There was not significantly different in condition factor between SCC group and control group ($P > 0.05$, Fig. 1E; Table 1).

3.2. Effects of SCC diet on the intestinal health of channel catfish

The effects of SCC diet on histological changes of intestine were investigated. As shown in Fig. 2, the intestinal structural integrity of channel catfish, as scored by comparing intestinal structural integrity and intestinal cells, was improved in SCC group compared with the control. Intestinal epithelial cells were more organized, and goblet cells were significantly increased (Fig. 2). Moreover, the genes expression related to intestinal health was detected. Compared with the control group, the expression of the hypoxia inducible factor (*HIF1 α*) in the intestine fed with SCC diet had a rising trend ($0.2 > P > 0.05$, Fig. 3A). The gene expression of pro-inflammatory mediator *NF- κ B* was

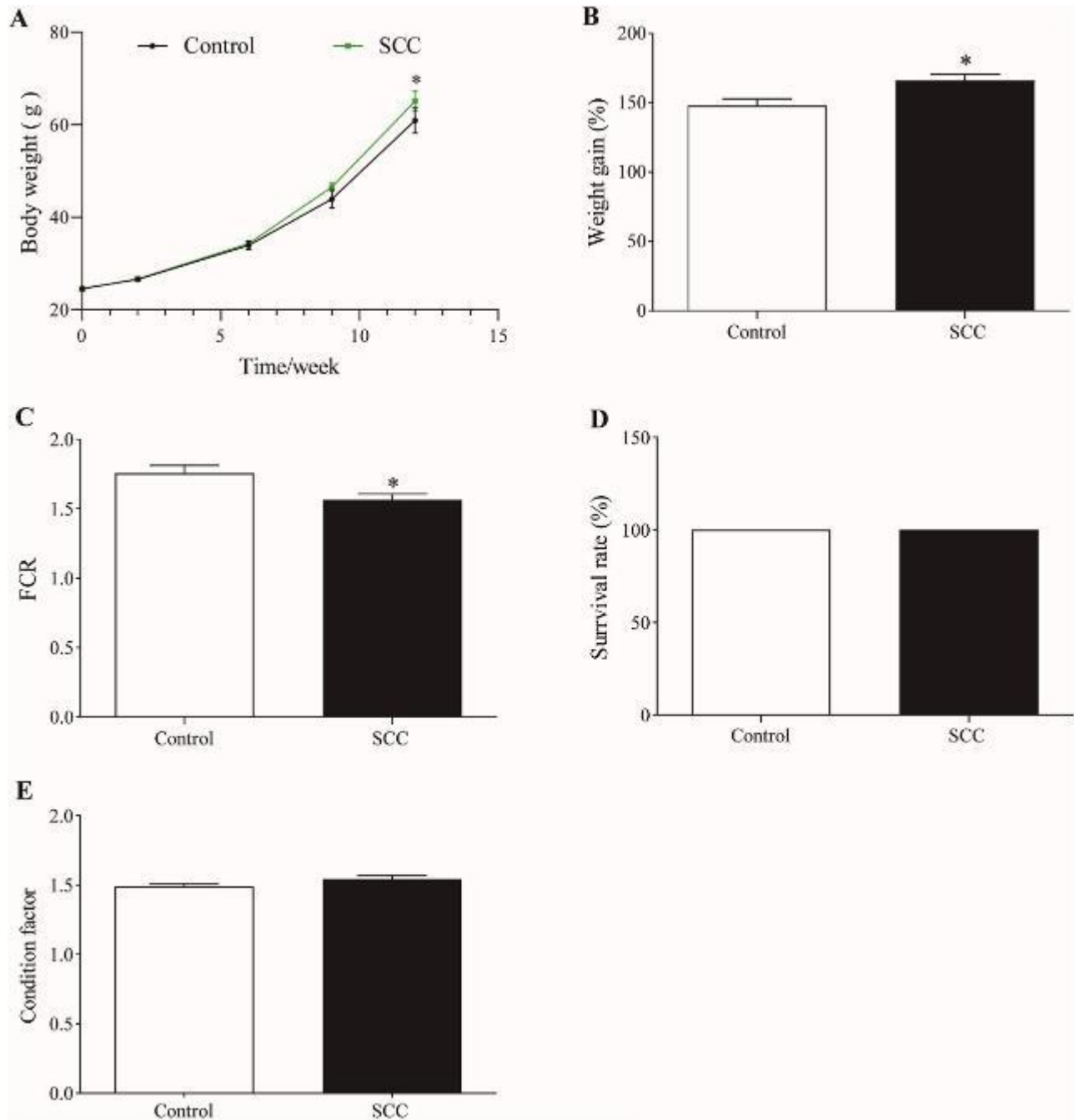


Fig. 1. Effects of SCC diet on the body weight, weight gain, FCR, survival rate and condition factor of channel catfish. (A) Body weight, (B) Weight gain (%), (C) FCR, (D) Survival rate, (E) Condition factor. Data are presented as the means ($\pm$ SEM) ($n = 6$). Differences are considered significant at $P < 0.05$ (*).

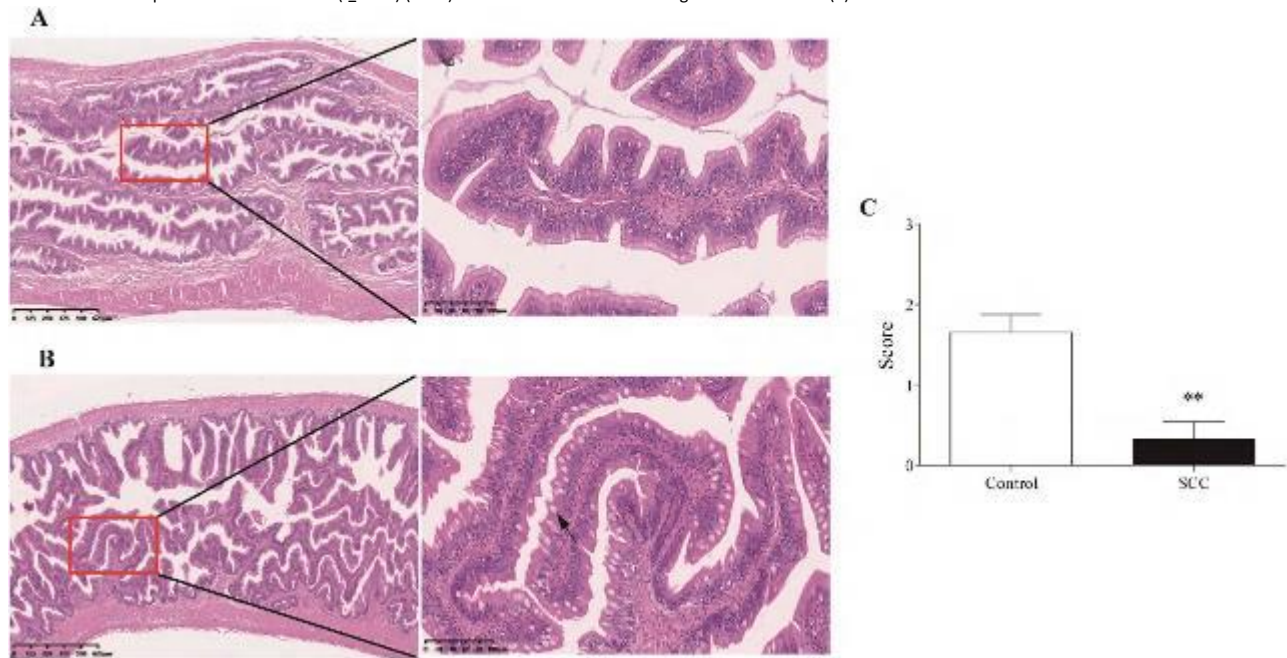


Fig. 2. Effects of SCC diet on the histology (H&E staining) of intestine of channel catfish. (A) Control, (B) SCC. (C) Score of intestinal histopathology (Evaluation parameters: abnormal lamina propria, loosening of enterocytes from basal membrane, edema and number of goblet cell). Data represent the means ($\pm$ SEM) ($n = 6$). Differences are considered significant at $P < 0.05$ (*) and $P < 0.01$ (**).

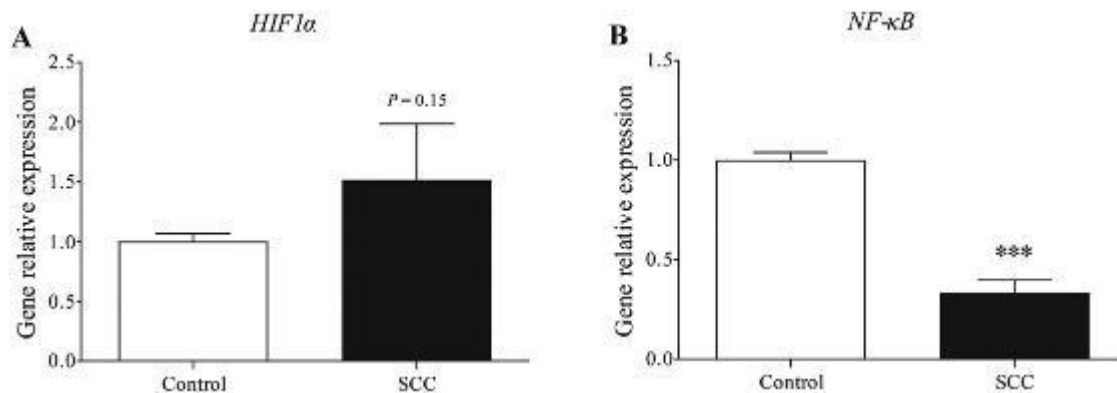


Fig. 3. Effect of SCC diet on the intestinal expression of *HIF1α* and *NF-κB* in channel catfish. (A) *HIF1α*, (B) *NF-κB*. Data are presented as the means ($\pm$ SEM) ($n = 6$). Black arrows indicated goblet cell. Differences are considered significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

significantly decreased in SCC group ($P < 0.05$, Fig. 3B). 3.3. The effect of

SCC diet on intestinal microbiota of channel catfish

The total amount of gut bacteria was detected after 12 weeks of feeding. As shown in Fig. 4A, there was no significant difference in the total number of bacteria between control group and SCC group. At the phylum level (Fig. 4B; Table 4), Firmicutes is dominant. Compared with the control group, the relative abundance of Firmicutes had an upward trend in SCC group ($0.2 > P > 0.05$). At the genus level (Fig. 4C; Table 5), the dominant bacteria were *Romboutsia*, *Turicibacter* and *Brvibacillus*. *Romboutsia* had a downward trend; *Turicibacter* had an upward trend ($0.2 > P > 0.05$) and *Brvibacillus* was significantly decreased ($P < 0.05$) after feeding the SCC diet. The results of PcoA analysis showed that the intestinal microbiotas of channel catfish fed the two diet was completely separated at the level of both phylum and genus (Fig. 4D, E).

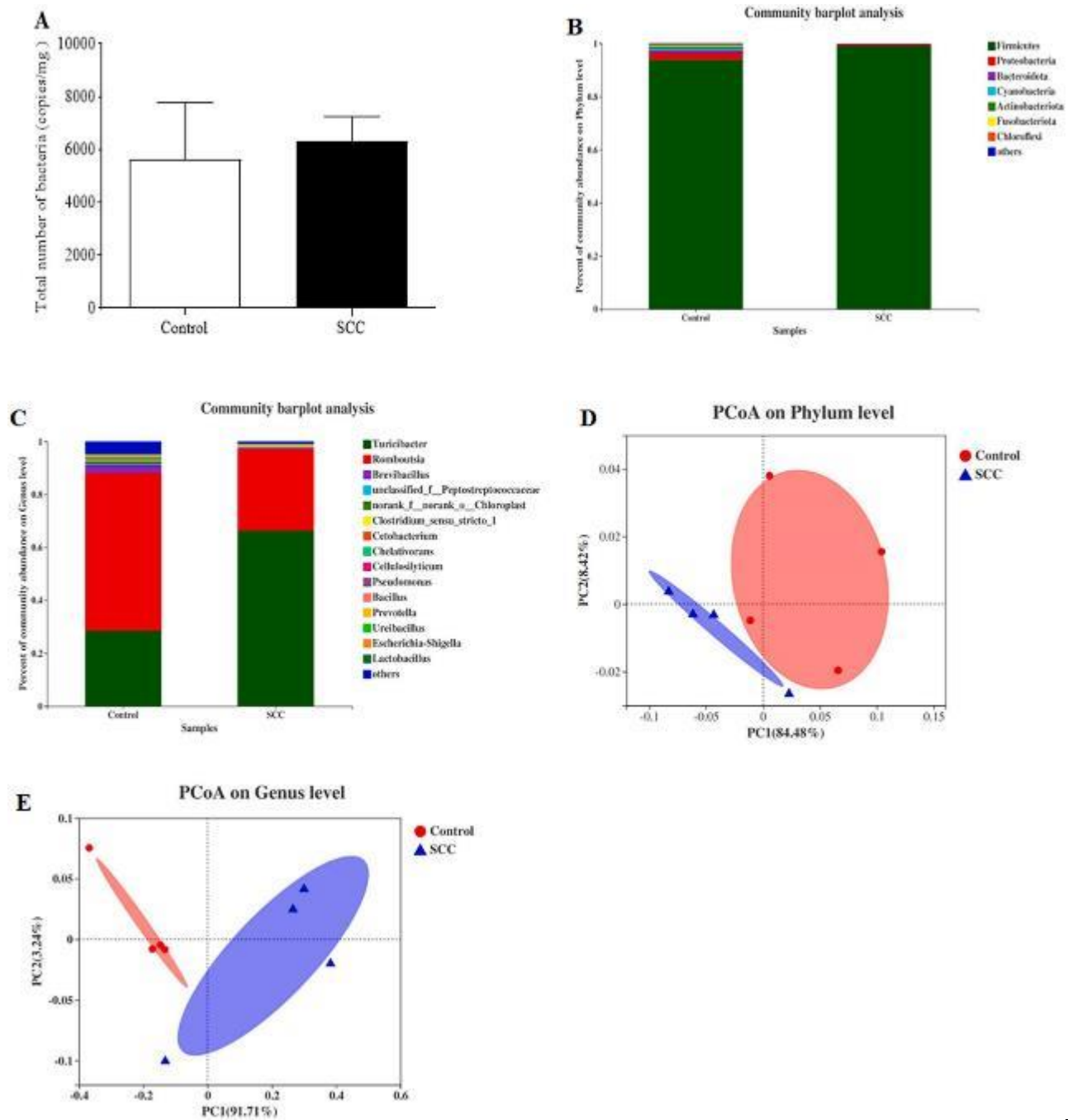
3.4. Effects of SCC diet on the liver health of channel catfish

Compared with the control group, SCC diet significantly reduced infiltration of inflammatory cells and the formation of necrosis (Fig. 5). Compared with the control group, the expression of pro-inflammatory mediator *NF-κB* significantly decreased in SCC group ($P < 0.05$, Fig. 6A). Consistently, western blot also showed that SCC diet significantly decreased the expression of *NF-κB* at protein level (Fig. 6B).

3.5. SCC diet protected channel catfish against bacterial infection

The disease resistance of the channel catfish in control group and SCC group was evaluated by intraperitoneal injection of *A. veronii* Hm091 and *A. hydrophila* NJ-1. At 28 h post challenge, all fish of the control group had died, while the mortality of the SCC group at 28 h was 62.5%. At 48 h, the mortality of SCC group was 66.6%. The survival rate of channel catfish against *A. veronii* Hm091 and *A. hydrophila* NJ-1 was significantly improved in SCC group ($P < 0.05$, Fig. 7).

Fig. 4. Effects of SCC diet on the intestinal microbiota of channel catfish. (A) The total number of bacteria in channel catfish fed with control diet and SCC diet. The relative phylum (B) and genus (C) abundance of the microbiota of channel catfish fed with control diet and SCC diet. PCoA of the intestinal microbiota on phylum (D)



and genus (E) level of the intestinal microbiota (n = 4).

Table 4

Intestinal microbiota at main phylum level of channel catfish fed with control diet and SCC diet.

Bacteria Phylum (%)	Control	SCC	P Value
Firmicutes Proteobacteria	93.97 ± 4.63	99.26 ± 0.50	0.11
Bacteroidetes	2.51 ± 2.10	0.23 ± 0.19	0.06
Cyanobacteria	0.82 ± 0.60	0.34 ± 0.44	0.31
Actinobacteriota	0.92 ± 1.79	0.01 ± 0.01	0.11
Fusobacteriota Chloroflexi	0.64 ± 0.50	0.05 ± 0.05	0.19
			0.06

0.56 ± 0.58 0.07 ± 0.09
0.16 ± 0.14 0.01 ± 0.01

4. Discussions

Yeast culture is a kind of microecological product which has good palatability and growth promoting effect. A large number of experiments have studied the mechanism of yeast culture improving animal production performance. It is generally believed that yeast culture can improve the gastrointestinal environment, accelerate the metabolism of microorganisms,

adjust the structure of bacteria in animals (Ernesto Cesena, ~ Vega-Villasante, Aguirre-Guzman, Luna-Gonzalez, and Campa-Cordova, 2021; Licona-Jain et al., 2020; Nimrat et al., 2021). Studies have reported that adding appropriate amount of yeast culture to aquatic feed can significantly improve the specific growth rate and

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Table 5

Intestinal microbiota at main genus level of channel catfish fed with control diet and SCC diet.

Bacteria Genus (%)	Control	SCC	P Value
<i>Turicibacter</i>	28.47 ± 11.05	67.67 ± 23.52	0.11
<i>Romboutsia Brevibacillus</i>	59.83 ± 3.91	29.73 ± 23.67	0.31
<i>unclassified_f__Peptostreptococcaceae</i>			0.03
<i>norank_f__norank_o__Chloroplast</i>	2.96 ± 4.05	0.08 ± 0.10	0.31
<i>Clostridium_sensu_stricto_1</i>	0.33 ± 0.62	0.65 ± 0.38	0.56
<i>Cetobacterium Chelativorans</i>	0.90 ± 1.77	0.01 ± 0.01	0.19
<i>Cellulosilyticum</i>			0.19
<i>Pseudomonas</i>	0.21 ± 0.20	0.46 ± 0.14	0.03
	0.56 ± 0.58	0.04 ± 0.04	0.06
	0.48 ± 0.62	0.01 ± 0.01	0.06
	0.05 ± 0.03	0.34 ± 0.31	
	0.31 ± 0.26	0.03 ± 0.03	

weight gain of white shrimp (Deng et al., 2013), Nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab, Abdel-Rahman, and Ismael, 2008; Islam et al., 2021), juvenile beluga (*Huso huso*) (Hoseinifar, Mirvaghefi, and Merrifield, 2011), rainbow trout (*Onchorhynchus mykiss*) (L. Wang et al., 2018) and rohu (*Labeo rohita*) (Jahan et al., 2021). In this experiment, similar effects were also observed. The results showed that adding an appropriate amount of yeast culture improved the growth performance and feed utilization of channel catfish. This may be attributed to the valuable nutrients of yeast culture, such as proteins, carbohydrates and nucleic acids, which play major nutritional roles in promoting fish growth (Ernesto Cesena et al., 2021; Licona-Jain et al., ~ 2020; S Nimrat et al., 2021).

The liver and intestines are the most important organs for absorbing nutrients and maintaining normal function (Fang et al., 2019; Vizcaino et al., 2014). Liver is an important metabolic organ that can regulate various physiochemical functions including synthesis, secretion and metabolism of xenobiotics (Tao et al., 2021). Damage to the liver could lead to these physiochemical function disorder. NF-κB is a ubiquitous transcription factor that plays an important roles in many biological processes including innate and adaptive immunity (W. Wang et al., 2019). In this study, compared with the control group, the expression of NF-κB in the liver of SCC group was significantly decreased, which indicated that the effect of *Saccharomyces cerevisiae* culture on the expression of NF-κB may contribute to its impact on hepatic health. The intestinal mucosa provides a physiologic and immunologic barrier to a wide range of microorganisms and foreign substances. The overlying mucus-gel layer is the first line of defense. Formation of the mucus gel is through goblet secretion of polymeric mucin glycoprotein and act as a medium for protection, lubrication, and transport between the luminal contents and the epithelial lining. In this present study, it was observed that the supplementation of SCC could increase goblet cell number of intestines, which is consistent with the results of previous (Islam et al., 2021). Previous studies showed that mannan oligosaccharides as a component of yeast

(*Saccharomyces cerevisiae*) have the capacity to increase villus length of white shrimp and rohu (Jahan et al., 2021; Yuan et al., 2019), and the addition of yeast polysaccharides increased the number of intestinal goblet cell (Zhu et al., 2012). Therefore, it can be speculated that prebiotic increased goblet cell number, resulting in improving intestinal defense. Tissue damage is usually

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accompanied with up-regulation of the expression of nuclear factor NF-κB (De Santis, Bartie, Olsen, Taggart, and Tocher, 2015). HIF1α is a transcription factor that can regulate the expression of the tight junctions and antimicrobial peptide-related genes of the intestine (Zhang et al., 2019). In this study, the supplementation of SCC significantly up-regulated the expression of HIF-1α and decrease the expression of NF-κB. These results were also consistent with the results of histological changes of intestine.

Intestinal mucosa is the first barrier for pathogen invasion, and intestinal bacteria is beneficial to maintain the barrier function of intestinal mucosal system (Tlaskalova-Hogenov' a et al., 2004'). Beneficial bacteria in intestine can regulate the balance of immune response,

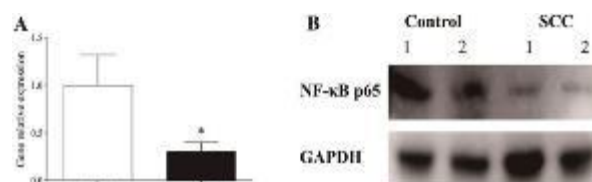


Fig. 6. Effect of SCC diet on the liver of expression of NF-κB gene and protein in channel catfish. (A) qPCR showing the gene expression of NF-κB in the liver, (B) Western blot showing the protein expression of NF-κB in the liver. Data are presented as the means (± SEM) (n = 6). Differences are considered significant at P < 0.05 (*).

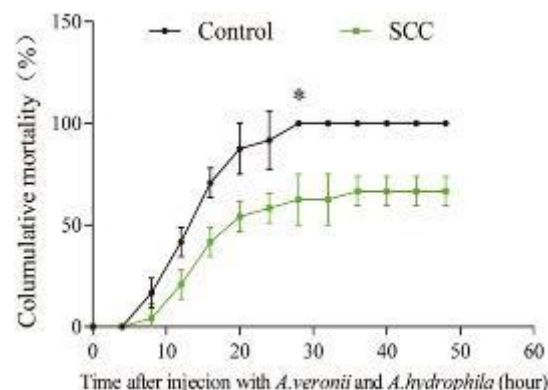


Fig. 7. Protection ability of SCC diet in channel catfish against *A. veronii* Hm091 and *A. hydrophila* NJ-1. Data are presented as the means (± SEM) (n = 3). Differences are considered significant at P < 0.05 (*).

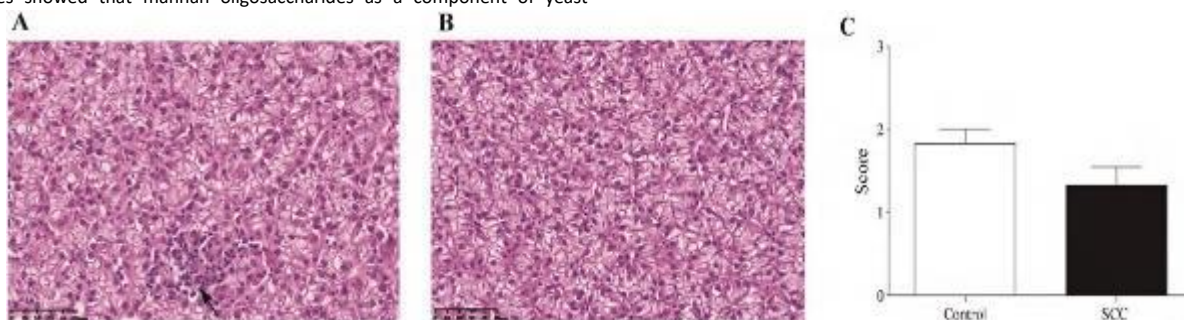


Fig. 5. Effects of SCC diet on the histology (H&E staining) of liver of channel catfish. (A) Control, (B) SCC. (C) Score of liver histopathology (Evaluation parameters: changes in the nucleus, changes in the cytoplasm, hepatocytes vacuolization). Black arrows indicated infiltration of inflammatory cell. Data are presented as the means (± SEM) (n = 6).

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nutrient absorption, internal environment stability and other beneficial functions of the host (Cahenzli, Koller, Wyss, Geuking, and McCoy, 2013; Hooper and Macpherson, 2010; Hooper, Midtvedt, and Gordon, 2002), but harmful bacteria can increase the permeability of the mucosal layer, thus allowing bacteria and macromolecules in food to pass through the mucosal barrier (Ma et al., 2021). In this study, the most abundant intestinal bacterial communities in channel catfish at the phyla level were Firmicutes, Proteobacteria and Bacteroidetes. Previous studies showed that Fusobacterium, Bacteroidetes, Firmicutes and Proteobacteria were the most abundant intestinal microbiota in grass carp fed with yeast culture (Yu et al., 2014). Firmicutes has been known to play a major role in the physiology of host health through metabolic activities, such as the fermentation of indigestible carbohydrates, which in turn produces short chain fatty acids (Yu et al., 2014). Butyrate is a key product of Firmicutes that promotes the health of the intestinal mucosa. The number of Firmicutes in the SCC group was higher than the control group, suggesting that the addition of SCC could improve the immune response of channel catfish.

Acute challenge experiment can directly observe the body's resistance to bacterial infection, thus reflecting the body's immune ability. *Aeromonas* is the pathogen of infectious disease outbreaks in freshwater fish. *Aeromonas* septicemia caused serious economic losses in freshwater aquaculture (Yu et al., 2014; Rathinam et al., 2021). Feeding freshwater fish with diet and then challenging them with bacteria pathogens have been a useful way to assess the resistance of fish to disease infections. Studies have reported that cell wall of baker's yeast (*Saccharomyces cerevisiae*) can improve the survival rate of apanese seabass (*Lateolabrax japonicus*) against *Aeromonas* (Yu et al., 2014). Dietary administration of Diamond V XP Yeast Culture® can protect shrimp (*Litopenaeus vannamei*) against a decline in resistance to *Vibrio* sp. 90–69B3 (Burgents, Burnett, and Burnett, 2004). Our results also showed that SCC diet enhanced the resistance of channel catfish to *A. veronii* and *A. hydrophila*. This may be attributed to the presence of β -glucan in the cell wall of yeast, and it contains specific phagocytic cells receptors that help to bind receptors molecules which will help to release signal molecules that finally stimulate the production of new WBCs, thereby contributing to the improvement of disease resistance of fish (Akhter, Wu, Memon, and Mohsin, 2015; Rohani et al., 2021).

In conclusion, diet supplemented with a certain proportion of *Saccharomyces cerevisiae* culture (ESTAQUA®) promoted the growth performance and feed utilization of channel catfish, improved the intestinal and hepatic health, modulated the structure of intestinal microbiota, and enhanced the ability of channel catfish to resist the infection of *A. veronii* and *A. hydrophila*.

CRedit authorship contribution statement

Zhigang Zhou and Zhen Zhang designed study and provided the laboratory resources; Rui Xia and Qiang Hao conducted the experiments and biochemical analyses; Yadong Xie, Qingshuang Zhang, Chao Ran, Yalin Yang, Wenhao Zhou, Fuwei Chu, Xiaomei Zhang and Yu Wang developed the methodology. Rui Xia, Qiang Hao and Zhen Zhang analyzed the data. All authors participated in writing draft, reviewing and editing the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests that could have appeared to influence the work reported in this paper.

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WILEY

ORIGINAL ARTICLE

Dietary yeast culture facilitates the growth, immune response, digestive enzyme activity, intestinal microbiota and disease

resistance against *Vibrio harveyi* of hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂)

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Abstract

The present study aimed to investigate the effect of yeast culture (YC) supplementation at different levels (0%, 2% and 4%) on the growth, immune response, digestive enzyme activity, intestinal morphology and microbiota of hybrid grouper. After 56 days feeding trial, the results showed that the weight gain rate, specific growth rate, survival rate and protein efficiency rate of hybrid grouper were significantly improved, and the feed conversion ratio of hybrid grouper in the 4% group ($p < 0.05$) was significantly reduced. The serum total protein, acid phosphatase, alkaline phosphatase and lysozyme of hybrid grouper in the 4% group were significantly higher than those in the control group, while the alanine transaminase, aspartate transaminase and malondialdehyde were significantly lower than those in the control group ($p < 0.05$). YC treatment significantly enhanced the trypsin, lipase and amylase activities ($p < 0.05$). *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* constituted the dominant phylum in grouper intestine. The relative abundance of *Sphingomonas*, *Stenotrophomonas* and *Phyllobacterium* in the YC groups was higher than that in the control group. The length and width of intestinal villi of grouper fed with YC diets were improved ($p < 0.05$). The protection against *V. harveyi* challenge in hybrid grouper was enhanced after YC treatment group, and the hybrid groupers fed with 4% YC diets experienced the highest cumulative survival rate of 67%. All these results suggested that the supplementation of 4% YC diets improved the growth performance, digestive enzyme activity, intestinal morphology, microbiota and disease resistance against *V. harveyi* of hybrid grouper.

KEYWORDS

antioxidant capacity, growth performance, hybrid grouper, intestinal morphology, serum immunity, yeast culture

Qi Wang and Stephen Ayiku Co- first author and contributed equally to the study.

1 | INTRODUCTION

Due to environmental pollution and overexploitation of marine fishery resources, the world fishery resources are diminishing in recent years. As a result, the demand for aquatic products worldwide can only be satisfied through aquaculture (Pauly et al., 2002). Currently, intensive aquaculture remains the development direction of culture mode in the future. However, high-density intensive aquaculture tends to cause disease outbreaks,

leading to the death of cultured animals (Nayak, 2010). In this context, promoting fish growth and enhancing the disease resistance have received wide concerns. It is essential for the sustainable development of the grouper industry to develop low content additives to improve disease resistance and immunity of grouper. As a new type of dietary supplements, some probiotics can replace antibiotics in aquaculture and thereby improve the health and growth of fish, which have attracted significant attention in recent years (Huang et al., 2015).

The use of yeast products to improve the growth and immune response of fish has become a research hotspot in recent years. Yeast products can not only reduce the number of harmful bacteria in the intestinal tract by identifying, adsorbing and eliminating harmful bacteria, but also promote the reproduction of beneficial bacteria and regulate the balance of micro-ecological zones in the digestive tract of animals, thus improving the functions and maintaining the integrity of the intestinal tract, as well as improving the immunity of the animal organism (Deng et al., 2013; Zhou et al., 2009). More encouragingly, the potential benefits of yeast products for aquaculture are increasing. Numerous scholars have revealed the positive effects of different yeast products on the growth performance and immune response of aquatic animals (Abass et al., 2018; Berto et al., 2016). Yeast culture (YC) is a compound yeast fermentation product composed of yeast and its growth medium, which is an ideal feed additive (Bu et al., 2019). Yeast fermentation products are rich in protein, amino acids, vitamins and trace elements, which are ideal protein sources for fish, shrimp and livestock feed. Currently, the main method used is high protein solid fermentation culture, that is adding non- protein nitrogen to the fermentation substrate to increase the protein content of the yeast fermentation products. In our study, non- protein nitrogen was added to the YC fermentation substrate, which contained 55% crude protein. YC contains protein, minerals, amino acids and immunomodulatory components such as mannan-oligosaccharides (MOS), β - glucan and nucleotides (Seo et al., 2010). The β -glucan ingredient in YC can activate macrophages and release numerous immunoglobulins, such as interleukins and cytokines, which help aquatic animals to defend against a variety of pathogens (Jayachandran et al., 2018). β - glucan has been demonstrated to exert a potent immunostimulatory effect on much freshwater and marine fish (Sealey et al., 2008; Vetvicka et al., 2013). Miest et al. (2016) have reported that the supplementation of β -glucan in the diet induces an immunomodulatory response, which can effectively increase the survival rate in turbot (*Scophthalmus maximus*) culture (Miest et al., 2016). Emerging *in vitro* studies have also unveiled that β - glucan activates a range of immune cells in mammals mainly through Toll-like receptors (TLRs), complement receptors (CR3) and Dectin- 1 receptors (Zhang et al., 2018). The addition of MOS to the diet can effectively improve the growth performance and intestinal histomorphology of fish (Dimitroglou et al., 2010; Ye et al., 2011). MOS also enhances immunity by modulating the intestinal microbiota (Ren et al., 2020; Torrecillas et al., 2011). Dietary 10^7 CFU/kg *Saccharomyces cerevisiae* can be colonized in the intestinal tract to improve the growth rate and feed efficiency of grouper, thus enhancing the resistance to streptococcus and iridovirus (Chiu et al., 2010). Besides, Ayiku et al. (2020) have shown that the addition of 2% YC in the diet can improve the growth performance, disease resistance and immune response of *Litopenaeus vannamei* (Ayiku et al., 2020b).

Hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) is a kind of marine carnivorous fish widely cultivated in southern China due to its delicious flavour and commercial importance (Ayiku et al., 2020b; Yin et al., 2018, 2020). Hybrid grouper bears the advantages of rapid growth, strong disease resistance and high nutritional value, but requires high content of fish meal in the feed because of its eating habits (Chen et al., 2020; Yang et al., 2019). Systematic studies have demonstrated the effect of YC on *Litopenaeus vannamei* (Ayiku et al., 2020a, 2020b), grass carp

(*Ctenopharyngodon idella*) (Liu et al., 2018), gibel carp (*Carassius auratus gibelio* CAS III) (Zhang et al., 2018) and Ussuri catfish (*Pseudobagrus ussuriensis*) (Bu et al., 2019). However, research on YC in hybrid grouper is relatively lacking. Previous studies have shown the beneficial of YC on the growth performance, serum biochemical parameters and antioxidant capacity of hybrid grouper (Wang et al., 2019). Based on the existing studies, the present study investigated the effect of YC with non- protein nitrogen added to the fermentation substrate on the growth performance, immune response, intestinal flora and resistance to *Vibrio harveyi* infection in hybrid groupers, hoping to confer a scientific basis for the research on the healthy culture of pearl grouper and the development of environment- friendly and energy- saving feed.

2 | MATERIALS AND METHODS

2.1 | Diet preparation

The ingredients of the nutrients of the experimental diets are shown in Table 1. Fish meal, casein and wheat gluten were used as protein sources. Three iso- nitrogenous (crude protein about 50%) and iso- lipidic (crude lipid about 11%) experimental diets containing fish oil and phospholipid were prepared. YC (55% protein, fermentation substrate with non- protein nitrogen) was provided by Enhakor Biotechnology Company. Firstly, brewer's yeast was seeded into YEPD liquid medium to obtain a seed culture, and then, the seed culture was seeded into molasses medium for aerobic fermentation to obtain a liquid fermentation broth. The liquid fermentation broth was mixed with the solid medium seeded with *Aspergillus niger*, and the fermentation product was dried to produce a yeast culture. The YC used in the present study was the same as that used by Ayiku

Ingredients	Treatment ^f		
	0%	2%	4%
Fish Meal ^a	34.00	32.50	31.00
Yeast culture ^d	0.00	2.00	4.00
Casein ^a	25.00	25.00	25.00
Wheat gluten ^a	6.00	6.00	6.00
Starch ^a	18.00	18.00	18.00
Fish oil ^a	8.00	8.00	8.00
Phospholipid ^a	1.00	1.00	1.00
Vitamin mixture ^b	0.50	0.50	0.50
Mineral mixture ^c	2.50	2.50	2.50
Choline chloride ^b	0.50	0.50	0.50
Antioxidants ^a	0.03	0.03	0.03
Calcium Dihydrogen Phosphate ^a	1.00	1.00	1.00

Vitamin complex ^b	0.05	0.05	0.05
Attractant ^e	0.10	0.10	0.10
Yttrium oxide Y ₂ O ₃ ^a	0.1	0.1	0.1
MCC ^a	3.22	2.72	2.22
Total	100	100	100
Nutrient levels			
Dry Matter	92.60	92.03	92.27
Crude Protein	50.57	50.36	50.72
Crude Lipid	11.25	11.34	11.50
Ash	10.89	10.17	10.18

^a Ingredients purchased from Zhanjiang HaiBao Feed Factory, Zhanjiang, Guangdong, China. MCC: Microcrystalline cellulose. ^b Vitamin mixture (g/kg mixture): vitamin B1, 17.00 g; vitamin B2, 16.67 g; vitamin B6, 33.33 g; vitamin B12, 0.07 g; vitamin K, 3.33 g; vitamin E, 66.00 g; retinyl acetate, 6.67 g; VD, 33.33 g, nicotinic acid, 67.33 g; D- calcium pantothenate, 40.67 g; biotin, 16.67; folic acid, 4.17 g; inositol, 102.04 g; cellulose, 592.72 g. All ingredients were diluted with corn starch to 1 kg.

^c Mineral mixture (mg g⁻¹ mixture): CaCO₃, 350 g; NaH₂PO₄·H₂O, 200 g; KH₂PO₄, 200 g; NaCl, 12 g; MgSO₄·7H₂O, 10; FeSO₄·7H₂O, 2 g; MnSO₄·7H₂O, 2 g; AlCl₃·6H₂O, 1 g; CuCl₂·2H₂O, 1 g; KF, 1 g; NaMoO₄·2H₂O, 0.5 g; NaSeO₃, 0.4 g; CoCl₂·6H₂O, 0.1 g; KI, 0.1 g; zeolite powder, 219.9 g. All ingredients were diluted with corn starch to 1 kg. (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China). ^d The yeast products were purchased from Enhalar Biotechnology Company (Beijing, China).

^e Attractant composition: taurine: glycine: betaine = 1:3:3.

^f Diets were supplemented with YC at 0%, 2% and 4% respectively.

et al. (2020b). The nutrient composition was given with reference to the literature of Ayiku et al. (2020b). The three diets were supplemented with 0%, 2% and 4% YC respectively. All feed materials were crushed and passed through a 60- mesh sieve and then evenly mixed with fish oil, phospholipid and water in a certain proportion. Thereafter, the mixture was processed into 2.5 mm particles by a twin- screw extruder (South China University of Technology, F- 26). Finally, the feed materials were air- dried to a moisture content of 10% and stored at- 20°C for use.

2.2 | Feeding trial

The hybrid groupers used in this study were obtained from a commercial hatchery (Zhanjiang, China). Prior to the feeding trial, the hybrid groupers were acclimatized to the experimental conditions and fed with a commercial feed (crude protein: 50%, crude lipid: 11%) for 2 weeks. All fish was subjected to a 24 h fasting before the experiment. There were 3 experimental groups with 3 replicates in each group. Briefly, 270 health hybrid groupers of similar size (initial weight: 17.56 ± 0.02 g) were randomly distributed into 9 fibreglass tanks (500 L), with 30 hybrid groupers in each

tank. The groupers underwent satiated feeding twice a day (9:00 and 17:00) and 60% water changes daily for 8 weeks. The culture temperature was maintained at 28.0 ± 2.0°C, and the dissolved oxygen (DO) was within the range of 6– 8 mg l⁻¹. Also, the culture water was used after precipitation, filtration, chlorine dioxide disinfection and aeration.

2.3 | Sample collection and calculation formula

All the fish were sampled after 24 h fasting at the end of the culture experiment. The specific growth rate (SGR), weight gain rate (WGR), survival rate (SR) and feed coefficient rate (FCR) were calculated. Two fish were randomly selected from each tank and stored at -20°C for whole- body composition analysis. The blood samples were collected from 6 groupers in each tank via tail vein using a 1 ml sterile syringe and transferred into the 1.5 ml Eppendorf tube. The blood samples were stored at 4°C for 12 h, followed by 3500 rpm centrifugation at 4°C for 10 min. The supernatant was collected and stored at -80°C for subsequent enzyme activity analysis. The midgut was taken from 3 groupers in each group under aseptic condition, transferred into the 1.5 ml Eppendorf tube and immediately put into liquid nitrogen for microflora analysis. Furthermore, the midguts of another 3 groupers were collected and stored in 10% paraformaldehyde for histological analysis. Finally, 2 groupers were randomly elected from each tank and their midguts were stored in liquid nitrogen for enzyme activity analysis. The corresponding calculation formulas were as follows:

Weight gain rate (WGR, %) = 100 × (final body weight-initial body weight)/(initial body weight).

Specific growth rate (SGR, %/d) = 100 × [ln (final weight)- ln (initial weight)]/days.

Feed conversion ratio (FCR) = diet intake/wet weight gain.

Survival rate (SR, %) = 100 × (final fish number)/(initial fish number).

Protein efficiency ratio (PER) = fish weight gain/fish protein intake.

Condition factor (CF, %) = 100 × body weight/body length³.

2.4 | Analysis of diets and fish body composition

The dry matter, crude protein, lipid and ash of diet and whole fish were determined according to the methods of AOAC (1995). The samples were dried in an oven at 105°C to a constant weight to determine the dry matter. The crude lipid content was determined using the Soxhlet extraction method, and the crude protein content was determined using the Kjeldahl method. Moreover, the crude ash content was determined by burning in a muffle furnace at 550°C for 6 h.

2.5 | Serum and intestinal enzyme activity analysis

The supernatant of the blood samples was placed on ice for enzyme activity analysis. The levels of serum glucose (Glu), triglyceride (TG), cholesterol (CHO), catalase (CAT), superoxide dismutase (SOD), acid phosphatase (ACP), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (AKP), lysozyme (LZM), total protein (TP), malondialdehyde (MDA) and immunoglobulin M (IgM) were tested using the commercial kits (Nanjing Jiancheng Bioengineering Institute,).

The intestinal tissue sample (0.5 g) was diluted with 0.9% NaCl solution at the ratio of 1:9 (w/v). The sample was then ground on ice and centrifuged at 4°C for 10 min. Afterwards, the supernatant was transferred into an Eppendorf tube for testing. Intestinal digestive enzymes (trypsin, lipase and amylase) were determined in line with the instructions of commercial kits (Nanjing Jiancheng Biological Engineering Institute,).

2.6 | Intestinal histological analysis

In the present study, the intestinal morphological indicators were examined using the method of Ayiku et al. (2020) to determine the effect of YC on the intestinal morphology of groupers. The fixed tissues were dehydrated with gradient alcohol, cleared in toluene, transparented in xylene and embedded in paraffin. Finally, the paraffin-embedded tissues were sectioned with a thickness of 5 µm. After haematoxylin–eosin staining, intestinal images were captured under a microscope (Olympus, BX51, serial number: 9K18395,). The height, width and muscle thickness of intestinal villi were measured using the Image-Pro Plus 6.3 software. A total of 12 measurements were obtained for each tissue.

2.7 | Intestinal microflora analysis

2.7.1 | DNA extraction and PCR amplification

The samples of intestinal contents were treated using the E.Z.N.A. Soil DNA kit, and the intestinal contents were extracted following the instructions of the kit. The concentration and purity of extracted DNA were quantified using the NanoDropND-2000 spectrophotometer, and the extracted DNA was used as a template. The V3-V4 sequences of 16S rRNA gene in each sample DNA were amplified by universal primers 314F (341F: 5'-CCTACGGGNGGCWGCAG-3') and 806R (806R: 3'-GGACTACHVGGGTATCTAAT-5'). PCR reaction system (30 µl) was as follows: 15 µl Phusion Master Mix (2×), 1.5 µl upstream and downstream primers (2 µmol/L), 10 µl template DNA and 2 µl ddH₂O. The reaction procedures were as follows: pre-denaturation at 98°C for 1 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s and final extension at 72°C for 5 min. The PCR products were electrophoretic with 2% agarose gel. The sequence bands between 400 and 500 bp were selected, and the gel was cut. In addition, the GeneJET (Thermo Scientific) Gel Extraction kit was used to recover and purify the target bands.

2.7.2 | Library construction and sequencing

Libraries were constructed using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs) library building kits for library construction and then sequenced by Illumina HiSeq 2500 sequencing system.

2.7.3 | Sequencing data analysis

The removal of low-quality Reads was conducted by using Cutadapt (V1.9.1, <http://cutadapt.readthedocs.io/en/stable/>) (Asshauer et al., 2015). The sample data were separated from the Reads obtained by barcode. The reads obtained from the original data after the preliminary quality control of barcode and primer sequences need to be processed to remove the chimera sequence. The read sequence was compared with the species annotation database to detect the chimera sequence (<https://github.com/torognes/vsearch/>) (Martin,), and finally, the chimera sequence was removed to obtain the final valid data (Clean Reads). All the Clean Reads of all samples were clustered using the Uparse software (Uparse v7.0.1, <http://www.drive5.com/uparse/>) (Hass et al., 2011), and the sequence was clustered into Operational Taxonomic Units (OTUs), with 97% consistency (Identity) by default. Moreover, the representative sequence of OTUs was selected. The sequence with the highest frequency in OTUs was selected as the representative sequence of OTUs according to the principle of its algorithm. The sequence of OTUs was annotated, and the Mothur method was used to analyse the species annotation with SILVA132's (<http://www.arb-silva.de/>) (Edgar, 2013) and SSUrRNA database (Wang et al., 2007). QIIME software (Version 1.9.1) was used to calculate Chao1, Shannon, Simpson and ace indexes. The dilution curve was drawn using the R software (Version 2.15.3). The differences of microflora structure in different samples were analysed and compared by using unweighted UniFrac distance algorithm and principal coordinate analysis (PCoA).

TABLE 2 Effect of yeast culture supplementation on hybrid grouper growth performance

Treatment	IBW(g/fish)	FBW(g/fish)	WGR(g/fish)	SGR(g/fish)	FCR	SR(%)	PER(%)	CF(%)
0%	17.86 ± 0.00	78.91 ± 0.68 ^b	341.90 ± 3.59 ^b	2.65 ± 0.01 ^b	1.03 ± 0.02 ^a	92.33 ± 1.17 ^b	1.54 ± 0.04 ^b	2.77 ± 0.05 ^b
2%	17.87 ± 0.01	81.53 ± 0.76 ^{ab}	356.29 ± 4.17 ^{ab}	2.71 ± 0.02 ^{ab}	0.94 ± 0.01 ^b	94.22 ± 0.89 ^b	1.73 ± 0.22 ^a	2.87 ± 0.02 ^{ab}
4%	17.85 ± 0.04	82.32 ± 0.66 ^a	361.00 ± 3.21 ^a	2.73 ± 0.01 ^a	0.92 ± 0.05 ^b	100 ± 0.00 ^a	1.72 ± 0.04 ^a	3.10 ± 0.07 ^a

Note: The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($p < 0.05$).

respectively.

Abbreviations: CF, Condition factor; FBW, Final body weight; FCR, Feed conversion ratio; IBW, Initial body weight; PER, Protein efficiency ratio; SGR, Specific growth rate; SR, Survival rate; WGR, Weight gain rate.

2.8 | Experimental challenge test

For the challenge test of hybrid groupers with *Vibrio harveyi*, the frozen *Vibrio harveyi* (Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Animal, Guangdong Ocean University,) was placed in a 250 ml flask containing Luria- Bertani (LB) culture medium and cultured in an incubator at 37°C and 180 r/min for 20 h. The supernatant was discarded after centrifugation at 4°C and 8000 r/min for 10 min. Subsequently, the cells were washed twice with aseptic phosphate buffer solution (PBS) and continuously diluted to 3.58×10^5 CFU/ml (LD50) with PBS. After 8 weeks of feeding trial, 12 groupers were randomly selected from each replicate group for the challenge test, with the saline injection group as the control. Each grouper was injected with 200 µl bacterial suspension and fed during the challenge period. Two fish were randomly selected 12 h after bacterial injection, and the blood samples were collected. The sample was centrifuged for 10 min at 4°C and 3500 r/min. The supernatant was collected and stored at -80°C for the determination of ACP, alkaline phosphatase (ALP), LZM and IgM. The survival rate of groupers in each experimental group was recorded 168 h after the challenge test. The formula for cumulative survival rate was as follows:

Cumulative survival rate(%)

Total survival rate in each treatment after challenge

3.2 | Whole- body compositions

The effects of YC at different levels on the whole-body composition of hybrid grouper are shown in Table 3. Compared with those of the controls, the dry matter, crude protein and crude lipid of hybrid groupers fed with YC were significantly enhanced with the increase in YC level ($p < 0.05$). The dry matter, crude protein and crude lipid of hybrid groupers in the 4% group were significantly higher ($p < 0.05$) than those in the 0% group. Also, the ash content of hybrid groupers in the 0% and 4% groups was significantly higher than that of hybrid groupers in the 2% group ($p < 0.05$).

$$=100 \times \frac{\text{Total number of grouper challenged for same treatment}}{\text{Total number of grouper challenged for same treatment}}$$

2.9 | Statistical analysis

Data analysis was performed using the SPSS23.0 (SPSS Inc.,). The results are presented as means ± standard error of mean (SEM). All data were analysed using one- way analysis of variance followed by a Duncan's multiple range test to determine significant differences between treatment groups. A value of $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Growth performance

The effect of YC supplementation on the growth performance of hybrid grouper is shown in Table 2. The WGR and SGR of hybrid groupers in the 4% group were significantly higher than those of hybrid groupers in the 0% group ($p < 0.05$), but there was no significant difference between the 2% and 4% groups ($p > 0.05$). The survival rate of hybrid groupers in the 4% group was significantly higher than that of hybrid groupers in the 0% and 2% groups ($p < 0.05$). The FCR of hybrid groupers in the 2% and 4% groups was significantly lower than that of hybrid groupers in the 0% group, but there was no significant difference between the 2% and 4% groups ($p > 0.05$). The PER and CF of hybrid groupers in the 4% group were significantly higher than those of hybrid groupers in the 0% group ($p < 0.05$). However, there was no significant difference between the 2% and 4% groups ($p > 0.05$).

3.3 | Serum biochemical parameters, antioxidant enzymes and immune enzyme activities

As shown in Table 4, the serum TP levels of hybrid groupers in the 4% group were significantly higher than those in the 0% group ($p < 0.05$), but no significant difference was observed between the 4% group and 2% group ($p > 0.05$). The serum ALT and AST levels of hybrid groupers in the 4% group were significantly lower than those in the 0% and 2% groups ($p < 0.05$). There were no significant difference in serum GLU, TG and CHO levels between the control group and experimental groups ($p > 0.05$).

The activities of SOD, MDA and CAT in the serum of hybrid groupers before and after the challenge test are shown in Figure 1. There was no significant difference in SOD among the treatment groups before the challenge test ($p > 0.05$). The serum SOD and MDA concentrations were significantly increased after the challenge compared with those before the challenge ($p < 0.05$). The serum SOD was raised with the increase in YC addition level, but there was no significant difference among all groups ($p > 0.05$).

0.05). There was no significant difference in serum CAT between the control and experimental groups before and after the challenge. In addition, the content of serum MDA in the 2% and 4% groups was significantly lower than that in the 0% group before and after the challenge ($p < 0.05$).

The activities of ACP, AKP, LZM and IgM in the serum of hybrid groupers before and after the challenge are shown in Figure 2. The activity of serum ACP was significantly increased after the challenge compared with that before the challenge ($p < 0.05$). The ACP activity of hybrid groupers in the 4% group was significantly higher than that in the control group ($p < 0.05$). After the challenge, the serum AKP activity in the 2% and 4% groups was significantly elevated compared with that in the 0% group ($p < 0.05$). The LZM activity in the 4% group was significantly higher than that in the 0% group after the challenge ($p < 0.05$). The IgM activity in the 2% group before the challenge was significantly higher than that in the 0% group ($p < 0.05$). However, there was no significant difference among the treatment groups after the challenge ($p > 0.05$).

3.4 | Intestinal digestive enzyme activity

As shown in Table 5, no significant differences were found in the trends of intestinal trypsin, lipase and amylase activities among all groups. Trypsin, lipase and amylase in the YC treatment groups were significantly higher than those in the control group ($p < 0.05$), with the 4% group showing the highest levels.

3.5 | Analysis of intestinal microflora

3.5.1 | Intestinal microbiota richness and species diversity

Figure 3a shows the sample rarefaction curve. It could be seen that the corresponding curve of each group of samples tended to be smooth with the increase in the sequence number, suggesting

Treatment	Dry matter	Crude protein	Lipid	Ash
0%	28.32 ± 0.40 ^b	17.19 ± 0.09 ^b	6.10 ± 0.01 ^b	4.28 ± 0.00 ^a
2%	28.98 ± 0.41 ^b	17.64 ± 0.05 ^a	6.19 ± 0.10 ^b	4.04 ± 0.03 ^b
4%	29.67 ± 0.20 ^a	17.89 ± 0.04 ^a	6.45 ± 0.21 ^a	4.37 ± 0.04 ^a

TABLE 3 Effects of dietary yeast culture on whole- body conventional components of hybrid grouper (% dry weight)

Note: The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($p < 0.05$). Diets were supplemented with YC at 0%, 2% and 4% respectively.

TABLE 4 Effects of dietary yeast culture supplementation on serum biochemical indices of grouper.

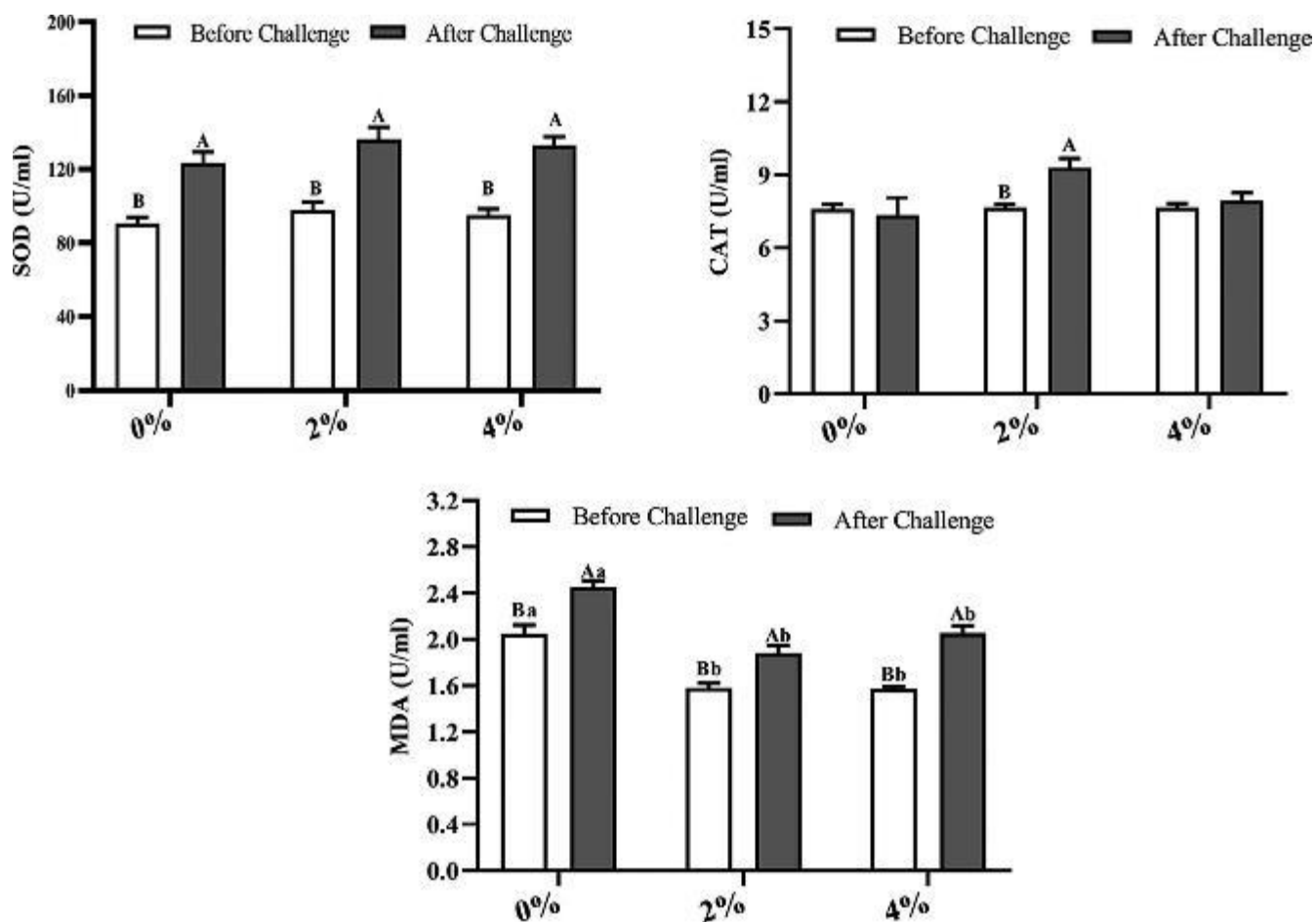
Treatment	TP (mmol/L)	GLU (mmol/L)	TG (mmol/L)	CHO (mmol/L)	ALT (U/L)	AST (U/L)
0%	44.21 ± 0.94 ^b	4.21 ± 0.17	0.64 ± 0.01	5.40 ± 0.06	4.42 ± 0.09 ^a	25.18 ± 0.47 ^a
2%	45.94 ± 0.50 ^{ab}	4.29 ± 0.06	0.67 ± 0.01	5.52 ± 0.14	4.64 ± 0.06 ^a	25.96 ± 1.00 ^a
4%	47.89 ± 0.36 ^a	4.31 ± 0.15	0.65 ± 0.00	5.41 ± 0.05	4.13 ± 0.03 ^b	23.96 ± 0.38 ^b

Note: The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($p < 0.05$). Diets were supplemented with YC at 0%, 2% and 4% respectively.

Abbreviations: ALT, Alanine transaminase; AST, Aspartate aminotransferase; CHO, Cholesterol; GLU, Glucose; TG, Triglyceride; TP, Total protein.

FIGURE 1 Yeast culture diet effects on serum antioxidant enzyme activity of hybrid grouper before challenge and after challenge with *Vibrio harveyi*.

Upright bars denote the mean ± SEM. Different capital letters indicate a comparison before and after challenge in the same group, and different lowercase letters indicate a comparison before and after challenge between different groups. The same letter indicates no significant differences among groups ($p > 0.05$). Diets were supplemented with YC at 0%, 2% and 4% respectively. SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde



that the amount of sequencing data was gradually becoming reasonable, with more data yielding only a small number of new species. The existing data can reflect the characteristics of microbial diversity in the samples. A species accumulation box plot chart was drawn to analyse the relationship between species diversity and sample size. As shown in Figure 3b, the box plot position rises sharply over a range, indicating the existence of large number of species was found in the community. When the box plot tended to be smooth, the species would not significantly increase with the increase in sample size, indicating that the sampling was sufficient and data analysis could be carried out. The OTUs and alpha diversity index of intestinal microflora operation taxon of hybrid groupers are shown in Table 6. The OTUs and Alpha diversity index (Chao, Ace, Shannon, Simpson) of different samples were calculated. The results showed that the OTUs and alpha diversity indexes had no significant difference between groups ($p > 0.05$). The Venn diagram (Figure 3c) was obtained after 97% consistency of OTUs clustering. Venn diagram analysis showed that there were 426 OTUs shared among the three groups. The mean OTUs of the 0%, 2% and 4% groups were 962, 340 and 594 respectively. The 0% and 4% groups shared the most OTUs at 390. In contrast, the 0% and 2% groups shared 124 OTUs.

3.5.2 | Taxonomic composition of intestinal microorganisms

The 4 kinds of intestinal bacteria with the most abundant relative abundance in each group were *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* at the phylum level (Figure 4).

Proteobacteria was the dominant phylum of all samples, with the 2% group (69.83%) showing the highest abundance, followed by the 4% group (60.64%) and 0% group (29.40%). *Firmicutes* (0% group, 22.35%; 2% group, 8.51%; 4% group, 16.47%), *Actinobacteria* (0 group %, 14.16%; 2% group, 4.81%; 4% group, 5.81%) and *Bacteroides* (0% group, 11.20%; 2% group, 5.29%; 4% group, 4.83%) came next. The intestinal microbial composition of the 3 groups at the genus level is shown in Figure 5. The 4 genera with the highest relative abundance in each group were as follows: group 0%: *Sphingomonas* (4.99%), *Stenotrophomonas* (3.20%), *Phyllobacterium* AKP,

4% 2768.15 ± 217.92^a 862.79 ± 68.52^a 537.17 ± 20.47^a

Note: The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($p < 0.05$). Diets were supplemented with YC at 0%, 2% and 4% respectively.

(4.61%) and *unidentified_Ruminococcaceae* (4.73%); group 2%: *Sphingomonas* (4.31%), *Perluclidibaca* (13.71%), *Stenotrophomonas* (16.84%) and *Phyllobacterium* (11.33%); group 4%: *Sphingomonas* (27.40%), *Stenotrophomonas* (6.57%), *Phyllobacterium* (14.29%) and

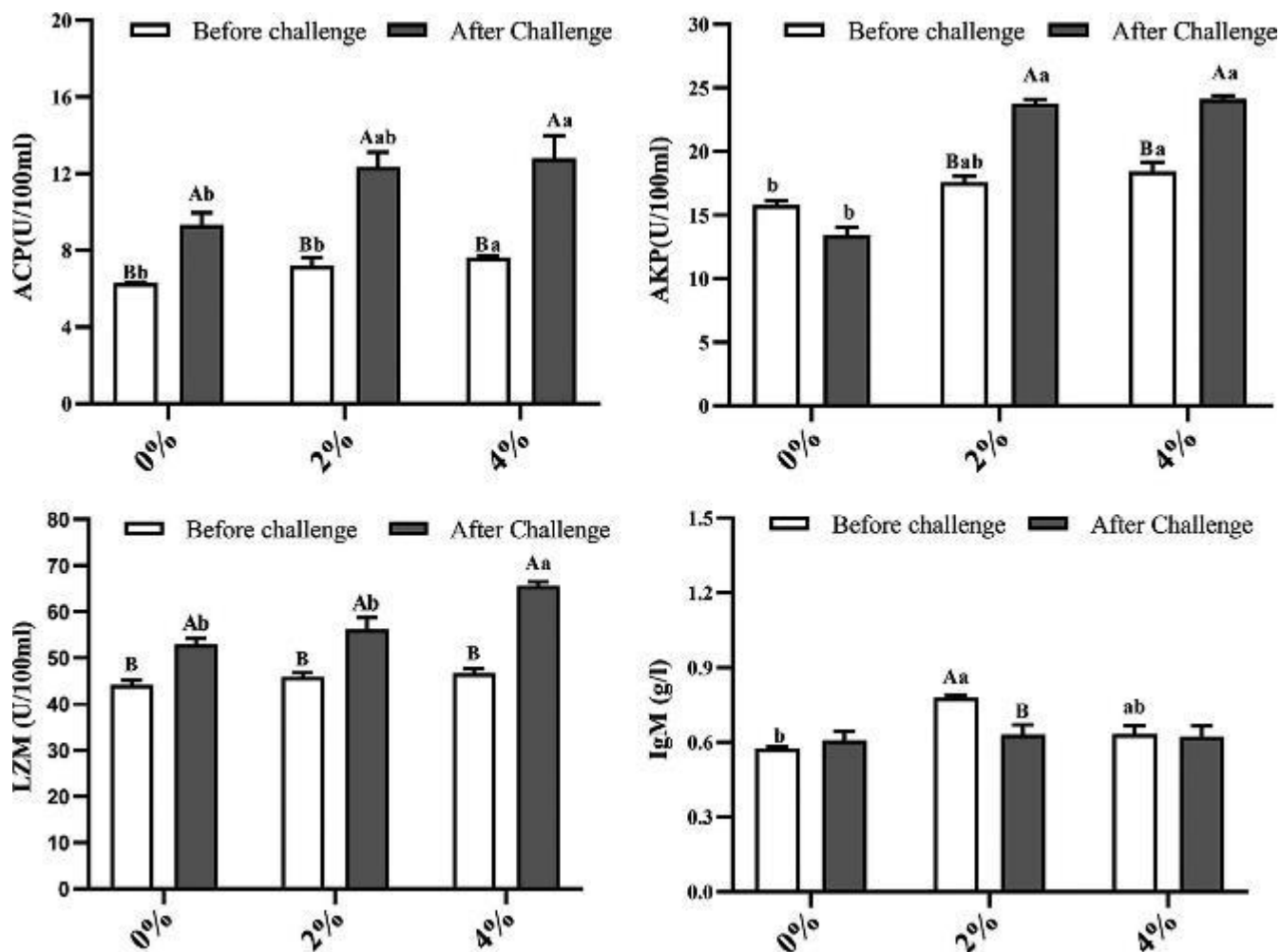


FIGURE 2 Yeast culture diet effects on serum immune enzyme activity of hybrid grouper before challenge and after challenge with *Vibrio harveyi*. Upright bars denote the mean ± SEM. Different capital letters indicate a comparison before and after challenge in the same group, and different lowercase letters indicate a comparison before and after challenge between different groups. The same letter indicates no significant differences among groups ($p > 0.05$). Diets were supplemented with YC at 0%, 2% and 4% respectively. ACP, acid phosphatase; AKP, alkaline phosphatase; LZM, lysozyme; IgM, immunoglobulin M

TABLE 5 Effects of dietary yeast culture supplementation on the intestinal digestive enzyme activities of hybrid grouper

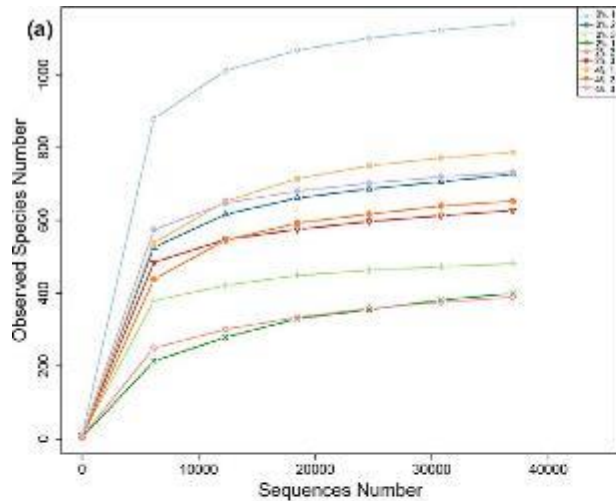
Treatment	Trypsin (U/mgProt)	Lipase (mU/mgProt)	Amylase (mU/mgProt)
0%	1352.58 ± 113.49 ^b	444.14 ± 20.29 ^c	318.81c ± 12.71 ^c
2%	2497.57 ± 146.34 ^a	631.08 ± 59.67 ^b	442.93c ± 16.40 ^b

Faecalibacterium (3.21%).

3.6 | Multi-sample comparative analysis

The principal coordinate analysis (PCoA) based on Weighted Unifrac at the OTU level is shown in Figure 6a. The YC group samples were separated from the control group samples, indicating the differences in the structural composition of intestinal microorganisms between the two groups. In contrast, the distance between the 2% and 4% groups was closer, indicating a high degree of community similarity between the two sample groups. Besides, all the groupers were assigned to two major clusters according to

the UPGMA method (Figure 6b). One of the clusters consisted of the members of the 2% and 4% groups, and the other cluster contained the members of the 0% group, which was consistent with the results of PCoA. It was indicated that the YC group samples had similar microbiota composition. *Proteobacteria* and *Firmicutes* were the two bacteria with the highest relative abundance in each group. Thus, the addition of YC to feed can influence the structure of the intestinal flora of hybrid grouper.



3.7 | Intestinal histological analysis

The intestinal tissue section is shown in Figure 7a. It could be seen that the intestinal mucosal folds of groupers in the 0% group were disorderly arranged and separated from the intestinal wall. The groupers in the 2% group had slight separation, and the groupers in the group 4% had no separation, with the intestinal epithelium neatly arranged. The groupers in

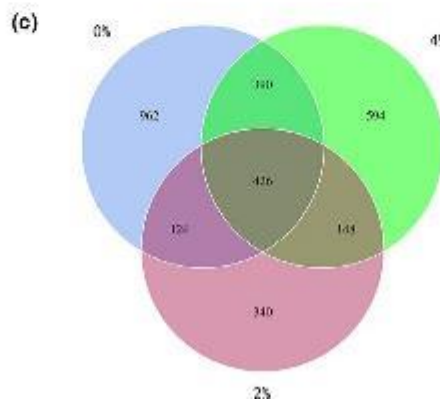
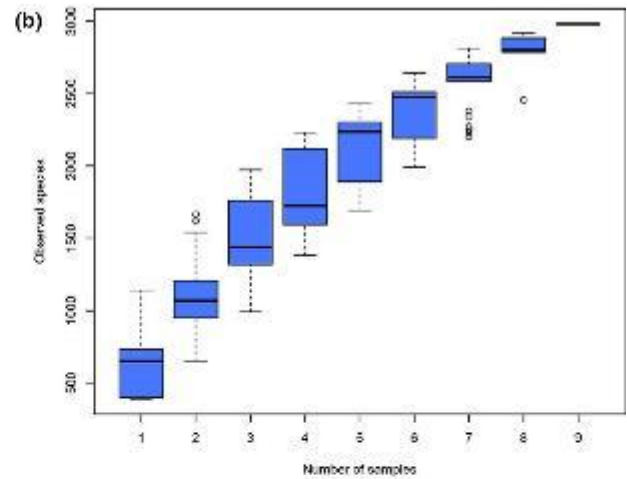


FIGURE 3 (a) Analysis of rarefaction curves of hybrid grouper gut samples. (b) The species accumulation box plot. The horizontal axis represents the number of samples, and the vertical axis represents the number of identified OTUs. (c) Venn diagram of OTUs comparison. Diets were supplemented with YC at 0%, 2% and 4% respectively

TABLE 6 Richness and diversity indexes of intestinal bacterial in hybrid grouper fed experimental diets

Index	Raw reads		Clean reads				
			OTUS	Chao	Ace	Shannon	Simpson
0%	73232	69616	814.67 ± 207.44	772.54 ± 268.52	650.56 ± 138.26	7.54 ± 0.70	0.98 ± 0.00
2%	81539	75850	542.67 ± 76.88	589.26 ± 93.86	587.24 ± 107.90	5.88 ± 1.75	0.88 ± 0.05
4%	86092	80143	777.33 ± 45.06	834.66 ± 21.64	815.21 ± 22.59	6.40 ± 0.89	0.76 ± 0.15

Note: The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($p < 0.05$). Diets were supplemented with YC at 0%, 2% and 4% respectively.

the 4% group had thicker muscles and wider villi compared with the groupers in the other two groups. The intestinal morphology of hybrid groupers was measured (Figure 7b). The villus height of groupers in the 4% group was significantly higher than that of groupers in the 0% group ($p <$

0.05). There was no significant difference in villus height between the 2% and 4% groups ($p > 0.05$). The villus width of groupers in the 4% group was significantly higher than that of groupers in the 0% and 2% groups. The

muscle thickness was increased with the increase in YC level, with the 4% group showing the highest muscle thickness. Besides, there was no significant difference in muscle thickness among these groups ($p > 0.05$).

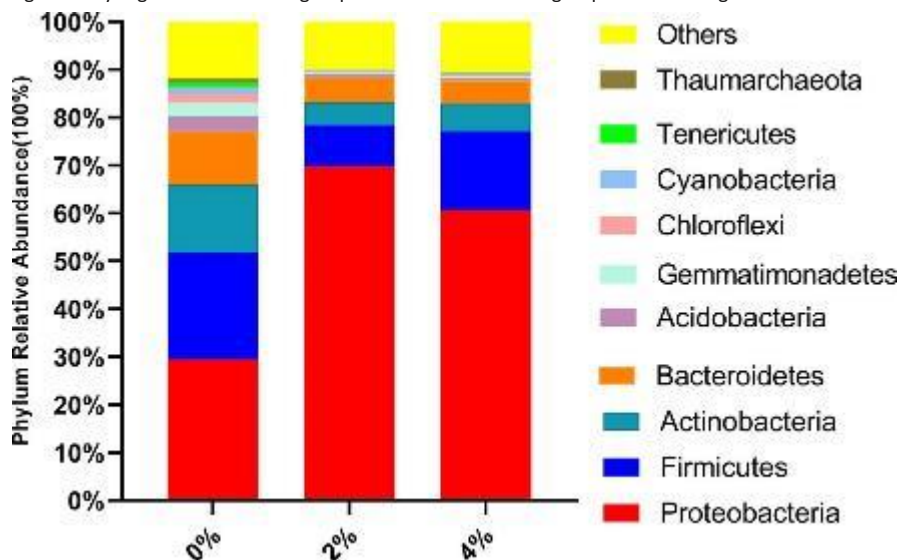


FIGURE 4 Histograms of intestinal bacterial community abundance at phylum level between three groups of hybrid grouper. Diets were supplemented with

YC at 0%, 2% and 4% respectively

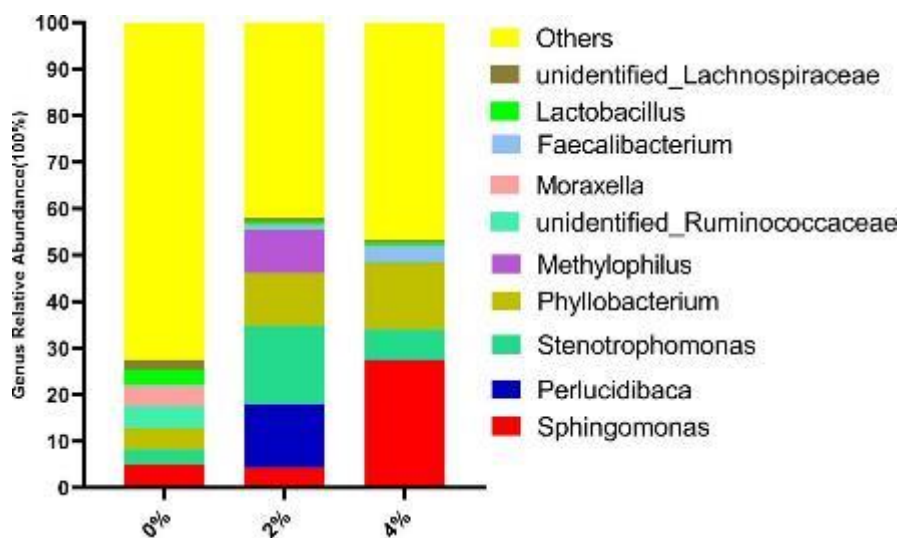


FIGURE 5 Histograms of intestinal bacterial community abundance at genus level between three groups of hybrid grouper. Diets were supplemented with

YC at 0%, 2% and 4% respectively

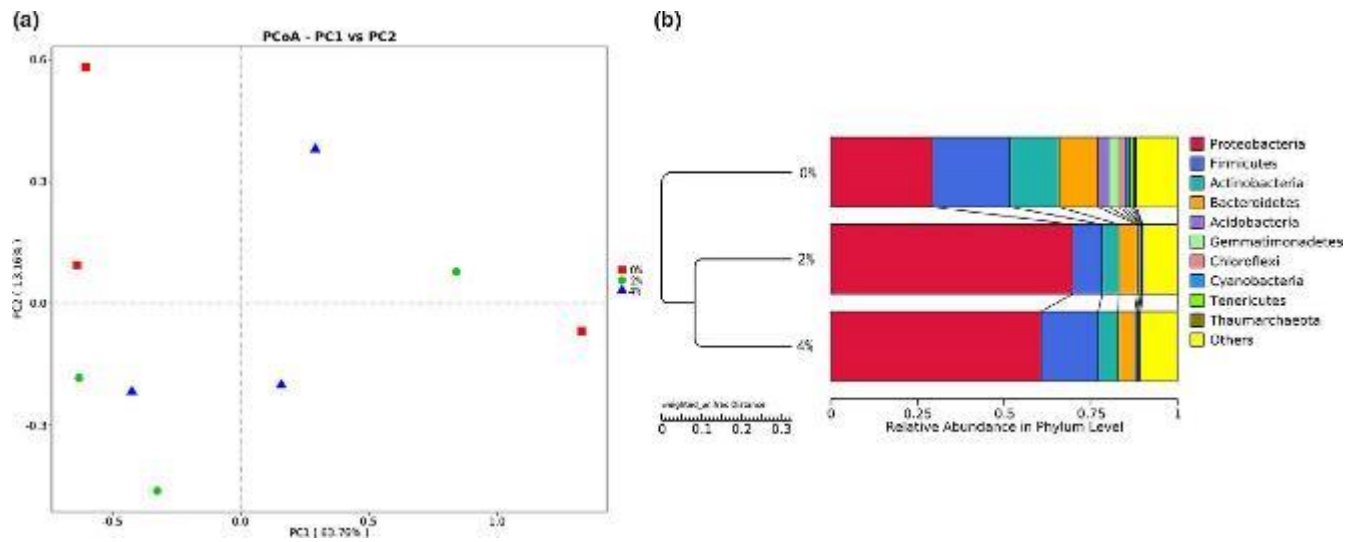


FIGURE 6 (a) Principal coordinate analysis based on Weighted UniFrac at OTU level. (b) Bacteria UPGMA clustering analysis. Diets were supplemented with YC at 0%, 2% and 4% respectively

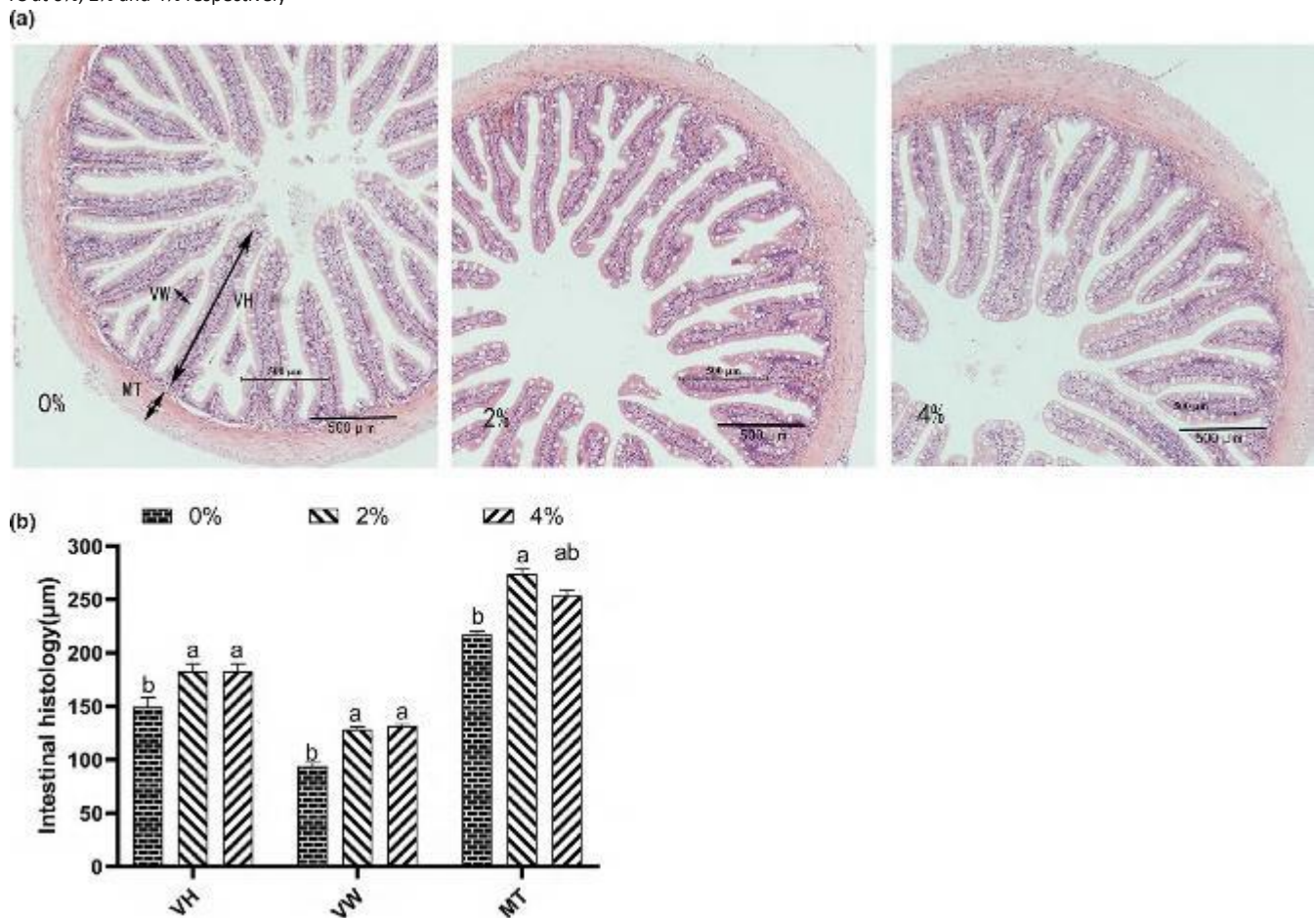


FIGURE 7 Effects of dietary yeast culture supplementation on intestinal tissue section (a) and morphological measurements (b). Upright bars denote the mean $\pm$ SEM. In Figure 8a, the VH, VW and MT double-headed arrows indicate intestinal villus height, villus width and muscle thickness respectively. Diets were supplemented with YC at 0%, 2% and 4% respectively

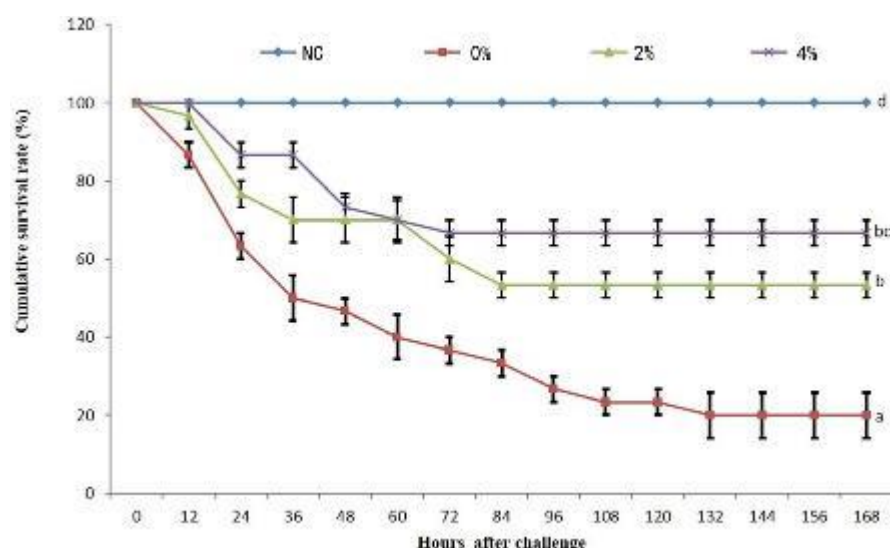


FIGURE 8 Cumulative survival rate of hybrid grouper fed yeast culture after 168 h of *Vibrio harveyi* infections. For each treatment, values with different letter suffix differ significantly ($p < 0.05$). Diets were supplemented with YC at 0%, 2% and 4% respectively

3.8 | Challenge test

The cumulative survival rate of groupers in each group was counted after 168 h of challenge (Figure 8). The mortality of groupers was not observed in the saline injection group. The cumulative survival rate of groupers in the 2% and 4% groups was significantly higher ($p < 0.05$) than that in the C0 (0%) group. The L2 (4%) group had the highest cumulative survival rate (67%), followed by the 2% group (55%) and 0% group (20%).

4 | DISCUSSION

YC is a product with complex composition, which contains the nutrients within the yeast cells and the yeast cell metabolites produced by fermentation (Liu et al., 2018). A series of studies have demonstrated that yeast extracts, yeast hydrolysates or YC can promote the health and growth of animals (Ayiku et al., 2020a; Berto et al., 2016; Jin et al., 2018). The present also provided supporting evidence that the supplementation of YC in the diets improved the growth performance and feed efficiency of hybrid groupers. Yeast products have been shown to improve the growth performance of the fish concerned (Berto et al., 2016; Liu et al., 2018; Yuan et al., 2019). For instance, Chiu et al. (2010) have found that the addition of *Saccharomyces cerevisiae* to the diet can significantly improve the weight gain and feed efficiency of groupers (Chiu et al., 2010). The addition of yeast extracts in the diet can contribute to the feed intake and specific growth rate of Nile tilapia (*Oreochromis niloticus*) (Berto et al., 2016). Consistently, this study showed that YC addition exerted positive effects on the feed efficiency of hybrid groupers. Therefore, it was suggested that improving feed utilization could be one of the important ways for YC to promote the growth of hybrid groupers. Accumulating studies have shown that the addition of 2% YC to the feed can significantly reduce the FCR of *Litopenaeus vannamei* (Ayiku et al., 2020b), and moreover, the addition of 1% and 2% brewer's yeast feed notably improves the feed efficiency of bass (*Morone chrysops* × *Morone saxatilis*) (Li et al., 2004), which may be due to the contribution of yeast cells and extracellular metabolites (proteins, amino acids, vitamins, nutrient metabolites and enzymes) in YC. These substances can enhance the activity of digestive enzymes and facilitate the absorption

and metabolism of nutrients, thus promoting growth (Ayiku et al., 2020a; Gao et al., 2008).

Intestinal digestive enzyme activity reflects the digestive capacity of fish. In this study, the intestinal digestive enzymes showed an increasing trend after YC treatment, which was consistent with the results of growth performance. Similar studies have been reported on Nile tilapia (*Oreochromis niloticus*) (Goda et al., 2012) and sea bass (*Dicentrarchus labrax*) (Tovar et al., 2002). It has also been demonstrated that the addition of 1% yeast hydrolysate and brewer's yeast to the shrimp diet significantly up-regulates mTOR-related gene expression, thereby promoting protein synthesis (Jin et al., 2018). However, the addition of *Saccharomyces cerevisiae* fermentation products to the feed has no significant effect on the growth performance of Nile tilapia cultured in cages (He et al., 2009; Zhang et al., 2018). The discrepancies in fish growth performance can be attributed to the differences in culture conditions, fish species and diet nutrient composition. In the present study, the rich nutrients of YC may promote the growth of hybrid grouper, but its exact mechanism of action remained to be further investigated.

Diet provides nutrients for fish, and the type of diet consumed by fish impacts the body composition. The nutritional value of fish mainly depends on the protein and lipid content of the muscle. The present study indicated that the level of YC added to the diet significantly affected the body composition of hybrid groupers. The supplementation of YC in the diet significantly increased the dry matter, crude protein, crude lipid and ash content of hybrid groupers, with the hybrid groupers fed with 4% YC showing the highest levels. Similarly, the supplementation of 2% YC in the diet improves the crude protein, crude lipid, dry matter and ash content of *Litopenaeus vannamei* (Ayiku et al., 2020a, 2020b). Abass et al. (2018) have also shown that the addition of 7% brewer's yeast to the diet significantly increases the crude protein and crude lipid in juvenile Nile tilapia (Abass et al., 2018). The supplementation of YC in the diet can enhance the activity of intracellular enzymes that contribute to growth performance and nutrient accumulation. For the present study, the addition of 4% YC to the diet effectively promoted the protein and lipid deposition in groupers.

Blood biochemical indicators can indirectly reflect the fish's metabolic condition and health status (Xie et al., 2021). As a kind of beneficial probiotics, the regulatory effect of YC on the animal body can be reflected by biochemical blood indexes. Aung (2019) and Hassaan et al. (2018) have

found that the addition of YC to the diet improves the blood biochemical parameters of animals (Aung et al., 2019; Hassaan et al., 2018). The serum TP content reflects the nutritional metabolism of the organism and is closely related to the immune system of the fish organism (Hossain et al., 2017; Tan et al., 2017). In the present study, we found that supplementation of YC in the diet affected the serum TP content. Similarly, the addition of yeast cell walls (β -glucan and mannan-oligosaccharides) to the diet can increase the total concentrations of protein and globulin in Nile tilapia (Abu-Elala et al., 2018), suggesting that YC may be an immune activator in hybrid groupers. As part of blood lipids, serum TG and CHO are essential indicators that reflect the lipid level of the fish body, indicating the metabolism and increase in lipids and energy storage in fish (Agrahar et al., 2007; Kumar et al., 2018; Xie et al., 2021). Blood GLU can be used as an indicator of stress in fish, with a high level of GLU indicating a high state of stress (Hossain et al., 2016). In the present study, the relevant results of GLU, TG and CHO levels confirmed that the addition of YC had no adverse effect on groupers. ALT and AST are essential indicators of liver health, both of which exist in the cytoplasm of hepatocytes. When hepatocytes are damaged, hepatocyte membrane permeability is increased, inducing the release of ALT and AST into the bloodstream and thus causing significant serum enzyme activity changes (Hassaan et al., 2018). The present study demonstrated that the addition of YC to the diet decreased the activities of AST and ALT, indicating that YC exerted a protective effect on the liver against free radical damage, which was consistent with existing studies' findings (Ayiku et al., 2020a, 2020b).

The oxidation reaction in the animal body produces harmful energy and reactive oxygen radicals, and excessive free radicals can damage the phospholipid bilayer of cell membranes (Wang, Zuo, et al., 2016a, 2016b, 2016c). SOD and CAT are essential components of the endogenous antioxidant defence system, reflecting the body's antioxidant strength. SOD converts harmful metabolites into hydrogen peroxide, while CAT decomposes hydrogen peroxide into oxygen and water to protect cells (Campa-Codova et al., 2002; Tavares-Sanchez et al., 2004). MDA is a primary marker of lipid peroxidation that reflects the extent of lipid oxidation (Xu et al., 2015). In the present study, YC supplementation in the diet decreased the serum MDA content, while the SOD content was increased with the increase in YC level after the challenge. The addition of YC had no significant effect on the CAT content. Similar findings have been reported on grass carp (*Ctenopharyngodon idella*) (Liu et al., 2018) and catfish (*Pseudobagrus ussuriensis*) (Bu et al., 2019). β -glucan in yeast products can reduce lipid peroxidation and protect the body from oxidative damage (Sener et al., 2005). In this study, the addition of 4% YC to the diet benefited the antioxidant capacity of hybrid groupers.

In the present study, ACP, AKP, LZM and IgM served as indicators of innate immune parameters in hybrid groupers. Innate immunity is the first line of defence against pathogen invasion in the host. LZM, a hydrolytic enzyme important for innate immunity, is commonly used to indicate the stress response and disease resistance of aquatic organisms (Saurabh et al., 2008). IgM, the main immunoglobulin in the serum of fish, can be used as a marker of immune status (Bag et al., 2009). ACP, a macrophage lysosomal marker enzyme, plays an essential role in the intracellular digestion of phagocytic antigens (Song et al., 2006). AKP is also an crucial component of the natural immune system that can dephosphorylate pro-inflammatory

compounds (Lalles., 2019). In the present study, there was an upward trend in innate immune parameters after the challenge. Zhang et al. (2018) have shown that the addition of 4 g of YC per 100 g of diet can improve the disease resistance and immunity of silver crucian carp (*Carassius auratus gibelio cas III*) partly through the TLR2 pathway (Zhang et al., 2018). MOS and β -glucan are the main functional components of the yeast cell wall (Abu-Elala et al., 2018). MOS can combine with bacteria and viruses to effectively alleviate the harm of bacteria, thus enhancing humoral and cellular immunity (Ren et al., 2020). Dietary supplementation of 0.2%-0.3% MOS significantly enhances the intestinal ACP, AKP and LZM activities of Chinese mitten crab (*Eriocheir sinensis*) (Zhang et al., 2019). As an immune response matrix, β -glucan activates the immune-related signal pathway, prompts immune cells to release downstream signal molecules and then improves the body's immune responses (Lee et al., 2014). β -glucan has been proven to enhance non-specific immunity in turbot (*Scophthalmus maximus*) (Miest et al., 2016), rainbow trout (*Oncorhynchus mykiss*) (Chettri et al., 2013) and Atlantic salmon (*Salmo salar L.*) (Engstad & Robertsen, 1993). Still, the mechanism of dietary YC supplementation in the innate immune system of groupers needs further exploration.

As an essential part of the intestinal mucosal barrier, intestinal microorganism forms a complex intestinal microbial system with intestinal epithelial cells, immune cells etc. Many microorganisms colonized in the intestinal tract can transform the nutrients in the diet into forms that can be used by the host, which contributes to the maintenance of the intestinal morphology, digestion and immunity of animals (He et al., 2021; Hooper & Gordon, 2001). In the current study, however, YC addition had no remarkable effect on OTUS and alpha diversity. The dominant phylum in healthy animals are *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroides* (Wang, Zuo, et al., 2016). In the present study, all the groups of hybrid groupers were dominated by *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroides* at the phylum level. These bacteria formed the core flora in the grouper intestine, which was consistent with the results of existing studies (Yang et al., 2019; Ye et al., 2020). The relative abundance of *Proteobacteria* in the YC treatment groups showed an increasing trend, while the relative abundance of *Firmicutes*, *Actinobacteria* and *Bacteroides* showed a decreasing trend. Consistently, Zhao et al. (2015) have revealed that the addition of non-protein nitrogen (nitrate) has no significant effect on ruminal alpha diversity and that *Proteobacteria* abundance is increased with nitrate addition (Zhao et al., 2015), which is consistent with the results of our study. The non-protein nitrogen in the YC fermentation substrate is converted to protein by the yeast, and furthermore, the YC contains a variety of metabolites. The present study may be the result of a combination of the two. At the genus level, the genera *Sphingomonas*, *Perluclidibaca*, *Stenotrophomonas* and *Phyllobacterium* made up the core microbial genera. YC supplementation in the diet had a positive effect on the relative abundance of *Sphingomonas*. Some studies have shown that healthy Atlantic salmon have a prosperous abundance of *Sphingomonas* in their gut microbes (Wang et al., 2017). Also, the carotenoids produced by *Sphingomonas* bear antioxidant properties and can scavenge free radicals produced in the body (Basta et al., 2004). *Stenotrophomonas* inhibits pathogenic microorganisms and acts as the dominant bacterium in the gut of healthy animals (Destoumieux-Garzon et al., 2016). In our study, the abundance of *Stenotrophomonas* and *Phyllobacterium* was elevated after YC treatment, which is consistent with

the findings of Liao et al. (2019). Our results also showed the presence of *Faecalibacterium* in the YC treatment groups. *Faecalibacterium* produces a short-chain fatty acid (SCFA) called butyric acid, which is the main nutrient for cell regeneration and repair of intestinal epithelial tissue cells (Miquel et al., 2014). *Faecalibacterium* has been identified as an important butyric acid-producing bacterium in the caecum of chickens (Duncan et al., 2002).

The animal intestine plays a vital role in nutrient absorption and metabolism. The increase in villi height and width expands the absorption area of the intestine, which contributes to the digestion and absorption of nutrients (Dimitroglou et al., 2010). Furthermore, the muscle thickness of the intestine determines the efficiency of absorption (Geda et al., 2012). In the present study, it was found that YC supplementation improved villi height, villi width and muscle thickness. The same findings have also been reported on *Litopenaeus vannamei* (Ayiku et al., 2020a, 2020b). Dimitroglou et al. (2008) have demonstrated that the supplementation of MOS in the diet can significantly increase the length of the intestinal villi of rainbow trout and affect the intestinal structure by increasing the density of microvilli. (Dimitroglou et al., 2008). Whether the changes in intestinal morphology of hybrid groupers in this study were also caused by MOS supplementation in the YC needs to be further verified.

Juvenile grouper is susceptible to bacterial infection. *Vibrio harveyi* is the most common pathogen causing bacterial diseases in groupers (Li et al., 2019). At present, there is a lack of effective methods to treat *Vibrio* disease in groupers. Through the challenge test, the survival rate of aquatic animals can evaluate the immunity and disease resistance of the body. The survival rate of groupers fed with YC was significantly higher than that of control groupers at the end of the challenge test. It has been previously reported that the addition of YC to the diet can significantly improve the survival rate of *Litopenaeus vannamei* (Jin et al., 2018; Tendencia et al., 2004). Both β -glucan and MOS, the primary nutrients of YC, activate the immune system and induce immune responses (Bu et al., 2019; Vetvicka et al., 2013). This study showed that the addition of 4% YC to the diet effectively improved the resistance of hybrid groupers to *Vibrio harveyi* infection.

5 | CONCLUSION

The present study demonstrates that the addition of YC to the diet improves the growth performance, immune response, digestive enzyme activity, intestinal microbiota and disease resistance against *Vibrio harveyi* of hybrid groupers. In summary, YC is an ideal diet additive, and 4% YC is recommended in the diet of hybrid groupers.

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AUTHOR CONTRIBUTIONS

All authors have actively contributed to the study. The author's contributions are as follows: H. Y. Liu designed the study; Qi Wang and S. Ayiku conducted the study and analysed the data; X. H. Dong participated in the interpretation of the results; Qi Wang and S. Ayiku wrote the manuscript; Q. H. Yang, S. Y. Chi, S. Zhang and W. H. Zhou purchased the

reagent supplies; H. Y. Liu revised the manuscript. All authors read and approved the final manuscript.

ETHICAL STATEMENT

The animal protocol was approved by the ethics review board of the Guangdong Ocean University. All procedures were performed by the standards of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978) and relevant Chinese policies.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Research Article

An Evaluation of Yeast Culture Supplementation in the Diet of *Pseudobagrus ussuriensis*: Growth, Antioxidant Activity, Nonspecific Immunity, and Disease Resistance to *Aeromonas hydrophila*

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An 8-week feeding trial was conducted to evaluate the effects of dietary yeast culture (YC) supplementation on growth performance, antioxidant activity, nonspecific immunity, and disease resistance of *Pseudobagrus ussuriensis* (average initial weight 6.01±0.01g). Four isonitrogenous and isolipidic diets were formulated to contain 0 (Y0), 10 (Y1), 20 (Y2), and 30 (Y3) g/kg YC, respectively. After the feeding experiment, the challenge test of injecting *Aeromonas hydrophila* was executed. Results showed that appropriate YC supplementation level in the diet could improve growth performance, digestive enzyme activities, nonspecific immunity capacity, antioxidant capacity, and disease resistance of *P. ussuriensis*. And the highest weight gain, feed intake, specific growth rate, and IGF-1 gene expression level were observed in fish fed the Y2 diet. The activities of protease and amylase in intestine in fish fed the Y2 diet were enhanced compared with that in fish fed the Y0 diet significantly ($P<0.05$). Simultaneously, fish fed the Y2 diet had significantly higher serum lysozyme activity and significantly lower serum alanine amino transferase activity ($P<0.05$). Dietary 20 g/kg YC supplementation increased the activity of catalase and total antioxidant capacity in liver and reduced malondialdehyde content in the liver and intestine of *P. ussuriensis* significantly ($P<0.05$). Fish fed the Y2 diet had the highest disease resistance under the condition of *A. hydrophila* challenge ($P<0.05$). The quadratic regression analysis based on weight gain against dietary YC levels indicated that the appropriate dietary YC supplementation level is 13.4 g/kg diet.

palatable protein source for fish feed formulation. However, due to increasing demand, unstable supply, and high price of the FM with the expansion of aquaculture, there is an increasing demand for searching more potential alternative protein sources in aquafeeds [1, 2]. Yeast culture (YC) is one kind of complicated yeast fermentation product, which has great prospects as a potential protein source to replace fish meal in aquaculture [3, 4]. Growth studies have confirmed that YC can improve growth performance of many fish species; for instance, the research on juvenile largemouth bass

1. Introduction

Fisheries play a critical role in making sure about food security and employment to millions of folks worldwide. Fishmeal (FM) is a highly nutritious and appropriately

(*Micropterus salmoides*) fed high-starch diet showed dietary 30g/kg YC supplementation could improve the growth performance, liver function, and intestinal barrier [5]. Liu et al. [6] observed that grass carp (*Ctenopharyngodon idellus*) fed diet with YC (120-160g/kg) significantly increased the growth performance.

YC have the ability to activate immune response, modulate intestinal microflora, and resist certain pathogenic bacteria and have been considered as potential “antibiotic alternatives” [7]. Some surveys found that the abovementioned effects may be attributed to the various functional elements in YC such as β -glucan, mannan-oligosaccharides, and digestible proteins [8, 9]. Previous reports have demonstrated that those elements take an important part of enhancing the immune function of different kinds of animals [10–13]. Zhang et al. [4] indicated that dietary YC addition could enhance the resistance to *Aeromonas hydrophila* in gibel carp (*Carassius auratus gibelio* CAS III). Moreover, Torrecillas et al. [14] found that mannan-oligosaccharides, one kind of yeast products constitute part, could facilitate antigen processing to activate immune response and then improve the host’s health capability.

Pseudobagrus ussuriensis is an important economic fish in China and East Asia [8]. It is very popular among consumers due to its high nutritional value. However, due to the large-scale and intensive fish farming, the shortage of fishmeal is prominent. To date, several studies have reported feasibility in replacing fishmeal with different proteins, including corn gluten meal [15], rapeseed meal [16], cottonseed meal [17], meat and bone meal [18] as well as mussel (*Cristaria plicata*) meat meal [19] in *P. ussuriensis*. But there is limited message regarding the potential role of dietary YC as partial FM substitute on growth performance and healthy status for *P. ussuriensis*. Thus, the aim of the present study is to evaluate the effects of dietary YC as partial FM substitute on growth performance, nonspecific immunity, and antioxidant capacity as well as disease resistance of *P. ussuriensis* through an 8-week feeding experiment with different level of YC and an *A. hydrophila* challenge test after feeding experiment.

2. Materials and Methods

2.1. Experimental Diet and Fish Preparation. Four isonitrogenous and isolipidic diets were formulated: a control diet and three experimental diets (recorded as Y0, Y1, Y2, and Y3, respectively) with 10, 20, and 30 g/kg of YC inclusion. And the YC was provided by the Beijing Enhalar Institute of Biotechnology (Beijing, China). *Saccharomyces cerevisiae* strain (Patent No. 201210349633.4) was obtained from the cooperative research production between the Institute of Microbiology Chinese Academy of Sciences (Beijing, China) and Beijing Enhalar Institute of Biotechnology (Beijing, China), and its growth conditions were pH at 6.5, temperature at $30 \pm 1^\circ\text{C}$, and dissolved oxygen at 20-60%. The compositions of the diets used in feeding trials are given in Table 1. The diets were produced as shown in the former study in our lab and stored at -20°C [8]. *P. ussuriensis* were

provided by the Fisheries Research Institute of Harbin Academy of Agricultural Sciences (Harbin, China). Fish were acclimated using the Y0 diet for 2 weeks prior to the experiment to adapt to the diet and environment. 480 healthy and unwounded fish with homogeneous size were selected and

Table 1: Diets composition (g/kg dry matter).

Ingredients	Y0	Y1	Y2	Y3
Fish meal	280	270	260	250
Soybean meal	300	300	300	300
Cottonseed meal	110	110	110	110
Corn gluten meal	70	70	70	70
Wheat meal	164	164	164	164
Soybean lecithin	10	10	10	10
Soybean oil	33	33	33	33
Vitamin premix and mineral premix ^a	10	10	10	10
Choline	3	3	3	3
Ca(H ₂ PO ₄) ₂	20	20	20	20
Yeast culture	0	10	20	30
<i>Proximate composition (g/kg dry matter)^b</i>				
Dry matter	932.7	926.0	928.0	931.0
Crude protein	458.3	453.5	455.5	450.4
Crude lipid	77.7	78.0	76.4	76.0
Gross energy (kJ/g) ^c	184.9	185.7	186.0	180.0
Ash	121.3	120.1	115.3	118.4

^aVitamin premix (IU or mg/kg dry diet): retinol (V_A) 3000 IU; cholecalciferol (V_D) 1500 IU; tocopherol (V_E) 40 mg; menadione (V_K)

4.5 mg; thiamin (V_{B1}) 8 mg; riboflavin (V_{B2}) 8.5 mg; pyridoxine (V_{B6}) 6.5 mg; cyanocobalamin (V_{B12}) 0.02 mg; nicotinic acid 45 mg; nicotinamide 45 mg; D-Ca pantothenate 17 mg; inositol 40 mg; biotin 0.15 mg; folic acid 1.3 mg; ascorbic acid 110 mg. Mineral premix (mg/kg dry diet): copper 6.5 mg; iron 45 mg; selenium 0.35 mg; zinc 70 mg; manganese 8.5 mg; magnesium 100 mg; cobalt 1 mg; iodine 1.2 mg. ^bProximate composition were measured values. ^cGross energy was determined using an adiabatic bomb calorimeter (Parr 6300, USA).

divided into 4 groups (Y0, Y1, Y2, and Y3), containing 4 repetitions each group. These fish were placed, respectively, in 16 aquariums (1.0×0.5×0.6m, water depth about 0.4m) with 30 fish per aquarium. The total body weight of the fish in each aquarium was measured, and the average weight was calculated as $6.01 \pm 0.01\text{g}$. Subsequently, fish were maintained in circulated, aerated, and filtered fresh water, and an 8-week feeding trial was conducted.

2.2. Feeding Trial. Fish were artificially fed to apparent satiation thrice daily (07:00, 13:00, and 19:00). The trial was conducted under steady pH (6.5-8.5), ammonia-N (<0.1mg/L), temperature ($22 \pm 2^\circ\text{C}$), water flow rate (2.5 L/min), dissolved oxygen (>5mg/L), and a light: dark cycle of 12:12 h condition.

2.3. Sampling and Chemical Analysis. At the end of the experiment, the fish were starved for 24 hours. The fish were then euthanized with eugenol (1:12 000) anesthetic. The total number and body weight of fish in each aquarium were measured to calculate the growth performance and nutrient

utilization. Three fish were randomly selected from each aquarium, and the body length and body weight were measured, and the weight of isolated muscle, viscera, intestine, and liver tissue were determined [17]. Caudal vein blood samples were collected by heparinized syringes from five fish per repetition, and plasma was collected after centrifuged (3000rpm, 10min, 4°C; Microfuge 22R centrifuge, Beckman Coulter, USA) and stored at -80°C. Another five fish per repetition were randomly obtained, and the liver, intestine, and muscle were immediately frozen with liquid nitrogen and stored at -80°C. This study was performed under the guidelines approved by the Animal Care and Use Committee of the Northeast Agricultural University, China.

The digestive enzymes' activities (amylase, lipase, and protease) were measured as described by Luo et al. [19] with the kits (No.: C016, A054, A080, respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The liver and intestine samples were homogenized in 0.9% (w/v) NaCl solution on the ice and then centrifuged at 3000rpm (Microfuge 22R centrifuge, Beckman Coulter, USA) for 10min. The approach used in this investigation is similar to that used by other researchers [8, 19]. The activities of catalase (CAT), SOD, and T-AOC and the content of malondialdehyde (MDA) in hepatic and intestinal tissues were measured with the reagent kits (No.: A007, A001, A015, A003, respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The total protein content was determined by Coomassie brilliant blue staining method (No.: A045; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum and liver as well as lysozyme (LZM) and alkaline phosphatase (AKP) in serum were also determined using the kits from the Nanjing Jiancheng Bioengineering Institute (No.: C009, C010, A050, A059, respectively; Nanjing, China).

2.4. IGF-1 Gene Expression. The method of IGF-1 gene expression was acted in accordance with those previously described in literatures of our laboratory [15, 16]. The primer sequences were displayed in Table 2, and β -actin was chosen as an internal reference. Total RNA was isolated from the muscle and liver samples using the Trizol method (TransGen Biotech Co., Ltd., Beijing, China), and then, cDNA was synthesized by PrimeScript® RT reagent Kit (Takara Biomedical Technology Co., Ltd., Beijing, China). Quantitative PCR (20 μ L) was conducted using TransStart® Green qPCR SuperMix kit (TransGen Biotech Co., Ltd., Beijing, China) on Applied Biosystems® 7500 real-time PCR system (USA). Briefly the real-time PCR began with 30s at 94°C, followed by 40 cycles at 94°C for 5s and 60°C for 30 s. The gene expression levels were calculated with $2^{-\Delta\Delta CT}$ method [20].

2.5. Bacterial Challenge. The detailed description of *A. hydrophila* used in this study and the preparation was presented by our preliminary experiments [17]. The sample of graded doses of *A. hydrophila* was carefully injected into enterocoelia to explore 96 h LD50, and results found that

3.48×10^3 CFU per fish was the 96 h LD50. At the end of the feeding trial, a total of 160 fish of all groups (40 fish from each group) were peritoneal injected with 0.1mL per fish of bacterial suspension of *A. hydrophila* (3.48×10^4 CFU/mL). During challenge trial, the regulation condition was the same as feeding experiment. The cumulative mortality was observed and recorded for 7 days.

2.6. Calculations and Statistical Analysis. The proximate composition of diets, feed ingredients, and fish samples were analyzed using the standard methods of AOAC [21]. The data was presented as the means $\pm$ SE. All data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. There was significant difference at the $P < 0.05$. All calculations were performed using SPSS 20.0 for Windows (SPSS, Inc., USA). The following variables were calculated:

$$\text{Weight gain WG, } \delta\%P = 100 \times \frac{W_t - W_0}{W_0}P,$$

$$\text{Feed intake FI, } \delta\%P = 100 \times \frac{W_t - W_0}{W_0}P,$$

$$\text{Specific growth rate SGR, } \delta\%P = 100 \times \frac{\ln W_t - \ln W_0}{t}P,$$

$$\text{Protein efficiency ratio PER, } \delta\%P = \frac{\text{crude protein intake}}{\text{wet weight gain}}P,$$

$$\text{Survival rate SR, } \delta\%P = 100 \times \frac{\text{final fish number}}{\text{initial fish number}}P,$$

$$\text{Feed conversion ratio FCR, } \delta\%P = \frac{\text{feed consumption}}{\text{weight gain}}P,$$

$$\text{Condition factor CF, } g \cdot cm^{-3} = 100 \times \frac{\text{body weight}_3}{\delta\text{body length}^3}P,$$

$$\text{Hepatosomatic index HSI, } \delta\%P = 100 \times \frac{\text{whole body weight}}{\text{liver weight}}P,$$

$$\text{Viscera somatic index VSI, \%} = \frac{\text{viscera weight}}{\text{whole body weight}} \times 100$$

where W_0 is the initial body weight (g), W_t is the final body weight (g), and t is the feeding duration (day).

3. Results

3.1. Growth Performance. The body weight and length of *P. ussuriensis* were measured after feeding YC diet with different levels for 56 days, and the growth performance was evaluated. It was found in this study that dietary YC supplementation could promote WG, SGR, and PER and reduce FCR of *P. ussuriensis* except for dietary 30 g/kg YC supplementation level. The best growth performance was observed in the Y2 group (Table 3). FI was obviously affected by dietary YC supplement, and fish in Y2 group had the highest FI value ($P < 0.05$) (Table 3). Besides, no significant difference was observed in HSI, VSI, CF, and survival rate among all groups ($P > 0.05$), although fish in Y2 group showed the lowest survival (94.4%) (Table 3). Furtherly, the IGF-1 gene expression in the muscle (Figure 1(a)) and liver (Figure 1(b)) were meaningfully elevated in Y1 and Y2 groups compared to Y0 and Y3 groups ($P < 0.05$). Based on the polynomial curve analysis of WG among all groups, Figure 2 suggests that the optimal level of YC supplementation for *P. ussuriensis* was 13.4 g/kg.

Table 2: Primers sequence.

Primer names	Sequence (5'-3')	Accession number	Annealing temperature (°C)
IGF-1-F	TTATTTCAGCAAGCCAACAGGC	KX257402.1	58
IGF-1-R	ACGGATCTTGGAGCTTTACCAG		
β -Actin-F	CCTCCGTCTGGATTGGCTG	Bu et al. [15]	60
β -Actin-R	TCAAGGGCGACGTAGCAGAG		

Table 3: Growth parameters of *P. ussuriensis* fed diet with different levels of YC.

Parameters	Y0	Y1	Y2	Y3
Initial body weight (g)	6:01±0:02	6:01±0:01	6:02±0:01	6:00±0:01
Final body weight (g)	13:59±0:11 ^b	13:82±0:12 ^b	14:94±0:13 ^a	12:36±0:08 ^c
Weight gain (%)	126:10±2:36 ^b	129:95±2:38 ^b	148:29±1:56 ^a	107:11±1:64 ^c
Feed conversion ratio	1:24±0:03 ^b	1:18±0:01 ^c	1:19±0:01 ^{bc}	1:30±0:01 ^a
Specific growth rate (% day ⁻¹)	1:46±0:02 ^b	1:49±0:02 ^b	1:62±0:01 ^a	1:30±0:01 ^c
Feed intake (% day ⁻¹)	1:49±0:02 ^b	1:44±0:03 ^{bc}	1:57±0:01 ^a	1:41±0:02 ^c
Survival rate (%)	96:67±1:92	98:89±1:11	94:44±2:94	95:83±3:15
Protein efficiency ratio	1:70±0:04 ^{bc}	1:79±0:02 ^a	1:78±0:02 ^{ab}	1:65±0:02 ^c
Hepatosomatic index (%)	2:30±0:01	2:31±0:24	1:98±0:10	2:20±0:21
Viscera somatic index (%)	11:38±0:73	10:70±1:11	10:14±0:12	10:68±0:32
Condition factor (g cm ⁻³)	0:97±0:09	0:96±0:09	0:86±0:03	1:02±0:04

Different superscripts within a line denote significant difference ($P < 0.05$). Data presented as mean±SE.

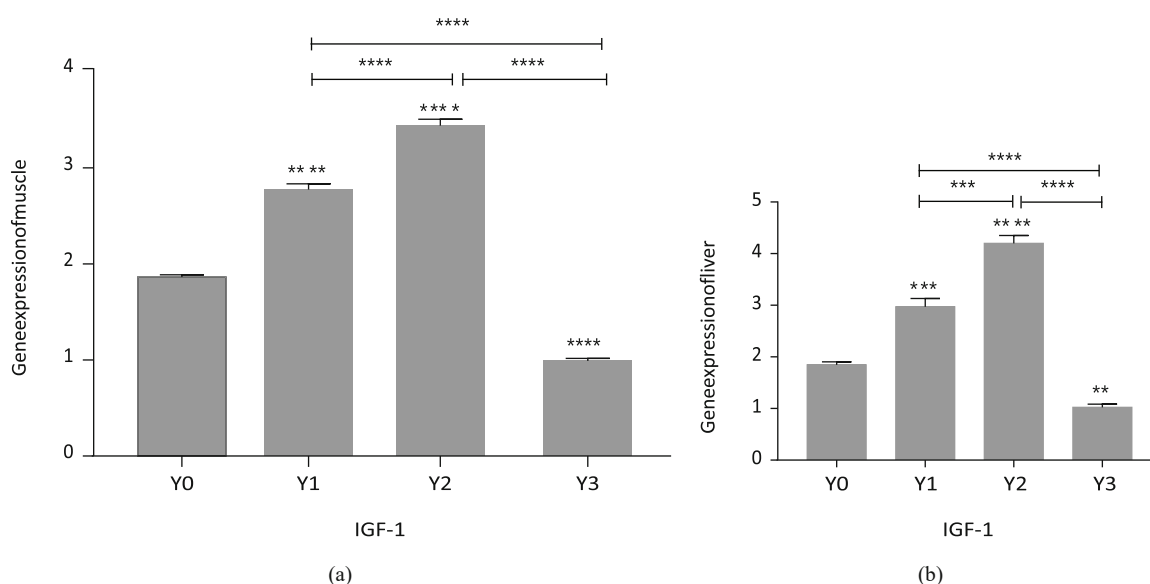


Figure 1: IGF-1 expression in liver and muscle of *P. ussuriensis* fed with test diets for 56 d ($n=4$). Bars with asterisks are significantly different from the control (** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$).

3.2. Digestive Enzyme Activity in Intestine. There is a close correlation between the activity of digestive enzymes and feed digestion and absorption capacity. Fish fed with Y1 and Y2 diets showed significantly higher protease and amylase activities than fish in Y0 group ($P < 0.05$) (Figures 3(b)

and 3(c)). However, lipase activity showed no obvious differences among all groups ($P > 0.05$) (Figure 3(a)).

3.3. Immune and Antioxidative Parameters. The effects of dietary YC supplementation on nonspecific immune and

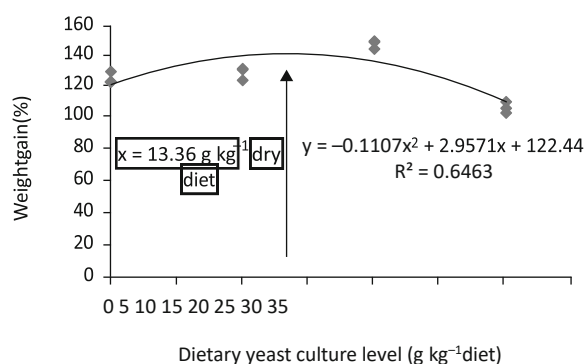


Figure 2: Polynomial curve model analysis based on weight gain against dietary YC levels for *P. ussuriensis* fed with test diets for 56 d.

antioxidant parameters could be used to evaluate the pathologic and nutritional status of *P. ussuriensis*. The immune (LZM) and antioxidative parameters (T-AOC, SOD, CAT, and MDA) were influenced significantly in the fish fed with 20 g/kg YC supplemented diet. The results revealed that the hepatic and intestinal CAT activities and T-AOC values were enhanced in dietary YC supplementation groups compared with the Y0 group, particularly in Y2 group which the highest T-AOC level was found ($P < 0.05$) (Table 4). The intestine MDA content was dramatically decreased in Y1 and Y2 groups compared with the control group ($P < 0.05$) (Table 4). The lowest value of hepatic MDA was observed in Y2 group, and the lowest hepatic SOD activity was found in Y3 group ($P < 0.05$) (Table 4). The significantly increased intestinal SOD and hepatic AST activities were observed in Y1 and Y2 groups ($P < 0.05$) (Table 4 and Table 5). The hepatic ALT and serum LZM activities were significantly raised in Y2 group compared with Y0 group ($P < 0.05$) (Table 5 and Figure 4(b)). There was significant reduction of serum ALT activity in Y2 group compared to Y0 group ($P < 0.05$) (Table 5). But no remarkable differences were obtained in serum AKP and AST activities among four groups ($P > 0.05$) (Table 5 and Figure 4(a)). The results in this section indicated that dietary 20 g/kg YC level could enhance nonspecific immunity and antioxidation capability of *P. ussuriensis*.

3.4. Challenge Test. At the ninth week after feeding, 30 fish were challenged with 96h LD50 dose of *A. hydrophila* (3.48×10^4 CFU/mL), and each fish was injected 0.1 mL *A. hydrophila* suspension. Figure 5 showed that feeding *P. ussuriensis* with different levels of YC enhanced its resistance to disease caused by *A. hydrophila*, and Y2 group had the highest cumulative survival rate among all groups ($P < 0.05$).

4. Discussion

YC are recently viewed as an exploitable replacement of fishmeal, with promising results in Pacific white shrimp (*Litopenaeus vannamei*) and gibel carp [3, 4, 22]. Stephen et al. [22] found that 10 g/kg and 20 g/kg YC addition in shrimp diet improved growth performance and decreased FCR significantly. Similarly, Chen et al. [23] stated that 10 g/kg

hydrolyzed yeast supplementation improved juvenile Nile tilapia's (*Oreochromis niloticus*) growth performance. Conversely, Zhang et al. [4] revealed that there was no influence in growth performance between different levels of dietary YC in gibel carp. It could be extrapolated from previous results that YC may have different effects on growth performance for different aquatic animal species. In the present study, the significantly higher growth performance was found in Y2 group by increasing FBW, WG, SGR, and FI as well as reducing FCR of *P. ussuriensis*. In addition, the inferior growth performance was observed in Y3 group compared with any other group. Similar results occurred in previous studies, that is, with the increase in the amount of yeast added, the weight gain of aquatic animals first increased and then remained stable or even decreased [24]. This may be due to the presence of nucleotides and indigestible polysaccharides (for example, β -glucans) in the yeast composition, which can act as antinutritional factors when used in high concentrations. This impairs the animal's digestive capacity and full nutrient utilization, which is then reflected in reduced livestock growth performance [25]. In our study, *P. ussuriensis* fed 30g/kg YC diet had the lower FBW, WG, and FI than fish fed control diets. It revealed that dietary YC supplemental level of 30g/kg is too high to be utilized effectively for *P. ussuriensis*. IGF-1, a hormone produced primarily by the liver which can be secreted into the blood, has influence on organismal growth, development, and anabolic protein [15, 26]. The IGF-1 gene expression level is one kind of indicator to appraise growth performance [16, 27]. Our results revealed that with the YC supplementation level rising, liver and muscle IGF-1 gene expression levels showed the "low-high-low" trend, and the highest level was observed in Y2 group, indicating that IGF-1 was positively correlated with the growth parameter.

The term "digestive enzymes" has been used to refer to enzymes which can enhance nutrient digestion and absorption. High-digestive enzyme activities could promote nutrition digestion and absorption, thereby affecting growth performance directly [28, 29]. Research showed that the activities of digestive enzymes (lipase, protease, and amylase) in aquatic animals could be affected by diet formula [28, 30]. Ran et al. [31] noted that the trypsin activity of Nile tilapia fed with yeast was improved. Moreover, it has been reported that the trypsinase and amylase activities of *L. vannamei* were enhanced by yeast extract, but the lipase activity was reduced [32]. In the current study, the significant difference was observed in protease activity between the Y2 and Y0 groups. Fish fed Y1 and Y2 diets had significantly higher amylase activity compared with fish fed Y0 and Y3 diets. These results were consistent with growth performance, implying that YC might increase growth performance by affecting digestive enzymes activities.

Oxidant stress, the state of imbalance between oxidation and antioxidation, plays a crisis element in disease development, such as metabolic and viral diseases [33–35]. T-AOC as an important antioxidant index could reflect the state of body oxidative stress [36]. Organisms can produce antioxidant enzymes (SOD and CAT, for example) to prevent

oxidative stress [37]. Simultaneously, MDA is a lipid

peroxidation

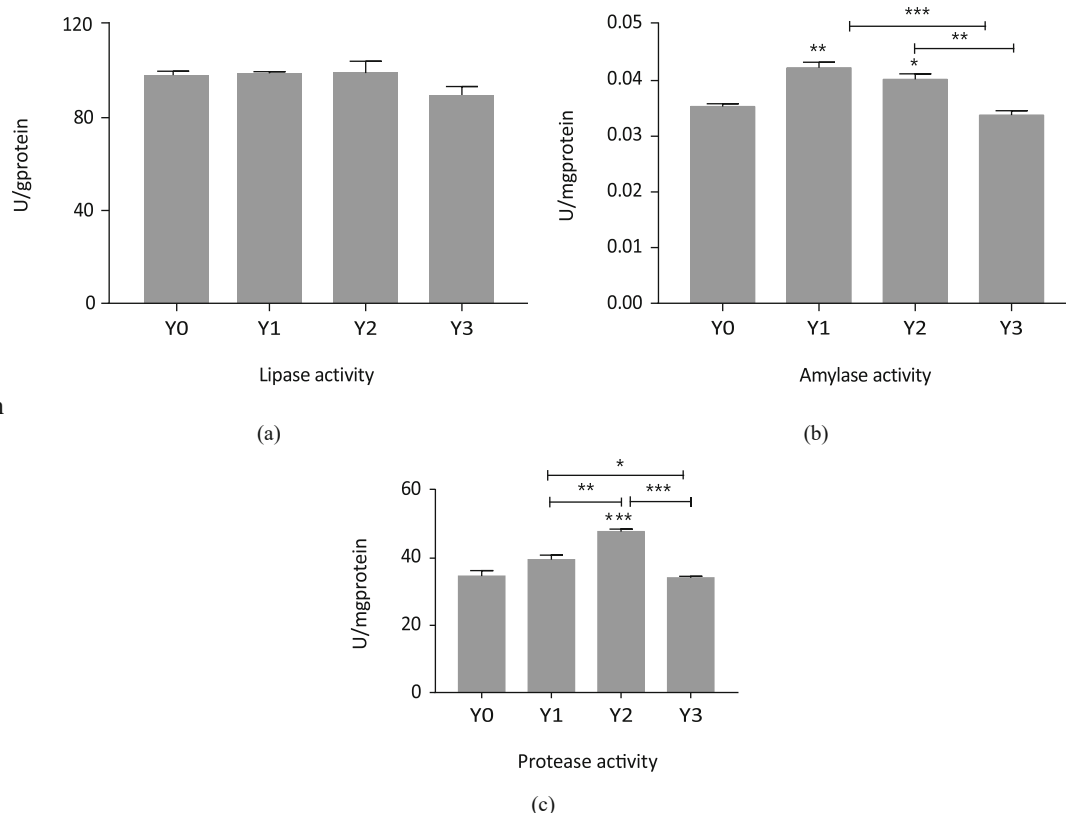


Figure 3: Digestive enzymes activities (amylase, lipase, and protease) in intestine of *P. ussuriensis* fed with test diets for 56 d (n=4). Bars with asterisks are significantly different from the control (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

Table 4: Effect of dietary YC on liver and intestine antioxidant parameters of *P. ussuriensis*.

Parameters	Y0	Y1	Y2	Y3
Liver				
Catalase (U mg ⁻¹)	6:41±0:63 ^c	9:41±0:24 ^a	8:04±0:21 ^b	7:13±0:17 ^{bc}
Superoxide dismutase (U mg ⁻¹)	113:28±5:84 ^a	117:81±7:58 ^a	126:41±1:65 ^a	80:26±2:92 ^b
Total antioxidant capacity (U mg ⁻¹)	0:42±0:03 ^c	0:78±0:05 ^b	1:57±0:12 ^a	0:54±0:05 ^c
Malondialdehyde (nmol mg ⁻¹)	1:16±0:15 ^{ab}	0:83±0:05 ^{bc}	0:76±0:06 ^c	1:24±0:11 ^a
Intestine				
Catalase (U mg ⁻¹)	14:39±1:13 ^c	18:42±0:70 ^b	23:16±1:53 ^a	15:41±0:83 ^{bc}
Superoxide dismutase (U mg ⁻¹)	195:63±3:55 ^c	230:03±5:03 ^b	287:37±6:41 ^a	181:13±2:71 ^c
Total antioxidant capacity (U mg ⁻¹)	0:31±0:03 ^b	0:39±0:02 ^{ab}	0:52±0:07 ^a	0:35±0:05 ^b
Malondialdehyde (nmol mg ⁻¹)	7:07±0:09 ^a	5:80±0:31 ^b	4:83±0:22 ^c	7:10±0:35 ^a

Data represented as mean±SE of triplicate aquariums. Means within rows with different superscript letters differ ($P<0.05$).

Table 5: Effect of dietary YC on alanine aminotransferase and aspartate aminotransferase activities in serum and liver of *P. ussuriensis*.

Parameters	Y0	Y1	Y2	Y3
Serum				
Alanine aminotransferase (U gprot ⁻¹)	34:85±3:32 ^a	40:05±3:06 ^a	21:44±1:45 ^b	32:56±2:70 ^a
Aspartate aminotransferase (U gprot ⁻¹)	86:74±7:31 ^{ab}	88:20±7:44 ^{ab}	66:92±10:36 ^b	97:62±4:74 ^a
Liver				
Alanine aminotransferase (U gprot ⁻¹)	200:54±8:93 ^b	204:86±12:31 ^b	257:66±6:97 ^a	167:75±2:17 ^c
Aspartate aminotransferase (U gprot ⁻¹)	156:57±3:67 ^b	182:73±7:83 ^a	193:93±6:24 ^a	144:22±3:17 ^b

Data represented as mean±SE of triplicate aquariums. Means within rows with different superscript letters differ ($P<0.05$).

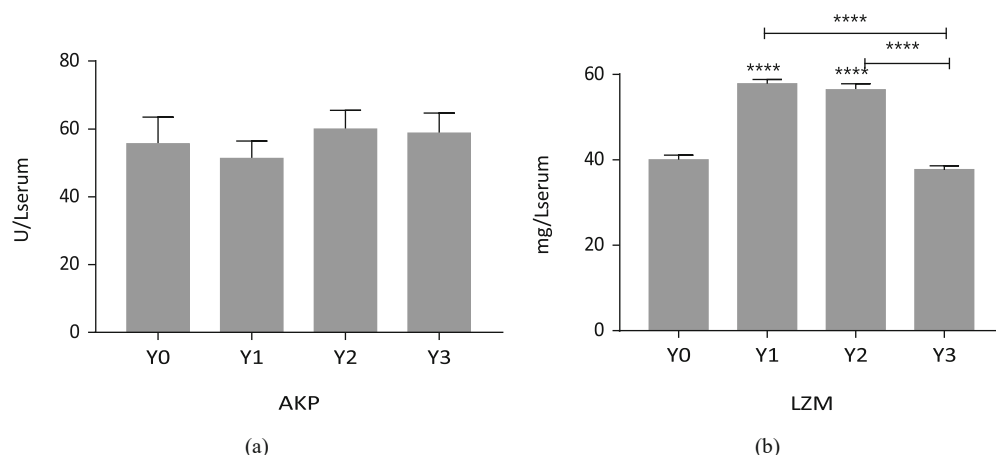


Figure 4: Serum immune related parameters of *P. ussuriensis* (AKP: alkaline phosphatase, LZM: lysozyme; $n=4$) fed with test diets for 56 d. Bars with asterisks are significantly different from the control (**** $P<0.0001$).

marker; its production will impair cell structure and function [38]. Yeast product applications were reported to enhance antioxidant capacity of some species such as Nile tilapia [39], blunt snout bream (*Megalobrama amblycephala*) [40], and *L. vannamei* [22]. Andriamialinirina et al. [39] stated that juvenile Nile tilapia fed 10g/kg yeast hydrolysate showed significantly higher hepatic SOD, CAT activities, and lower MDA content than the control group. Our data suggested that the diet with 20g/kg YC addition could enhance the activities of T-AOC, SOD, and CAT and reduce the value of MDA in liver and intestine of *P. ussuriensis* compared with the diet without YC addition. But dietary 30g/kg YC addition increased the MDA content and decreased the activities of SOD and T-AOC significantly in liver of *P. ussuriensis* compared with dietary 20g/kg YC addition. As we know, yeasts contain higher levels of nucleic acids and indigestible polysaccharides [25], but too high levels of nucleic acids and polysaccharides cannot be safely metabolized in fish to generate harmful hydroxyl radicals, which can destroy antioxidant system and contribute to oxidative stress [41, 42]. The results in the present study manifested that the proper YC supplementation (20g/kg) in diet would enhance the antioxidase activities, thereby inhibiting the activity of oxygen free radicals and preventing oxidative damage; however, excessive YC addition may lead to an adverse impact for antioxidant capacity.

As an essential organ for the body, the liver has wide range of functions such as digestion, metabolism, immunity, and detoxification [33, 43]. AST and ALT, as the sensitive markers of liver integrity, can provide the assessment of animals' liver damage [44]. The ALT and AST content raised in serum are related to impair of hepatocytes, and its organelles caused by oxidative stress with endogenous antioxidant enzymes such as SOD and CAT values decreased [45–47]. It was noticed that YC had protective effect to the health of *P. ussuriensis*, and fish fed the 20 g/kg YC diet showed the lower serum AST and ALT activities and higher hepatic ALT and AST activities. An implication of this is the

possibility that YC may help hepatic cell metabolism without negative effects on liver cells considering the truth of that serum ALT and AST activities rise when hepatocyte membrane permeability enhanced at the condition of liver cells damaged [40]. The results denoted that YC has certain prevention and treatment effects on liver lesions for *P. ussuriensis*, so that fish could better adapt to the complex environment, ultimately the culture efficiency being improved.

It is universally acknowledged that immunological parameters are useful tools to investigate health status of aquatic animals. *A. hydrophila* is one kind of important bacterium causing huge mortality of farmed fish worldwide [38, 48]. Zhang et al. [4] discovered that dietary YC addition improved disease resistance of *A. hydrophila* in gibel carp. Similarly, Stephen et al. [22] found that shrimps fed YC reduced mortality after *Vibrio harveyi* challenge, and the lowest mortality was found in the 20 g/kg yeast hydrolysate group with a survival rate of 70%. The results of this trial found that YC supplementation could obviously increase the survival rate of *P. ussuriensis* exposed to *A. hydrophila*. Lysozyme can hydrolyze bacteria cell wall's peptidoglycans which make it becoming a momentous bacteriolytic component in immune system [49]. Our study found that fish in 10 g/kg and 20 g/kg YC groups had obviously enhanced serum lysozyme activity than fish in 0g/kg and 30 g/kg YC groups. β -Glucans present in the yeast cell walls can stimulate innate immune system, increasing nonspecific responses. However, excess yeast could lead to immune hyperstimulation, showing decreased lymphocyte numbers, which may be associated with the changes in metabolism and oxidative stress due to the intake of large amounts of polysaccharides and nucleic acids [24, 25]. Our results indicated that dietary proper YC supplementation enhanced the immunity of juvenile *P. ussuriensis*, and 20 g/kg was the suitable level. Also, Stephen et al. [22] found that *L. vannamei* fed diet 20g/kg YC reached the peak for LZM content in serum. The highest survival rate was found in

20g/kg YC group after *A. hydrophila* challenge in the present study; presumably, for one thing, the improved activity of LZM could further lyse the bacteria; for another, the enhanced SOD and CAT activities in liver and intestine produced synergistic effect to remove excessive free radicals and raised the body's disease resistance. Furtherly, this could be explained by the fact that β -glucan, MOS, and nucleotide from dietary YC

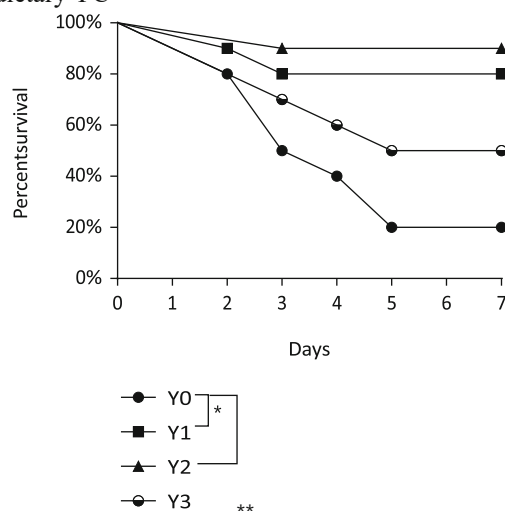


Figure 5: Survival rate of *P. ussuriensis* fed with test diets after challenge with *A. hydrophila* for 7 days ($n=4$). Bars with asterisks are significantly different from the control (* $P<0.05$; ** $P<0.01$).

supplementation may have favorable impact on immunity of aquatic animal [4, 50]. It is known that AKP plays significant roles in regulate nonspecific immunity, nutrient metabolism, and signal transduction [51, 52]. However, the present study showed that dietary YC supplementation had no significant influence on the AKP activity among all the groups. As the symbolic innate immunity factors, LZM and AKP could help body degrade exogenous substances [53, 54]. Our results showed that dietary YC addition improved LZM activity but did not affect AKP activity, indicating that the effect of YC on nonspecific immunity comes from the action of LZM rather than AKP.

This experiment is the first attempt to use yeast culture instead of fishmeal in the feed of *P. ussuriensis*, and our results showed that fish fed diet with YC supplemented levels from 10 to 20g/kg had better growth performance, digestive enzyme activities, nonspecific immunity capacity, antioxidant capacity, and bacterial resistance; quadratic regression analysis based on weight gain against dietary YC levels indicated that the appropriate dietary YC supplementation level is 13.4 g/kg diet.

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

Conflicts of Interest

We declare no conflicts of interest related to the submitted work.

Authors' Contributions

Xuying Hou, Liujian Sun, and Zhiqiang Li contributed equally to this work.

Acknowledgments

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Yeast culture supplementation alters the performance and health status of juvenile largemouth bass (*Micropterus salmoides*) fed a high- plant protein diet

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Abstract

The present study was conducted to investigate the effects of yeast culture on the growth, hepatic antioxidant capacity, intestinal barrier and microflora of fish fed a high- cottonseed protein diet. The largemouth bass (initial body weight: 12.17 ± 0.02 g) were randomly allotted into three treatments, fish meal-based diet (FM), cottonseed protein concentrate-based diet (CPC, 36% of the fish meal replaced by cottonseed protein concentrate) and CPC diet with yeast culture (CPCY, CPC+3% yeast culture) for 8 weeks, respectively. The results indicated fish fed 3% yeast culture diet could enhance specific growth rate (SGR), protein efficiency ratio (PER) and feeding rate (FR), while the hepatosomatic index (HSI) and liver glycogen content were significantly decreased compared with the CPC group ($p < .05$). Compared with the FM group, the CPC diet resulted in liver tissue damage accompanied by upregulation of pro-inflammatory factor (*IL-1 β* and *TNF α*) expression, while adding yeast culture significantly increased the relative expression of liver anti-inflammatory factor (*IL-10*) and antioxidant enzyme (*SOD*, *CAT* and *GSH-Px*) ($p < .05$). Meanwhile, yeast culture can reduce the accumulation of liver glycogen and improve the disorder of liver glucose metabolism caused by CPC. Furthermore, compared with CPC group, CPCY group significantly up-regulated the expression of *ZO-1*, *Claudin* and *Occludin* and the activities of antioxidant enzymes (*SOD*, *CAT*) in intestine ($p < .05$) as well as down-regulated diamine oxidase (DAO) activity, D-lactate (D-lac) and lipopolysaccharide (LPS) contents in serum ($p < .05$). In addition, the abundance of probiotics (*Lactobacillus*) increased significantly, and the abundance of intestinal potential pathogenic bacteria (*Vibrio*, *Brevundimonas*) decreased in CPCY group ($p < .05$). In conclusion, yeast culture can alleviate the adverse effects of concentrated cottonseed protein on the growth of largemouth bass and might be used as an effective probiotic for farmed fish.

KEYWORDS

antioxidant capacity, cottonseed protein concentrate, intestinal microbiota, *Micropterus salmoides*, yeast culture

1 | INTRODUCTION

Recently, plant proteins are increasingly being used as an economical and versatile alternative replacing animal source in fish nutrition. Animal protein presents growing costs and limited supply, and has been highly associated with resources depletion, biodiversity loss and environmental pollution. Therefore, reducing fishmeal in aquafeeds is becoming necessary for ensuring aquaculture sustainability, being particularly relevant in the production of carnivorous fish. As one of the most important aquaculture species in China, the largemouth bass (*Micropterus salmoides*) has received great attention, and several studies have been focused on alternative protein sources but have led to reduced feed intake, growth and impaired health status when included at high levels (He, Li, et al., 2020; He, Yu, et al., 2020; Jiang et al., 2018; Wu et al., 2021).

Proteins are essential macronutrients for fish nutrition, and the nutritional quality of a protein source varies substantially depending upon their bioavailability, digestibility, amino acid profile, purity, antinutritional factors and processing effects. Plant proteins are often recognized as incomplete or nutritionally inferior to animal proteins (Hughes et al., 2011). However, plant proteins certainly play a very valuable role in fish nutrition. The suitability of the plant protein sources in terms of growth performance has resulted to be highly variable among fish species and experimental conditions. Generally, carnivorous fish showed a much lower ability to accept high- plant protein diets even with balanced essential nutrients (Krogdahl et al., 2010). How to improve the utilization of plant protein sources in aquafeeds remains to be an important aspect. Therefore, specific strategies and techniques to increase the use of plant feedstuffs in aquafeeds and limit potentially adverse effects of bioactive compounds on farmed largemouth bass are worth research.

At present, yeast culture (YC) as synbiotics is widely used in aquaculture (Ayiku et al., 2020; Barnes et al., 2009). Studies have shown that yeast culture can not only be used as a protein source to replace fish meal (Barnes et al., 2006; Zhang et al., 2018) but also can be used as an immune modulator for fish due to it contains immunomodulatory components such as yeast polysaccharides (β - glucan and mannoooligosaccharide), peptides and nucleotides (Choudhury et al., 2005; Jensen et al., 2008; Selvaraj et al., 2005; Torrecillas et al., 2007). Furthermore, it was also documented that yeast culture can alleviate intestinal tissue damage caused by high- plant protein in yellow catfish (*Pelteobagrus fulvidraco*) (Sun, 2020) and turbot (*Scophthalmus maximus*) (Zhao, 2008). In addition, yeast cultures can alleviate gossypol induced inflammatory response in liver tissue of Ussuri catfish (*Pseudobagrus ussuriensis*) (Bu et al., 2020). Up to now, whether dietary yeast culture supplementation can mitigate detrimental effects of plant proteins and modulate antioxidant status still remains to be elucidated. Therefore, we aimed to explore the effect of yeast culture dietary intervention in largemouth bass fed a high- cottonseed protein diet for an 8- week randomized clinical trial. It provides valuable reference for the daily use of yeast culture in aquaculture.

TABLE 1 Formulation and proximate chemical composition of experimental diets (g kg⁻¹)

Experiment diets
CPCY

Ingredient (g kg ⁻¹)	FM	CPC	
Fish meal ^a	400	256	256
Domestic chicken powder	100	100	100
Cottonseed protein concentrate	0	157.8	157.8
Yeast culture	0	0	30
Soybean meal	70	70	70
Concentrated soy protein	110	110	95
Wheat gluten	30	30	30
Cassava starch	90	90	90
Fish oil	20	31	31
Soy lecithin	15	15	15
Soybean oil	40	40	40
Vitamin premix ^b	15	15	15
Mineral premix ^c	15	15	15
Choline chloride	5	5	5
Ca(H ₂ PO ₄) ₂	15	15	15
Microcrystalline fibre	75	47.8	32.8
L- lysine-HCL	0	1.7	1.5
DL- methionine	0	0.7	0.9
Chemical composition (g/kg,	lry matter basis)		
Crude protein	481	482	484.2
Crude lipid	120.6	120.6	121.2
Ash	115.8	105.1	105.1

Abbreviations: CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture; FM, fish meal- based diet.

^a Imported from Peru.

^b Vitamin premix(mg kg of diet): VA, 18; VD₃, 5; VE, 150; VC (350 g kg⁻¹), 500; VB₁, 16; VB₆, 20; VB₁₂, 6; VK₃, 18; riboflavin, 40; inositol, 320; calcium- D- pantothenate, 60; niacinamide, 80; folic acid, 5; biotin, 2; ethoxyquin, 100.

^c Mineral premix (mg kg of diet): Na, 30; K, 50; Mg, 100; Cu, 4; Fe, 25; Zn, 35; Mn, 12; I, 1.6; Se, 0.2; Co, 0.8.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

Three isonitrogenous (crude protein 480 g/kg) and isolipidic (crude lipid 120 g/kg) diets included a fish meal- based diet (named as FM group, control),

a diet that FM with 36% fish meal protein replaced by cottonseed protein concentrate (named as CPC group), and a diet supplementing 3% yeast culture (Beijing Enhalar International Tech Co., Ltd) into the CPC diet (named as CPCY group) (Table 1). The CPC and CPCY diets were supplemented with methionine and lysine to approximate the amino acid composition of FM. The ingredients were weighed and mixed in a commercial food mixer YGH- 150 (Guibao Group), then blended with the oils. Pellets were prepared using a dry power press MUZL180 (Muyang Group), then air- dried and stored at 4°C until used.

2.2 | Feeding trial

Largemouth bass juveniles were obtained from a commercial farm (Changshou, Chongqing, China) and acclimated to the experimental conditions with a commercial feed (Guangzhou Jieda Feed Co. LTD) for 2 weeks. Two hundred and seventy juveniles of largemouth bass (initial average weight: 12.17 ± 0.02 g) were randomly distributed into 9 cylindrical plastic tanks (capacity: 180 L) for the growth trial (30 fish per tank). Each dietary treatment was randomly assigned to three tanks. Fish were fed to apparent satiation by hand three times (08:30, 13:00 and 18:00) daily for 8 weeks. The water source for aquaculture is fully aerated tap water. During the experiment, water temperature ranged from 23.2 to 28.5°C, ammonia-N was below 0.1 mg L⁻¹, dissolved oxygen was above 6.5 mg L⁻¹, and pH was around 7.5. The photoperiod was 12 L:12 D, with the light period from 08:00 to 20:00 h. Water is exchanged every 2 days and is a quarter the size of the aquarium.

2.3 | Sample collection

At the end of the experiment, fish were fasted for 24 h before harvest, and then weighed and counted separately after being anaesthetized with 0.1 g L⁻¹ MS- 222 (Sigma). The growth characteristics and survival rate of largemouth bass in each experimental tank were calculated. Twenty- one animals per treatment (7 fish per tank) were individually used for determining hepatosomatic index (HSI), viscera somatic index (VSI), condition factor (CF), and for collecting Liver and intestinal tissues for histology and function assays. All the experiments were conducted under the standard code of protocol for the Care and Use of Laboratory Animals in China. The study protocol and all experimental procedures were approved by the Experimental Animal Ethics Committee of Southwest University.

Six hours after feeding, four fish from each tank were randomly selected and anaesthetized with 0.1 g L⁻¹ MS- 222 (Sigma), and blood sample was obtained from the caudal vein using the 1 mL syringe. After standing for 12 h, serum was obtained by centrifugation. The bloodless fish were then dissected to obtain liver and intestinal tissues for RNA isolation, and samples with the protector (RNA/DNA, TaKaRa, Type 9750) were fast frozen in liquid nitrogen. At the same time, the digesta from the whole intestine of each fish was taken. The digesta samples were mixed, collected in sterilized tubes and immediately frozen in liquid nitrogen for microbiome sequencing.

2.4 | Chemical analysis

Chemical composition analysis of diets and whole body were conducted by standard methods (AOAC, 2005). Moisture was determined by oven drying to a constant weight at 105°C; crude protein was determined by the micro- Kjeldahl method using the $N \times 6.25$ factor; lipid was determined by ether extraction (without acid hydrolysis) using FOSS Soxtec 2050 (Foss Analytical Instruments Co. Ltd.), liver lipid was determined by chloroform– methanol (2:1); ash was determined by combustion at 550°C for 12 h in a muffle furnace. Liver glycogen content was assayed using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Serum D- lactate, diamineoxidase and endotoxin were assayed using attached kit (Shanghai Youxuan Biotechnology Co., Ltd.). The activities of superoxide dismutase (SOD) and catalase (CAT), glutathione peroxidase (GSH- Px) and malondialdehyde (MDA) contents in liver and intestine were assayed with a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of glucokinase (GK), phosphofructokinase (PFK), fructose- 1,6- bisphosphatase (FBPase) and glucose- 6- phosphatase (G6Pase) in liver were determined using a commercial kit (SinoBest Biological Technology Co., Ltd.). All enzyme assays were performed in triplicate. The liver and intestinal samples were stained with haematoxylin and eosin (H&E). Subsequently, the liver and intestinal morphology was observed under a light microscope (OLYMPUS, DP73, Nikon Corporation).

2.5 | Quantitative real- time PCR analysis

Total RNA in liver and intestine was extracted using RNAISO Plus reagent (Takara) according to the manufacturer's instructions. The RNA quality was assessed by formaldehyde agarose gel electrophoresis, and the concentration of RNA was quantified with a spectrophotometer Nano Drop 2000 (Thermo Scientific). Complementary DNA (cDNA) was synthesized from RNA with a NovoScrip Plus All- in- one 1st Strand cDNA Synthesis Supersis Super Mix (TaKaRa) and stored at -80°C for later use. Quantitative real- time PCR (qPCR) was carried out on the Bio- Rad CFX96 (Bio- Rad) in a total volume of 20 µL with the NovoStart SYBR qPCR Super Mix Plus (Novoprotein) following the manufacturer's protocol. The specific primers of the β - actin gene and target genes were synthesized by Beijing Qingke Biotech Co., Ltd. (shown in Table 2). The relative mRNA expression of target genes was normalized to the β - actin mRNA and determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.6 | Intestinal microbiota

Intestinal microorganism index entrusts biological company (Biomarker Technologies Co, LTD) to determine, and the data results were analysed on the BMKCloud. Total bacterial community DNA was extracted using a PowerFecal™ DNA Isolation kit (MO BIO Laboratories), and then quality and quantity of extracted genomic DNA were estimated by agarose gel electrophoresis and a NanoDrop 8000 spectrophotometer (Thermo Fisher

Scientific). The V3- V4 region of bacterial 16S rRNA gene was amplified by

TABLE 2 Primer sequences for real- time PCR

Genes	Primers	Sequence 5'–3'	TM (°C)
SOD	F _a	CCACCAGAGGTCTCACAGCA	60
	R _a	CCACTGAACCGAAGAAGGACT	
CAT	F	TGGTGTTACGGATGAGATGG	60
	R	GGAGAAGCGGACAGCAATAGG	
GSH- Px	F	ATACCAAGTCTCTCCCTCTGT	59
	R	CGTCCACCACTTTGCCATT	
IL-8	F	CGTTGAACAGACTGGGAGAGATG	64.9
	R	AGTGGGATGGCTTCATTATCTTGT	
IL-10	F	CGGCACAGAAATCCAGAGC	62.1
	R	CAGCAGGCTCAGAAATAACATCT	
IL-1 β	F	CGTGACTGACAGCAAAAAGAGG	59.4
	R	GATGCCAGAGCCACAGTTC	
TGF- β 1	F	GCTCAAAGAGAGCGAGGATG	59
	R	TCCTCTACCATTGCAATCC	
TNF- α	F	CTTCGTCTACAGCCAGGCATCG	63
	R	TTTGGCACACCGACCTCACC	
GK	F	CATTCGGAGACAACGGGGAG	60.46
	R	ATTGAGGTCTCGTCCACCAC	
PFK	F	ACAACACACCACTGACACCT	59.39
	R	GCAGCATCGAGCAGAACATGA	
G6Pase	F	GCCTACTGCGTAACATGGGT	60.11
	R	ATCCTGCGCTTGAACTGGCA	
FBPase	F	CACCATGACCCGGTTCGTTA	60.04
	R	CAGGAAATTGCCTTCACCGC	
ZO1	F	ATCTCAGCAGGGATTGACG	59.61
	R	CTTTGCGGTGGCGTTGG	
claudin	F	CCAGGGAAGGGGAGCAATG	60.08
	R	GCTCTTTGAACAGTGCGAC	
Occludin	F	GATATGGTGGCAGCTACGGT	59.61
	R	TCCTACTGCGGACAGTGTG	

β - actin	F	AAAGGGAAATCGTGCCTGAC	60
	R	AAGGAAGGCTGGAAGAGGG	

Abbreviations: CAT, catalase; FBPase, fructose- 1,6- bisphosphatase; G6Pase, glucose- 6-p phosphatase; GK, glucokinase; GSH- Px, glutathione peroxidase; IL- 10, interleukin- 10; IL-8 , interleukin- 8; PFK, phosphofructose kinase; SOD, superoxide dismutase; TGF- β 1, transforming growth factor- β 1; TNF- α , tumour necrosis factor- α ; ZO1, zonula occludens- 1.

^a F, forward primer; R, reverse primer.

PCR and purified. Then, the high- throughput sequencing was performed using a HiSeq platform (Illumina) at Biomarker Technologies Corporation. Sequences with similarity $\geq 97\%$ were assigned to the same OTUs. The taxonomic annotation of OTU sequences with reference database to obtain the species classification information corresponding to each OTU and then the community composition of each sample can be counted at each level (phylum, class, order, family, genus and species). The table of species abundance at different taxonomic levels was generated Using a QIIME software (Zaura et al., 2009), and the alpha diversity index of samples was evaluated Using a QIIME2 software (Grice et al., 2009). Meanwhile, phenotypic predictive analysis was performed using the BugBase tool (Ward et al., 2017).

2.7 | Calculations and statistical methods

All data were presented as Mean $\pm$ S.E.M. The level of significance was determined by one- way analysis of variance (ANOVA) with SPSS 18.0 (IBM) for Windows. Multiple comparisons were performed using Tukey multiple range tests. The statistical significance level was set at $p < .05$. The graphics were drawn using GraphPad Prism6.0 (GraphPad Software Inc. USA).

3 | RESULTS

3.1 | Growth performance

The Lower WG and SGR, and higher FCR were observed in fish fed the FM diet (control) ($p < .05$, Table 3). Compared with CPC group, the CPCY diet significantly enhanced the WG, SGR, PER and FR ($p < .05$), while no significant differences were observed between the CPCY group and the FM group. Furthermore, the VSI and HSI values, liver lipid and glycogen content of CPCY group were significantly lower than CPC group ($p < .05$, Table 4). No significant differences were found in the whole body moisture, protein, lipid and ash contents among dietary treatments ($p > .05$, Table 4).

3.2 | Hepatic parameters

As indicated in Figure 1, the yellowing livers were observed in CPC group, and the hepatic tissue was also disordered including diffused lipid vacuolization in some hepatocytes. However, normal liver colour and liver structure were found when yeast culture was added (CPCY group).

Furthermore, compared with the FM group, the activities of antioxidant enzymes (SOD and CAT) in fish liver of CPC group were significantly decreased ($p < .05$), but there was no significant difference between CPCY group and FM group ($p > .05$, Figure 2a). Meanwhile, the gene expression of *SOD*, *CAT* and *GSH-Px* in CPC group was significantly lower than the other group ($p < .05$, Figure 2b).

In addition, the expression of pro-inflammatory factors (*IL-1 β* and *TNF- α*) in liver was significantly up-regulated in CPC group compared with FM group, while it was down-regulated in CPCY group ($p < .05$, Figure 3). Interestingly, this study found that glycolytic enzyme (PFK and GK) activity was significantly reduced in the CPC group compared with the FM group ($p < .05$, Figure 4a). However, adding YC to CPC diet significantly increased the activity of glycolysis enzymes (GK) and decreased the activity of gluconeogenesis enzymes (FBPase). At the same time, the expression of glycolysis-related genes (*PFK* and *GK*)

TABLE 3 Growth performance of *M. salmoides* fed experiment diets for 8 weeks

Final body weight (g)	83.55 $\pm$ 0.32 ^b	75.35 $\pm$ 1.07 ^a	86.12 $\pm$ 0.45 ^c
WG (%) ^a	586.34 $\pm$ 4.78 ^b	511.02 $\pm$ 3.99 ^a	614.56 $\pm$ 2.12 ^c
SGR (%) ^b	3.21 $\pm$ 0.01 ^b	3.02 $\pm$ 0.11 ^a	3.45 $\pm$ 0.03 ^c
PER ^c	2.10 $\pm$ 0.04 ^a	1.99 $\pm$ 0.17 ^a	2.34 $\pm$ 0.05 ^b
FR (% per day) ^d	2.14 $\pm$ 0.05 ^{ab}	1.98 $\pm$ 0.18 ^a	2.31 $\pm$ 0.06 ^b
FCR ^e	0.86 $\pm$ 0.04 ^a	1.04 $\pm$ 0.04 ^b	0.78 $\pm$ 0.03 ^a
Survival (%) ^f	100	100	100

Note: Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each row with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture. ^aWeight gain (WG) = [final weight (g) - initial weight (g)] $\times$ 100/initial weight (g). ^bSpecific growth rate (SGR%) = [ln (mean final weight) - ln (mean initial weight)]/56 days $\times$ 100. ^cProtein efficiency ratio (PER) = total weight gain (g)/protein intake (g). ^dFeeding ratio (FR) = 100 $\times$ total feed intake in dry basis (g)/[final weight (g) + initial weight (g)] $\times$ 2/56 days. ^eFeed conversion ratio (FCR) = total feed intake in dry basis (g)/weight gain (g). ^fSurvival rate (SR) = 100 $\times$ final fish number/initial fish number.

Items	Experiment diets		
	FM	CPC	CPCY
Morphological measurements			
CF (%) ^a	2.27 $\pm$ 0.02 ^a	2.19 $\pm$ 0.02 ^a	2.46 $\pm$ 0.03 ^b
VSI (%) ^b	8.80 $\pm$ 0.42 ^{ab}	9.92 $\pm$ 0.15 ^b	8.34 $\pm$ 0.08 ^a
HSI (%) ^c	2.24 $\pm$ 0.02 ^b	2.38 $\pm$ 0.06 ^b	1.69 $\pm$ 0.01 ^a
Body composition			
Moisture (%)	70.27 $\pm$ 0.12	71.17 $\pm$ 0.00	71.11 $\pm$ 0.01
Crude lipid (%)	6.27 $\pm$ 0.04	6.10 $\pm$ 0.03	6.16 $\pm$ 0.02
Crude protein (%)	18.25 $\pm$ 0.30	17.99 $\pm$ 0.08	18.24 $\pm$ 0.05
Crude ash (%)	4.32 $\pm$ 0.02	4.29 $\pm$ 0.07	4.24 $\pm$ 0.01
Liver content			
Liver lipid (%)	3.76 $\pm$ 0.18 ^a	5.96 $\pm$ 0.33 ^b	4.25 $\pm$ 0.04 ^a
Liver glycogen (mg/g)	75.16 $\pm$ 0.15 ^a	85.00 $\pm$ 0.39 ^b	72.55 $\pm$ 0.02 ^a

TABLE 4 Morphological measurements, body composition, liver lipid and liver glycogen content of *M. salmoides* fed experiment diets for 8 weeks

Items	Experiment diets		
	FM	CPC	CPCY
Initial body weight (g)	12.17 $\pm$ 0.04	12.25 $\pm$ 0.12	12.08 $\pm$ 0.01

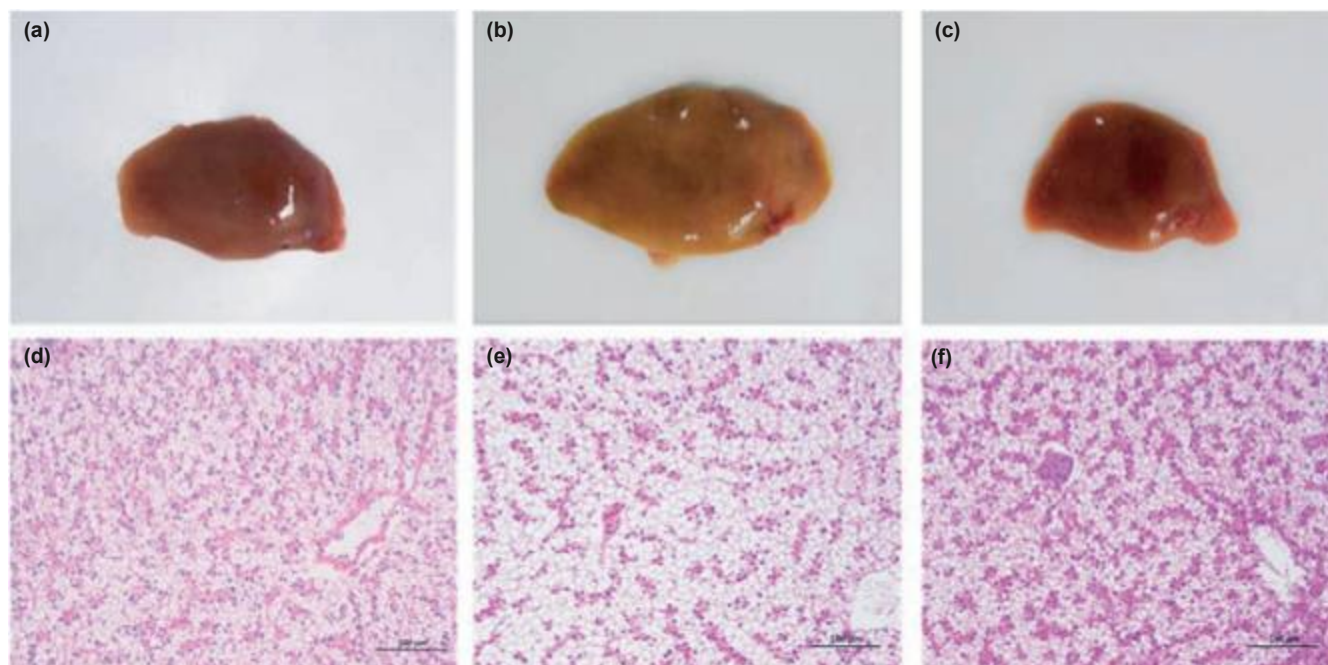


FIGURE 1 Histology of liver in juvenile largemouth bass fed the experiment diet. (a), (b) and (c) present different liver colours of FM, CPC and CPCY groups, respectively, and the below picture (d), (e) and (f) is part histology of (a), (b) and (c) under 200× magnification, respectively. (H. E 200×). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

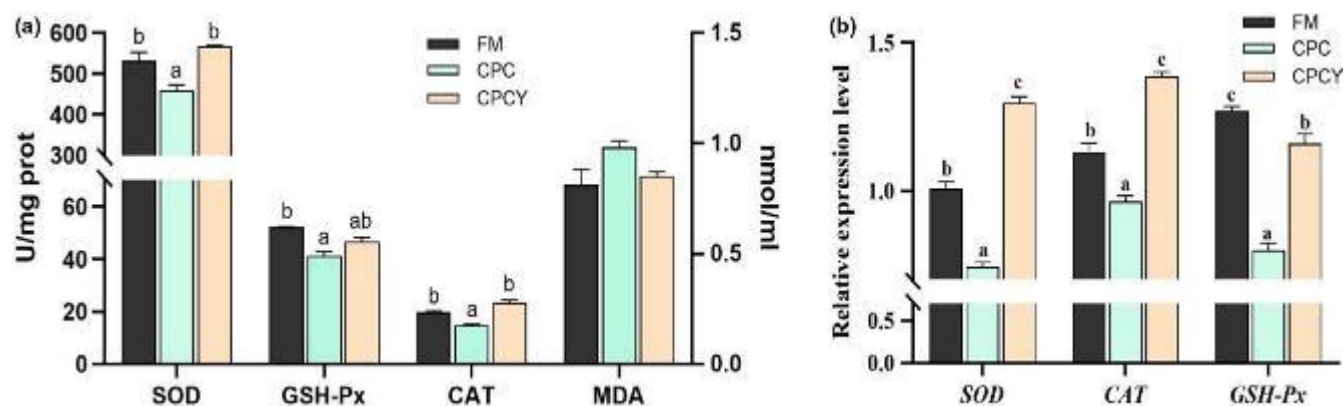


FIGURE 2 Effects of experiment diets on hepatic antioxidant capacity (a) and related gene expression (b) of *M. salmoides*. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

was significantly up-regulated and the expression of gluconeogenesis related genes (*G6Pase*) was down-regulated in the CPCY group compared with the CPC group ($p < .05$, Figure 4b).

3.3 | Intestinal health parameters

Compared with FM group, the relative gene expression of intestinal tight junction protein (*ZO-1*, *Claudin* and *Occludin*) in CPC group was

Note: Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each row with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture. aCondition factor (CF) =

significantly down-regulated ($p < .05$, Figure 5), while the contents of D-lactate (D-lac) and lipopolysaccharide (LPS) in serum were significantly increased as well as the activities of diamine oxidase (DAO) in serum were significantly enhanced ($p < .05$, Figure 6).

However, the addition of YC to the CPC diet significantly up-regulated intestinal tight junction protein-related gene (*ZO-1*, *Claudin*, *Occludin*) expression and reduced the content of LPS and the activity of DAO in serum ($p < .05$). Meanwhile, CPC diet significantly up-regulated the expression of pro-inflammatory factors (*IL-1 β* , *IL-8*

[body weight (g)/standard length³ (cm)] $\times 100$. bViscera somatic index (VSI) = $100 \times \text{viscera weight (g)/body weight (g)}$. cHepatosomatic index (HSI) = $100 \times \text{hepatopancreas weight (g)/body weight (g)}$.

and $TNF-\alpha$) and down-regulated the expression of anti-inflammatory factors ($IL-10$) compared with FM group ($p < .05$, Figure 7), while adding YC in CPC diet significantly down-regulated the expression of pro-inflammatory factors ($IL-1\beta$ and $IL-8$).

In addition, compared with FM group, the activity of antioxidant enzyme (CAT) in CPC group was significantly decreased, and the level of MDA was significantly increased ($p < .05$, Figure 8a), but there was no significant difference between CPCY group and FM group ($p > .05$). Meanwhile, the expression of antioxidant genes (SOD , CAT and $GSH-Px$) in CPC group was significantly lower than those in FM group and CPCY group ($p < .05$, Figure 8b).

3.4 | Intestinal microbial

A total of 239,263.17 sequences were obtained from fish intestinal bacteria. All OTUs were from samples with 97% similarity and sample coverage $\geq 99.7\%$. Compared with CPC group, the Shannon index and Simpson index of CPCY group had no significant difference, while the Chao1 index increased significantly ($p < .05$; Table 5). Beta-diversity index of intestine microbiota was presented by principal coordinate analysis (PCoA) by comparing the microbiota similarity of samples. As shown in Figure 9, the

clusters of FM, CPC and CPCY group samples separated from each other on the coordinate axis.

At the phylum level, the intestinal bacterial profiles of largemouth bass were dominated by *Proteobacteria* and *Firmicutes*, followed by *Bacteroidetes* and *Actinobacteria* (Figure 10a). Notably, the relative abundance of *Proteobacteria* in the CPC group increased from 29.85% to 36.73%, while that in the CPCY group decreased to 24.84% compared with the FM group. Meanwhile, the relative abundance of *Bacteroidetes* in the CPC group decreased from 14.99% to 6.68%, while that in the CPCY group increased to 12.81%. At the genus level, *Plesiomonas* and *Cetobacterium* were the dominant genera in the FM group, while *Lactobacillus*, *Sphingomonas* and *Bacteroides* were the dominant genera in the CPC group and the CPCY group (Figure 10b). Further Metastat analyses showed that the relative abundances of *Lactobacillus*, *Ruminococcaceae* UCG-005, *Akkermansia* and *Candidatus Udaobacter* increased, and the relative abundance of *Vibrio*, *Brevundimonas*, *Thermomonas* and *Kytococcus* decreased significantly in the CPCY group compared with the CPC group ($p < .05$; Figure 11).

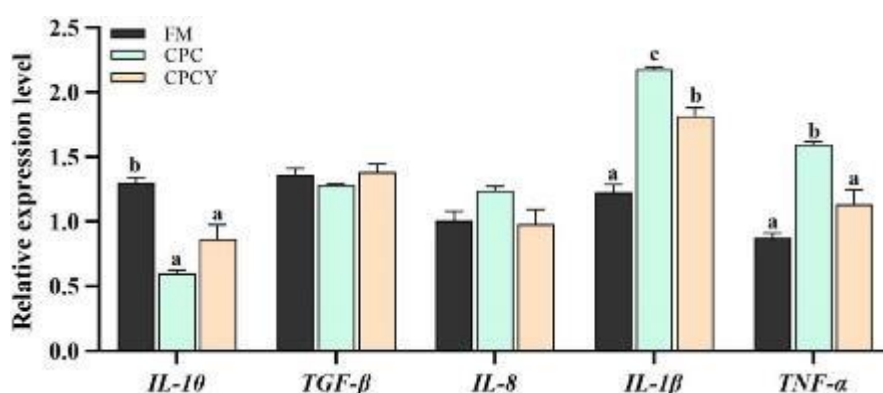


FIGURE 3 Hepatic expression levels of inflammatory and anti-inflammatory cytokines genes of juvenile largemouth bass fed the experiment diet. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

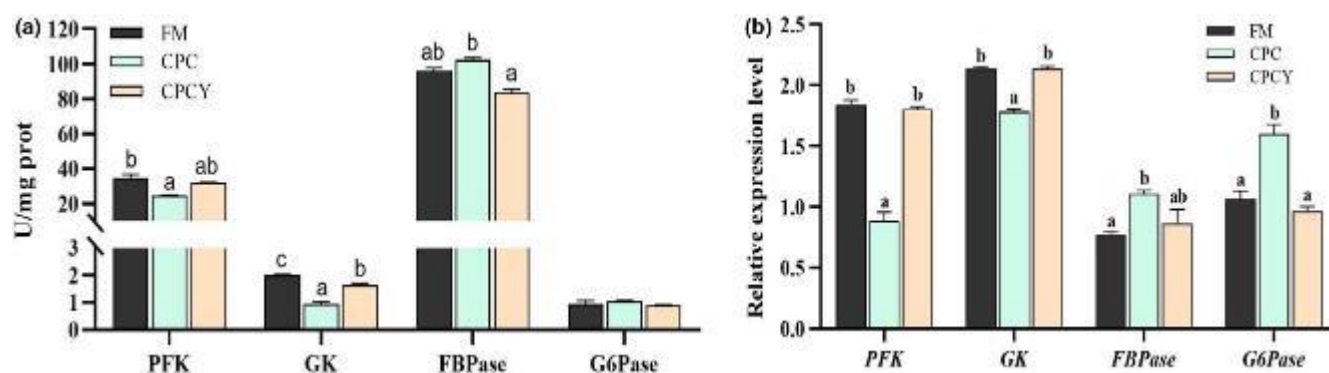


FIGURE 4 Effects of experiment diets on hepatic glycometabolism enzyme activities (a) and related gene expression levels (b) of juvenile largemouth bass. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

In addition, we used BugBase tools to predict microbial phenotypes (Figure 12). The results showed the relative abundance of intestinal gram-negative bacteria and potential pathogenic bacteria

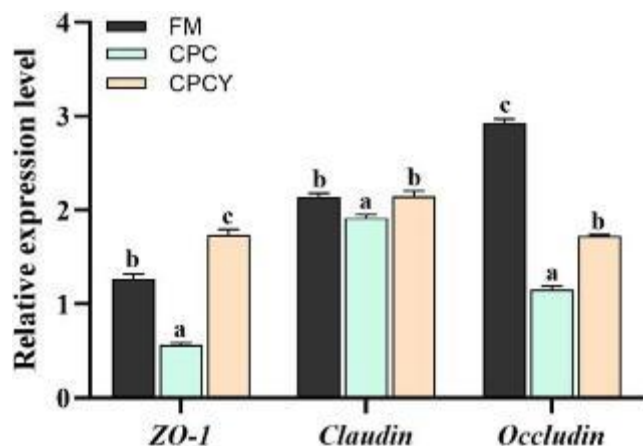


FIGURE 5 Intestinal expression levels of tight junction-related genes of juvenile largemouth bass fed the experiment diet. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

increased significantly in CPC group ($p < .05$), whereas decreased in CPCY group compared with FM group ($p < .05$).

4 | DISCUSSION

The results of the present study clearly show that the plant proteins significantly affected the growth of largemouth bass. Fish fed the diet containing 15.8% CPC showed lower growth and PER compared with the control group, which indicate that incorporating more than 15.8% CPC in the diet has been shown to depress growth of largemouth bass. Similar results were also reported by He, Yu, et al. (2020); He, Li, et al. (2020), in which the substitution level of FM with SBM was only 15% (16.2% SBM) in largemouth bass diet. In recent years, the nutritive value and potential of plant proteins for fish feeds have been examined in numerous studies (Jiang et al., 2018; Lin et al., 2010; Xu et al., 2017).

A large number of studies have shown that high dietary inclusion levels of CPC result in decreased growth performance and feed efficiency in fish (Liu et al., 2020; Ye et al., 2020). Noteworthy, the present study shows that replacement of a part of fish meal by increasing levels of yeast culture in diets significantly results in growth improvement, indicating a better valorization of the feed as shown by the better PER observed with CPCY. Previous study also has demonstrated that yeast production is a suitable protein to elevate the nutritive values of diets for fish (Vidakovic et al., 2016). In fact, there are numerous studies that demonstrated that yeast and its derivatives may have beneficial effects on growth performance and health (Shurson, 2018). Accordingly, this suggested that the yeast culture supplementation can achieve balanced nutritional composition and

improve the palatability to further reduce the use of fishmeal cost-effectively in aquafeeds (Guo et al., 2019; Wang et al., 2017).

Surprisingly, the results of the present study show that yeast incorporation in aquafeeds appears as a promising solution to decrease both lipid and glycogen content in liver. At the same time, the activities of hepatic glycolytic enzymes (GK and PFK) increased, whereas the hepatic gluconeogenic enzyme activities (FBPase and G6Pase) decreased significantly when fish fed with yeast culture, thus resulting in the decrease in glycogen content in the liver. High glycogen content in the liver will induce fat synthesis and increase hepatic fat deposition (Brauge et al., 1994). These investigations showed that the changes in HSI might result from the increase in lipid content and glycogen content in the liver. The results of this study suggested that endogenous glucose synthesis was particularly depressed by the yeast culture supplementation, even though further studies would be necessary to prove it.

The current study further found that dietary high-plant protein level significantly affected the activities of liver antioxidant defence enzymes (SOD, CAT and GSH-Px), which was also observed in tilapia (Lin et al., 2010). This suggests that the antioxidant system of largemouth bass is damaged because of certain constituents of plant proteins, and which could be partly responsible for the changes in hepatic morphology in this study. Previous studies have shown that anti-nutrition factors (gossypol) in cottonseed protein accumulate in the liver tissue of fish and cause inflammation (Jensen et al., 1982; Lee et al., 2002; Soares et al., 2012). However, adding yeast culture to feed can reduce gossypol induced liver inflammation of Ussuri catfish (*Pseudobagrus ussuriensis*) (Bu et al., 2020). Similarly, effects of YC supplementation on antioxidant capacity are observed in weaning pigs (Yang et al., 2016). Additionally, we also found that yeast culture down-regulated the expression of pro-inflammatory cytokines (*IL-1 β* and *TNF α*) and increases the antioxidant capacity of liver in largemouth bass. Therefore, fish preferred diet with yeast culture supplementation had lower occurrences of histopathological symptoms in response to high-plant protein diet, suggesting the mitigative effect of yeast culture on the hepatic lesion induced by high-plant protein for largemouth bass. This may be related to the presence of β -glucans and mannan oligosaccharides in yeast cultures (Ganner & Schatzmayr, 2012). Studies have shown that β -glucans in yeast culture can inhibit the excessive activation of the inflammatory response during the later phase of infection (Sherif et al., 2019). Apart from increasing the anti-inflammatory cytokine transcripts, β -glucans can prevent the degradation of *NF- κ B* inhibitor to minimize the expression of pro-inflammatory cytokines (*tnf- α* , *il-1 β* and *il-8*) in the fish (Ren et al., 2019). Moreover, studies indicate that mannan oligosaccharides can improve the antioxidant capacity of the liver of European sea bass and repair the liver tissue structure (Torrecillas et al., 2007). These results show that yeast cultures are effective against liver inflammation. Clearly, more research is needed in this area to elucidate the repeatability of these findings.

The intestine is one of the organs for fish to digest and absorb nutrients, which play an essential role in maintaining multiple physiological and pathological processes, and its health status is closely related to the growth of fish. Previous studies have shown that high- plant protein often causes intestinal damage in aquatic animals such as largemouth bass (He, Li, et al., 2020; He, Yu, et al., 2020) and amur sturgeon (*Acipenser schrenckii*) (Wei et al., 2019). In this investigation, fish fed the high CPC diet led to the increase in intestinal permeability and the upregulation of pro- inflammatory factor (*IL- 1 β* , *IL- 8* and *TNF α*). Unexpectedly, however, dietary yeast culture supplementation to a plant protein- based diet improved the health status of intestine in this study. On one hand, the yeast- containing diets can improve the integrity of intestinal tight junction proteins and inhibit the expression of pro- inflammatory factor (*TNF α*) (Wang et al., 2020). On the other hand, dietary supplementation of yeast culture could up- regulate the express of the intestinal barrier- related genes (*Occludin* and *Claudin 1*) (Zhang et al., 2020). Additionally, the current experiment also confirmed that yeast supplementation enhances the antioxidant capacity of the gut. These data suggest that yeast supplementation may be able to reduce inflammatory reactions induced by high- plant protein during the receiving period. The main reason evoked to explain the efficiencies of yeast culture is the presence of some yeast components, such as MOS, may have a strong beneficial effect on gut

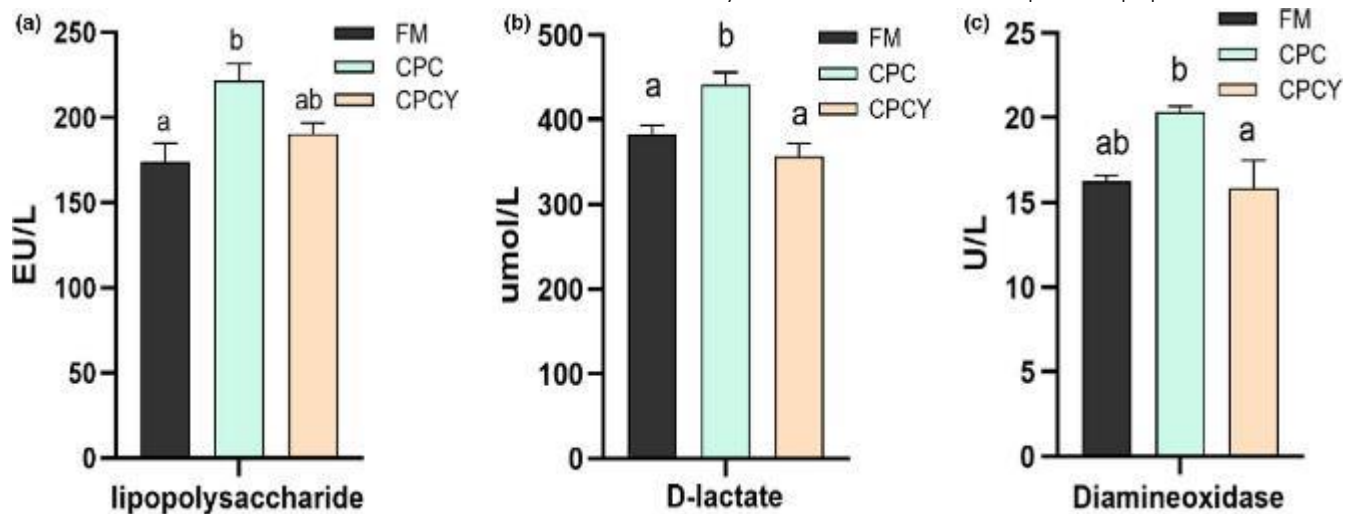


FIGURE 6 Parameters of intestinal permeability of juvenile largemouth bass fed the experiment diet. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal- based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

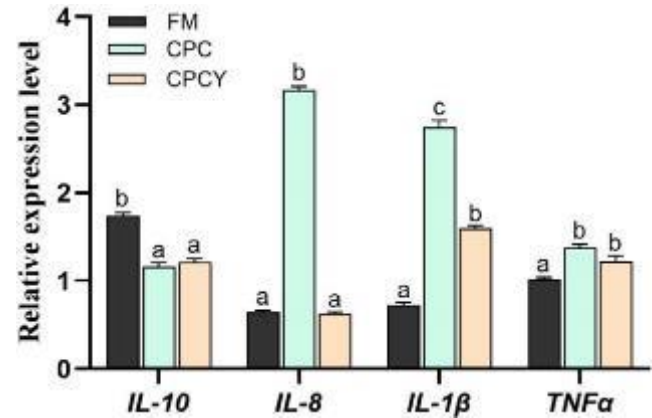


FIGURE 7 Intestinal expression levels of inflammatory and anti-inflammatory cytokines genes of juvenile largemouth bass fed the experiment diet. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal- based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

health condition in fish fed yeast- containing diets. Thus, it is possible to hypothesize that yeast culture supplementation in high- plant protein diets may have contributed to enhance the protective properties of the intestinal

epithelium and facilitate nutrient absorption, which is consistent with the better growth of largemouth bass of this group. It seems that a period of adaptation is needed before the effects of yeast culture supplementation can be significant because the changes in intestinal morphology and immune responses take time.

Notably, the current study found that diet structure can modify intestinal bacterial communities, and high- plant protein diet decreased the abundance of intestinal microflora of largemouth bass.

Interestingly, the supplementation of yeast culture could restore both ACE and Chao1 indices, which indicated that yeast culture can regulate the stability of intestinal microflora in this study, consistent with the result of Liu

et al. (2018). In addition, the present results also pointed out that *Proteobacteria* and *Firmicutes* were the two dominant phyla of largemouth bass. This is in agreement with previous reports of the same species (Zhou et al., 2018). Meanwhile, we found that the abundance of *Proteobacteria* increased caused by feeding CPC diet. Previous studies have shown that an increase in *Proteobacteria* is often regarded as a marker of microbial community instability (Shin et al., 2015), which may lead to nutritional and metabolic disorders in the host. More importantly, the bacterial phylum *Proteobacteria* is closely correlated with expression of *IL-10* and *TNF- α* , and trigger intestinal inflammatory responses (Larsen et al., 2010; Livak et al., 2001; Zhu et al., 2013). However, the supplementation of yeast culture in CPC diet not only decreased the abundance of *Proteobacteria*, but also increased the abundance of *Bacteroidetes*. It is of note that elevated *Bacteroidetes* conducted to the protection against gut inflammation (Ferreira et al., 2011). These results suggested that yeast cultures may be able to improve intestinal inflammation by regulating intestinal microbiota.

Further analysis at the genus level showed that CPCY diet increased the abundance of *Lactobacillus*, *Ruminococcaceae UCG-005* and *Akkermansia*. Studies indicate that *Lactobacillus* could reduce the intestinal permeability and exert a protective effect on intestinal epithelium via inhibiting the pro-inflammatory factor (*IL-6*, *TNF- α* , *TLR4* and *NF- κ B*), and activating *IL-10* expression (Liu et al., 2011). The *Ruminococcaceae UCG-005* has been reported to be butyrate-producing bacteria that may protect healthy subjects from chronic intestinal inflammation (Mancabelli et al., 2017). The metabolites synthesized by *Akkermansia muciniphila* modulate intestinal barrier integrity in vitro by strengthening the pore pathway and regulating tight junction protein expression (Cruz-Lebron et al., 2021). These

Chao1	1,004.60 $\pm$ 19.01 ^b	899.38 $\pm$ 16.40 ^a	970.12 $\pm$ 15.40 ^a
Simpson	0.85 $\pm$ 0.01	0.86 $\pm$ 0.06	0.94 $\pm$ 0.02
Shannon	5.32 $\pm$ 0.28 ^a	5.72 $\pm$ 0.38 ^{ab}	7.05 $\pm$ 0.12 ^b
Goods coverage	99.82 $\pm$ 0.08	99.79 $\pm$ 0.11	99.81 $\pm$ 0.09

Note: Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each row with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture.

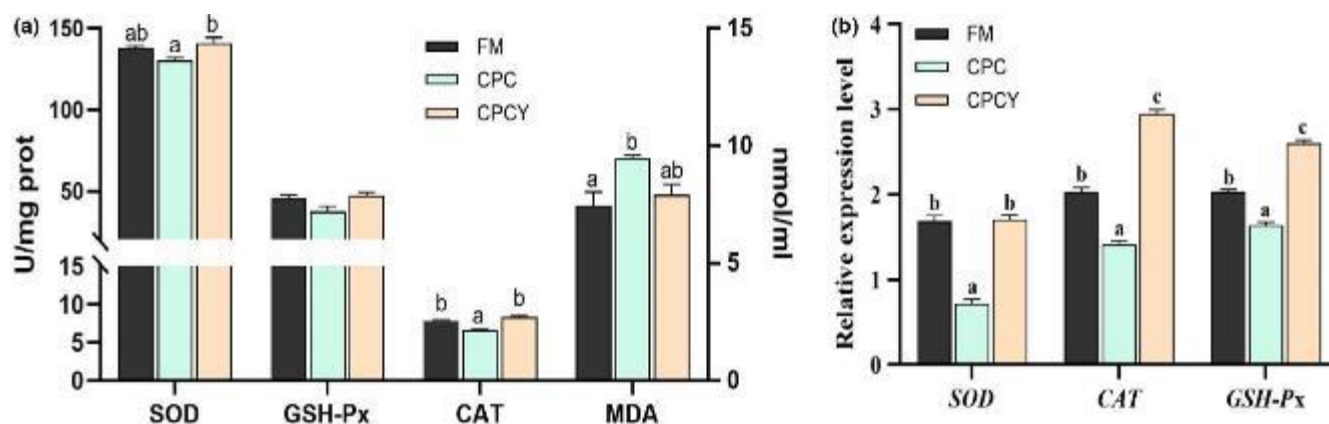


FIGURE 8 Effects of experiment diets on intestinal antioxidant capacity (a) and related gene expression levels (b) of juvenile largemouth bass. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

Items	Experiment diets		
	FM	CPC	CPCY
OTUs	708.50 $\pm$ 0.87 ^a	697.33 $\pm$ 18.41 ^a	873.33 $\pm$ 23.34 ^b
ACE	1,363.17 $\pm$ 10.01 ^c	941.25 $\pm$ 7.63 ^a	985.25 $\pm$ 7.03 ^b

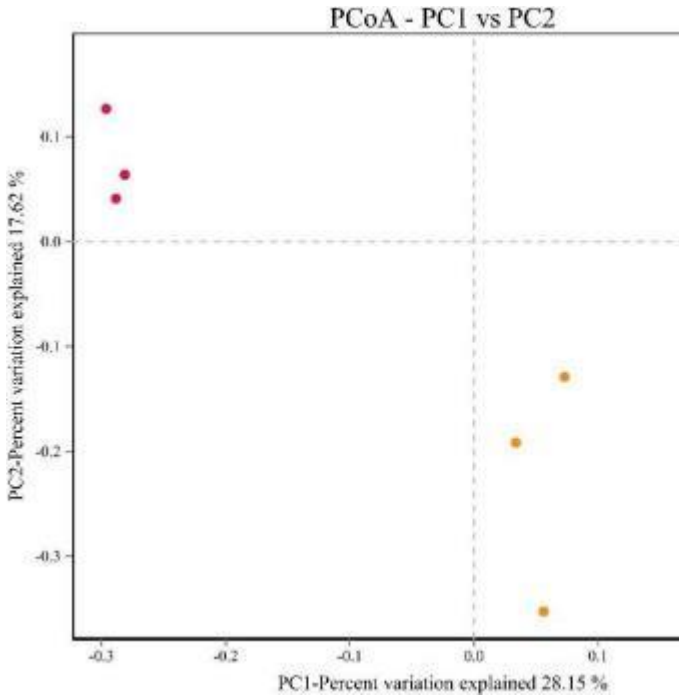


TABLE 5 Effects of experimental diets on OTUs and Alpha diversity statistics of the microbial sequencing of *M. salmoides*

FIGURE 9 Beta- diversity of intestinal microbiota by principal coordinates analysis. Abscissa and ordinate represent the first and second principal component, respectively. The percentage represents the contribution of the principal component to the sample difference ($n = 3$). FM, fish meal- based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

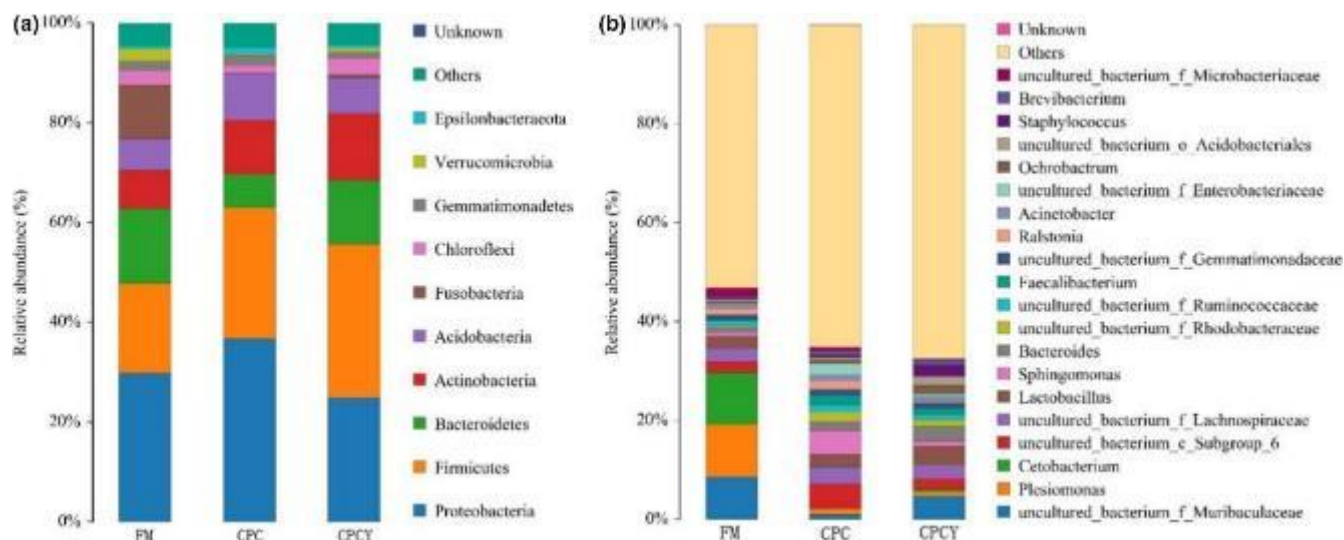
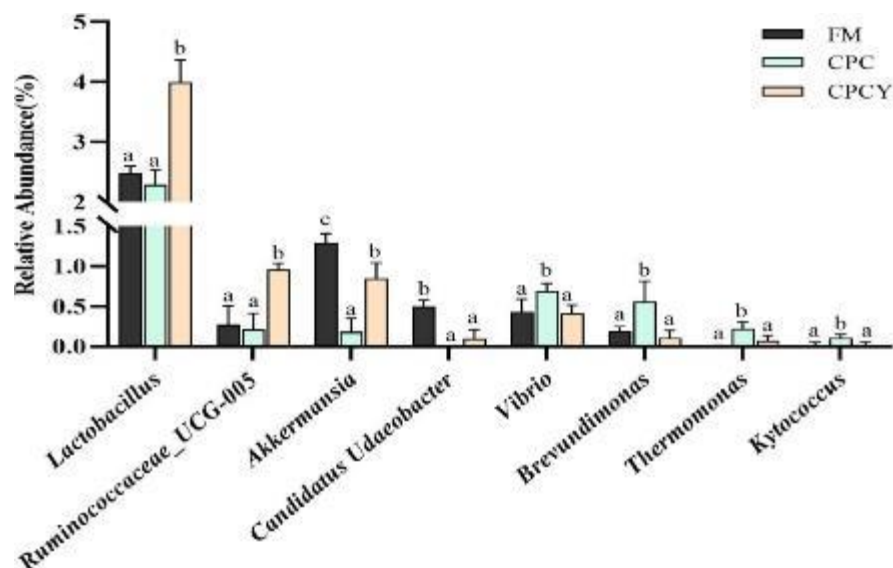


FIGURE 10 Intestinal microbial composition at phylum (a) and genus (b) levels in FM group, CPC group and CPCY group. The phylum level lists the top 10 most abundant (Based on relative abundance) bacterial phyla and the genus level lists the top 20 most abundant bacteria. Other phyla genera were all assigned as 'Others'. FM, fish meal- based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

FIGURE 11 Differences in microbial community abundance (at genus level) between the three groups were analysed by Metastats software. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal-based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture



results suggested that yeast cultures may strengthen the intestinal barrier and enhance intestinal immunity by increasing the abundance of these beneficial bacteria. On the contrary, supplement the yeast culture in diet decreased the abundance of *Vibrio*, *Brevundimonas* and *Kytococcus*. Noteworthy, *Vibrio* is one of the most common bacterial infections in fish, which causing inflammation and mortality (Pan et al., 2017). Studies have shown that *Vibrio anguillarum* induces inflammation and apoptosis of silvery pomfret (*Pampus argenteus*) epithelial cells (Gao et al., 2016). Moreover, *Brevundimonas* and *Kytococcus* are increasingly identified as an opportunistic pathogen in infections leading to bacteremia, especially among immunosuppressed patients (Chi et al., 2004) (Blennow et al., 2012; Chi et al., 2004). Subsequently, the Bugbase phenotype analysis again demonstrated that the supplementation of yeast culture could reduce the abundance of harmful bacteria and increase the abundance of beneficial bacteria, thus improving the intestinal ecosystem. This suggested that the

yeast culture has positive effects on key microbial populations and their metabolism, and consequently mediating intestinal immune function and stabilizing intestinal environment. However, the specific mechanisms and interactions between yeast culture and intestinal bacteria in largemouth bass remains unclear.

In conclusion, the present study successfully confirmed that dietary supplementation of yeast culture improved the performance of largemouth bass fed with high- plant protein diet.

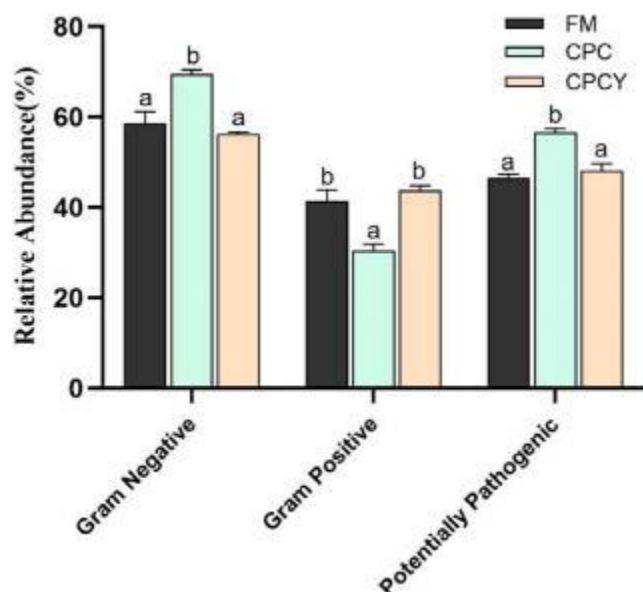


FIGURE 12 Phenotypic predictive of three groups was analysed by BugBase tool. Data are expressed as mean $\pm$ S.E.M ($n = 3$). Values in each column with different superscripts have significant differences ($p < .05$). FM, fish meal- based diet; CPC, 36% of the fish meal replaced by cottonseed protein concentrate; CPCY, CPC diet with 3% yeast culture

Furthermore, the positive effects of yeast culture on the performance of largemouth bass were associated with enhanced hepatic antioxidant capacity and specific immune- inflammatory biomarkers. Moreover, the improvement in the intestinal health by dietary yeast culture supplementation was related to the upregulation of intestinal barrier-related genes and positive effects on intestinal bacterial populations. Overall, these findings provide a potential explanation for the mechanisms that mediate the positive effects of yeast culture on largemouth bass. Thus, these findings will be beneficial for the nutritional management of largemouth bass.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the results of this study can be obtained from corresponding author upon reasonable request.

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WILEY

ORIGINAL ARTICLE

Yeast culture improve CCl₄ - induced liver damage, inflammatory response via inhibition of TLR2/NF- κ B signaling pathway expression in *Pseudobagrus ussuriensis*

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1 | INTRODUCTION

Liver is an important organ that may regulate various physiochemical functions including synthesis, secretion and metabolism of xenobiotics. Damage to the liver may lead to these physiochemical function disorder. Many risk factors in aquaculture may induce such damage, including environmental pollution and the abuse of antibiotics and pesticides. At present, there are few breakthroughs in the preventive and control of fish liver damage (Yin et al., 2011). Therefore, research identifying improve hepatic damage agents is urgently necessary. And the hepatic injury models induced by chemical substances were often used to investigate its pathogenesis and the underlying protective effect of drugs. These chemical substances mainly include carbon tetrachloride (CCl₄), alcohol, acetaminophen (Jaeschke et al., 2011) and thioacetamide (Ming et al., 2006). Presently, carbon tetrachloride (CCl₄) is widely used as a well- established hepatic injury model chemical with the merits of convenient accessibility, easy model making and little damage to other organs. Previous reports demonstrated that the metabolites of CCl₄ such as toxic trichloromethyl free radical (•CCl₃) and trichloromethyl peroxy (•OOCCl₃) free radicals may lead to severe damage to the function and structure of liver cells in the grouper and jian carp (*Cyprinus carpio* var. Jian) (Al- Harbi et al., 2014; Cao et al., 2015; Singh et al., 2008; Sun et al., 2019). Therefore, researchers always used carbon tetrachloride (CCl₄) as a classic liver hepatotoxicant to gain an insight into the pathophysiological processes in aquaculture (Zou et al., 2018).

In recent years, it is gaining increasing attention that probiotics was used as dietary supplements for the improvement of liver damage and diseases in animals. (Ayiku et al., 2020; Banerjee & Ray, 2017; Bu et al., 2019; Kong et al., 2020). Yeast culture (YC) a member of probiotic family possesses many immunomodulatory constituents (β- glucan, nucleotide and mannose

Abstract

The aim of this study was to investigate the underlying hepatoprotective effects of yeast culture (YC) against carbon tetrachloride (CCl₄)- induced hepatic damage in *Pseudobagrus ussuriensis*. The fish were randomly divided into three experimental groups (Control, CCl₄ and YC+CCl₄) with three replicates of 30 fish in each replicate. Firstly, the Control and CCl₄ groups were fed basal diet without yeast culture, and the YC+CCl₄ group was fed diet with 20 g/kg YC for 8 weeks. After the end of feeding experiment, Control group was intraperitoneally injected olive oil with 0.05 ml/15 g fish body weight, while CCl₄ and YC+CCl₄ groups were intraperitoneally injected CCl₄ olive oil solution (CCl₄: olive oil = 3:7) with 0.05 ml/15 g fish body weight for 48 h. The results indicated that fish fed with 20 g/kg yeast culture not only ameliorated injured hepatic cell, as evidenced by well- preserved liver architecture, but also significantly decreased plasma AST activity in the CCl₄- induced hepatic injury model. Next, we found that dietary 20 g/kg YC supplementation could improve hepatic antioxidant activity and inhibit lipid peroxidation induced by CCl₄. Fish fed 20 g/kg YC could suppress the decrease of plasma IgM and plasma ACH50 content caused by CCl₄ ($p < .05$). In addition, we also found that fish treated with CCl₄ up- regulated the expression of immune- related genes (TLR2, MyD88 and NF- κBp65), proinflammatory cytokines (IL- 1β and IL- 8) and Hsp70 mRNA expression in liver compared with the Control group; meanwhile, fish fed with 20 g/kg YC down- regulated the above- mentioned genes expressions in liver compared with CCl₄ group. In general, the results mentioned above suggested that the dietary yeast culture could relieve the oxidative stress, immune damage and liver injury induced by CCl₄ and could also suppress CCl₄- induced inflammation through inhibiting the TLR2/NF- κB signalling pathway.

KEYWORDS

antioxidant capacity, carbon tetrachloride, NF- κB signalling pathway, *Pseudobagrus ussuriensis*, TLR2, yeast culture

oligosaccharide) and antioxidant substances (B- vitamin, glutathione) (Jensen et al., 2008; Liu et al., 2004; Xiong et al., 2017), respectively. Previous studies have demonstrated that yeast culture or yeast hydrolysate as dietary supplements could improve growth performance, immune response, intestinal health, antioxidant capacity and disease resistant in aquatic animals (Ayiku et al., 2020; Bu et al., 2019; Cheng et al., 2019; Jin et al., 2018; Zhang et al., 2018). The above- mentioned positive effect was probably attributed to the β- glucan and mannose oligosaccharide in YC, which could inhibit the colonization of pathogens in the intestine, improve the structure of intestinal flora. In addition, β- glucan as immune regulatory ligand could co- act with immune- receptors such as Toll- like receptors (TLRs) to elicit a series of immune cells containing dendritic cells, macrophages, monocytes, natural killer cell and neutrophils resulting in various immune responses (Dalmo & Bøgwald, 2008; Goodridge et al., 2009). TLRs identified as a mediator of inflammatory response may associate with their corresponding adaptor molecules myeloid differentiation factor 88 (MyD88) to active downstream signal, eventually stimulate the transcription of nuclear factor κB (NF- κB), thereby inducing the expression of proinflammatory cytokines such as interleukin- 1β (IL- 1β) and interleukin- 8 (IL- 8) (Sun et al., 2017;

Thompson & Locarnini, 2007; Zhang et al., 2017). TLR2 plays a crucial role in innate initiating immune responses and influences subsequent adaptive immune responses (Fan et al., 2015). Growing number of researches have proved that the liver damage induced by CCl₄ may be correlated with the up-regulation of TLR2/NF- κ B signalling pathway (Gan et al., 2018). And the inhibition of this pathway by dietary yeast culture supplementation could alleviate liver injury and inflammatory response induced by gossypol in *Pseudobagrus ussuriensis* (Bu et al., 2019). Nevertheless, it is still lack of enough information about the effectiveness and the associated mechanisms of YC as dietary additives to relieve hepatic damage.

Pseudobagrus ussuriensis is an important indigenous species in China and East Asia because of its great economic value and better productive performance. However, this fish is facing the problem of immune suppression and liver damage with the development of intensive farming. To date, some experiments about dietary nutrition requirement of this fish have been conducted and the effect of different vegetable protein (soybean meal, vegetable meal, cotton meal and corn protein) and animal protein (meat and bone meal, mussel meal) replacing fish meal had been studied in our laboratory (Bu et al., 2017; Luo, 2019; Wang et al., 2020). Moreover, a preliminary study about the health function of YC on *Pseudobagrus ussuriensis* has been explored (Bu et al., 2019). But little information is available on YC against liver damage of *Pseudobagrus ussuriensis*. Thus, the objectives of this study were to investigate the effects of dietary yeast culture against CCl₄-induced liver damage in *Pseudobagrus ussuriensis*.

2 | MATERIAL AND METHODS

2.1 | Reagents

Yeast culture was purchased from Beijing Enhalar International Tech Co., Ltd. (Beijing, China). CCl₄ (analytical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and dissolved in olive oil to generate CCl₄ solution (CCl₄: olive oil = 3: 7).

2.2 | Diets and feeding management

To investigate the underlying protective mechanism of yeast culture against CCl₄ challenge, growth experiment was conducted ahead of the CCl₄ challenge trial. The basal diet and experimental diet with 20 g/kg yeast culture were formulated (Table 1), and referred as control and YC, respectively. The diet was produced according to the method described by Bu et al. (2019) and stored at -20°C until used. The diets ingredients were determined based on AOAC procedures. And the dry matter of diet was measured by desiccation at 105°C until constant weight. Crude protein was measured by Auto Kjeldahl System (2300- Auto- analyzer; FOSS, USA) according to the Kjeldahl method. Crude lipid was measured using Soxhlet extraction. Ash was measured by placing the sample in a muffle furnace at 550°C for 24 h. Gross energy was measured by the adiabatic bomb calorimeter (Parr 6300, USA).

TABLE 1 Formulation and chemical composition of the test diet (g kg⁻¹ dry matter)

Ingredients	Diet	Diet
	Control	YC
Fish meal ^a	280	260
Soybean meal ^b	300	300
Cottonseed meal ^c	110	110
Corn gluten meal ^d	70	70
Wheat meal	164	164
Soybean lecithin	10	10
Soybean oil	33	33
Vitamin premix ^e	5	5
Mineral premix ^f	5	5
Choline	3	3
Ca(H ₂ PO ₄) ₂	20	20
Yeast cultured ^g	0	20
Proximate composition (g kg ⁻¹ dry matter)		
Dry matter	932.7	928.0
Crude protein	458.3	455.5
Crude lipid	77.7	76.4
Gross energy (kJ g ⁻¹)	184.9	186.0
Ash	121.3	115.3

^a Fish meal: crude protein: 645.0 g kg⁻¹, crude lipid: 85.0 g kg⁻¹.

^b Soybean meal: crude protein: 467.9 g kg⁻¹, crude lipid: 31.4 g kg⁻¹.

^c Cottonseed meal: crude protein: 482.0 g kg⁻¹, crude lipid: 14.7 g kg⁻¹.

^d Corn gluten meal: crude protein: 602.0 g kg⁻¹, crude lipid: 14.7 g kg⁻¹. These ingredients were supplied by Huada feed Co., Ltd. (Harbin, China).

^e Vitamin premix (IU or mg kg dry diet): retinol (VA) 3000 IU; cholecalciferol (VD) 1500 IU; tocopherol (VE) 40 mg; menadione (VK) 4.5 mg; thiamin (VB1) 8 mg; riboflavin (VB2) 8.5 mg; pyridoxine (VB6) 6.5 mg; cyanocobalamin (VB12) 0.02 mg; nicotinic acid 45 mg; nicotinamide 45 mg; D- Ca pantothenate 17 mg; inositol 40 mg; biotin 0.15 mg; folic acid 1.3 mg; antiscorbic acid 110 mg.

^f Trace mineral mixture use providing the following concentration (mg kg⁻¹ dry diet): copper 6.5 mg; iron 45 mg; selenium 0.35 mg; zinc 70 mg; manganese 8.5 mg; magnesium 100 mg; cobalt 1 mg; iodine 1.2 mg.

^g Yeast culture: crude protein: 487.0 g kg⁻¹, crude lipid: 42.1 g kg⁻¹, supplied by Beijing Enhalar International Tech Co., Ltd. (Beijing, China).

Fish were obtained from Fisheries Research Institute of Harbin Academy of Agricultural Sciences (Harbin, China), and were fed the control diet for 2 weeks to acclimate to the experimental diets before starting the feeding

trial. After fasting for 24 h, the fish randomly allotted to three groups (Control, CCl₄ and YC+CCl₄) in triplicate (1.0 × 0.5 × 0.8 m, water depth 50–60 cm) with 30 fish to each aquarium. The Control and CCl₄ groups were fed control diet, and YC+CCl₄ group were fed YC diet. All fish were hand-fed two times (08:30 and 16:30) a day to apparent satiation for 8 weeks. During the experimental period, the flow rate of water in each tank was maintained at 2.4 L min⁻¹ and the water conditions were dissolved oxygen >6 mg L⁻¹, temperature 24 ± 1°C pH 7.0 ± 0.5, respectively.

The experimental photoperiod was set at 12- h light and 12- h dark.

2.3 | CCl₄ challenge and Sample collection

After the 8- week feeding trial, the fish in each tank were weighed and collected for CCl₄ challenge. Fish in the Control group were intraperitoneally injected with olive oil (0.05 ml/15 g bodyweight) for 48 h. Fish in CCl₄ and YC+CCl₄ groups were intraperitoneally injected with CCl₄ solution (0.05 ml/15 g bodyweight) for 48 h. At the end of challenge trial, three fish from each tank were anaesthetized with eugenol (1:12,000) (Shanghai Reagent Corporation, Shanghai, China) and used for the collection of blood and liver samples. The blood samples were collected via caudal vein into tubes containing 32 g/kg sodium citrate and then centrifuged at 4000 g for 10 min and stored at -80°C until used. The liver was immediately frozen in liquid nitrogen and stored frozen at -80°C until analysed.

2.4 | Liver histology

One portion of liver tissue per fish was obtained and stained according to the method of Chunhua and Hongyan (2017). The slides were stained with the conventional haematoxylin– eosin (HE) protocol, mounted with neutral resin and examined with optical microscopy.

2.5 | Plasma biochemical index

The alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AKP), albumin (ALB) and lysozyme (LZM) were determined by commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, China). The plasma immunoglobulin M (IgM) and alternative complement pathway activity (ACH50) level were measured by the kit of enzyme- linked immunosorbent assay as described by Yu et al. (2014).

2.6 | Hepatic antioxidant status analysis

Liver tissues were homogenized and centrifuged according to the method of Bu et al. (2019). Superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities, and hepatic malondialdehyde MDA content were determined using commercial kits according to the instructions of the kit (Nanjing Jiancheng Bioengineering Institute, China).

2.7 | Real- time polymerase chain reaction

(PCR) analysis

The total RNA from each liver was extracted with *TransZol Up Plus Kit* (TransGen Biotech, China) according to the manufacturer's instructions. The quantity and concentration of RNA were measured on an ultra- micro spectrophotometer (Implen, Germany), respectively. The cDNA was synthesized using *TransScript® One- Step gDNA Removal and cDNA Synthesis SuperMix* (TransGen Biotech, China) according to the manufacturer's instructions. According to the reference (Bu et al., 2019), the primers were designed and showed in Table 2. β - actin was used as the internal control gene. Real- time PCR assays were performed on Applied Biosystems® 7500 (USA) in a final volume of 20 μ l with 2 × *TransStart® Top Green qPCR SuperMix* (TransGen Biotech, China) and ROX Reference Dye II following the manufacturer's instruction. The real- time PCR condition uses the following temperature profile: 30 s at 94°C, 5 s at 94°C for 40 cycles, 30 s at 60°C. The gene expression level was determined by 2^{- $\Delta\Delta$ CT} method (Livak & Schmittgen, 2001).

2.8 | Statistical analysis

Data in this paper were statistical analysed by SPSS 25, and the results are presented as means ± standard error of means (SEM) with superscript letters indicating differences between groups. All data were checked for homogeneity of variance using Levene's equal variance test. One- way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was chosen to determine the significant differences among the means when the data met the homogeneity of variance. In addition, the Kruskal– Wallis test was used to determine the significant differences among the means when the data did not pass homogeneity of variance test. The value of statistical significance was set at $p < .05$.

3 | RESULTS

3.1 | Growth

The growth performance parameters in different groups are shown in Table 3. As expected, there was no difference in the initial body weight among three groups. After feeding trial, final body weight, weight gain (WG), feed intake (FI) and specific growth rate (SGR) in YC+CCl₄ group were significantly higher than Control and CCl₄ groups ($p < .05$). And the survival rate (SR) and feed conversion ratio (FCR) were not significantly different among three groups ($p > .05$).

3.2 | Histological observation of liver

Regular morphological structure of liver tissue was observed in the control group (Figure 1A). The administration of CCl₄ caused histopathological changes in the liver such as hepatocyte swelling, vacuolar degeneration, necrosis and nuclei shifting to the cellular periphery. (Figure 1B). Meanwhile, the structure of cell membrane was incomplete in CCl₄ group. However, fish fed yeast culture showed a lower occurrence rate of above histopathological symptoms compared with those fish in CCl₄ group (Figure 1C).

3.3 | ALT and AST activities

Plasma ALT and AST activities are shown in Figure 2. After treated with CCl₄ for 48 h, the plasma activities of AST ($p > .05$) and ALT ($p < .05$) in CCl₄ group were higher than that in Control group, and ALT activity was significantly different between the above-mentioned two groups. Compared with CCl₄ group, the AST and ALT activities in YC+CCl₄ group were reduced, but AST activity in YC+CCl₄ group was significantly lower than that in CCl₄ group ($p < .05$).

3.4 | Antioxidant capacity

As shown in Table 4, the MDA content in CCl₄ group was significantly increased than the Control group after treated with CCl₄ ($p < .05$).

MDA content in YC+CCl₄ group was reduced than that in CCl₄ group, but there was no significant difference ($p > .05$). The determination of liver SOD activity showed that the activity in CCl₄ group was significantly reduced compared with that in Control group ($p < .05$). But the activity of SOD in YC+CCl₄ group was significantly higher than that in CCl₄ group ($p < .05$). Simultaneously, there was no significant difference in the GPX activity among three groups ($p > .05$), but there was an improvement for that of fish in YC+CCl₄ group compared with that of fish in CCl₄ group. Those results showed that dietary yeast culture supplementation could alleviate *Pseudobagrus*

Primer names	Sequence (5'-3')	Product (bp)	Tm (°C)	Reference or accession number
TLR2- F TLR2- R	TTGTACAGCTGGATGAGTTG TGTCGTCACTGAAATGTCTC	206	54	Bu et al. (2019)
MyD88- F MyD88- R	TCAGACAGCTGGAGCAGACA CGCTGGTGATGGTCCAAACA	93	59	Bu et al. (2019)
NF- κB p65- F NF- κB p65- R	AAGAACCAGCCATACAAGCCACAC TCAGGCAGGTCCGCTTCGTAG	83	60	Bu et al. (2019)
IL- 1β- F IL- 1β- R	CCTGAACACCTTCGAGTCGG AGGTGGCTGGTTTGCTGATT	102	58	Bu et al. (2019)
IL- 8- F IL- 8- R	ATCGAAGGAAAAGCAGAGCG CTTTCACAGGAGCCACTTG	111	57	Bu et al. (2019)
Hsp70- F Hsp70- R	GACTGTCCTGATCAACGCAAC TGGCTCTTTCACCTCATACACG	116	59	XM027173973
β- actin- F β- actin- R	CCTCCGCTCGGATTTGGCTG TCAAGGGCGACGTAGCAGAG	141	60	Bu et al. (2019)

Abbreviations: Hsp70, heat shock protein 70; IL- 1β, interleukin- 1β; IL- 8, interleukin- 8; MyD88, myeloid differentiation factor 88; NF- κB p65, nuclear factor kappa- B p65; TLR2, toll- like receptor 2.

TABLE 3 Growth performance of *Pseudobagrus ussuriensis* in different groups

Index	Control	CCl ₄	YC+CCl ₄
IBW (g) ^a	180.15 ± 0.55	180.7 ± 0.00	180.46 ± 0.38
FBW (g) ^b	409.43 ± 5.13 ^b	405.30 ± 1.00 ^b	448.10 ± 3.77 ^a
WG (%) ^c	127.28 ± 3.54 ^b	124.29 ± 0.55 ^b	148.29 ± 1.56 ^a
SGR (%) ^d	1.47 ± 0.03 ^b	1.44 ± 0.04 ^b	1.62 ± 0.01 ^a
SR (%) ^e	95.00 ± 1.67	98.33 ± 1.67	94.44 ± 2.93
FI (%) ^f	1.47 ± 0.01 ^b	1.50 ± 0.04 ^b	1.57 ± 0.01 ^a
FCR ^g	1.22 ± 0.02	1.27 ± 0.03	1.19 ± 0.01

Note: Data represent as mean ± SEM. Mean with different superscripts in the same row is significantly different ($p < .05$). ^a IBW, initial body weight. ^b FBW, final body weight.

^c WG, weight gain (%) = $100 \times (\text{final body weight} - \text{initial body weight}) / (\text{initial body weight})$. ^d SGR, Specific growth rate (%) = $100 \times (\ln(\text{final body weight}) - \ln(\text{initial body weight})) / \text{days}$. ^e SR, Survival Rate (%) = $100 \times \text{final fish number} / \text{initial fish number}$.

^f FI, Feed intake (% per day) = $100 \times \text{dry feed intake} / [(\text{initial body weight} + \text{final body weight}) / 2 \times t]$. ^g FCR: Feed conversion ratio = feed consumed / weight gain.

ussuriensis liver damage induced by CCl₄ to a certain extent, which may be related to its functions of anti- stress ability and anti- lipid peroxidation.

3.5 | Immune ability

Compared with control group, the lysozyme activity in CCl₄ group was significantly increased ($p < .05$), while the activity in YC+CCl₄ group was significantly decreased compared with that in CCl₄ group ($p < .05$) (Figure 3). The IgM ($p < .05$) and ACH50 ($p > .05$) content in CCl₄ group was decreased compared with the control group. But compared with CCl₄ group, the plasma ACH50 in YC+CCl₄ group was significantly increased and IgM

content also increased with no significant difference. There were no significant differences in AKP activity among three groups ($p > .05$).

3.6 | Gene expression

After treated with CCl₄, the expression levels of hepatic TLR2, MyD88 and NF- κ Bp65 gene in CCl₄ group were significantly increased compared with Control group ($p < .05$) (Figure 4). The

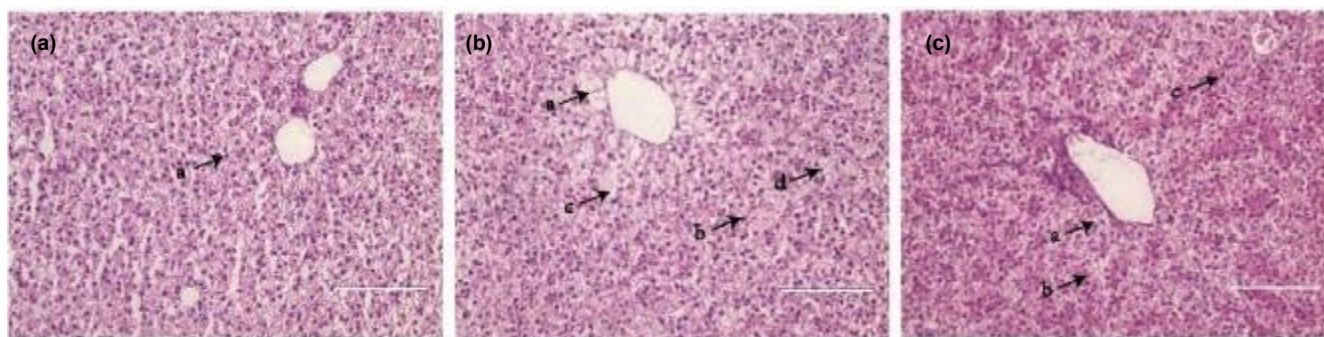


FIGURE 1 (A) Liver structure of fish in control group: (a) hepatocyte (H & E). (B) Liver structure of fish in CCl₄ group: (a) cell membrane lysis, (b) karyolysis, (c) hydropic degeneration, (d) nuclei shifting to the cellular periphery. (C) Liver structure of fish in YC+CCl₄ group: (a) hydropic degeneration, (b) nuclei shifting to the cellular periphery, (c) karyolysis. Scale bar = 100 μ m

FIGURE 2 The effect of dietary YC on ALT and AST activities in plasma of CCl₄-treated *Pseudobagrus ussuriensis*. Vertical bars represented the means $\pm$ SEM of three replicates. Different letters in each figure represented significant difference among dietary treatments ($p < .05$). ALT, alanine transaminase; AST, aspartate transaminase

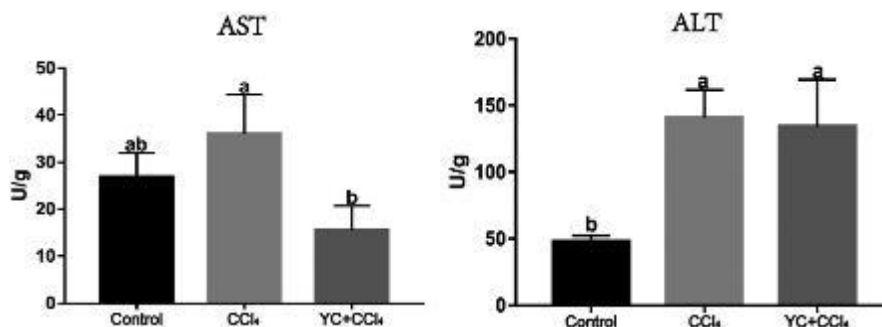


TABLE 4 The effect of YC on antioxidant capacity in liver of CCl₄-treated *Pseudobagrus ussuriensis*

Index	Control	CCl ₄	YC+CCl ₄
SOD (U mg ⁻¹)	23.39 $\pm$ 0.69 ^a	20.46 $\pm$ 0.86 ^b	23.32 $\pm$ 0.80 ^a
MDA (nmol mg ⁻¹)	0.24 $\pm$ 0.02 ^b	0.37 $\pm$ 0.02 ^a	0.32 $\pm$ 0.01 ^a
GPX (U mg ⁻¹)	20.56 $\pm$ 0.90	17.62 $\pm$ 0.61	19.46 $\pm$ 2.43

Note: Data represent as mean $\pm$ SEM. Mean with different superscripts in the same row is significantly different ($p < .05$).

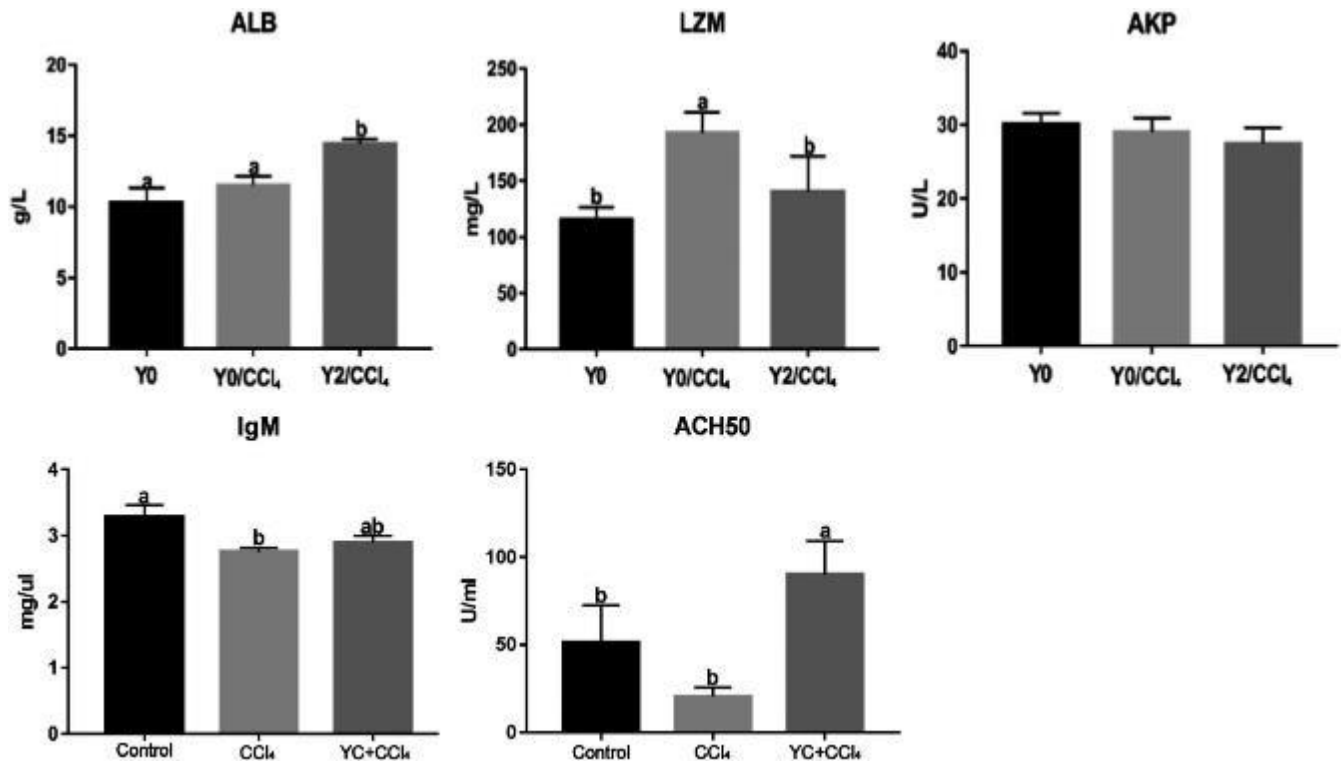


FIGURE 3 The effect of YC on immune ability of hepatic injury induced by CCl₄ in *Pseudobagrus ussuriensis*. Vertical bars represented the means $\pm$ SEM of three replicates. Different letters in each figure represented significant difference among dietary treatments ($p < .05$). ACH50, alternative complement pathway activity; ALB, albumin; AKP, alkaline phosphatase; IgM, immunoglobulin M; LZM, lysozyme

decrease of TLR2, MyD88 and NF- κ Bp65 was found in YC+CCl₄ group compared with CCl₄ group, but hepatic NF- κ Bp65 gene expression level in YC+CCl₄ group was significantly reduced ($p < .05$). The expression levels of hepatic IL-8 and Hsp70 gene in CCl₄ group was significantly increased compared with the Control group ($p < .05$). But compared with CCl₄ group, the hepatic IL-8 and Hsp70 gene in YC+CCl₄ group were significantly decreased ($p < .05$). The hepatic IL-1 β gene expression level was not significantly different among three groups ($p > .05$).

The aim of this study was to investigate the hypothesis that yeast culture as an additive may have a protective effect against CCl₄-induced liver damage in *Pseudobagrus ussuriensis*. CCl₄ is one of the most commonly used hepatotoxins in the study of acute liver injury. Metabolism of CCl₄ produces the trichloromethyl free radical (CCl₃•) and trichloromethyl peroxy radical (CCl₃OO•) (Dong et al., 2013). And CCl₃OO• attacks and destroys polyunsaturated fatty acids, which initiates the chain reaction of lipid peroxidation resulting in the exudation of soluble cytoplasmic enzymes and increased serum AST and ALT (Al-Harbi et al., 2014). Serum enzymes such as AST and ALT are employed in the evaluation of hepatic disorders, and the increase of these enzyme activities in serum reflects acute liver damage and inflammatory hepatocellular disorder. The present

4 | DISCUSSION

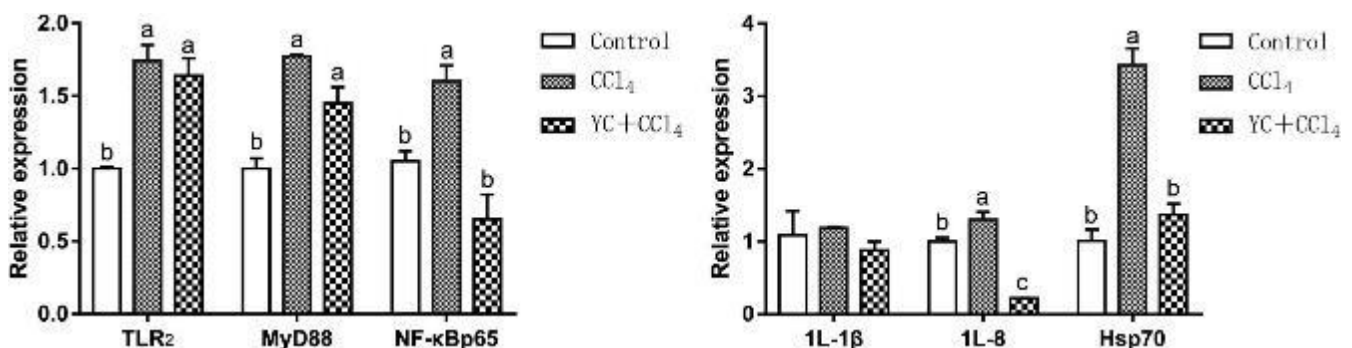


FIGURE 4 The effect of YC on hepatic inflammation related genes expression in CCl₄-treated *Pseudobagrus ussuriensis*. Vertical bars represented the means $\pm$ SEM of three replicates. Different letters in each figure represented significant difference among dietary treatments ($p < .05$). IL-1 β , interleukin-1 β ; IL-8, interleukin-8; MyD88, myeloid differentiation factor 88; NF- κ Bp65, nuclear transcription

results showed that CCl₄ administration caused severe acute liver damage in *Pseudobagrus ussuriensis* as evidenced by elevation of serum ALT ($p < .05$) and AST ($p > .05$) activities and classical histopathological changes, which is similar with previous reports that CCl₄ solution (3 ml/kg b.w., 1:1 in groundnut oil) can increase the activities of serum ALT and serum AST and change the hepatic histopathology in rat (Singh et al., 2008). However, when YC was supplemented in groups exposed to CCl₄, it was seen that serum AST activities dropped to a level similar to that of control in this study. Similar results were also reported by Bu et al. (2020), in which Ussuri factor κB p65; TLR2, toll-like receptor 2

catfish (*Pseudobagrus ussuriensis*) prefed diet with 10 g/kg yeast culture significantly decreased serum AST activities and alleviate the gossypol-induced liver damage.

The first line of defence against free radical consists of various endogenous antioxidants such as SOD, GPX, which maintains a complex immune response in animals. Their activities could reflect the antioxidant capacity for removing superoxide radicals and assisting the recovery of normal physiological functions of the cells (Yousefi et al., 2020). CCl₄ metabolite CCl₃O₂• could suppress SOD, GPX activities, resulting in the accumulation of large amounts of radicals and the lipid peroxidation product MDA, which lead liver cell necrosis, further aggravating liver damage. Previous study reports that the fish (*Cyprinus carpio* var. Jian) treated with CCl₄ can significantly increase hepatic MDA level and reduce SOD activity (Cao et al., 2015). In this study, CCl₄ had a decreasing effect on the activities of hepatic SOD and GPX; nevertheless, incorporation of yeast culture into diets restored the activities of antioxidant enzymes and resulted in a decline in the MDA content. Some studies have indicated that oligosaccharides, glutathione, vitamins and β -glucan could increase the antioxidant activity and regulate immune response (Liu et al., 2020; Lu et al., 2019; Takavar & Mandieh, 2019). Thus, it can be presumed that the protective effect of YC against CCl₄-induced liver damage in *Pseudobagrus ussuriensis* may be related to the mannose oligosaccharides (MOS), vitamins, β -glucan, glutathione and other antioxidants in yeast culture. The antioxidative effect of those substances (oligosaccharides, vitamins, β -glucan and glutathione) has been reported in crabs (*Eriocheir sinensis*), gibel carp (*Carassius auratus gibelio* CAS) and yellow drum (*Nibea albiflora*), and these studies have demonstrated that the above-mentioned substances can improve antioxidant ability by increasing SOD and GPX activities and reducing MDA production (Liu et al., 2020; Lu et al., 2019; Wang et al., 2019; Zhang et al., 2018).

The innate immune system of fish is an essential defence mechanism providing the first line of defence to against pathogens for the host (Ke & Zhang, 2019). Recently, in order to maintain fish health and improve disease resistance, immunostimulants, probiotics and nucleotides have been used as dietary additives to improve the immunity and resist the diseases (Vallejos-Vidal et al., 2016; Zhao et al., 2020). In this paper, indicators of innate immunity including plasma IgM, ACH50, LZM, AKP and ALB were evaluated to explore the adverse influence of CCl₄ on immunity

of *Pseudobagrus ussuriensis* and the effects of yeast culture against immune damage induced by CCl₄.

IgM level and ACH50 activity as good biomarkers for evaluating the immune status may be adversely affected by toxicants in aquatic animals (Sharifian et al., 2015; Wang et al., 2014). In the current study, a reduction in IgM and ACH50 was found in the group exposed to CCl₄ alone, which may be considered as a damage to immune system. These results provide further support for the fact that CCl₄ may lead to the disorder of immune system in *Pseudobagrus ussuriensis*. Furthermore, fish in YC+CCl₄ group had higher IgM levels ($p > .05$) and ACH50 activities ($p < .05$) than fish in CCl₄ group, suggesting that fish prefed yeast culture could relieve the immune damage response induced by CCl₄ for *Pseudobagrus ussuriensis*. These results probably were demonstrated by the positive effect from the dietary addition of β -glucan as the main component of yeast culture, which could stimulate the complement activities. Some studies showed that the fish (*Oreochromis niloticus*) fed β -glucan had significantly higher ACH50 and IgM contents than the fish in control group (Amphan et al., 2018; El-Murr et al., 2019).

Albumin, the most abundant plasma protein, is primarily synthesized in liver. In this paper, fish prefed 20 g/kg yeast culture had a higher ALB level than fish in Control and CCl₄ groups. Similar results were also reported by Hardy et al. (2020), in which juvenile Nile tilapia (*Oreochromis niloticus*) fed diet with 10 or 30 g/kg yeast hydrolysate had markedly higher ALB than those of fish fed diets without yeast hydrolysate. Albumin also acts as a leading carrier for some cations, nutrients, hormones and metabolites and plays a role in tissue repairment. The body's physiological function would be affected by the concentration of ALB in plasma with the transportation of nutrients and hormones to the tissues and organs according to the requirement. Thus, we assume that the elevation of ALB was also responsible for the alleviation of liver damage, where it would accelerate the transportation of nutrients and hormones to meet the requirements of metabolic activities.

Moreover, lysozyme as a bactericidal enzyme is an important component of the immune system that can degrade pathogenic bacteria by breaking down their structure of cell wall (Hauge et al., 2002; Lushchak et al., 2001). In this study, the fish in CCl₄ group had higher LZM activity than fish in Control group. Similar results were also reported by Demers and Bayne (1997), in which the plasma lysozyme activity was significantly increased briefly in Rainbow trout (*Oncorhynchus mykiss*) treated with handling stressor, and the reason might be attributed to that the acute stress may help humoral components of innate defences where it is most needed.

Several researches have indicated that TLRs interact with myeloid differentiation factor 88 (MyD88), an adaptor molecule shared by all the members of TLRs except for TLR3, 21, 22 to activate NF- κB and induce several types of cytokines, such as interleukin, tumour necrosis factor and chemokines (Chou et al., 2019; Montero et al., 2008; Seki et al., 2005). Previous study indicated that TLR2 and its downstream signalling are stimulated by CCl₄-induced oxidative stress, as determined by the remarkable up-regulation of TLR2, MyD88 and NF- κB mRNA expression in rat (Gan et al., 2018). In present study, we found that the expression of TLR2, MyD88 and NF- κB p65 mRNA was significantly enhanced in CCl₄ group compared with Control group, and YC supplementation reversed the CCl₄-induced up-regulations of TLR2, MyD88 and NF- κB p65. Those data

indicated that dietary yeast culture supplementation may relieve the hepatic damage of Ussuri catfish through inhibiting TLR2/NF- κ B signalling pathway. NF- κ B a transcription factor could up-regulate the release of inflammatory cytokines including IL-8 and IL-1 β , which may further perpetuate the inflammatory cascades resulting in liver damage (Jung et al., 1995). In this paper, fish in CCl₄ group had higher hepatic IL-1 β ($p > .05$) and IL-8 ($p < .05$) mRNA expression than fish in Control group; however, hepatic IL-1 β ($p > .05$) and IL-8 ($p < .05$) mRNA expressions of fish in YC+CCl₄ group were lower than fish in CCl₄ group, suggesting that CCl₄ may have a stimulatory action upon proinflammatory processes and dietary yeast culture supplementation, to some extent, inhibited the inflammatory response.

Heat shock proteins 70 (HSP70), a member of heat shock proteins (HSPs) family, are engaged in overall proteome maintenance while under conditions of environmental alterations (Song et al., 2007). At an early stage after CCl₄ administration, transient increases in the gene expression of the stress-inducible HSP70 are preceded (Schiavonati & Tiberio, 1997). Furthermore, Song et al. (2007) reported that HSP70 knock out mice injected with CCl₄ showed higher alanine transaminase level and a more severe degree of neutrophilic infiltration and necrosis than those of wild-type mice injected with CCl₄. In this study, the significant increase was observed in case of hepatic HSP70 mRNA in CCl₄ group ($p < .05$), and hepatic HSP70 mRNA expression of fish in YC+CCl₄ group was significantly lower than fish in CCl₄ group ($p < .05$). These data suggested that yeast culture could have protective role against the hepatic damage induced by CCl₄.

5 | CONCLUSIONS

In conclusion, fish injected CCl₄ olive oil solution could induce inflammatory response, reduce antioxidant capacity and destroy hepatocyte structure. Dietary yeast culture supplementation could relieve the oxidative stress, immune damage and liver injury induced by CCl₄ and could also suppress CCl₄-induced inflammation through inhibiting the TLR2/NF- κ B signalling pathway.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Effects of dietary ESTAQUA® yeast culture supplementation on growth, immunity, intestinal microbiota and disease-resistance against *Vibrio harveyi* in hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*E. lanceolatus*)

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ABSTRACT

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This study investigated the effects of dietary supplementation of ESTAQUA® yeast culture (YC) on the growth, immunity, intestinal microbiota, and disease-resistance against *Vibrio harveyi* in hybrid grouper (*♀Epinephelus fuscoguttatus* × *♂E. lanceolatus*) at 0% (C0), 2% (N1), and 4% (N2). After 8 weeks of the feeding trial, the results indicated that the survival rate (SR), weight gain rate (WGR), and specific growth rate (SGR) of hybrid grouper were significantly higher in the N1 (2%) and N2 (4%) group than those in the control group ($P < 0.05$). Serum superoxide dismutase (SOD), catalase (CAT), total protein (TP), alkaline phosphatase (AKP), acid phosphatase (ACP), and immunoglobulin (IgM) activities were significantly higher in the N2 (4%) group ($P < 0.05$). Contrary to the control, aspartate aminotransferase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA), and glucose (GLU) were notably decreased in the N2 (4%) group ($P < 0.05$). The activity of intestinal digestive enzymes was significantly higher in the experimental groups than that in the control group ($P < 0.05$). Furthermore, at the phylum level, the relative abundance of *Actinobacteria* and *Bacteroidetes* was remarkably higher in the N2 (4%) group than that in the control group ($P < 0.05$). The relative abundance of *Blautia* and *Lactobacillus* was notably higher in the N2 (4%) group at the genus level ($P < 0.05$). Hybrid grouper in the N1 (2%) and N2 (4%) groups effectively improved intestinal villus height and width ($P < 0.05$). Lastly, the protection against *V. harveyi* challenge in hybrid grouper was enhanced in the YC treatment group, while N2 (4%) experienced the highest cumulative SR of 80%. In conclusion, the N2 (4%) treatment group is suggested as the best to improve the growth and immunity of hybrid grouper.

1. Introduction

Due to high market demand, grouper farming has been well- established in China (He et al., 2017). Unfortunately, intensive grouper farming may lead to the outbreak of diseases, which causes substantial economic losses to the grouper farming industry (Amaya et al., 2007; Jin et al., 2018). Using antibiotics is a common method to prevent and control fish infectious diseases. Nevertheless, the application of antibiotics could increase the growth and bioaccumulation of

resistant pathogens (Teuber, 2001; Yuan et al., 2019), which eventually leads to the transfer of resistant bacteria and the related factors from fish to humans (Leung et al., 2020). Therefore, people pay more attention to the use of natural immunostimulants and probiotics (such as yeast) as substitutes for traditional disease-control therapies has gained attention (Yuan et al., 2019). Probiotics, as a non-digestible feed ingredient, may influence the immunity of the host by regulating the gastrointestinal flora. It facilitates host health mainly through enzymatic digestion, host immunity enhancement, and the promotion of growth factors (Gupta

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et al., 2019).

The use of yeast products to improve fish growth and immune response has been a research hotspot in recent years. Yeast products can not only reduce the number of harmful bacteria in the intestinal tract by identifying, adsorbing, and eliminating the harmful bacteria, but also promote the reproduction of beneficial bacteria; moreover, it can and regulate the balance of micro-ecological zones in the animal's digestive tract, thus improving the intestinal tract's function, maintaining the intestinal tract's integrity, and improving the immunity of the animal organism (Zhou et al., 2009; Deng et al., 2013). Yeast culture (YC) is a complex yeast fermentation product composed of yeast and its growth medium. Currently, the high protein content solid fermentation culture method is generally used in the research of yeast protein feed. No non-protein nitrogen was added to the YC fermentation substrate in this study and the protein content was 50%. YC contains not only high levels of amino acids, protein content, and minerals, but also immunomodulatory factors (such as mannan oligosaccharides, β -glucan, and nucleotides) (Pongpet et al., 2016). The structural components of mannan-oligosaccharide (MOS) and β -glucan in the yeast cell wall help to promote immunity. β -glucan in YC can effectively lower cholesterol (CHO) and activate macrophages to release various immunoglobulins, such as cytokines and interleukin (Jayachandran et al., 2018). When fed with YC (12%) diet, the grass carp (*Ctenopharyngodon idella*) showed notably affected the metabolic capacity of intestinal microflora, along with good growth performance (Liu et al., 2018). MOS can block the recognition and adhesion mechanism of potential pathogens to host tissue surface molecules (competitive attachment sites), thereby improving the integrity and performance of the intestinal epithelial barrier (Gainza and Romero, 2020). Previous researchers have proved that MOS can enhance fish growth and regulate intestinal microbiota and gut morphology (Dimitroglou et al., 2010; Yuan et al., 2019). Moreover, it has

shown that the addition of YC exerts a positive impact on the digestibility, feed intake, growth rate, and feed efficiency of aquatic animals (Essa et al., 2011; Berto et al., 2016). The study showed that dietary addition of 1% yeast hydrolysate or beer yeast to shrimp could improve the innate immunity and promote the growth of shrimp, as well as strengthen the resistance of shrimp to ammonia nitrogen stress (Jin et al., 2018). The addition of YC to feed helps to regulate the intestinal bacterial community of grass carp, thereby increasing growth performance and immune response (Liu et al., 2018).

Hybrid grouper is a carnivorous marine fish, which is a cross-breed between the giant grouper (*Epinephelus lanceolatus*) and brown- marbled grouper (*Epinephelus fuscoguttatus*) (Wang et al., 2020; Yin et al., 2020). Due to the high feed conversion ratio, fast growth rate, high palatability, and good market value, it has been widely cultured in many Asian countries, including China (Ye et al., 2019). A study has validated that mannan oligosaccharides could improve immunity response, growth, and resistance to grouper disease (Reyes-Becerril et al., 2011). However, it remains elusive whether the dietary addition of YC can improve grouper's growth performance, immune response, and intestinal health. Against this backdrop, the present study aimed to evaluate the effects of dietary supplementation of YC on the growth performance, immune response, intestinal morphology, intestinal microbiota, and disease- resistance against *V. harveyi* attacks in hybrid grouper.

2. Materials and methods

2.1. Diet preparation

The components and nutrient contents of the basal diet are shown in Table 1. In the present study, there were three iso-nitrogenous and iso- lipidic experimental diets with increasing YC levels at 0%, 2%, and 4%, designated as

C0 (0%), N1 (2%), and N2 (4%), respectively. The YC (fermentation substrate free of non-protein nitrogen; 50% protein content) was provided by Enhalar Biotechnology Company (Beijing, China). Wheat gluten, casein, and fishmeal (60% crude protein) were used as

Table 1

Ingredients and proximate composition of the experimental diets (DM %).

Ingredients	C0 (0%)	N1 (2%)	N2 (4%)
Fish meal ^a	34.00	32.50	31.00
Yeast culture ^d Casein ^a	0.00	2.00	4.00
Wheat gluten ^a	25.00	25.00	25.00
Starch ^b	6.00	6.00	6.00
Fish oil ^a	18.00	18.00	18.00
Phospholipid ^a	8.00	8.00	8.00
Vitamin mixture ^b	1.00	1.00	1.00
Vitamin mixture ^b	0.50	0.50	0.50
Mineral mixture ^c	2.50	2.50	2.50
Choline chloride ^a	0.50	0.50	0.50
Antioxidants ethoxyquin ^a	0.03	0.03	0.03
Calcium Dihydrogen Phosphate ^a	1.00	1.00	1.00
Vitamin complex ^b	0.05	0.05	0.05
Attractant ^e	0.10	0.10	0.10
Yttrium oxide Y ₂ O ₃ ^a	0.1	0.10	0.10
Microcrystalline cellulose MCC ^a	3.22	2.72	2.22
Total	100	100	100
Nutrient levels Dry matter			
Crude protein	92.60	92.17	92.27
Crude lipid	50.57	50.91	50.61
Ash	11.25	11.13	11.11
	10.89	10.23	10.32

^a Ingredients purchased from Zhanjiang HaiBao Feed Factory, Zhanjiang, Guangdong, China. ^b Vitamin mixture (g/kg mixture): vitamin B1, 17.00 g; vitamin B2, 16.67 g; vitamin B6, 33.33 g; vitamin B12, 0.07 g; vitamin K, 3.33 g; vitamin E, 66.00 g; retinyl acetate, 6.67 g; VD, 33.33 g; nicotinic acid, 67.33 g; D-calcium pantothenate, 40.67 g; biotin, 16.67; folic acid, 4.17 g; inositol, 102.04 g; cellulose, 592.72 g (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China). All ingredients were diluted with corn starch to 1 kg.

^c Mineral mixture (mg/g mixture): CaCO₃, 350 g; NaH₂PO₄·H₂O, 200 g; KH₂PO₄, 200 g; NaCl, 12 g; MgSO₄·7H₂O, 10; FeSO₄·7H₂O, 2 g; MnSO₄·7H₂O, 2 g; AlCl₃·6H₂O, 1 g; CuCl₂·2H₂O, 1 g; KF, 1 g; NaMoO₄·2H₂O, 0.5 g; NaSeO₃, 0.4 g; CoCl₂·6H₂O, 0.1 g; KI, 0.1 g; zeolite powder, 219.9 g. All ingredients were diluted with corn starch to 1 kg. (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, and China). ^d The yeast culture was provided by Enhalar Biotechnology Company (Beijing, China). ^e Attractant composition:

taurine: glycine: betaine = 1:3:3.

protein sources, fish oil and phospholipid oil were used as lipid sources. After crushing all the coarse ingredients, they were sieved using a 60- mesh sieve, and then gradually stirred and mixed in a V-mixer type machine. Next, fish oil and phospholipid oil were added to the mixture, followed by further mixing by hand. After that, choline chloride- contained water was mixed into the mixture (nearly 30% of the net weight of the ingredient), which was then adjusted to a dough shape using a Hobart-type mixer. Next, it was processed into 2.5 mm particles with a diameter of 2.5 mm by a twin-screw extruder (F-26, South China University of Technology), dried (room temperature, 3–4 days) until the moisture was reduced to about 10%, and then refrigerated (20 °C) until use.

2.2. Fish and feeding regime

Hybrid groupers were purchased from a local fish farm hatchery (Zhanjiang, China) and transferred to the Marine Biological Research Base of Guangdong Ocean University. They were kept in fiberglass tanks for two weeks and fed with a commercial diet to adapt to the experimental conditions. After that, all fish fasted for 24 h. A total of 270 hybrid groupers (17.56 g ± 0.02) were randomly placed into 9 fiberglass tanks (250 L), 30 fish per tank. They were fed twice a day (9:00 and 17:00) with a feed intake of 5–8% of their average body weight in three replicates to apparent satiation for 8 weeks. About 60% of the water was refreshed every day to maintain water quality. During the feeding trial, was within 6the water temperature was 28.0–8 mg/L. ± 2.0 °C and the dissolved oxygen (DO)

2.3. Sample collection and calculations

At the end of the 8-week feeding trial, all fish fasted for 24 h before sampling. The total number of fish from each tank was recorded and they were weighed collectively. Two fish were randomly selected from each tank and stored at 20 °C and their dry matter, crude protein, ash, and lipid were calculated. Six fish were randomly selected from each tank and their blood was taken using a 1 mL syringe. The blood samples were stored at 4 °C for 12 h, followed by 3500 rpm centrifugation at 4 °C for 10 min. The supernatant was collected and stored at 80 °C for subsequent enzyme activity analysis. The midgut (0.8–1.2 cm) of three fish from each tank was taken under aseptic conditions and preserved in 10% paraformaldehyde for histological analysis. Moreover, three fish were randomly selected from each group and their midguts were taken, which were then carefully loaded into the 1.5 mL Eppendorf tube and immediately stored in liquid nitrogen for microflora analysis. The growth performance formulas were as follows:

Weight gain rate(WGR,%)=100

$$\times \frac{\text{final body weight} - \text{initial body weight}}{\text{initial body weight}} \times 100$$

$$\text{Feed conversion ratio(FCR)} = \frac{\text{final body weight} - \text{initial body weight}}{\text{feed intake(g)}}$$

Specific growth rate(SGR,%)=100

$$\times \frac{[\ln(\text{final weight}) - \ln(\text{initial weight})]}{\text{days of experiment}}$$

$$\text{Protein efficiency ratio(PER)} = 100 \times \frac{\text{fish weight gain}}{\text{crude protein intake(g)}}$$

$$\text{Survival rate(SR,\%)} = 100 \times \frac{\text{final fish number}}{\text{initial fish number}}$$

$$\text{Condition factor(CF,\%)} = 100 \times \frac{\text{body weight(g)}}{\text{body length(cm)}^3}$$

2.4. Analysis of diets and whole-body composition

The dry matter, crude protein, lipid, and ash of diet and whole fish were determined according to the methods of Lee (1995). The samples were dried in an oven at 105 °C to a constant weight to determine the dry matter. The crude lipid content was determined using the Soxhlet extraction method (YSXT-06, XiYang instrument co., Ltd., Shanghai, China), and the crude protein content was determined using the Kjeldahl method (Kjeltc 8400, FOSS Technology and Trade Co., Ltd., Beijing, China). Moreover, the crude ash content was determined by burning in a muffle furnace (SX2-2.5-10, QiuZuo Instrument and Equipment Co., Ltd., Shanghai, China) at 550 °C for 6 h.

2.5. Serum biochemical parameters and intestinal enzyme activity analysis

Fish serum was collected. Total protein (TP, A045-2-2), malondialdehyde (MDA, A003-1-2), aspartate aminotransferase (AST, C010-2-1), alanine aminotransferase (ALT, C009-2-1), lysozyme (LZM, A050-1-1), alkaline phosphatase (ALP, A059-2-2), immunoglobulin M (IgM, H109), acid phosphatase (ACP, A060-2-2), catalase (CAT, A007-1-1), superoxide dismutase (SOD, A001-3-2), glucose (GLU, A154-1-1), triglyceride (TG, A110-1-1), and

cholesterol (CHO, A111-1-1) were detected using commercial kits (Nanjing Jiancheng Biological Engineering Institute, China) as per the kit instructions.

Intestinal tissue homogenates were centrifuged and the supernatant was taken for the determination of digestive enzyme activities in the intestine using commercial kits (Jiancheng Biological Engineering Institute) according to the manufacturer's instructions. Digestive enzymes to be measured are amylase (C016-1-1), lipase (A054-2-1), and trypsin (A080-2-1). 2.6. *Intestinal microbiota analysis*

As described previously (Suo et al., 2017), microbiota analysis was performed by identification of V3-V4 region of 16S rRNA gene using high throughput sequencing. In this study, the procedure was slightly altered. Total DNA was performed from the enteric bacterial communities using a soil DNA kit. Sample DNA concentration was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Science, Wilmington, DE, USA). The corresponding region of the primer was 16S V3-V4 (341F and 806R). The primer sequence of 341F was 5'-CCTACGGGNGGCWGCAG-3'; and the primer sequence of 806R was 3'-GGACTACHVGGGTATCTAAT-5'. The system of PCR was 30 µl, and high-throughput sequencing was performed through Illumina HiSeq 2500 sequencing. Following this, 2% agarose gel was used to separate the amplicons. Next, they were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) as per the manufacturer's protocol. The library was constructed using NEB Next® Ultra library DNA Library Prep Kit for Illumina construction kit, and then sequenced by Illumina HiSeq 2500 sequencing system. The data were filtered by noise sequence, and the chimera sequence was detected using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Quast et al., 2013). OTU clustering was performed with 97% sequence similarity as the threshold. The UPARSE software (UPARSE v7.0.1001, <http://drive5.com/parse/>) (Edgar, 2013) was used for sequence analysis. Next, the rarefaction curve was plotted and the alpha-diversity metrics were calculated (Simpson, Shannon, ACE, and Chao). The endemic species between different groups are shown on the Venn diagram. The PCoA of unweighted UniFrac distance was drawn in WGCNA under R version 2.15.3 and the differences among multiple samples were compared.

2.7. Histological analysis of fish intestine

Following the method of Bullerwell et al. (2016), intestinal morphological indexes were detected to determine the YC diet's effect on fish intestinal morphology (Bullerwell et al., 2016). The fixed tissues were dehydrated with gradient alcohol, cleared in toluene, transparented in xylene, and embedded in paraffin. Finally, the paraffin-embedded tissues were sectioned with a thickness of 5 µm. Intestinal tissues were stained with hematoxylin-eosin (H&E), and intestinal images were collected using a microscope (BX51 type, serial number: 9K18395; Olympus, Tokyo, Japan). A total of 12 measurements were obtained for each tissue. The data of intestinal villus height (VH), villus width (VW), and muscle thickness (MT) under 100x magnification were obtained through electronic measurement using Image-Pro Plus 6.3 software (Media Cybernetics, Inc., Rockville, USA).

2.8. Bacteria challenge test

Vibrio harveyi for challenge test was obtained from the Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Animal, Guangdong Ocean University, China. They were initially isolated from a diseased hybrid grouper and stored in 40% glycerol at 80 °C. Upon challenge test, the frozen stock culture was revived. Bacteria were cultured in Luria-Bertani (LB) medium in a flat-bottomed flask (250 mL) by shaking at 180 rpm in an incubator (37 °C, 20 h). Afterward, the cells were centrifuged (8000 rpm, 4 °C, 10 min), with the supernatant discarded, followed by 2 washes with sterile phosphate-buffered saline (PBS). Next, they were serially diluted with PBS to attain 3.58×10^5 CFU/mL (LD 50). After the 8-week feeding trial, 12 fish were randomly selected from each replicate tank for the challenge test, and each

fish was injected with 200 µl of bacterial cell suspension at LD50 (3.58×10^5 CFU/mL). In addition, 12 fish were randomly selected from each group and injected with the same dose of saline, which served as the blank control group. During challenge test, fish was not offered feed. Following the bacteria injection for 12 h, blood was sampled from two fish, which was then centrifuged (3500 rpm/min, 4 °C, 10 min), separated, and stored (80 °C) for later analysis of ALP, ACP, LZM, and IgM activities. Fish were kept under observation. The mortality rate was recorded up to 168 h.

2.9. Statistical analysis

Data analysis was performed using SPSS23.0 (SPSS Inc., Chicago, IL, USA). The results are presented as means $\pm$ standard error of mean (SEM). All data were analyzed using a one-way analysis of variance followed by Duncan's multiple range test to determine significant differences between treatment groups. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Growth, feed utilization, and survival rate of hybrid grouper

The effects of different YC protein diets on the growth and feed utilization of hybrid grouper were illustrated in Table 2. The results showed that YC-treated fish exhibited notably improved growth performance than the control fish. FBW, WGR, and SGR were the best in the N2 (4%) groups ($P < 0.05$). FCR was notably less in the N1 (2%) and N2 (4%) groups than that in the control group, with the lowest in the N2 (4%) group ($P < 0.05$). The dietary supplementation of YC significantly improved the SR of hybrid grouper ($P < 0.05$). The excellent SR was recorded in the N1 (2%) and N2 (4%) groups. PER and CF were remarkably increased in hybrid grouper fed with N1 (2%) and N2 (4%) as compared to those in the control hybrid fish ($P < 0.05$).

3.2. Whole-body composition of fish

The YC protein effects on fish carcass analysis were illustrated in Table 3. A significant improvement in dry matter, crude protein, and ash content in fish fed with YC treatment diets were observed ($P < 0.05$), with the N2 (4%) group showing the highest significant improvement. Crude lipid content was found notably increased in the N1 (2%) group ($P < 0.05$).

3.3. Serum biochemical parameters and intestinal digestive enzyme activity

The effect of dietary supplementation of YC on serum biochemical parameters of hybrid grouper is presented in Table 4. It was observed that serum GLU was decreased in the YC group compared to the control group, with the N2 (4%) group showing the lowest serum GLU ($P < 0.05$). Dietary addition of YC increased hybrid grouper serum TP contents ($P < 0.05$), with the N2 (4%) group showing the significantly high TP content ($P < 0.05$). In our study, when hybrid grouper was fed with YC, no significant difference was found among other groups except for the significant increase of TG content in the N1 group. There was no significant difference in CHO content among groups ($P > 0.05$). The

Table 2

Table 3

Effects of dietary yeast culture supplementation on whole body composition of hybrid grouper.

Treatment	Dry matter (%)	Crude protein (%)	Lipid (%)	Ash (%)
C0 (0%)	28.32 $\pm$ 0.40 _a	17.19 $\pm$ 0.09 _a	6.10 $\pm$ 0.01 _a	4.28 $\pm$ 0.00 _a
N1 (2%)				
N2 (4%)	31.02 $\pm$ 0.76 _c	19.04 $\pm$ 0.12 _c	6.93 $\pm$ 0.01 _{ab}	4.52 $\pm$ 0.012 _c

32.92 ± 0.21 19.92 ± 0.08 6.50 ± 0.12 4.73 ± 0.05

The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($P < 0.05$).

activities of serum AST and ALT in fish fed with N2 (4%) diet were lower than that of the control ($P < 0.05$).

The results of intestinal digestive enzyme activities are presented in Table 5. Intestinal trypsin, lipase, and amylase activities showed the same trends, which was significantly higher in the N1 (2%) and N2 (4%) groups than those in the control group ($P < 0.05$). However, there was no significant difference between the N1 (2%) and N2 (4%) groups ($P > 0.05$).

3.4. Serum antioxidant capacity

The results of antioxidant parameters in the serum are presented in Fig. 1. The serum SOD activity in the N2 (4%) group was notably improved after the challenge ($P < 0.05$). The increased SOD activity in the fish N2 (4%) group was dramatically higher as compared to that in the control group ($P < 0.05$). Fish in the N1 (2%) and N2 (4%) groups had dramatically increased CAT activity after challenge ($P < 0.05$), with the greatest in the N2 (4%) group. Serum MDA contents were decreased in the YC treatment groups, and it was significantly

Treatment	IBW (g/fish)	FBW (g/fish)	WGR (%)	SGR (%)	FCR	SR (%)	PER (%)	CF (g/cm ³)
C0 (0%)	17.86 ± 0.00 ^a	78.91 ± 0.68 ^b	343.49 ± 3.59 ^b	2.65 ± 0.01 ^b	1.03 ± 0.02 ^b	92.33 ± 1.17 ^b	1.54 ± 0.04 ^b	2.77 ± 0.05 ^c
N1 (2%)	17.85 ± 0.01 ^a	87.15 ± 0.43 ^b	387.13 ± 2.16 ^b	2.83 ± 0.01 ^b	0.86 ± 0.00 ^a	100.00 ± 0.00 ^b	1.85 ± 0.03 ^b	3.12 ± 0.00 ^b
N2 (4%)	17.86 ± 0.00	89.54 ± 0.50	± 2.90	2.87 ± 0.01	0.84 ± 0.00	± 0.00	1.87 ± 0.01	3.01 ± 0.01

The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($P < 0.05$). IBW, initial body weight; FBW, final body weight;

WGR: weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; PER: protein efficiency ratio; CF, condition factor.

Table 4

Effects of different yeast culture supplementation on hybrid grouper serum biochemical activity.

Treatment	TP (g/L)	GLU (mmol/L)	TG (mmol/L)	CHO (mmol/L)	ALT (U/L)	AST (U/L)
C0 (0%)	44.21 ± 0.94 ^b	4.21 ± 0.17 ^{ab}	0.64 ± 0.01 ^b	5.40 ± 0.06 ^a	4.42 ± 0.09 ^{ab}	25.18 ± 0.47 ^c
N1 (2%)	51.72 ± 0.81 ^b	3.85 ± 0.04 ^a	0.75 ± 0.02 ^a	5.64 ± 0.13 ^a	3.93 ± 0.06 ^a	21.64 ± 0.41 ^a
N2 (4%)	52.92 ± 0.88	3.58 ± 0.02	0.66 ± 0.01	5.53 ± 0.09	3.70 ± 0.02	18.25 ± 0.67

The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($P < 0.05$). TP, total protein ; GLU, glucose; TG, triglyceride;

CHO, cholesterol; ALT, alanine transaminase; AST, aspartate aminotransferase.

Table 5

Effects of dietary yeast culture supplementation on the intestinal digestive enzyme activities of hybrid grouper.

Treatment	Trypsin (U/mgProt)	Lipase (mU/ mgProt)	Amylase (mU/ mgProt)
C0(0%)	1352.58 ± 113.49 ^b	444.14 ± 20.29 ^b	318.81 ± 12.71 ^b
N1(2%)	1914.67 ± 149.13 ^b	722.32 ± 63.32 ^b	404.73 ± 39.47 ^b
N2(4%)	1894.54 ± 150.75	± 21.37	433.94 ± 9.47

analysis (Fig. 4). As shown in Venn diagram analysis, a different superscripts indicate significant difference ($P < 0.05$). total of 540 OTUs were identified across the three treatments. The N2 (4%) group had the highest number of unique OTUs (1031), followed by C0 (0%) (771) and N1 (2%) (584). Alpha indices were represented by

lower in the N2 (4%) group before and after the challenge test (compared to the control group) ($P < 0.05$). 3.5. Serum innate immune indexes

The activities of serum ACP, AKP, LZM, and IgM of hybrid grouper before and after the challenge test are illustrated in Fig. 2. Serum ACP activity was significantly higher after the challenge, which was remarkably higher in the N2 (4%) group relative to that in the control group ($P < 0.05$). After the challenge, AKP serum activities of the N1 (2%) and N2 (4%) groups were dramatically enhanced ($P < 0.05$), with the highest improvement observed in the N2 (4%) group. Serum LZM activity was increased in the N1 (2%) and N2 (4%) groups after the challenge, which was significantly higher in the N1 (2%) group than that in the control group ($P < 0.05$). Serum IgM activity was dramatically increased in the N1 (2%) and N2 (4%) groups after the challenge, as compared to the control group ($P < 0.05$).

3.6. Microbiota analysis in hybrid grouper intestine

3.6.1. Intestinal microbiota richness and species diversity

The rarefaction curve analysis of the samples is shown in Fig. 3A. It can be seen from the figure that rarefaction curves tended to be flat with the continuous increase in sequencing amount. The sequencing results could reflect the structure and composition of the intestinal microbial community of the grouper. As shown in Fig. 3B, the box plot position

found in the community. When the boxplot tended to be smooth, the species would not significantly increase as the sample size increased, indicating that the sampling was sufficient and data analysis could be

rises sharply over a range, indicating that a large number of species were

carried out. Operation taxonomic units (OTUs) and Alpha diversity results in hybrid grouper intestinal microbiota are illustrated in Table 6. OTUs in groupers fed with N2 (4%) diet were dramatically higher than that in groupers fed with C0 (0%) and N1 (2%) diets ($P < 0.05$). All the

OTUs made up with the mean abundance of more than one were selected The data are presented as mean ± SEM. Means in the same column with for Venn diagram

analysis (Fig. 4). As shown in Venn diagram analysis, a different superscripts indicate significant difference ($P < 0.05$). total of 540 OTUs were identified across the three treatments. The N2 (4%) group had the highest number of unique OTUs (1031), followed by C0 (0%) (771) and N1 (2%) (584). Alpha indices were represented by ACE, Chao, Shannon, and Simpson. Results showed that the N2 (4%)

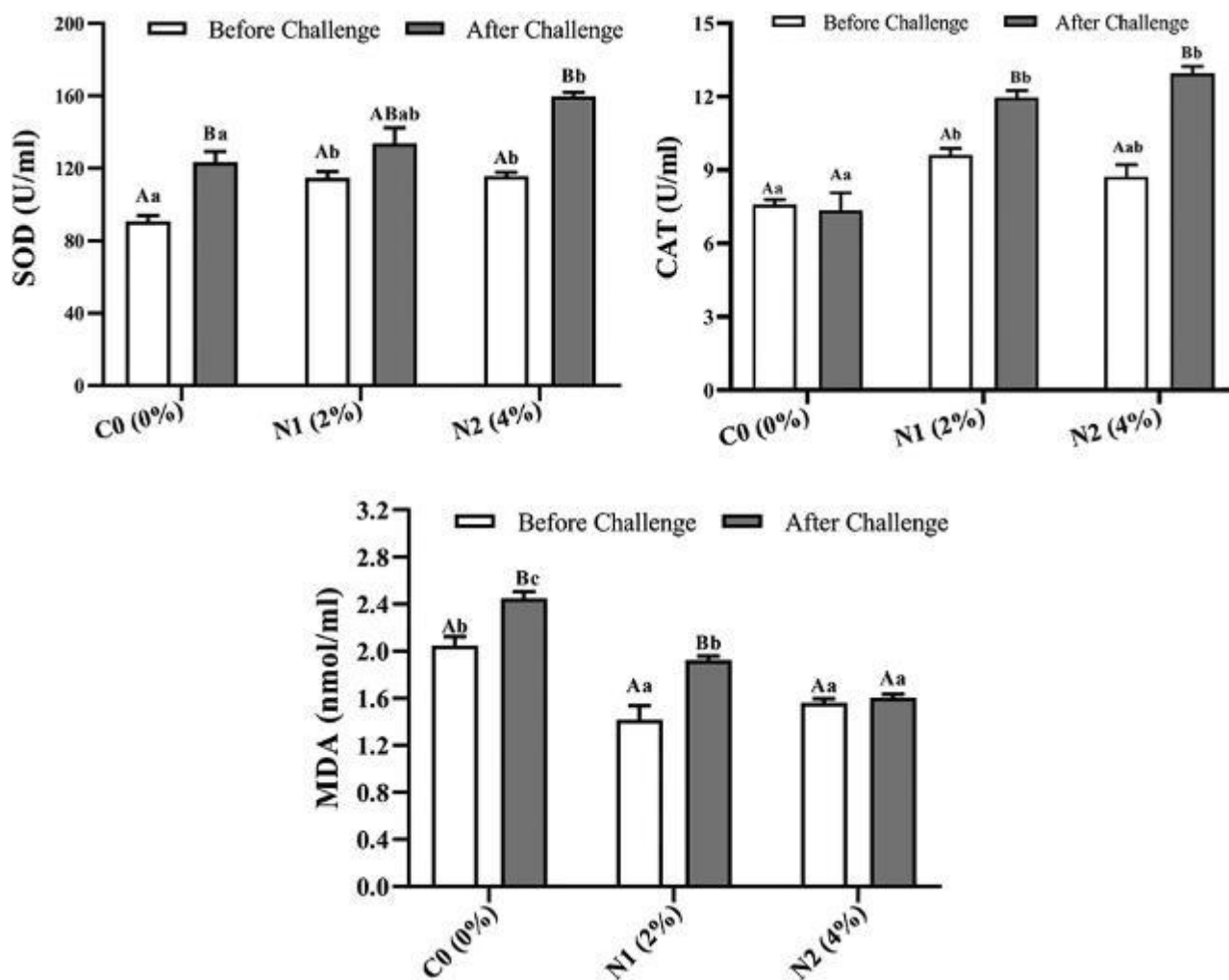


Fig. 1. Yeast culture diets effects on serum antioxidant capacity of hybrid grouper before challenge and after challenge with *V. harveyi*. The data were shown in Mean $\pm$ SEM. Different capital letters indicate significant differences before and after the challenge within the same treatment group ($p < 0.05$), and different lowercase letters indicate significant differences before and after the challenge between groups ($p < 0.05$). SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

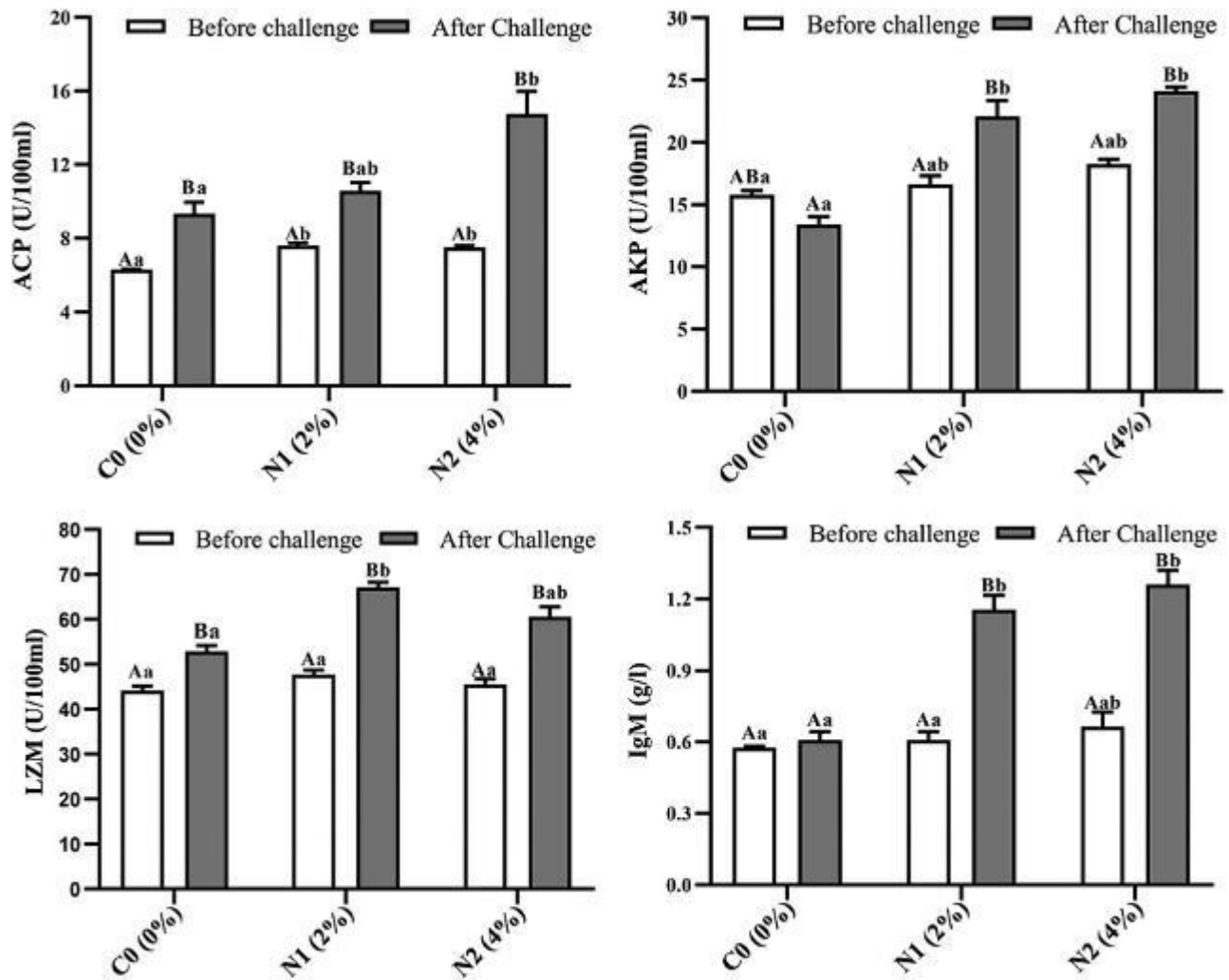


Fig. 2. Yeast culture diets effects on serum innate immune response of hybrid grouper before challenge and after challenge with *V. harveyi*. The data were shown in Mean $\pm$ SEM. Different capital letters indicate significant differences before and after the challenge within the same treatment group ($p < 0.05$), and different lowercase letters indicate significant differences before and after the challenge between groups ($p < 0.05$). ACP, acid phosphatase; AKP, alkaline phosphatase; LZM, lysozyme; IgM, immunoglobulin M.

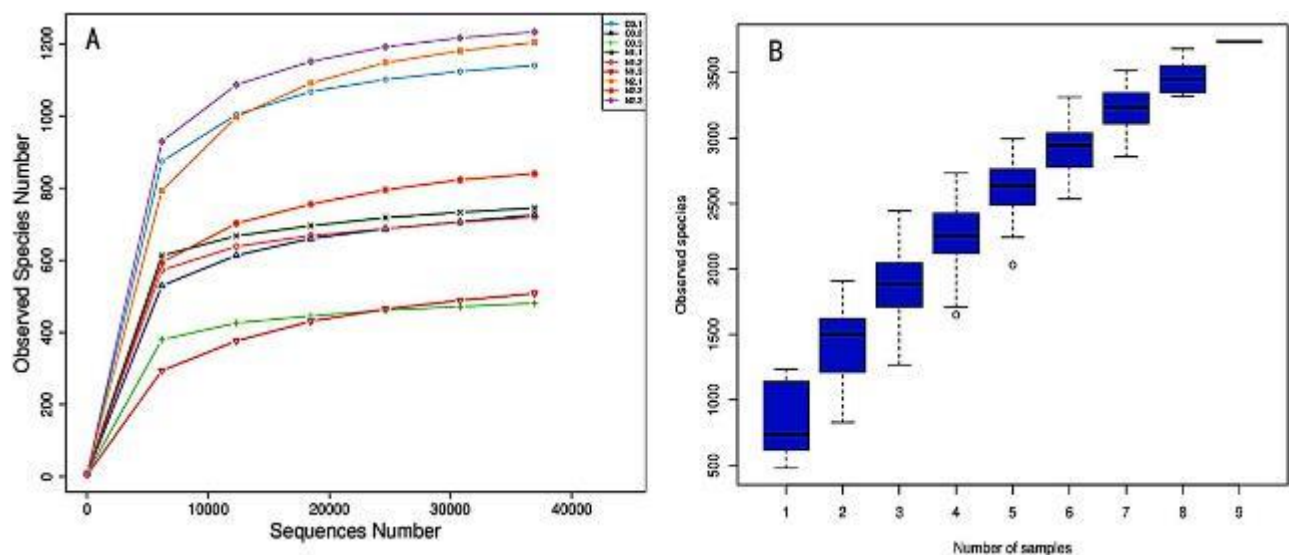


Fig. 3. A: Rarefaction curve analysis of intestinal samples in hybrid grouper from different treatment groups. B: The species accumulation boxplot. The horizontal axis represents the number of samples, and the vertical axis represents the number of species found.

Table 6

Richness and diversity indexes of intestinal bacterial in hybrid grouper fed experimental diets.

Treatment	Raw reads	Clean reads	OTU	Chao	Ace	Shannon	Simpson
C0 (0%)	76,860	69,616	770.33 ± 44.33 ^a	1136.28 ± 66.88 ^b	753.78 ± 34.88 ^a	6.91 ± 0.07 ^a	0.98 ± 0.00 ^a
N1 (2%)	85,025	80,121	797.00 ± 2.00 ^b	798.12 ± 2.87 ^b	796.00 ± 3.20 ^b	7.32 ± 0.13 ^a	0.98 ± 0.01 ^a
N2 (4%)	84,743	80,144	1292.00 ± 3.00	1258.61 ± 12.29	1258.43 ± 11.79	7.14 ± 0.05	0.97 ± 0.00

The data are presented as mean ± SEM. Means in the same column with different superscripts indicate significant difference ($P < 0.05$).

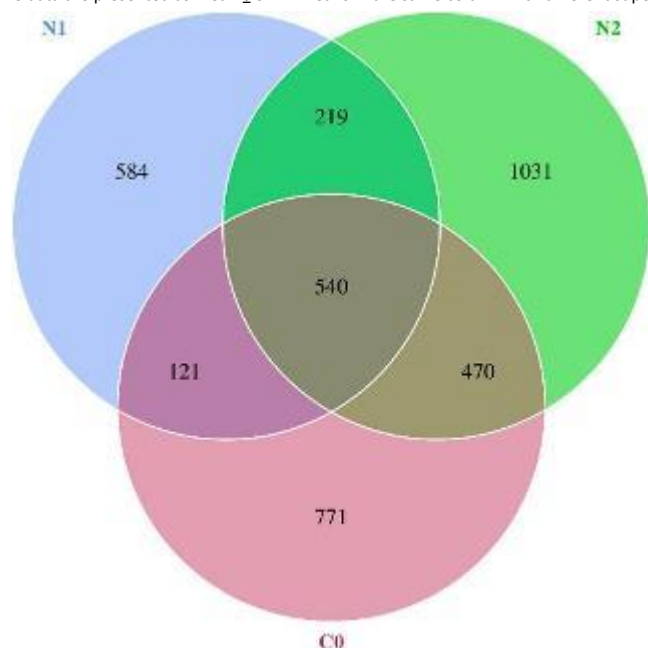


Fig. 4. Venn diagram of OTUs comparison.

group had the highest Chao index, but there was no significant difference as compared to the control group ($P > 0.05$). The ACE index was notably higher in the N2 (4%) group than that in the C0 (0%) and N1 (2%) groups ($P < 0.05$). The bacterial diversity indexed by Shannon was numerically increased in the N1 (2%) and N2 (4%) groups, but there was no significant difference among groups

($P > 0.05$). According to Simpson's results, no difference was found between the treated and untreated groups ($P > 0.05$).

3.6.2. Composition characteristics of intestinal microbiota

The ten bacteria with the highest relative abundance at the phylum and genus level were shown in Fig. 5 and Fig. 6, respectively. Four dominant intestinal bacteria at the phylum level were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Fig. 5). The relative abundance of *Proteobacteria* was noticeably higher in the N2 (4%) group than that in the N1 (2%) group ($P < 0.05$), but not significantly different from the C0 (0%) group ($P > 0.05$). The abundance of *Bacteroidetes* in the N2 (4%) group was notably higher than that in the control group ($P < 0.05$). The relative abundance of *Actinobacteria* in hybrid grouper in the N1 (2%) and N2 (4%) groups were significantly higher than that in the control group, with no significant difference between the N1 (2%) and N2 (4%) groups ($P > 0.05$). The N1 (2%) and N2 (4%) groups had a higher relative abundance of *Firmicutes* than the C0 (0%) group, and it was significantly higher in the N1 (2%) group than that in the N2 (4%) group. At the genus level, the main intestinal bacteria were *Sphingomonas*, *Blautia*, *Lactobacillus*, and *Stenotrophomonas* in all the treatment groups (Fig. 6). The relative abundance of *Sphingomonas* was high in the N2 (4%) group, but there was no significant difference between the three groups ($P > 0.05$). *Blautia* abundance was significantly higher in the N2 (4%) group than that in the other two groups ($P < 0.05$). The hybrid grouper intestinal *Lactobacillus* were abundant in YC-fed grouper than untreated grouper ($P < 0.05$). The abundance of *Stenotrophomonas* was significantly higher in the N1 (2%) group than that in the control group, but not significantly different than the N2 (4%) group. Based on Principle Coordinate Analysis (PCoA) (Fig. 7) of unweighted UniFrac distances from all samples (microbiota composition clustered by feedstuff type), there were distinct microbial community structure in the C0 (0%), N1(2%), and N2 (4%) groups, which indicated that the addition of YC may influence the composition of the intestinal microbial community.

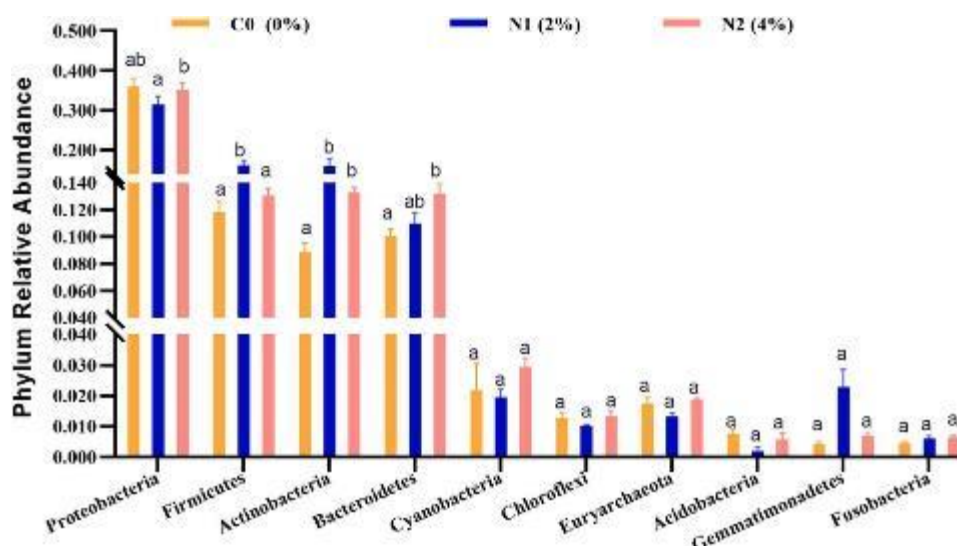


Fig. 5. Relative abundance of intestinal bacterial of hybrid grouper at the phylum level. Upright bars denote the mean ± SEM. ($P < 0.05$). Different superscript letters indicate significant differences between groups ($P < 0.05$).

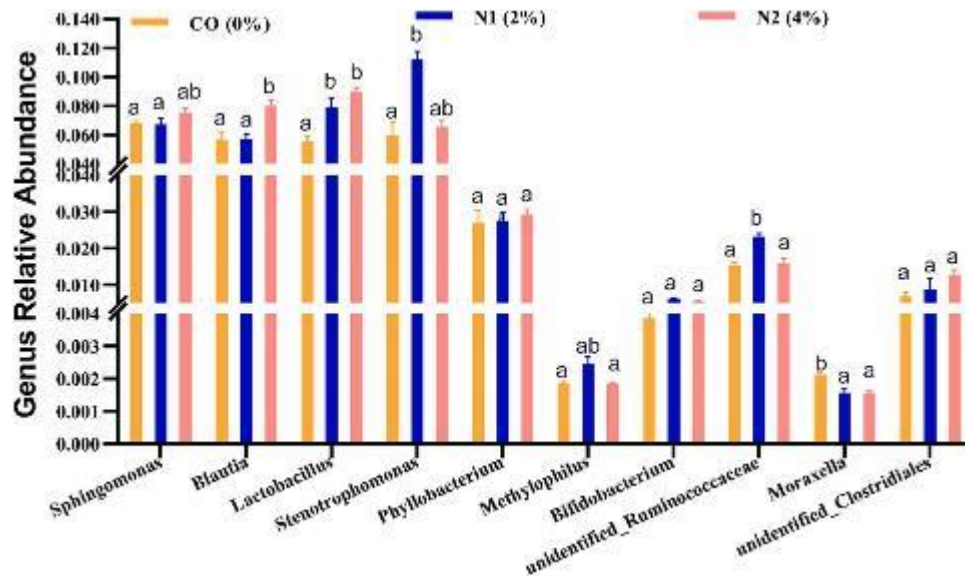


Fig. 6. Relative abundance of intestinal bacterial of hybrid grouper at the genus level. Upright bars denote the mean $\pm$ SEM. ($P < 0.05$). Different superscript letters indicate significant differences between groups ($P < 0.05$).

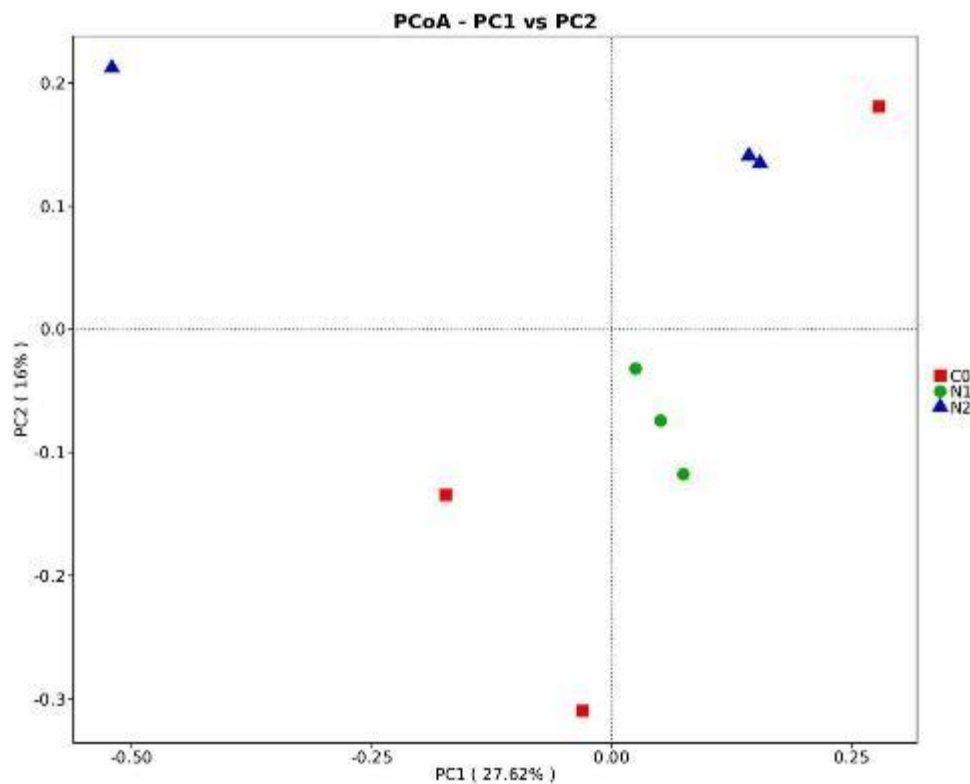


Fig. 7. Principal coordinate analysis (PCoA) of intestinal microbial community of grouper.

3.6.3. Bacterial challenge test

The cumulative fish SR after challenge with hybrid grouper for 168 h is illustrated in Fig. 8. No mortality of grouper was observed in the saline injection group. At the end of 168 h of challenge, the cumulative SR of hybrid grouper with dietary supplementation of YC was notably higher than that of the control. The highest SR of fish was recorded in the N2 group (80%), and the least registered number was found in fish fed with the control diet (20%).

3.7. Intestinal morphometric measurement

The measurement of intestinal morphometric parameters is represented in Fig. 9. The intestinal mucosal fold separation from the intestinal wall was seen in control groupers, whereas fusion of mucosal folds was observed in groupers fed with N1 (2%) and N2 (4%) diets (Fig. 9A). YC supplementation resulted in a significant increase in VH and VW ($P < 0.05$). VW and VH were significantly higher in the hybrid group fed

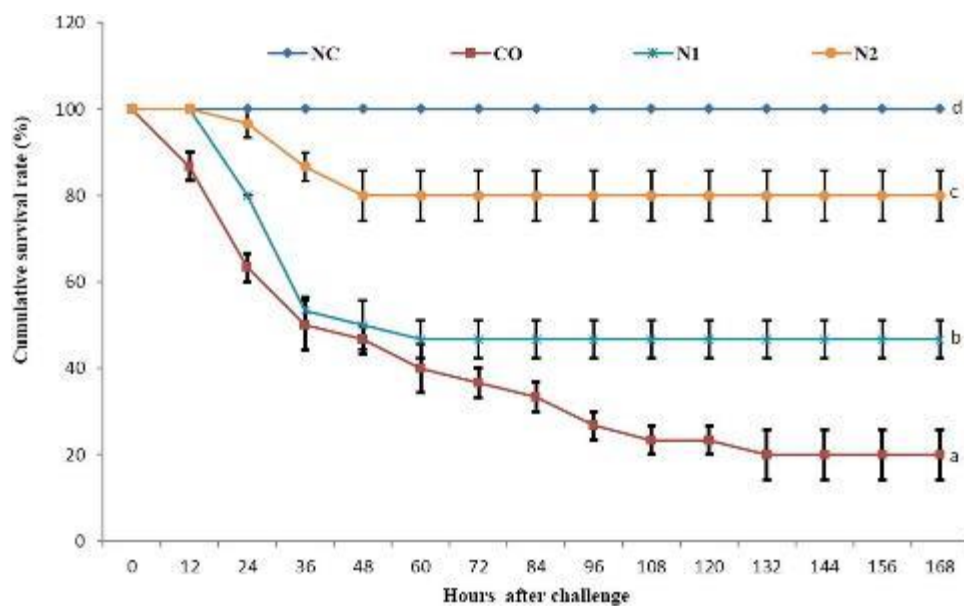


Fig. 8. Cumulative survival rate (%) of hybrid grouper after challenge with *V. harveyi*. For each treatment, values with different letter suffix differ significantly ($P < 0.05$).

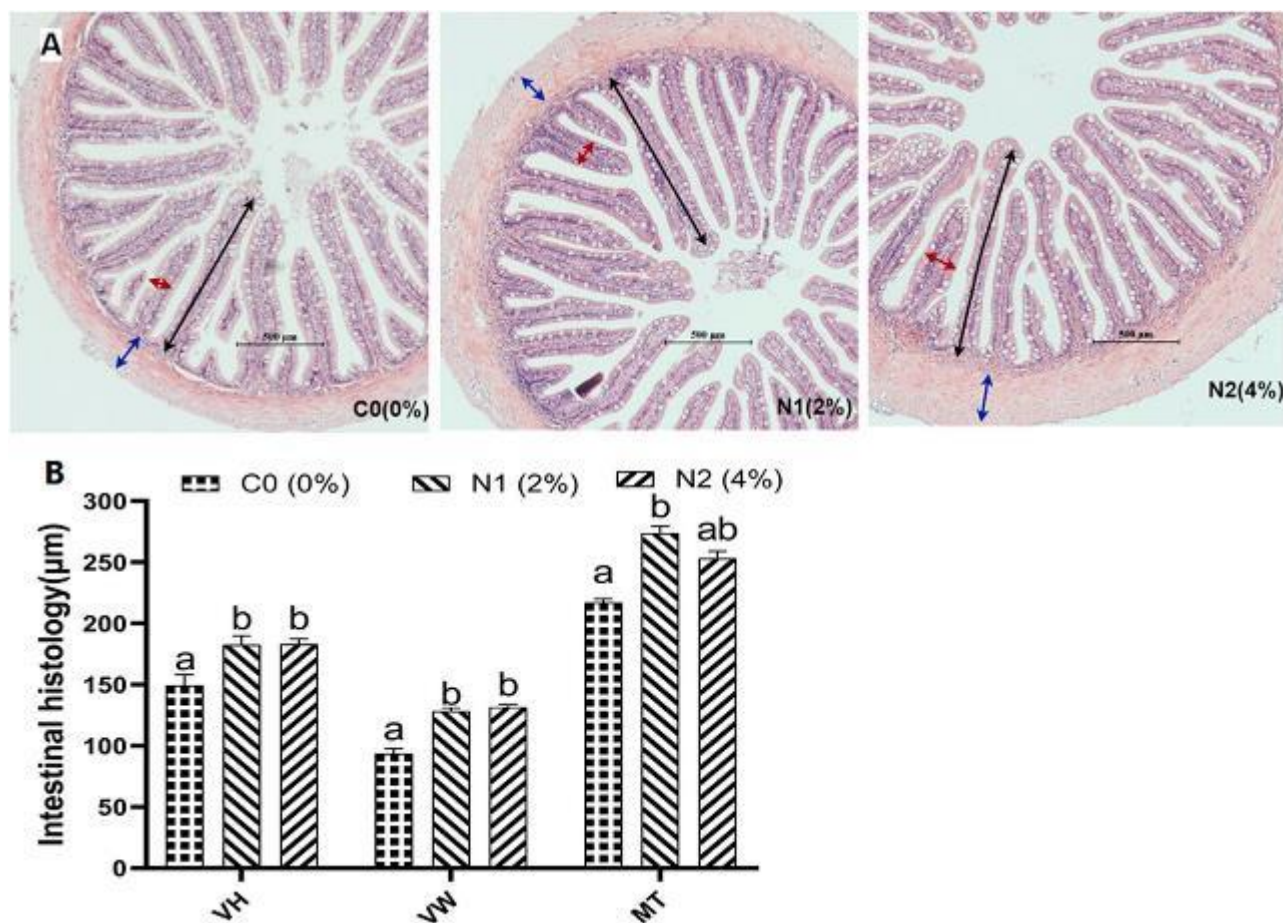


Fig. 9. Effects of supplementation of yeast culture on villus height, villus width and muscle thickness of hybrid grouper. (A) Photomicrographs. Black arrow: villus height; Red arrow: villus width; Blue arrow: muscle thickness. (B) Morphological measurements. Values are presented mean $\pm$ SEM. Different letters indicate significant differences ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with YC N2 (4%) showed than those in the control group, while no improvement was found between the N1 (2%) and N2 (4%) groups ($P < 0.05$).

Among all the experimental groups, only the N1 (2%) group showed significant MT changes, as compared to the control group ($P < 0.05$) (Fig. 9B).

4. Discussion

Recently, it has been of interest to improve the growth and immune response of fish using yeast products. The evaluation of several yeast products has shown a positive influence on the growth performance and immune response of aquatic animals (Dimitroglou et al., 2010; Zhao et al., 2011; Sado et al., 2014). A previous study has proved that juveniles' leopard grouper fed with yeast diet shows improved growth and immune response (Reyes-Becerril et al., 2011). In the present study, the benefits of dietary supplementation of different levels of YC (fermentation substrate free of non-protein nitrogen; 50% protein content) in hybrid grouper were evaluated from the aspects of growth performance, intestinal morphology, digestive enzymes, intestinal microbiota, immune response, and disease resistance.

After 8 weeks of feeding trials with dietary supplementation of YC, the SR of hybrid grouper was enhanced, which may be due to the diet's nutritional fulfillment. The dietary inclusion of YC could help to increase the growth performance and feed utilization of hybrid grouper in the current study. However, FBW, WGR, and SGR were notably improved, and FCR was decreased in the dietary N1 (2%) and N2 (4%) groups, which indicated that YC could enhance the growth performance and feed utilization of hybrid grouper. This beneficial influence may be due to the bioactive compounds secreted by YC, including nucleotides, proteins, amino acids, fatty acids, vitamins, and polysaccharides. Nucleotides are especially important, which can serve as precursors of DNA and RNA in rapidly growing cells (such as intestinal tissue) in the diet via the salvage pathway, thereby sparing the organism from the cost of de novo synthesis. Moreover, the increased digestive enzyme activities are an important factor in promoting growth and feed utilization (Zhang et al., 2018). In the present study, fish in the N1 (2%) and N2 (4%) groups showed high digestive enzyme activities and improved feed utilization. Consistently, previous studies have demonstrated a similar increase in digestive enzyme and feed utilization in Seabass (*Dicentrarchus labrax*) (Tovar et al., 2002). Furthermore, intestinal microorganisms are closely involved in the absorption, digestion, and metabolism of several nutrients in aquatic animals. Specifically, the abundance of intestinal *Bacteroides* in fish in the N2 (4%) group was increased. *Bacteroides* decompose polysaccharides into simple sugars, which can be easily absorbed into the host to synthesize proteins and play mutualistic roles in fish intestine (Xia et al., 2014). Therefore, the enhanced feed utilization and growth performances in this study may be related to the effect of dietary YC on the intestinal microbiota and digestive enzymes. Based on the changes in the intestinal microbiota and digestive enzymes of hybrid grouper, it's indicated that YC could increase exogenous bacterial enzyme activities, which may lead to the increased growth performance of hybrid grouper.

Grouper whole-body composition analysis is a good indicator for studying the physiological condition of hybrid grouper. The change of feed composition will affect the nutritional composition of the whole body of fish. In this study, the YC diet had a positive influence on the protein, lipid, and ash content in the whole body of fish, which may be due to the addition of YC. YC promotes the breakdown, synthesis, digestion, and absorption of diet nutrition in the gastrointestinal tract, improving the utilization of nutrition and resulting in increased deposition of nutrition. Changes in protein and lipid content may be related to the synthesis of nutrition components and the rate of deposition, which in turn affects the changes in ash content. Similar results were observed in tilapia fed with yeast diets (Abdel-Tawwab et al., 2008), which further indicated that yeast could enhance the physiology of aquatic animals. From all above, it was plausible to state that dietary supplementation of YC could increase intracellular enzyme production, and then promote the nutrient composition and growth performance of hybrid grouper.

Serum analysis plays an essential role in the nutrition, physiology, and health of aquatic organisms, which can indirectly reflect the health degree of aquatic animals (Hoseinifar et al., 2011). GLU can be used as a stress indicator of fish, with high blood GLU often indicative of a high level of stress (Berto et al., 2016). In the present study, dietary addition of YC decreased the serum GLU content, which may be due to the presence of β -glucan (an immunostimulant) in the YC diet. In animals, β -glucan could lower serum GLU by activating the PI3K/AKT pathway (Ma et al., 2015). β -glucans stimulated several PI3K/AKT receptors (such as Dectin-1, complement receptor 3, and toll-like receptors) to induce specific signal pathways as a sign of serum GLU reduction (Huth et al., 2000). When β -glucan was administered to fish diet,

GLU content was not elevated, which invariably shows that the dietary application of β -glucan has no stress effect on fish (Misra et al., 2006). Serum TG and CHO, as components of blood lipids, are important indicators of lipid levels in fish, which reflects the metabolism, and increases energy storage of lipids in fish. High contents of TG and CHO can cause damage to the hepatopancreas of fish (Agrahar et al., 2007). In our study, when hybrid grouper was fed with YC, no significant difference was found among the other groups except for the significant TG increase in the N1 group. There was no significant difference in CHO among groups. Previous research has demonstrated that the application of yeast products contributes to decreasing both CHO and TG content in aquatic animals (Bell et al., 1999), which contradicts our results. It may be due to the non-significant effect of YC on lipid anabolism in hybrid grouper. However, the specific mechanism needs to be further investigated.

Oxidative stress, as a frequent stress response in fish, leads to a series of destructive processes (such as membrane damage and accumulation of lipid peroxidation products). As an endogenous antioxidant enzyme, SOD can improve the level of immune response and enhance the body's resistance (Reyes-Cerpa, 2018). SOD scavenges superoxide anions to produce H_2O_2 , which constitutes the first line of defense against antioxidant enzymes (Li et al., 2015). CAT, as an antioxidant enzyme, can further convert SOD-produced H_2O_2 into water and oxygen in aquatic animals' immune systems (Wang et al., 2017). MDA, the primary marker of lipid peroxidation, reflects the extent of lipid oxidation (Xu et al., 2015). Fish serum CAT and SOD activities were significantly high, while serum MDA content was significantly lower in the N2 (4%) group than those in the control group. Conforming with this study, the addition of YC to Ussuri catfish (*Pseudobagrus ussuriensis*) feed could improve the antioxidant capacity, as characterized by the enhanced serum CAT and SOD activity, and decreased MDA content (Bu et al., 2019). In addition, it has been shown that feeding grass carp (*Ctenopharyngodon idella*) with yeast hydrolysate can enhance the activity of CAT and SOD in the fish (Yuan et al., 2019). Yeast cells contain vitamins (Ghazanar et al., 2015), which may enhance the immune system and promote the growth of aquatic animals (Zhang et al., 2019). Vitamin B6 compounds inhibit lipid peroxidation by scavenging oxygen free radicals (Kannan and Jain, 2004). AST and ALT are soluble enzymes present in the cytoplasm of liver cells, and their elevated activity in serum reflects liver damage (Du et al., 2017). As shown by the results of this study, YC could effectively reduce serum ALT and AST activity, which corresponds to the results of grass carp fed with YC (Liu et al., 2018). A series of active metabolism in the liver may account for this result. According to this study, dietary addition of YC can not only protect the liver, but also improve the antioxidant, and immune system of hybrid grouper.

Improving the innate immune system of fish plays a significant role in protecting them against disease infections (Lu et al., 2019). Innate immune parameters (such as LZM, AKP, ACP, TP, and IgM) were used to monitor the non-specific immunity of fish. TP, as a crucial component of the innate immune system, helps to protect fish from potentially invasive organisms (Soltanzadeh et al., 2016). In our study, serum TP activity was remarkably increased in the N2 (4%) group, which was consistent with the report that notably enhanced serum TP modulated immune activities in gibel carp (*Carassius auratus gibelio cas III*) (Zhang et al., 2018). ACP and AKP, as important lysosomal enzymes in fish, play a crucial role in the immune response through hydrolysis and digestion of invaded pathogen (He et al., 2017). In fact, elevated ACP and AKP activities in the fish are indicative of strong immunity. In many cases, ACP and AKP are considered to be powerful markers for macrophage activation of the immune system (Jin et al., 2018). In agreement with our study, dietary supplementation of MOS in hybrid grouper stimulates AKP activity in fish (Ren et al., 2020). LZM, a useful innate immune enzyme, can lyse and digest infectious bacteria. Numerous studies related to aquatic animals have shown that yeast products exert positive effects on innate immune responses (Abu-Elala et al., 2013; Jin et al., 2018; Bu et al., 2019). In Ussuri catfish (*Pseudobagrus ussuriensis*), dietary YC supplemented at 10 g/kg improved plasma ACP, AKP, and serum LZM (Bu et al., 2019), which indicated improved innate immune response. Tukmechi and Bandboni (2014) reported that dietary supplementation of two combined yeast products in rainbow trout (*Oncorhynchus mykiss*) could improve the innate immune response (Tukmechi and Bandboni, 2014). Moreover, IgM, a useful immunoglobulin marker, has been used in evaluating fish immune response (Ortuno et al., 2002). In our results, the notably elevated serum IgM

activity in the N2 (4%) group also indicated that dietary supplementation of 4% YC could enhance the non-specific immune response in hybrid grouper. Similarly, dietary addition of yeast may help to enhance the non-specific immune response in Atlantic salmon (*Salmo salar*) (Reyes-Cerpa et al., 2018) and gilthead seabream (*Sparus auratus*) (Ortuno et al., 2002). This beneficial effect could attribute to the immunostimulant property exhibited by the YC diet. Functional components of yeast (such as β -glucan and mannan-oligosaccharide) have been identified to stimulate fish immunity (Jayachandran et al., 2018). β -glucan can bind to receptor molecules on the surface of tissue phagocytes. This increases the phagocytic activity of killing and digesting bacteria (Abu-Elala et al., 2013). Dietary supplementation of β -glucan improves the innate immune response in Atlantic salmon (Vetvicka et al., 2013). Our results demonstrated a significant increase in serum ACP, AKP, and IgM activity in fish in the N2 treated group after the challenge, which suggested that dietary supplementation of 4% YC could improve the non-specific immune defense capacity of the hybrid grouper.

It is essential to understand the relationship between the host and its intestinal bacteria for the successful and efficient production of cultured fish. The intestinal microbiota composes of pathogenic and beneficial bacteria that influence fish growth and physiology. In our study, the alpha diversity indexes (OTU and ACE) were significantly higher in the N2 (4%) group than those in the control group. Consistently, dietary addition of yeast products has been shown to increase the gut microbial diversity of red drum (*Sciaenops ocellatus*) and fathead minnow (*Pimephales promelas*) (Burr et al., 2008; Lochmann et al., 2011). Dominant bacteria detected in all treatment groups at the phylum level were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, which is consistent with the results of existing studies (Liu et al., 2020). *Proteobacteria*, the largest phylum bacteria in the gut of marine fish, have been involved in metabolic activities (such as immune regulation, and nitrogen and carbon fixation) (Vikram et al., 2016). The rumen microbes in *Firmicutes* could degrade the fiber content of the ration (Backhed et al., 2004). *Actinobacteria* and *Bacteroidetes* not only promote the absorption of nutrients by the host but also inhibit the colonization of other harmful bacteria in the gut (Binda et al., 2018; Yang et al., 2020). In this experiment, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* were found higher in the YC treatment group than those in the control group. Similar results have been reported in grass carp (Liu et al., 2018) and mice (Geurts et al., 2014). In this study, beneficial bacteria in the YC treatment group showed remarkably higher relative abundance than those in the control group at the genus level. *Sphingomonas* was high in the N2 (4%) group, with no significant difference among the three groups ($P > 0.05$). *Sphingomonas*, the beneficial bacteria, was used as a probiotic to inhibit the virulence of *Vibrio anguillarum* in *Labeo rohita* (Liu et al., 2020). From all above, the YC diet can promote the growth of *Sphingomonas* bacteria, thus producing antimicrobial substances to inhibit pathogens infection in the hybrid grouper. *Blautia*, a Gram-positive bacterium, produces metabolites such as acetic acid and lactic acid, and its increased level is associated with host intestinal health (Liu et al., 2008). In addition, *Lactobacillus* was identified significantly higher in fish in the YC supplemented groups, especially in the N2 (4%) treatment group. As a probiotic, *Lactobacillus* produces sticky substances in the intestine that protects the host from pathogenic infection (Reunanen et al., 2012; He et al., 2017). Furthermore, *Lactobacillus* could produce exogenous enzymes, which could increase the digestion and growth performance of fish (Ziaei-Nejad et al., 2006; Akter et al., 2019). Our results showed that dietary addition of YC can promote the growth of beneficial microorganisms in the gut of hybrid grouper, which provides a safeguard for the feed utilization, growth performance, and immunity protection of the fish. In general, the result of the intestinal microbiota composition of hybrid grouper demonstrated that significant change was associated with the dietary protein source, which may influence the digestion and immune responses. A higher level of YC protein diet had a positive influence on the digestion and immune response of hybrid grouper.

The outbreak of infectious diseases is a fundamental problem affecting the fish aquaculture industry (Flores-Miranda et al., 2011). *Vibrio* is the pathogen of the outbreak of grouper infection, and its high mortality rate has caused severe economic losses to farmers in intensive farming (Fan et al., 2019). It is a valuable method to evaluate fish's resistance to diseases by feeding fish with specific feeds and challenging them with bacterial pathogens. In this study, fish fed with dietary YC indicated significant defense against *V. harveyi* infection, particularly in the N2 (4%) group. These outcomes confirm with those who fed YC to shrimps at a 1% diet (Burgents et al., 2004). The results of Burgents et al. (2004) also show that dietary administration of YC could protect shrimp against

a decline in resistance to bacterial disease (Burgents et al., 2004), which indicates that YC could enhance shrimp immune response to pathogenic infection. In another study, no mortality after 9 weeks in hybrid striped bass yeast-contained diet was found ((Morone chrysops Li and Gatlin, 2003×M. saxatilis) fed with a 4% brewer).

The intestinal tract's morphology and structure are crucial for the maintenance of normal intestinal function. The villus length and muscle thickness reflect the function of the intestinal wall to a certain extent (Al-Fataftah and Abdelqader, 2014; Fang et al., 2019). YC diet notably increased villus height and width of hybrid grouper, thus promoting the growth. In related work, dietary yeast hydrolysates in juvenile Jian carp increased villus height and enhanced fish growth (Yuan et al., 2019). Besides, the intestinal villus length of *L. vannamei* was increased due to the dietary addition of MOS (Zhang et al., 2012), which indicated that MOS in feed provided nutrients and promoted the growth of intestinal villi. In this study, the growth of intestinal villi surface area may be attributed to MOS components in YC.

5. Conclusion

In conclusion, this study indicated that YC products in a hybrid grouper diet could significantly improve the growth performance and serum immune responses against *V. harveyi* attacks. Furthermore, it positively influences intestinal bacterial composition and morphology. In summary, dietary supplementation of N2 (4%) YC is recommended for hybrid grouper (*Epinephelus fuscoguttatus*♀×*E. lanceolatus*♂).

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Author contributions

All authors have actively contributed to the study. The author's contributions are as follows: H.Y. Liu designed the study; Qi Wang and S. Ayiku conducted the study and analyzed the data; X.H. Dong participated in the interpretation of the results; Qi Wang and S. Ayiku wrote the manuscript; Q.H. Yang, S.Y. Chi, S. Zhang and W.H. Zhou purchased the reagent supplies; H.Y. Liu revised the manuscript. All authors read and approved the final manuscript.

Ethics statement

The animal protocol was approved by the ethics review board of the Guangdong Ocean University. All procedures were performed by the standards of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978) and relevant Chinese policies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Yeast cultures alleviate gossypol induced inflammatory response in liver tissue of Ussuri catfish (*Pseudobagrus ussuriensis*)

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ABSTRACT

The present study explored the mechanisms of gossypol induced inflammatory response in liver tissue of Ussuri catfish (*Pseudobagrus ussuriensis*), and investigated whether the yeast culture could relieve the inflammatory response of fish. For this purpose, Ussuri catfish were fed two diets with or without yeast culture for 8 weeks, and then fish were treated with 300 mg gossypol/kg body weight by intragastric administration for 15 days. Fish pre-fed diet without yeast culture supplementation and treated with gossypol oral administration (Gos group) had higher interleukin-1 β (IL-1 β) and interleukin 8 (IL-8) mRNA levels compared with fish pre-fed diet without yeast culture supplementation and treated with sodium carboxymethylcellulose solution oral administration (control group) and pre-fed yeast culture diet and treated with gossypol oral administration (Gos/YC group), but had lower interleukin 10 mRNA level than the control group ($P < .05$). Simultaneously, hepatic toll-like receptor 2 (TLR2), myeloid differentiation factor 88 (MyD88) and nuclear transcription factor kappa B p65 (NF- κ B p65) mRNA levels of fish in Gos group were also up-regulated. However, dietary yeast culture supplementation remarkably prevented the increase of IL-1 β and IL-8 mRNA levels and the decrease of IL-10 mRNA level by inhibiting the excessive activation of TLR2-MyD88-NF- κ B signaling pathway through down-regulating TLR2, NF κ B p65 ($P < .05$) and MyD88 ($P > .05$) mRNA levels. Besides, the lowest serum lysozyme and alkaline phosphatase activities were observed in fish of Gos group. There was an increased trend in immunoglobulin M content among the groups. Fish in Gos group had significantly lower hepatic catalase, superoxide dismutase and total antioxidant capacity compared with those fish in the control group ($P < .05$). Higher occurrences of the dilatation in sinusoids, karyolysis, hydropic degenerations and nuclei shifting to the cellular periphery were detected in Gos group compared with those in the control group, coupled with increased serum aspartate aminotransferase activity. Lower occurrences of these histopathological symptoms were observed in the Gos/YC group compared with those of fish in the Gos group. Overall, the results mentioned above indicated that gossypol could result in immune and oxidative damage, liver injury and inflammatory response and dietary yeast culture supplementation could alleviate these negative responses for Ussuri catfish.

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1. Introduction

The yeast culture (YC) is a complicated yeast fermentation product composed of the yeast and its growth media. In the process of yeast cultures production, yeast cells are only used for the production of their metabolites. Recently, YC is widely used in animal feed including aquafeed due to the fact that it contains protein, lipid, B-vitamins, which may possibly serve as an alternative protein source to fish meal (Sanderson and Jolly, 1994; Cheng et al., 2004; Barnes et al., 2006). Moreover, YC possessed immunomodulatory constituents such as yeast

cell walls (β -glucans and mannan-oligosaccharides), peptides, nucleotides, cell solubles, and oligosaccharides (Jensen et al., 2008). These immunostimulants have been used in aquatic animal culture such as carp (*Cyprinus carpio*) (Selvaraj et al., 2005), European sea bass (*Dicentrarchus labrax*) (Torrecillas et al., 2007) and rohu (*Labeo rohita* L.) (Choudhury et al., 2005), altering the immune response and inducing protection against a wide range of diseases (Soltanian et al., 2007). Based on these observations, it could be hypothesized that YC might be a potential immune modulator for fish. Moreover, previous study has shown that YC also has anti-inflammatory property (Jensen et al., 2008).

Ussuri catfish (*Pseudobagrus ussuriensis*) is an omnivorous fish widely distributed in inland rivers in China and East Asia. The farming production of this fish has spread all over China, and it is well popular with Chinese consumers due to its high nutritional value, high resistance against diseases and availability of reproduction technology. It lives at the bottom of freshwater, the most suitable growth temperature of 25 to 28 °C and the pH range of 7.0 to 8.5. Up to now, the study of fish meal replacement with cottonseed meal in

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diets for Ussuri catfish has been well assessed in our laboratory, and it was noteworthy that dietary too high proportion of cottonseed meal may result in the inhibition of the growth, immune response, disease resistance and liver function of fish, speculating that dietary free gossypol toxicity may be the main reason responsible for the above-mentioned negative effects (Bu et al., 2017). Previous study demonstrated that dietary free gossypol can cause toxic effects on fish. It is reported that dietary 431.3 mg kg⁻¹ free gossypol supplementation could damage the growth performance, while dietary 647.0 mg kg⁻¹ free gossypol supplementation could impair the liver for common carp (*Cyprinus carpio*) (Wang et al., 2014a, 2014b). Bian et al. (2016) demonstrated that high levels of free gossypol in diets could cause liver fibrosis and stimulate chemokine and proinflammatory cytokine secretions for turbot (*Scophthalmus maximus* L.). Toll-like receptors (TLRs) are considered to mediate the inflammatory reaction, which interact with their corresponding adaptor molecules such as myeloid differentiation factor 88 (MyD88) to elicit downstream signaling events, resulting in nuclear transcription factor kappa B (NF-κB) activation and the induction of several types of cytokines (Thompson and Locarnini, 2007). Several previous studies have suggested that TLR2 activation could initiate a pro-inflammatory response, which depends on the activation of NF-κB (Hajishengallis et al., 2009). It is reported that signaling molecules NFκB could regulate various cytokines (Jiang et al., 2017), and up-regulation of proinflammatory cytokines can be directly toxic on hepatocytes (Kono et al., 2001). Moreover, fish immunity is generally associated with cytokines, which were regulated by NF-κB signaling pathway. However, up to data, the possible mechanisms of dietary free gossypol toxicity and whether the yeast culture could relieve the inflammatory response of Ussuri catfish (*Pseudobagrus ussuriensis*) have not been well carried out, and deserves deep investigation.

Therefore, we investigate the effects of YC on gossypol induced inflammatory response in liver tissue of Ussuri catfish. And based on these effects, the potential mechanisms of YC-modulated immune and anti-inflammatory responses were evaluated.

2. Materials and methods

2.1. Ethical considerations

All animal care and handling procedures in this study were conducted under the Guidance of the Care and Use of Laboratory Animals in China and were approved by the Animal Care Committee of Northeast Agricultural University of China.

2.2. Experimental diets and chemicals

Two isonitrogenous (crude protein, 450 g kg⁻¹) and isolipidic (crude lipid, 77 g kg⁻¹) experimental diets were formulated, which contained 0 or 10 g kg⁻¹ YC (Table 1). Ingredients were finely ground through a 320-μm mesh and fully mixed, then the oil was added to the mixture as needed, next the mixture was dissolved by adding deionized water (100 mL kg⁻¹ diet) to produce dough. The dough was then extruded by a laboratory extruder (QL, Henan, China) of diameter 1.5 mm at room temperature. The resultant moist pellets were dried in a convection oven at 45 °C for approximately 6 h, and then allowed to cool overnight at room temperature. Finally, the sinking diets were stored at -20 °C until used.

Table 1
Formulation and proximate composition of the experimental diets (g kg⁻¹ dry matter).

Ingredients	Diet	Diet
	Control	YC
Fish meal ^a	280	270
Soybean meal ^a	300	300
Cottonseed meal ^a	110	110
Corn gluten meal ^a	70	70

Wheat meal	164	164
Soybean lecithin	10	10
Soybean oil	33	33
Vitamin premix ^b	5	5
Mineral premix ^c	5	5
Choline	3	3
Ca(H ₂ PO ₄) ₂	20	20
Yeast culture ^d	0	10
Proximate composition (g kg ⁻¹ dry matter)		
Dry matter	932.7	926.0
Crude protein	450.3	458.3
Crude lipid	77.7	77.0
Gross energy (kJ g ⁻¹)	184.9	185.7
Ash	121.3	118.1
Crude fiber	31.5	31.6
Nitrogen free extract	243.9	243

^a Fish meal: crude protein: 645.0 g kg⁻¹, crude lipid: 85.0 g kg⁻¹; soybean meal: crude protein: 467.9 g kg⁻¹, crude lipid: 31.4 g kg⁻¹; cottonseed meal: crude protein: 482.0 g kg⁻¹, crude lipid: 14.7 g kg⁻¹; corn gluten meal: crude protein: 602.0 g kg⁻¹, crude lipid: 14.7 g kg⁻¹; These ingredients were supplied by Huada feed Co., Ltd. (Harbin, China).

^b Vitamin premix (IU or mg kg⁻¹ dry diet): retinol (V_A) 3000 IU; cholecalciferol (V_D) 1500 IU; tocopherol (V_E) 40 mg; menadione (V_K) 4.5 mg; thiamin (V_{B1}) 8 mg; riboflavin (V_{B2}) 8.5 mg; pyridoxine (V_{B6}) 6.5 mg; cyanocobalamin (V_{B12}) 0.02 mg; nicotinic acid 45 mg; nicotinamide 45 mg; D-Ca pantothenate 17 mg; inositol 40 mg; biotin 0.15 mg; folic acid 1.3 mg; ascorbic acid 110 mg. ^c Trace mineral mixture use providing the following concentration (mg kg⁻¹ dry diet): copper 6.5 mg; iron 45 mg; selenium 0.35 mg; zinc 70 mg; manganese 8.5 mg; magnesium 100 mg; cobalt 1 mg; iodine 1.2 mg. ^d Yeast culture: crude protein: 487.0 g kg⁻¹, crude lipid: 42.1 g kg⁻¹, supplied by Beijing Enhalor International Tech Co., Ltd. (Beijing, China).

Gossypol-acetic acid (1 mg = 0.8962 mg gossypol) is the naturally occurring acetic acid form of gossypol and was purchased from Yuanye Biotechnology Co. (purity ≥98%, Shanghai, China). Sodium carboxymethylcellulose (CMCNa) was purchased from Yongda Reagent Corporation (Tianjin, China).

2.3. Feeding trial procedures and oral gavage with gossypol

Experimental fish were obtained from Fisheries Research Institute of Harbin Academy of Agricultural Sciences (Harbin, China). Prior to starting the feeding trial, all fish were fed the diet without yeast culture for 2 weeks to acclimate to the experimental diet and experimental conditions. After fasting for 24 h, fish of homogeneous size (initial average weight 7.39 ± 0.32 g) were randomly allotted to 6 aquaria (1.0 × 0.5 × 0.8 m, water depth 50–60 cm) with 20 fish to each aquarium. Diet with YC and diet without YC were randomly assigned to triplicate aquaria respectively. Fish were hand-fed to apparent satiation twice daily (08:00 and 16:00). During the experimental period, the flow rate of water was maintained at 2.5 L min⁻¹ and each aquarium was provided with continuous aeration to maintain the dissolved oxygen level above 8 mg L⁻¹, the temperature was 25 ± 1 °C, pH 7.5–8.0, ammonia-N was < 0.1 mg L⁻¹, the photoperiod was set at 12-h light and 12-h dark. The feeding experiment lasted for 8 weeks.

At the end of the feeding trial, the experimental fish were anesthetized with eugenol (1: 12000) (Shanghai Reagent Corporation, Shanghai, China). We collected the fish and created three different

groups: i.e., Control, Gos and Gos/YC groups. There were three replicates per group and 10 fish per replicate (For a total of 30 fish in each group). Fish pre-fed diet without YC without gossypol oral gavage or with gossypol oral gavage was referred as the control group and Gos group, respectively. Fish pre-fed diet with YC with gossypol oral gavage was referred as Gos/YC group. In the Gos and Gos/YC groups, fish were given a single dose of 200 μ L of 1% CMC-Na solution containing gossypol, namely a final dose of 300 mg gossypol / kg body weight. In the control group, fish fed diet without YC were given a single dose of 200 μ L of 1% CMC-Na solution without gossypol addition. Gossypol solution or CMC-Na solution was forced into the stomach of the fish using a 1-mL syringe fitted with an infusion tube. The oral administration experiment was conducted once daily (18:00) and lasted for 15 days.

2.4. Sample collection

At the end of the oral administration experiment, all experimental fish were anesthetized with eugenol (1: 12000) (Shanghai Reagent Corporation, Shanghai, China) before sampling. Serum samples were collected from the fish caudal vein using 1 mL syringe and withdrawn into Eppendorf tubes without anticoagulant. Blood samples in Eppendorf tubes were allowed to clot for 6 h at 4 °C. Following centrifugation (3000 g for 10 min), serum was stored at -80 °C for analyses of lysozyme (LZM) and alkaline phosphatase (AKP) activities and immunoglobulin M (IgM) content. After serum samples were collected, the liver tissues were selected on ice pack and were immediately frozen in liquid nitrogen and stored at -80 °C prior to analysis. Liver tissue samples were fixed in Bouin's solution for histological analysis.

2.5. LZM, AKP and AST activities and IgM level measurement

The serum LZM activity was determined by commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, China) as described by [Yin et al. \(2015\)](#). A LZM activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min⁻¹ at 530 nm. AKP activity unit is defined as 15 min per 100 mL plasma at 37 °C and substrate effects, produce 1 mg of phenol by a unit of enzyme activity as described by our previous study ([Bu et al., 2017](#)). The serum IgM level was determined by the kit of enzyme linked immunosorbent assay (ELISA) as described by [Yu et al. \(2014\)](#). Serum AST activity was also determined by the commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, China).

2.6. Hepatic antioxidant status analysis

The liver tissues were homogenized in ice-cold 0.86% (w / v) NaCl solution in tissue homogenizer and centrifuged at 3000 g 4 °C for 10 min. The supernatants of liver homogenates were used as enzyme source for measuring the hepatic total antioxidant capacity (TAC), activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and hepatic malondialdehyde (MDA) content by using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) as described by our previous study ([Bu et al., 2017](#)). The protein content in the liver sample was also determined using a total protein quantification kit (Nanjing Jiancheng Bioengineering Institute, China) with bovine serum albumin as the standard.

2.7. Real-time polymerase chain reaction (PCR) analysis

RNA extraction, reverse transcription and quantitative real-time PCR were conducted according to the previously established methods in our laboratory ([Bu et al., 2017](#)). Total RNA was extracted from liver using TransZol Up Plus Kit (TransGen Biotech, China) according to the manufacturer's instructions followed by DNase I treatment. Quantity and quality of the RNA was assessed by spectrophotometric (A260:

Table 2
Primers used in this study.

Primer names	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
TLR2-F	TTGTACAGCTGGATGAGTTG	206	54
TLR2-R	TGTCGTCAGTGAATGTCTC		
MyD88-F	TCAGACAGCTGGAGCAGACA	93	59
MyD88-R	CGCTGGTGATGGTCCAAACA		
NF- κ B p65-F	AAGAACCAGCCATACAAGCCACAC	83	60
NF- κ B p65-R	TCAGGCAGGTCCGCTTCGTAG		
IL-1 β -F	CCTGAACACCTTCGAGTCGG	102	58
IL-1 β -R	AGGTGGCTGTTTGTCTGATT		
IL-8-F	ATCGAAGGAAAAGCAGAGCG	111	57
IL-8-R	CTTGACACAGGAGCCACTTG		
IL-10-F	TCATACGCCGTCATCCGAGA	161	58
IL-10-R	CTGACTGCACTGGGCAACAC		
β -actin-F	CCTCCGCTGGATTGGCTG	141	60
β -actin-R	TCAAGGGCGACGTAGCAGAG		

TLR2-F	TTGTACAGCTGGATGAGTTG	206	54
TLR2-R	TGTCGTCAGTGAATGTCTC		
MyD88-F	TCAGACAGCTGGAGCAGACA	93	59
MyD88-R	CGCTGGTGATGGTCCAAACA		
NF- κ B p65-F	AAGAACCAGCCATACAAGCCACAC	83	60
NF- κ B p65-R	TCAGGCAGGTCCGCTTCGTAG		
IL-1 β -F	CCTGAACACCTTCGAGTCGG	102	58
IL-1 β -R	AGGTGGCTGTTTGTCTGATT		
IL-8-F	ATCGAAGGAAAAGCAGAGCG	111	57
IL-8-R	CTTGACACAGGAGCCACTTG		
IL-10-F	TCATACGCCGTCATCCGAGA	161	58
IL-10-R	CTGACTGCACTGGGCAACAC		
β -actin-F	CCTCCGCTGGATTGGCTG	141	60
β -actin-R	TCAAGGGCGACGTAGCAGAG		

280 nm ratio) analysis and electrophoresis in 1.2% agarose gel. Subsequently, the first strand cDNA synthesis was performed using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China). Reaction conditions were recommended by the manufacturer. Quantitative PCR (20 μ L) was performed with TransStart® Green qPCR SuperMix kit (TransGen Biotech, China) using Applied Biosystems® 7500 Real-time PCR System (USA) according to the manufacturer's protocol. There were no significant differences in transcript levels of β -actin gene among dietary treatments and was used as internal control. The real-time PCR began with 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, 30 s at 60 °C. The melting curve analysis showed only one peak for each PCR product. The gene expression levels were calculated by 2^{- $\Delta\Delta$ CT} method ([Livak and Schmittgen, 2001](#)). The primers used are shown in Table 2.

2.8. Histological analysis

Liver samples were preserved in Bouin's solution fixative for 24 h, dehydrated in a graded ethanol series, embedded in paraffin. The sections (5 μ m) were stained with hematoxylin-eosin (HE) and observed with an Axioskop microscope (BX51, Olympus, Tokyo, Japan).

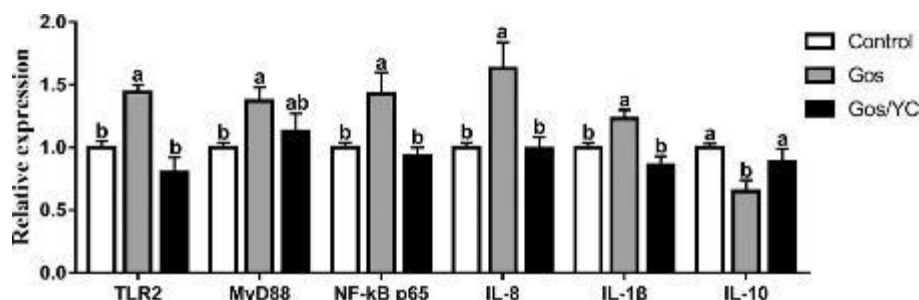
2.9. Statistical analysis

Results are presented with means $\pm$ S.E.M (standard error of the means) with superscript letters indicating differences between groups. Data was subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. A significant level of $P < .05$ was employed in all cases. All statistical analyses were performed using SPSS 22.0 for Windows (SPSS, Inc., USA).

3. Results

3.1. Inflammation-related gene expression analysis

As shown in Fig. 1, the mRNA levels of hepatic TLR2, NF- κ B p65, IL1 β and IL-8 were significantly up-regulated in Gos group compared with the other groups ($P < .05$), but no significant differences were observed in these parameters between the Gos/YC and the control groups ($P > .05$). The highest hepatic MyD88 mRNA level was also found in the Gos group, but no significant difference was observed between the Gos and the Gos/YC groups ($P > .05$). The lowest hepatic IL-10 mRNA level was found in the Gos



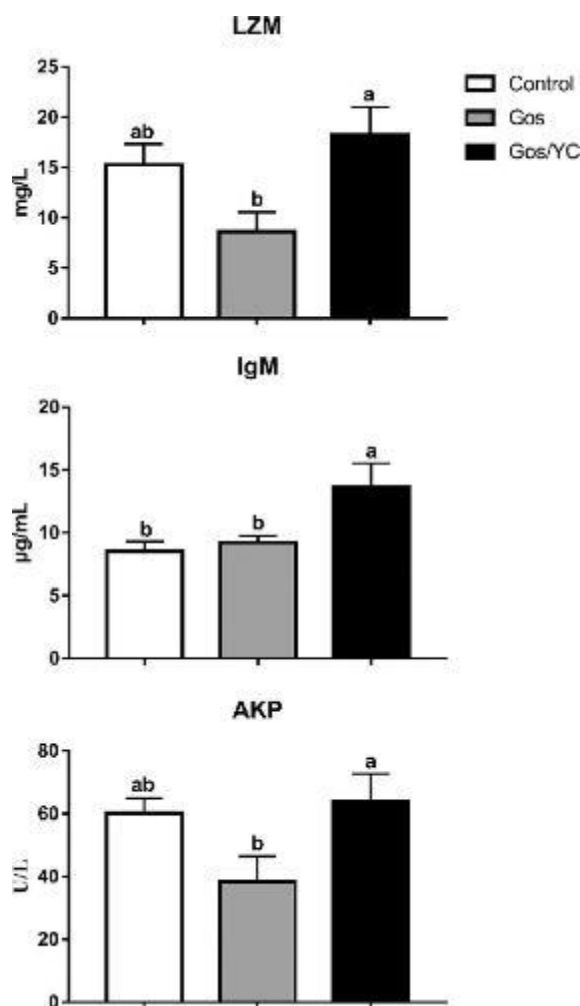
group, and fish in the Gos/YC group had significant higher IL-10 mRNA level than fish in the Gos group ($P < .05$).

difference among dietary treatments ($P < .05$). LZM: lysozyme; IgM: immunoglobulin M; AKP: alkaline phosphatase; S.E.M: standard error of the means.

3.2. Non-specific immunity parameters

Serum LZM and AKP activities and serum IgM content data are shown in Fig. 2. Fish in Gos/YC group had significantly higher serum LZM and AKP activities than fish in Gos group ($P < .05$). Simultaneously, there was no significant difference in serum IgM content for fish in Gos group compared with fish in control group ($P > .05$), but there was a sharp rising for that of fish in Gos/YC group compared with that of fish in Gos group ($P < .05$).

β terleukin 8; IL-10: interleukin 10; S.E.M: standard error of the means.



3.3. Hepatic antioxidant status analysis

As shown in Fig. 3, fish in Gos and Gos/YC groups had significantly lower hepatic CAT activity than the control group ($P < .05$), and there was no significant difference between fish in Gos and Gos/YC groups ($P > .05$). The lowest hepatic SOD and TAC activities were observed in Gos group, and the hepatic TAC in Gos/YC group was significantly higher than that in Gos group. No significant differences were observed in hepatic GPx activity and MDA content among the groups ($P > .05$), but it was noteworthy that the highest MDA content was found in Gos group.

3.4. Liver histology and aspartate aminotransferase activity

No markedly histopathological changes were observed in the liver tissues of fish in the control group (Fig. 4). In the liver tissues of fish pre-fed diet without yeast culture supplementation and treated with gossypol oral administration, higher occurrences of the dilatation in sinusoids, karyolysis, hydropic degenerations and nuclei shifting to the cellular periphery were detected compared with the fish in the control group. However, lower occurrences of these histopathological symptoms were observed in the Gos/YC group compared with those of fish in the Gos group. Meanwhile, fish in Gos group had significantly higher serum AST activity than the other groups ($P < .05$).

4. Discussion

Free gossypol is a yellow polyphenolic compound in the pigment glands of cotton plants *Gossypium* spp., which is known to be toxic to monogastric animals including fish. Herman (1970) reported that gossypol had toxic effects on growth, hematocrit, hemoglobin and total plasma protein of rainbow trout (*Salmo gairdneri* Richardson). Moreover, Bian et al. (2016) found that dietary

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Fig. 1. The effects of dietary pre-supplementation with YC on the hepatic inflammation-related related genes expression in response to gossypol for Ussuri catfish. The immune related genes expression of TLR2, MyD88, NF-κB p65, IL-1β, IL-8 and IL-10 are determined in the liver of Ussuri catfish. Vertical bars represented the means $\pm$ S.E.M of three replicates. Different letters in each figure represented significant difference among dietary treatments ($P < .05$). TLR2: toll-like receptor 2; MyD88: myeloid differentiation factor 88; NF-κB p65: nuclear transcription factor kappa B p65; IL-1 : interleukin-1 ; IL-8: in-

Fig. 2. The effects of dietary pre-supplementation with YC on serum LZM and AKP activities and IgM content in response to gossypol for Ussuri catfish. Vertical bars represented the means $\pm$ S.E.M of three replicates. Different letters in each figure represented significant

gossypol could cause liver fibrosis and simulated proinflammatory cytokine secretions. Previous studies suggested that gossypol accumulated mainly in the liver (Jensen et al., 1982; Kim et al., 1996; Lee and Dabrowski, 2002). Thus, at

present we evaluated the effect of free gossypol on the gene expression levels of three cytokines (IL-1 β , IL-8 and IL-10) in liver tissue of Ussuri catfish. Cytokines, such as IL-1 β and IL-8 have a fundamental role in regulation of the pro-inflammatory response in fish (Jung et al., 1995). In the present study, fish in Gos group had significantly higher hepatic IL-1 β and IL-8 mRNA expression than fish in control group, however, hepatic IL-1 β and IL-8 mRNA expression of fish in Gos/YC group was significantly lower than fish in Gos group, suggesting that free gossypol may have a stimulatory action upon proinflammatory

processes and dietary yeast culture supplementation inhibited the inflammatory response. In addition, IL-10 is regarded to have an anti-inflammatory role, potentially inhibiting the capacity of cells to secrete inflammatory mediators (Fiorentino et al., 1991). In the present study, dietary yeast culture supplementation upregulated IL-10 mRNA expression level. Taken together, these results suggested that yeast culture could have potentially protective role against gossypol-induced inflammatory response,

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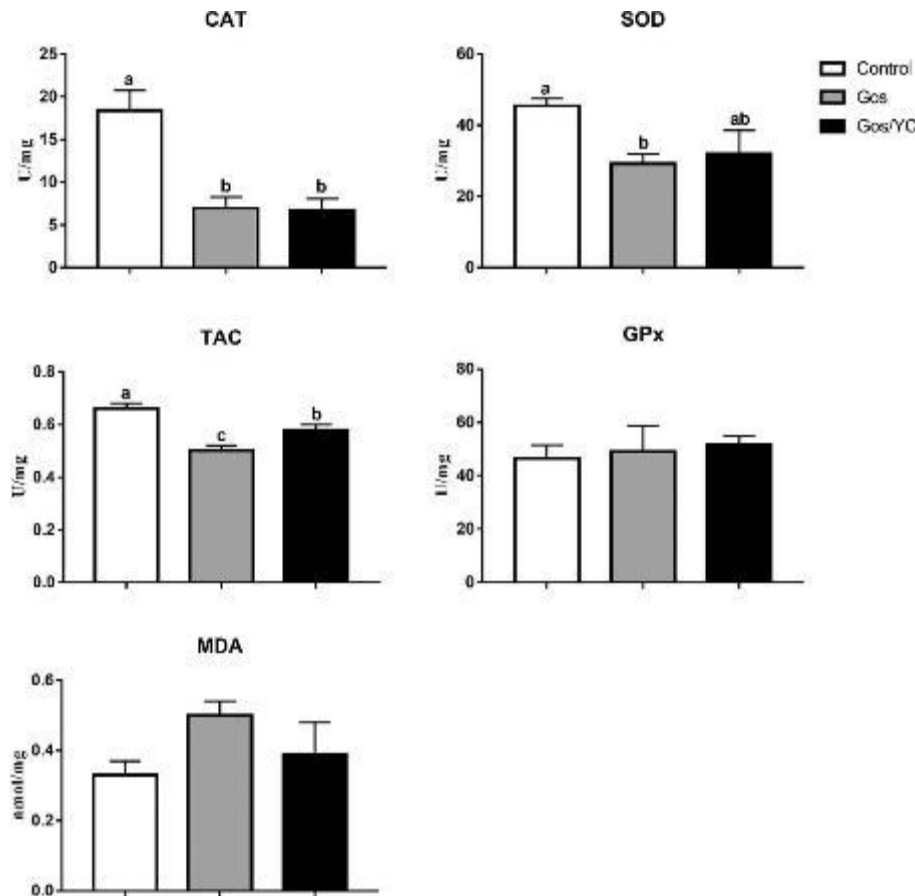


Fig. 3. The effects of dietary pre-supplementation with YC on hepatic antioxidant capacity parameters in response to gossypol of Ussuri catfish. Vertical bars represented the means $\pm$ S.E.M. of three replicates. Different letters in each figure represented significant difference among dietary treatments ($P < .05$). CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; TAC: total antioxidant capacity; MDA: malondialdehyde; S.E.M: standard error of the means.

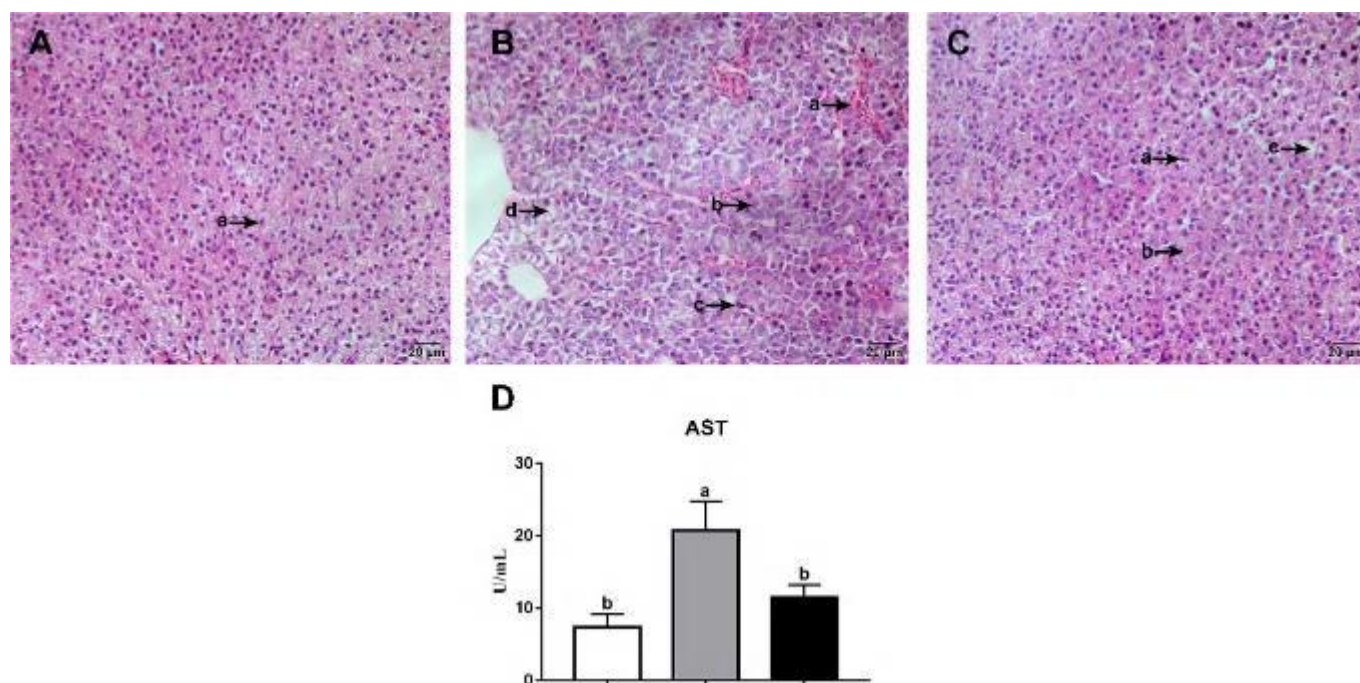


Fig. 4. The effects of dietary pre-supplementation with YC on histological analysis of liver sections and aspartate aminotransferase activity (AST) in response to gossypol for Ussuri catfish. (A) Liver structure of fish in control group: (a) hepatocyte (H & E). (B) Liver structure of fish in Gos group: (a) dilatation in sinusoids, (b) karyolysis, (c) nuclei shifting to the cellular periphery and (d) hydropic degenerations. (C) Liver structure of fish in Gos/YC group: (a) nuclei shifting to the cellular periphery, (b) karyolysis and (c) hydropic degenerations. (D) Aspartate aminotransferase activity. Scale bar = 20 µm.

and this is the first attempt to investigate whether yeast culture could relieve gossypol-induced inflammatory responses in fish.

Bian et al. (2016) also reported that TLRs act an important role in the activation of hepatic immune and inflammatory responses. TLRs are found on immune cells and recognize molecular sequences found on microbes (Kigerl et al., 2007), which serve as major pattern recognition receptors that detect pathogen-associated molecular patterns (PAMPs). The PAMPs could trigger NF-κB signaling pathway and production of pro-inflammatory (Nguyen et al., 2002). TLR2 is one type of pattern recognition receptors, which has been confirmed to have a strongly associated with immunity and inflammatory response of fish (Liu et al., 2016). Moreover, previous study demonstrated that dietary gossypol could induce inflammation of liver (Soares et al., 2012). Similarly, the present results showed that fish pre-fed the control diet and accompanying with gossypol oral gavage had significantly higher hepatic TLR2 mRNA level than that in the control group, suggesting that gossypol exposure may induce the production of inflammatory cytokine through the TLR2 signaling pathway, causing liver inflammation response of Ussuri catfish. However, the hepatic TLR2 mRNA level of fish in Gos/YC group was decreased significantly than that in Gos group. This result again verified that yeast culture may have a mitigative effect on gossypol-induced inflammatory response. To illuminate the regulation mechanisms of inflammation-related cytokine production after the fish was treated with gossypol oral gavage, we evaluated the effects of the main signaling molecules such as TLR2, MyD88 and NF-κB p65 in TLR2-MyD88-NF-κB signaling pathway. It was well known that overactivation of this signaling pathway would aggravate inflammatory reaction and then have negative effects on organism (Jiang et al., 2015). Accordingly, the present results demonstrated that the hepatic MyD88 and NF-κB p65 mRNA levels were up-regulated in response to gossypol, and fish pre-fed yeast culture prevented the increase in the MyD88 and NF-κB p65 mRNA levels. These results suggested that yeast culture may relieve gossypol induced inflammatory response via altering TLR2-MyD88-NF-κB signaling pathway in liver tissue of Ussuri catfish. However, it is necessary to point out that so far it has not been defined in fish how yeast culture regulates the TLR2-MyD88-NF-κB signaling pathway or cytokines gene expression and the mechanisms need to be further investigation.

In fish, the innate immune system is the essential defense mechanism because fish is continuously exposed to numerous opportunistic pathogens and this part of immune response provides the first line of defense for the host (Staykov et al., 2007; Ke and Zhang, 2019). Currently, several approaches were employed to enhance the immunity and control the diseases of fish, such as the using of immunostimulants, probiotics and nucleotides (Selvaraj et al., 2005; Yin et al., 2015; Li et al., 2019). Our previous study also showed that dietary yeast culture supplementation could improve the immunity of Ussuri catfish (Bu et al., 2019). In the present study, three indicators of innate immunity (serum LZM, AKP and IgM) were tested to evaluate the harmful effect of gossypol on immunity of Ussuri catfish and the alleviative effect of yeast culture on gossypol-induced immune damage. Lysozyme, a bactericidal enzyme, is known to act as an important component of the immune defense in fish (Lushchak et al., 2001). It is responsible for breaking down the peptidoglycan layer of the bacterial cell wall, causing lysis and thus stimulating the phagocytosis of bacteria (Hauge et al., 2002). Moreover, alkaline phosphatase is an important lysosomal enzyme with potential protective role in fish (Ghahderijani et al., 2015). In the present study, fish in Gos group had lower serum LZM and AKP activities than the control group though there were no significantly differences between these two groups. These results suggested that the gossypol, to some extent, may cause the disorder of the immune system of fish and reduce the antibacterial ability of Ussuri catfish. Similarly, dietary gossypol could decrease intestinal innate and adaptive immune components of grass carp (*Ctenopharyngodon idella*) (Wang et al., 2019). Furthermore, fish in Gos/YC group had higher serum LZM and AKP activities than fish in Gos group, indicating that fish pre-fed yeast culture could alleviate the gossypol-induced immune damage response for Ussuri catfish. Moreover, IgM is the most widely studied immunoglobulin in fish (Estensoro et al., 2012; Purcell et al., 2012) and it is the earliest produced antibody in the initial humoral immune response, which could be a good biomarker for evaluating the immune status of fish. In the current study, fish pre-fed yeast culture could induce the secretion of IgM. Presumably, the functional components (i.e. β-glucan, mannan-oligosaccharide, peptides) in yeast culture possibly induced B lymphocytes to produce antibodies to activate immune response of fish. But it is unclear why gossypol stress did not cause a decrease in serum IgM level of fish. The mechanisms await further characterization.

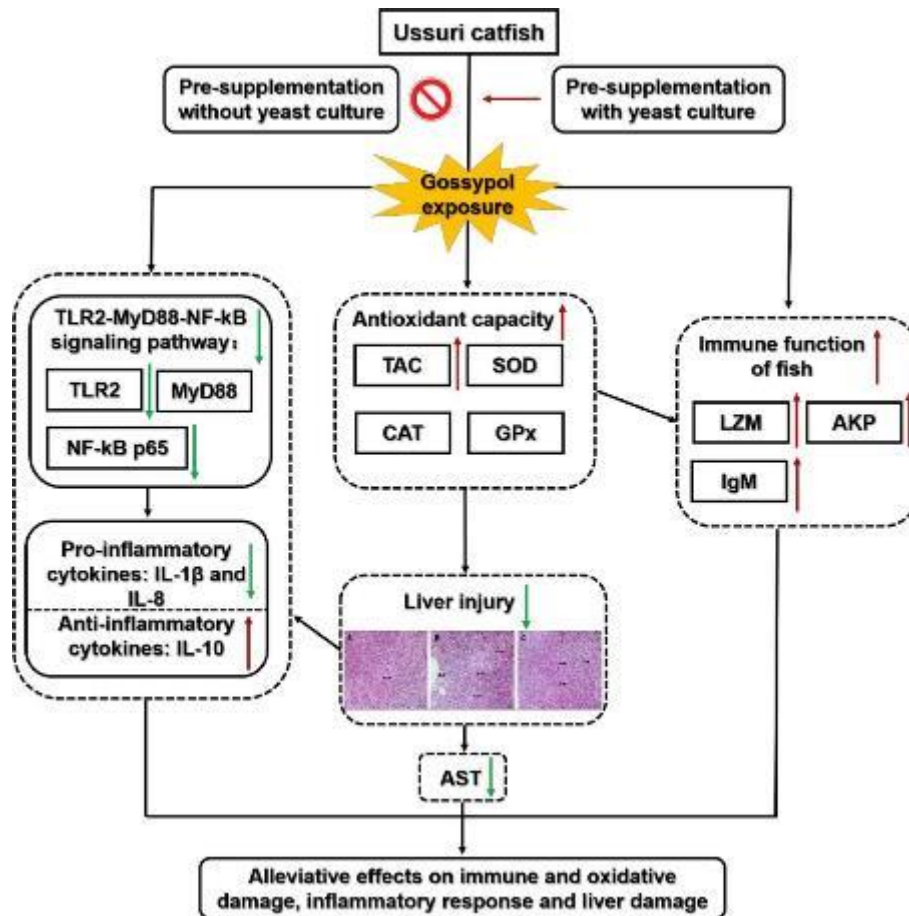


Fig. 5. Potential action pathways of yeast culture relieved gossypol induced immune and oxidative damage and inflammatory response of Ussuri catfish. Fish pre-fed yeast culture could alleviate the immune damage and oxidative stress induced by gossypol oral administration. Meanwhile, yeast culture could alleviate the liver injury through down-regulating TLR2MyD88-NF-κB signaling pathway. TLR2: toll-like receptor 2; MyD88: myeloid differentiation factor 88; NF-κB p65: nuclear transcription factor kappa B p65; IL-1β: interleukin-1β; IL-8: interleukin 8; IL-10: interleukin 10; CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; TAC: total antioxidant capacity; LZM: lysozyme; IgM: immunoglobulin M; AKP: alkaline phosphatase.

The immunity of fish was also related with the antioxidant system. Under normal physiological or stressful conditions, free radicals and reactive oxygen species (ROS) are continuously generated and organisms possess adequate protection systems such as key enzymatic antioxidant defenses to avoid/repair the ROS induced damage (Liu et al., 2018). However, oxidative stress occurs when the balance between the generation and removal of ROS are disrupted. In the current study, fish in Gos group down-regulated the hepatic TAC, CAT and SOD activities significantly, suggesting that gossypol may reduce the antioxidant capacity of Ussuri catfish. In addition, the possible mechanisms of dietary free gossypol toxicity include the generation of oxygen metabolites such as superoxide and hydrogen peroxide radicals (Rinchart et al., 2003). Furthermore, fish in Gos/YC group had significantly higher TAC than the Gos group. Meanwhile, the hepatic SOD activity of fish also had a slightly increased trend in Gos/YC group compared with the Gos group though there was no significantly difference between these two groups. These results demonstrated that fish pre-fed yeast culture, to some extent, had an enhanced effect of antioxidant capacity for Ussuri catfish.

Generally, liver is the vital organ for detoxification, and histological changes in liver are considered an important indicator for evaluating nutritional condition (Van der Oost et al., 2003; Rašković et al., 2011). Thus, the present study investigated the effect of gossypol on liver histology in Ussuri catfish, and found that gossypol oral gavage could cause abnormalities of liver, including dilatation in sinusoids, karyolysis, hydropic degenerations and nuclei shifting to the cellular periphery. Likewise, previous studies reported that these histopathological changes in liver revealed the liver injury or degenerative necrotic condition, which may affect liver function of fish (Zhang et al., 2019; Wang et al., 2014). Moreover, AST, as a sensitive marker of hepatocellular integrity, is generally considered important diagnostic indicators, which is used to estimate the health of fish (Wang et al., 2014). According to the present results, elevated serum AST level of fish in Gos group was also evidence of liver injury. Simultaneously, liver is the preferred organ for evaluating the status of antioxidant defense, and is also highly relevant for biomonitoring researches (Zhang et al., 2019). Therefore, the reduction in antioxidative capacity and upregulated proinflammatory

cytokines may also indirectly verified the liver damage of fish. Moreover, fish pre-fed diet with yeast culture supplementation had lower occurrences of histopathological symptoms in response to gossypol oral gavage, suggesting the mitigative effect of yeast culture on the hepatic lesion induced by gossypol for Ussuri catfish.

In conclusion (Fig. 5), fish treated with gossypol could induce inflammatory response, resulting in up-regulation IL-1β and IL-8 mRNA levels in liver tissue of fish. Dietary yeast culture supplementation could alleviate gossypol-induced inflammatory response via altering TLR2MyD88-NF-κB signaling pathway and could also relieve immune damage, oxidative stress and liver injury of Ussuri catfish.

Declaration of Competing Interest

No potential conflict of interest.

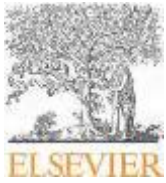
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Effects of dietary yeast culture on shrimp growth, immune response, intestinal health and disease resistance against *Vibrio harveyi*Stephen Ayiku^{a,c}, Jian-fei Shen^{a,c}, Bei-ping Tan^{a,b,c}, Xiao-hui Dong^{a,b,c}, Hong-yu Liu^{a,b,c,*}^a Laboratory of Aquatic Animal Nutrition and Feed, College of Fisheries, Guangdong Ocean University, Zhanjiang, Guangdong, 524088, PR China^b Key Laboratory of Aquatic, Livestock and Poultry Feed Science and Technology in South China, Ministry of Agriculture, Zhanjiang, Guangdong, 524000, China ^c

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Vibrio harveyi

ABSTRACT

The current study was conducted to evaluate the effects of different levels of yeast culture (YC) supplementation at 0% (YC 0%), 1% (YC 1%), and 2% (YC 2%) on growth, feed conversion ratio, body composition, intestinal morphology, microflora, immune response, and resistance to *Vibrio harveyi* infection in *Litopenaeus vannamei*. After 8-weeks feeding trial, the results showed significant improvement ($p < .05$) in the final weight, weight gain rate, specific growth rate, survival rate and low feed conversion ratio in YC groups than the control. Serum total protein, superoxide dismutase, catalase, alkaline phosphatase, acid phosphatase, lysozyme, and phenol oxidase in shrimps fed diet YC (2%) were significantly higher ($p < .05$), whereas significantly decreased trend in serum cholesterol, triglyceride, aspartate aminotransferase, and alanine aminotransferase ($p < .05$) were observed in YC (2%) diet. Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes were the core phylum bacteria found in the shrimp intestines. At the genus level, opportunistic pathogenic bacteria, *Vibrio* was significantly decreased ($p < .05$) while beneficial bacteria *Pseudoalteromonas* was increased in YC (2%) group. Intestinal villus height and width in shrimps fed YC diets were significantly improved than the control diet ($p < .05$). YC groups challenged test significantly showed ($p < .05$) improved shrimps immune response against *V. harveyi* infections with YC (2%) recording the highest percentage survival rate (70%). The present study demonstrated that supplementing YC (2%) can improve growth, intestinal microbiota, intestinal morphology, and immune response against *V. harveyi* infections in *L. vannamei*.

1. Introduction

Litopenaeus vannamei (Pacific white shrimp) is a worldwide cultured animal among the crustacean species because of its rapid growth, tender flesh, and high nutritional value [1]. For the past years, disease occurrences have led to substantial economic loss in the shrimp aquaculture industry [1–3]. Preventing pathogenic microorganisms using antibiotics have been widely associated with problems such as development and bioaccumulation of antibiotic resistant pathogens [4,5]. As an alternative to traditional disease-control, the use of probiotics, prebiotics, and natural immunostimulants has been of growing interest [4]. Non-digestible feed ingredient, prebiotics has the potentials of affecting host immunity by exclusively modulating metabolic activities in the gut, therefore, stimulating the host intestinal balance [6]. Due to abundant vitamins, proteins, pigments nucleotides, β -glucan content of single-cell proteins such as microalgae, yeast, and bacteria, they are often used as feed additives in aquaculture [7].

Single cell protein of some yeast, for example, *Saccharomyces cerevisiae* and *Candida* species have been used as probiotics [8,9]. The potential probiotics effects of *S. cerevisiae* on several aquatic species are enhanced immunity, growth,

and protection against pathogen infections [10–12]. Yeast culture (YC) is a complex fermented product, which contains yeast cell and its metabolic products. YC possesses high protein, lipid, and B-vitamins [13], and as a source of prebiotics, it contains β -glucan, chitin, nucleic acid, and oligosaccharides. Mannan oligosaccharides (MOS) and β -glucan structural components of yeast cell walls have the capacity of enhancing immunity. The B-glucan component in yeast culture activates the macrophage and releases various immunoglobulins, interleukin and cytokines to defend the fish against various pathogens [14]. Fish, when fed yeast culture diet, increase the abundance of Actinobacteria in the intestinal microbiota [15]. Actinobacteria maintain intestinal permeability, down regulate inflammation by IL-4 and IL-13, and induces regulatory T-cells [16]. The complex carbohydrate (MOS) content of yeast in the intestine inhibit pathogen colonization by blocking pattern recognition molecules and serves as binding site of potential pathogen instead of directly colonizing the gut walls [17]. Proven effects have shown that (MOS) can improve fish growth performance, gut morphology, modulating the intestinal microbiota [11,18], and elevating immunity and stress-resistance ability [19,20]. Reports have shown that yeast β -1,3-glucan has immunostimulatory capacity in shellfish against viral or bacterial

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infections [21]. A further report on β -glucan fed to *Penaeus monodon* postlarvae enhanced vibriosis resistance in shrimp [21]. Studies have demonstrated positive effects of YC on feed intake and nutrient digestibility, which in turn improved feed efficiency and growth of aquatic animals [22,23]. Dietary 1% yeast hydrolysate or brewer's yeast (Guangdong Hinabiotech Co., Ltd and Tech-bank Aquatic Feed Co., Ltd respectively) supplementation in diet improved growth performance, immune response, and ammonia nitrogen resistance of Pacific white shrimp [1]. Zhang et al. [24], reported that dietary supplementation of YC (4 g/100 g diet) (Enhalor Biotechnology Company (Beijing, China)) resulted in better growth performance, enhanced immune response, and improved the protection against *Aeromonas hydrophila* in gibel carp (*Carassius auratus gibelio*). A range of studies have shown that dietary YC supplementation improved growth, immune response, and diseases resistance in aquatic animal, however, information regarding YC effect in *L. vannamei* is limited.

Therefore, this research was conducted to evaluate the effect of YC supplementing fish meal (FM) concentration at 1% and 2% in *L. vannamei* diets by assessing growth performance, serum immune response, intestinal microbiota, intestinal morphology, and resistance against *V. harveyi*. The findings from this study might provide useful information on the formulation of immune response and disease resistant diets for *L. vannamei* production.

2. Materials and methods

2.1. Ingredients and experimental diet preparation

The ingredients and the nutrient profile of the basal diet are displayed in Table 1. Three experimental diets were formulated by supplementing YC at 0%, 1%, 2% designated as YC (0%), YC (1%) and YC (2%) respectively. The YC derived from *S. cerevisiae* was provided by Enhalor Biotechnology Company (Beijing, China) and contains 50.0% crude protein, 0.29% crude lipid, 4.0% moisture, and 9.3% ash on dry matter basis. The basal diet contains fishmeal (60% crude protein), soybean meal, peanut meal and shrimp shell powder meal as a protein source and fish oil, soybean oil, and phospholipid oil as lipid sources. Prior to diet preparation, all the coarse ingredients were milled into powder using a hammer mill machine, sieved through 80 mm mesh before mixing thoroughly with minerals and vitamins in a V-mixer machine. Phospholipid oil, soybean oil, and fish oil were added to mixtures and mixed by hand. Water containing Choline Chloride was blended in the mixture (approximately 25% of the total ingredient weight) to dough form which was divided into 1 kg and 3 kg. They were separately pelleted using meat grinder machines containing different discs with holes sizes of 1.0 mm and 1.5 mm to make spaghetti pellet. Pellets were oven-dried at 90 °C for 15 min, followed by air drying using electrical fan until moisture content reduced to about 10%. Pellets were broken into pieces, sieved into a proper length of 2.00 mm and 3.0 mm. After packaging pellets into seal bags, they were then stored at -20 °C until feeding.

2.2. Shrimp and rearing conditions

Juveniles of *L. vannamei* with the same genetic background, free from pathogens were obtained from Zhanjiang Allied Pacific Aquaculture Limited to Marine Biological Research Base of Guangdong

Table 1
Ingredients and proximate composition of the experimental diets (%).

Ingredients	CO (0%)	YC (1%)	YC (2%)
Fish meal ^A	20.00	19.26	18.51
Yeast culture	0.00	1.00	2.00
Soybean meal ^A	18.00	18.00	18.00
Peanut meal ^A	18.00	18.00	18.00
Shrimp shell powder ^A	7.00	7.00	7.00
High-gluten flour ^A	20.00	20.00	20.00
Fish oil ^A	1.80	1.80	1.80
Soybean oil ^A	1.80	1.80	1.80
Phospholipid oil ^A	1.80	1.80	1.80
Vitamin mixture ^B	0.20	0.20	0.20
Mineral element mixture ^C	0.50	0.50	0.50
Attractant ^A	0.10	0.10	0.10
Antioxidants ethoxyquin ^A	0.03	0.03	0.03
Choline chloride 99% ^A	0.50	0.50	0.50
92% monobasic calcium phosphate ^A	1.00	1.00	1.00

Vc polyphosphate 35% ^E	0.09	0.09	0.09
Antimony trioxide ^D	0.50	0.50	0.50
CMCC ^D	8.86	8.32	8.02
Total	100.00	100.00	100.00
Nutrient levels Dry matter	89.16	89.06	89.67
Crude protein	42.20	42.19	42.21
Crude lipid	8.01	7.95	7.90
Ash	8.94	8.89	9.05

A. Ingredients purchased from Zhanjiang HaiBao Feed Factory, Zhanjiang, Guangdong, China.

B. Vitamin mixture supplied the following per kg of the diet: vitamin A, 22,500 IU; vitamin D3, 6,000 IU; vitamin E, 200 mg; vitamin K3, 40 mg; vitamin B1, 30 mg; vitamin B2, 45 mg; vitamin B6, 35 mg; vitamin B12, 0.25 mg; calcium pantothenate, 150 mg; niacin, 225 mg; folic acid, 12.5 mg; biotin, 0.5 mg; inositol, 500 mg (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China).

C. Mineral element mixture provided the following per kilogram of diet: Fe, 60 mg; Zn, 24 mg; Mn, 16 mg; Cu, 1.4 mg; Co, 0.2 mg; Se, 0.1 mg; I, 0.2 mg (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China).

D. Obtained from Shanghai Macklin Biochemical Co. Ltd., 1288 Guangdong Rd., Shanghai, China.

E. Obtained from Shantou Xilong Chemical Factory, Guangdong, China.

Ocean University where the experiment was conducted. Shrimps were conditioned in indoor rearing fiberglass tanks for four weeks and fed with a commercial diet. Having starved shrimps for 12 h, those with an average weight of 0.36 ± 0.002 were selected for the experiment. A group of 40 healthy shrimps was stocked into each fiberglass tank containing 200 L of sieved seawater. They were offered diet four times daily (7.00, 11.00, 17.00 and 21.00) at 5–8% of their average body weight in four replicates. Before first feeding, 70% of water in each tank was replaced with new seawater every day. Feed intake, mortality, and water quality parameters were monitored. The rearing temperature was 28.0 ± 2.0 °C while dissolved oxygen (DO) was within 6–8 mg l⁻¹.

2.3. Sample collection

After feeding trials for 8 weeks, all shrimps were starved feed for 24 h before sampling. The total numbers, as well as body weight of shrimps from each tank, were recorded. Per tank, two shrimps were randomly picked, stored at -20 °C for carcass analysis. Hemolymph samples were drawn from the first abdominal segment of ten shrimps per tank with the aid of 1 mL sterile syringe into 1.5 Eppendorf tubes. The tubes were kept at 4 °C overnight before centrifuging at 3500 rpm/ min for 10 min at 4 °C, supernatants were separated and stored at -80 °C for enzymatic activities analysis. Mid-guts (0.8–1.2 cm) were aseptically removed from three shrimps in each tank and fixed into 10% paraformaldehyde for histological analysis. Furthermore, another three mid-guts per tank were randomly sampled gently loaded into empty 1.5 Eppendorf tubes and immediately stored in liquid nitrogen for

microbiota analysis.

2.4. Growth performance analysis

Shrimp were weighed from each tank at the initial stage and 8 weeks after feeding trial. The total amount of diet fed to each of the experimental groups and the control group was calculated after 8 weeks of the feeding trials. The final weight (FW), weight gain rate (WGR), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated using the following formula;

$$\begin{aligned}
 &= \frac{\text{SR (\%)} \times 100 \times \text{Shrimp final number}}{\text{Shrimp initial number}}; \text{FCR} \\
 &= \frac{\text{Total dry feed intake}}{\text{Total wet weight gain by shrimp (g)}}; \\
 &= \frac{\ln \left[\frac{\text{Final body weight g}}{\text{Initial body weight g}} \right]}{\ln \left[\frac{\text{Final body weight g}}{\text{Initial body weight g}} \right] - \ln \left[\frac{\text{Initial body weight g}}{\text{Initial body weight g}} \right]}; \\
 &= \frac{\text{SGR (\%)} \times 100 \times \text{number of culture days}}{\text{number of culture days}}
 \end{aligned}$$

$$\text{WGR (\%)} = 100 \times \frac{[\text{Final body weight g}] - [\text{Initial body weight g}]}{[\text{Initial body weight g}]}$$

2.5. Chemical analysis of feed and whole body composition

Proximate compositions of formulated feed and shrimp carcass samples were examined using a standard method [25]. Dry matter was assessed by oven-drying samples at 105 °C until a constant weight was obtained. Crude protein was established following the Kjeldahl method which involves using boric acid to trap NH₃ and was calculated as N x 6.25. The quantity of ash was tested by burning 2 g of samples in a muffle furnace at 550 °C for 4 h. Crude lipid was examined by the ether extraction method using Soxhlet System HT.

2.6. Serum immune analysis

The serum collected from shrimp were used to analyze parameters such as Glucose (Glu), Triglyceride (TG), Cholesterol (CHO), Catalase (CAT), Superoxide dismutase (SOD), Acid phosphatase (ACP), Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Lysozyme (LZM), and Phenoxidase (PO). These parameters were determined using commercial kits (Nanjing Jiancheng Biological Engineering Institute, China) following the company's protocol. A unit of SOD activity was described as the quantity of enzyme required to yield 50% inhibition of the nitroblue tetrazolium reduction (NBT) rate measured at 550 nm using ELISA microplate reader and is expressed as unit/millilitre (U/ml). A unit of CAT activity was defined as the amount of enzyme needed to decrease absorbance of 0.01/min at 405 nm in a microplate reader and is expressed as unit/millilitre (U/ml). AST and ALT activities were determined following the calorimetric method of Reitman and Frankel's [26] decided by standard curve acquired by contrasting assay between experimental method and Carmen's unit (1 Carmen's unit = 0.482 IU/L, 25 °C) at an absorbance peak of 510 nm. ACP and AKP activities of the serum were determined by spectrophotometer at 520 nm. A unit of AKP activity was defined as the volume of an enzyme that reacted with the matrix to produced 1 mg phenol in 30 min at 37 °C. A unit of ACP activity was defined as the amount of enzyme that reacted with the matrix and produced 1 mg phenol in 30 min at 37 °C. A unit of LZM activity was defined as the sum of the enzyme required to cause decrease absorbance rate of 0.001 min⁻¹ mL⁻¹. The PO activity was well-defined as the amount of enzyme required to produce an increase in absorbance of 0.001 min⁻¹. TP was quantified as described by Ref. [27] using bovine serum albumin as a standard.

2.7. Histological analysis of shrimp intestine

In order to determine the effect of the YC diet on shrimp intestinal morphology, we examined intestinal morphological indexes as described by Ref. [28]. Briefly, after the fixation process, intestinal segments retrieved from Bouin's liquid were dehydrated with graded concentrations of alcohol, cleared in toluene, equilibrated in xylene, and inserted in paraffin to form wax blocks. About 5 µm of wax blocks with tissue segments were sliced by using a rotary microtome and then stained with hematoxylin-eosin (H&E). Intestinal images were captured by using a microscope (Olympus, model BX51, Serial number: 9K18395, Tokyo, Japan). Electronic measurements were taken using the software, Image-Pro Plus 6.3 (Media Cybernetics, Inc., Rockville, USA) to obtain data on intestinal villus height (VH), villus width

(VW) and muscle thickness (MT) at 100× magnifications. From each tissue, 12 measurements were taken.

2.8. Microbiota analysis

Using high-throughput sequencing to identify the V3–V4 region of 16S rRNA gene, microbiota analysis was performed according to Ref. [29] with slight changes. Bacterial genomic DNA was extracted from the stool sample using Soil DNA Kit by following the manufacturer's instructions. Sample DNA concentration was quantified using NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The 16S ribosomal V3–V4 hypervariable region of bacteria gene was amplified by polymerase chain reaction (PCR) (95 °C for 2 min, followed by 27 cycles at 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 30 s with final elongation at 68 °C for 10 min) using universal bacteria primers (341F: 5'-CCTACGGGNGGCWGCAG-3'; 806R: 3'-GGACTACHVGGGTATCTAAT-5'). PCR reactions were carried out in triplicate 25 µL mixture containing 2.5 µL of TransStart Buffer, 2 µL of dNTPs, 1 µL of each primer, and 20 ng of template DNA. Through Illumina HiSeq 2500 sequencing, high-throughput sequencing was performed. Amplicons were then separated by 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) by following the manufacturer's protocol before being quantified with QuantiFluor-ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2×250) on an Illumina platform according to the standard protocols.

Quality filtering on the raw reads was performed by filtering to obtain the high-quality clean reads according to the Cutadapt. The reads were compared with the reference database (Silva database, <https://www.arb-silva.de/>) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) [30] to detect chimera sequences, and then the chimera sequences were removed and finally, clean reads were obtained. Sequence analysis was performed by Uparse software (Uparse v7.0.1001, <http://drive5.com/uparse/>) [31] where ≥ 97% similarities were assigned to the same OTUs. Alpha diversity was applied in analyzing the complexity of species diversity for samples through 4 indices, including Chao1, Shannon, Simpson, and ACE. Unique species among groups were shown by Venn analysis.

2.9. Challenge test

Bacteria *Vibrio harveyi* (HY99) was acquired from Guangdong provincial key laboratory of pathogenic biology, Fisheries College, Guangdong Ocean University. They were grown in Luria-Bertani (LB) media in 250 ml flat bottom flask by shaking in an incubator at 37 °C in 180 rpm for 20 h. The cells were centrifuged at 8,000 rpm for 10 min at 4 °C and supernatant discarded. The cells were washed twice with sterile phosphate-buffered saline (PBS). Using serial dilutions method, graded concentrations (10⁶, 10⁷, 10⁸ and 10⁹ CFU/ml) of *V. harveyi* was used to conduct preliminary bacteria challenge experiment. 0.1 ml of each concentration of *V. harveyi* was intramuscularly injected into the 288 third abdominal segment of ten shrimps (average weight 9.7 ± 0.1 g) in two replicates each in tanks containing 50 L of seawater. Mortalities were observed for 168 h, and calculation of best median lethal dose 50 (LD50) was determined (10⁸ CFU/ml).

At the end of the feeding trial, 40 shrimps from each treatment were randomly redistributed into four tanks for bacteria resistance test. Another ten shrimps per each experimental group were randomly selected and intramuscularly injected with PBS which was indicated as the negative control (NC). The other four replicate tanks were challenged with 0.1 ml of *V. harveyi* cell suspensions (10⁸ CFU/ml) by intramuscular injection. During the challenge trials, shrimps were still fed with their normal experimental diets. From ten shrimps per each treatment, hemolymph was sampled after 24 h of bacteria injection and centrifuged at 3500 rpm/min at 4 °C for 10 min, sera were separated and stored at -80 °C for subsequent analysis of ACP, ALP, PO, and LZM activities. The PBS and the other three bacteria challenge groups per treatment were kept under monitoring, and mortality was recorded up to 168 h. Cumulative mortalities were calculated using the formula shown below. Cumulative mortality, % = 100

Total mortality in each treatment after challenge
Total number of shrimp challenged for same treatment

2.10. Statistical analysis

The results in this experiment were presented as Mean $\pm$ Standard Error (SE). Statistical significant differences were established by using One-Way Analysis of Variance (ANOVA) at 5% level of probability and differences between means were compared using Tukey Range Test. Statistical analysis was carried out in SPSS for Windows, version 22 (SPSS Inc.) Chicago, IL, USA.

3. Results

3.1. Shrimps growth performance, feed conversion ratio and survival rates

The growth performance, feed conversion ratio, and survival rates of *L. vannamei* juveniles fed experimental diets are shown in Table 2. Diet supplemented with YC significantly showed improved growth performance and feed conversion ratio than the control group (YC (1%)) ($p < .05$). No significant differences ($p > .05$) had occurred between YC (1%) and YC (2%) supplemented diets in terms of FW, WGR, SGR, and FCR. YC supplemented groups improved survival rate during the experimental trial period, however, no significant differences ($p > .05$) have been found among all the experimental groups.

3.2. Proximate whole body composition of *L. vannamei*

Proximate whole body compositions of *L. vannamei* fed experimental diets are presented in Table 3. There was a significant improvement in the dry matter ($p < .05$) of the YC (2%) group but not Y

Table 2

Table 3

Effects of YC on *L. vannamei* proximate whole body composition.

Treatment	YC (0%)	YC (1%)	YC (2%)
Dry Matter (%)	21.45 $\pm$ 0.48 ^a	22.60 $\pm$ 0.35 ^{ab}	23.68 $\pm$ 0.25 ^b
Crude Protein (%)	15.12 $\pm$ 0.07 ^a	15.65 $\pm$ 0.02 ^b	16.00 $\pm$ 0.10 ^c
Crude Lipid (%)	2.26 $\pm$ 0.15 ^a	2.26 $\pm$ 0.01 ^a	2.76 $\pm$ 0.10 ^b
Ash (%)	2.55 $\pm$ 0.02 ^a	2.82 $\pm$ 0.04 ^b	2.89 $\pm$ 0.01 ^b

Effects of YC on growth performance and feed conversion ratio in *L. vannamei*.

Treatment	YC (0%)	YC (1%)	YC (2%)
IW(g shrimp ⁻¹)	0.36 $\pm$ 0.001	0.36 $\pm$ 0.002	0.36 $\pm$ 0.000
FW(g shrimp ⁻¹)	9.79 $\pm$ 0.08 ^a	10.66 $\pm$ 0.06 ^b	10.54 $\pm$ 0.05 ^b
WGR%	2617.31 $\pm$ 43.05 ^a	2824.37 $\pm$ 49.94 ^b	2789.75 $\pm$ 32.32 ^b
SGR%day ⁻¹	6.17 $\pm$ 1.35 ^a	6.36 $\pm$ 1.29 ^b	6.34 $\pm$ 1.24 ^b
FCR	1.35 $\pm$ 0.02 ^b	1.29 $\pm$ 0.02 ^{ab}	1.24 $\pm$ 0.01 ^a
SR%	85.00 $\pm$ 85.00 ^a	91.25 $\pm$ 91.25 ^a	87.50 $\pm$ 87.50 ^a

Values are presented as means $\pm$ SE. The values in the same row with different superscripts are significantly different ($p < .05$) ($n = 4$).

The values are presented as the mean $\pm$ SE. The values in the same row with different superscripts are significantly different ($p < .05$) ($n = 4$).

Table 4

Effects of YC on serum biochemical indexes in *L. vannamei*.

Treatment	YC (0%)	YC (1%)	YC (2%)
TP(mmol/L)	22.43 $\pm$ 0.77 ^a	23.15 $\pm$ 0.62 ^{ab}	25.14 $\pm$ 0.41 ^b
GLU(mmol/L)	2.53 $\pm$ 0.03 ^b	2.46 $\pm$ 0.06 ^b	2.07 $\pm$ 0.44 ^a
TG(mmol/L)	0.78 $\pm$ 0.01 ^{ab}	0.81 $\pm$ 0.00 ^b	0.75 $\pm$ 0.01 ^a
CHO(mmol/L)	1.24 $\pm$ 0.02 ^a	1.29 $\pm$ 0.01 ^a	1.23 $\pm$ 0.00 ^a
CAT(U/ml)	3.33 $\pm$ 0.06 ^a	3.65 $\pm$ 0.03 ^b	3.91 $\pm$ 0.07 ^c
SOD(U/ml)	938.76 $\pm$ 35.0 ^a	1148.99 $\pm$ 24.88 ^b	1210.66 $\pm$ 27.13 ^b
AST(U/L)	3.24 $\pm$ 0.06 ^{ab}	3.37 $\pm$ 0.01 ^b	3.09 $\pm$ 0.03 ^a
ALT(U/L)	5.21 $\pm$ 0.02 ^b	5.10 $\pm$ 0.06 ^b	4.83 $\pm$ 0.05 ^a

The values are presented as the mean $\pm$ SE. The values in the same row with different superscripts are significantly different ($p < .05$) ($n = 4$).

(1%) when compared to the YC (0%) group. Crude protein and ash were significantly high ($p < .05$) in YC (2%) group, followed by YC (1%) compared to the YC (0%) group. In addition, crude lipid was significantly higher ($p < .05$) in YC (2%) group than the YC (1%) and YC (0%) treatment groups.

3.3. Evaluation of serum biochemical indexes

Serum biochemical and antioxidant activities are presented in Table 4. Significantly improved ($p < .05$) TP (but not higher than YC (1%)) was detected in YC (2%) treatment in comparison to the YC (0%). There was no significant difference ($p > .05$) between the GLU of YC (0%) and YC (1%), however, GLU of YC (2%) decreased significantly ($p < .05$). TG concentration in serum was significantly low ($p < .05$) in YC (2%) (but not lower than YC (0%)) supplemented group. CHO showed no significant difference ($p > .05$) among all the treatment groups when compared to the untreated YC (0%) group. All YC treated groups (YC (1%) and YC (2%)) showed a significant rising tendency ($p < .05$) of SOD and CAT activities with the highest been observed in YC (2%) fed group. The activities of serum ALT and AST in shrimp fed YC (1%) and YC (2%) diet were clearly lower than the YC (0%) diet ($p < .05$).

3.4. Serum innate immune responses

Serum innate immune response in shrimps before and after 24 h *V. harveyi* challenge test is presented in Fig. 1. Before the challenge test, the result showed significant improvement ($p < .05$) in serum ACP and LZM activity with shrimps fed YC (1%) and YC (2%) supplemented diet

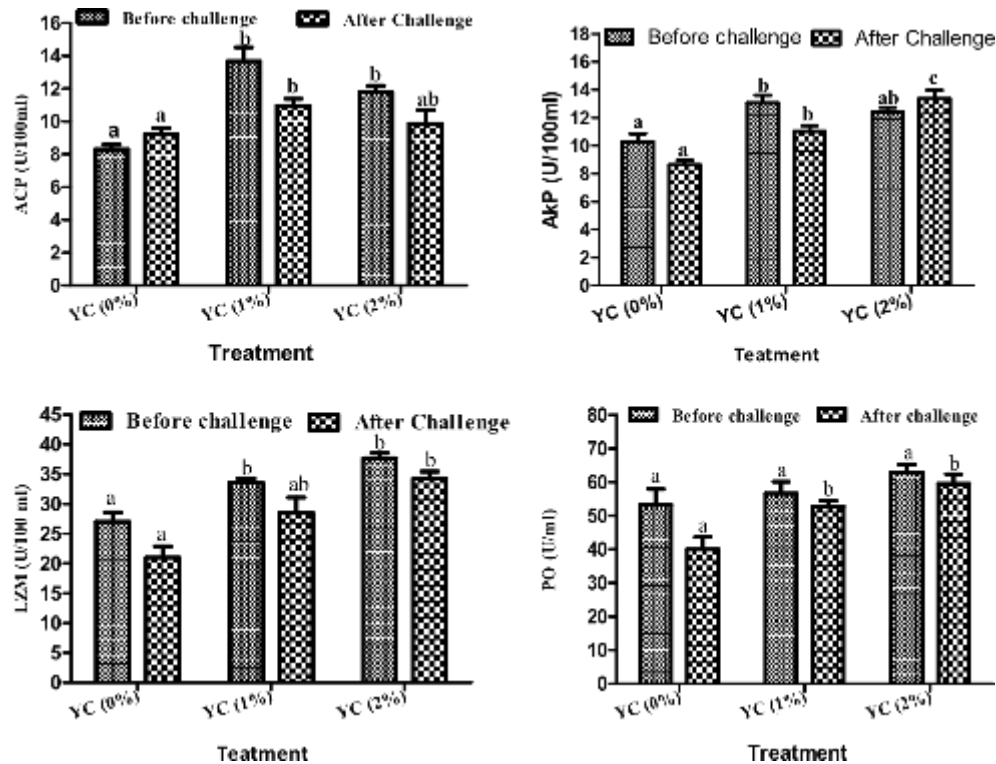


Fig. 1. Effects of YC on serum AKP ACP LZM and PO activity in *L. vannamei* before the challenge, and after 24 h post-challenge with *V. harveyi*. Upright bars denote the mean $\pm$ SE. Bars labeled with different letters denote significant difference ($p < .05$) ($n = 4$) among the treatment groups.

when compared to the YC (0%) diet. After 24 h bacteria challenge, serum AKP, ACP (but not YC (2%)), PO and LZM (but not YC (1%)) were also increased ($p < .05$) in YC (1%) and YC (2%) treated groups (Fig. 1) than the YC (0%) group.

3.5. Microbiota analysis in shrimp gut

3.5.1. Intestinal microbiota richness and species diversity

Operation taxonomic units (OTUs) and Alpha diversity data in *L. vannamei* intestinal microbiota are presented in Table 5. OTUs in shrimp fed YC (1%) and YC (2%) diet were significantly ($p < .05$) higher than the YC (0%). Venn diagram analysis showed that a total of 224 OTUs were shared among all the treatments. The individual OTUs were abundant in YC (2%) (535) group, followed by YC (1%) (177) with YC (0%) (64) having the least (Fig. 2). YC (1%) and YC (2%) treatment groups significantly improved ($p < .05$) chao1 and ACE estimator indices than the YC (0%) group. In addition, the Shannon and Simpson estimator index ranged between 5.08 to 5.82 and 0.86 to 0.91 respectively.

Table 5

Richness and diversity indexes of intestinal bacterial in *L. vannamei* fed experimental diets.

Treatment	YC (0%)	YC (1%)	YC (2%)
Raw Read	85597.39	92351.69	96510.46
Clean Read	80134.65	90462.33	94354.54
Tags	70600.16	89633.33	91688.00
OTU	342.35 $\pm$ 22.05 ^a	425.77 $\pm$ 19.67 ^a	632.91 $\pm$ 26.24 ^b
Chao	382.34 $\pm$ 9.67 ^a	463.78 $\pm$ 21.07 ^b	666.96 $\pm$ 15.97 ^c
ACE	424.96 $\pm$ 9.48 ^a	483.85 $\pm$ 8.34 ^b	728.97 $\pm$ 13.51 ^c
Shannon	5.08 $\pm$ 0.03 ^a	5.46 $\pm$ 0.02 ^b	5.82 $\pm$ 0.05 ^c
Simpson	0.86 $\pm$ 0.07 ^a	0.88 $\pm$ 0.00 ^a	0.91 $\pm$ 0.01 ^b

The data are presented as Mean $\pm$ SE. Means in the same row with different superscripts indicate significant difference ($p < .05$) ($n = 4$).

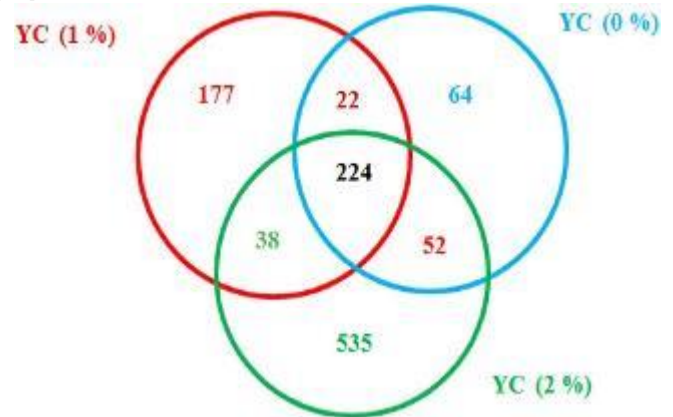


Fig. 2. Venn diagram indicating OTUs shared among *L. vannamei* fed with YC diets.

3.5.2. Gut microbiota composition of *L. vannamei* fed with YC supplemented diets

The top ten most abundant intestinal bacteria at phylum and genus levels are displayed in Figs. 3 and 4. The four dominant intestinal bacteria at the phylum level were Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Fig. 3). Proteobacteria was significantly decreased ($p < .05$) in YC (2%) fed groups while Bacteroidetes and Actinobacteria increased. Although Firmicutes was abundant in YC (1%) and YC (2%) fed group, no significant increase had occurred in any of the experimental groups. At the genus level (Fig. 4), *Vibrio*, *Motilinas*, *Pseudoalteromonas*, and *Candidatus_bacilloplasma* were abundant in all the experimental groups. However, *Vibrio* was significantly decreased ($p < .05$) while *Pseudoalteromonas* was abundant in the YC (2%) groups than in YC (0%) group ($p < .05$).

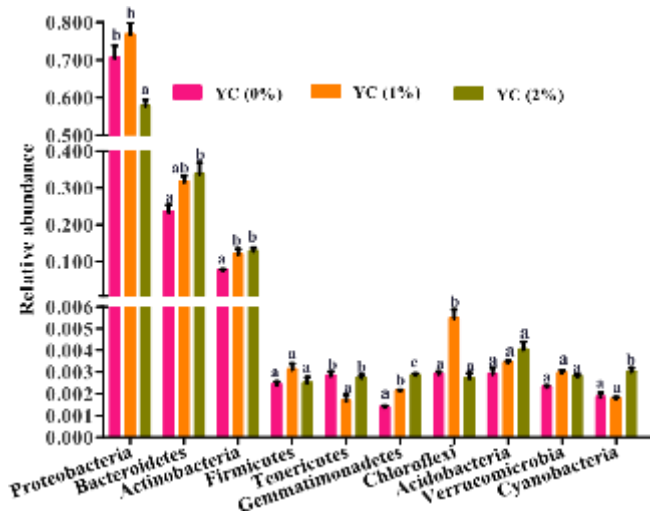


Fig. 3. Effects of YC on intestinal bacterial structure and composition in shrimps at the phylum level of the taxonomy. Upright bars denote the mean $\pm$ SE. ($p < .05$) ($n = 4$). Bars labeled with different letters denote significant differences ($p < .05$) among treatment groups.

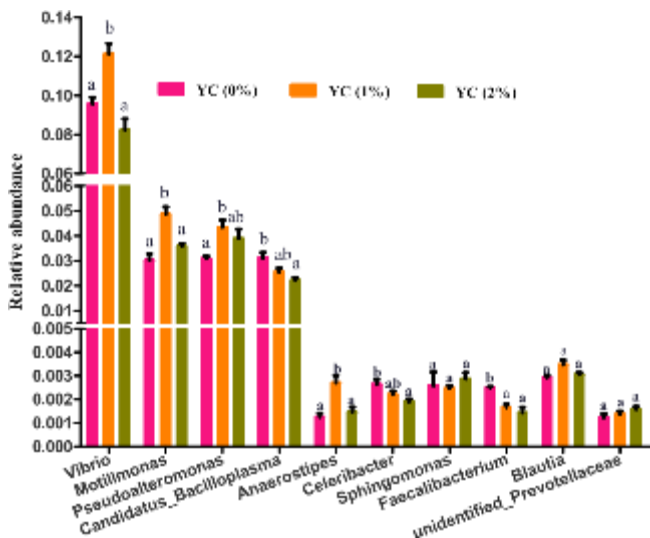


Fig. 4. Effects of YC on the relative abundance of intestinal bacteria in *L. vannamei* at the genus level. Upright bars denote the mean $\pm$ SE. ($p < .05$) ($n = 4$). Bars labeled with different letters denote significant differences ($p < .05$) among treatment groups.

3.6. Histological analysis of shrimp intestine

Mid-intestine photomicrographs and morphological measurements of *L. vannamei* fed with experimental diets are displayed in Fig. 5. No morphological distortions of villus attachment to their intestinal muscle wall were detected in shrimps fed the YC (1%) and YC (2%) diets (Fig. 5A). Taller and wider villi were found in shrimp fed with YC (1%) and YC (2%) diets than the YC (0%) (Fig. 5B). VH was not significantly high ($p > .05$) in YC (1%) compared to YC (2%) while VW was significantly high ($p < .05$) in YC (2%) compared to YC (1%) group.

3.7. Challenge test

At the end of 168 h challenge test, no mortality of *L. vannamei* was observed in the PBS-injected group. However, *L. vannamei* post-challenge with

V. harveyi in the experimental group's started dying before 12 h (Fig. 6). YC (1%) and YC (2%) diet fed groups showed low cumulative mortality rates than the YC (0%) diet (Fig. 6) with the least recorded in YC (2%) fed group. The mean cumulative mortality rates in YC (0%), YC (1%), and YC (2%) groups were 80%, 50.0%, and 30.0%, respectively.

4. Discussion

Yeast culture is a fermented yeast product which contains yeast cell wall and its metabolic fermentation products [32]. A range of studies has established that baker's yeast (*S. cerevisiae*), YC or yeast extract has the capacity of improving growth and animal health [33–35]. The present study demonstrated improved significant FBW, WGR, and SGR, and decreased FCR among shrimps fed YC (1%) and YC (2%) diets than the YC (0%). In agreement with the current study, Jin et al. [1], reported that supplementation of 1% yeast products enhanced growth and feed conversion ratio in *L. vannamei*. The addition of 1%–3% yeast products in the diet of shrimp showed an improvement in the final body weight and specific growth rate [1,36]. Similar growth performance and feed conversion ratio were reported in *L. vannamei* fed with yeast-based additive [33]. Both 1% and 2% dietary supplementation of yeast nucleotides or baker's yeast improved growth and feed efficiency of *Babylonia areolata* [34]. In contrast, hybrid tilapia fed YC diets under cage culture system did not significantly improve growth and feed conversion ratio [37]. The discrepancies in fish growth and feed conversion ratio might be due to the difference in culture systems, fish species and nutrient composition in the diets. The improved growth performance observed in the present study could be attributed to the valuable nutrients in YC such as protein, carbohydrate, vitamins, and nucleic acids, which might have played major nutritional roles in improving shrimp growth. A different reason may be that YC triggered digestive enzyme activities which could influence the nutrient digestibility in shrimp [38–40]. Shrimps fed YC (1%) and YC (2%) groups had a high survival rate without any significant differences being observed when compared with YC (0%) group. Hybrid striped bass fed both (1% and 2%) supplemented brewer's yeast or the prebiotic Grobiotick AE diet was reported to have improved survival rate [41].

Analysis of whole-body composition is a good marker of shrimp physiology and change in feed composition can adversely affect nutrient content in shrimp whole body. In this experiment, positive influence of the YC diet on protein, lipid, and ash content in shrimp's whole body has been observed. Also, Abdel-Tawwab et al. [42], observed similar results in tilapia fed with yeast diets, indicating yeast could enhance the physiology of aquatic animals. From the above results, it could be said that dietary YC supplementation increase intracellular enzyme production which promotes the growth and nutrient composition of Pacific whiteleg shrimps.

Serum analysis provides information on the wellbeing of aquatic animals as it has an essential role to play on the physiological, nutritional, and pathological status of aquatic organisms [43]. TP is a crucial component of the innate immune system, protecting shrimp from potentially invasive organisms [44–46]. In our study, dietary YC, particularly 2% significantly improved serum TP. Zhang et al. [47], reported a significant enhancement in serum TP in grass carp which modulated immune activities. Yeast nucleotide supplemented diet enhanced serum TP in shrimp thus may serve as an immunostimulant for shrimp [35]. TG and CHO are part of the shrimp's body fat, which activities in serum have been used in determining lipid metabolism [48]. YC (2%) diets displayed a decreased trend of serum TG and CHO which contradict the findings reported by Ref. [1]. Normally, an increase in serum glucose is an indicator of stress in aquatic animals [49]. YC (1%) and YC (2%) groups in the present study demonstrated low glucose content than the YC (0%) group; this unveiled that feeding Pacific whiteleg shrimp with YC (1%) and YC (2%) may not expose the shrimps to stress. Metabolic activities in animals produce several Reactive Oxygen Species (ROS) which may lead to oxidative stress. SOD have been used as a molecular marker for assessing oxidative stress condition in shrimps, owing to its

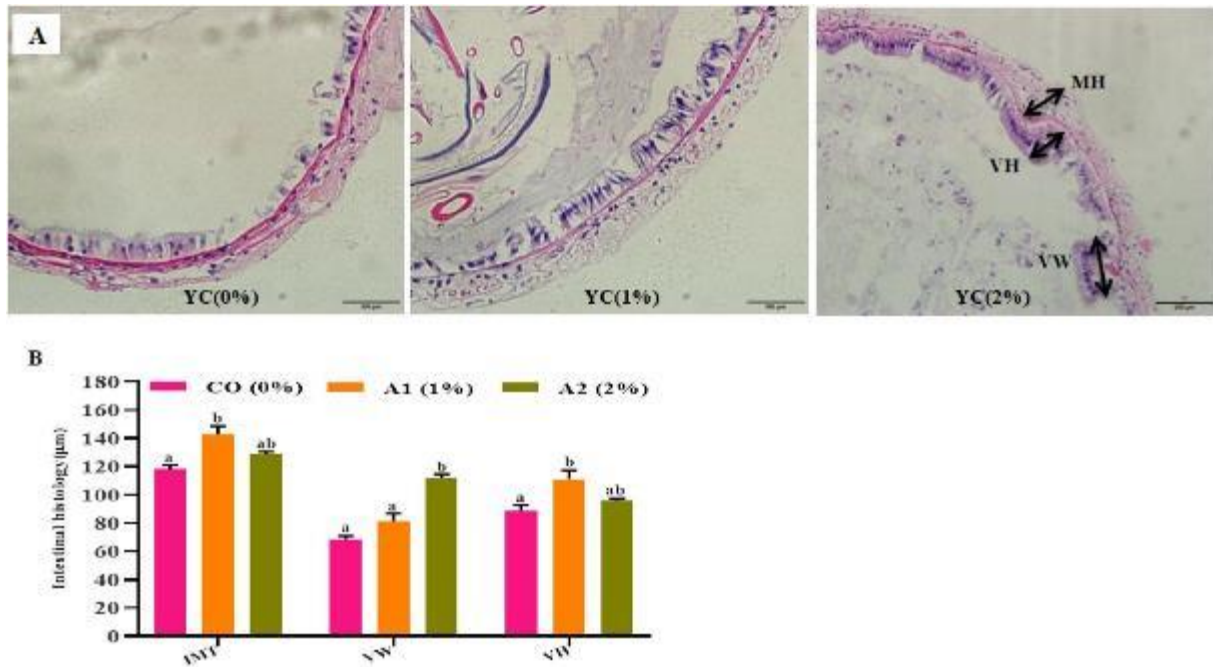


Fig. 5. Photomicrographs (A) and Morphological measurements (B) of the mid-intestine of *L. vannamei* fed YC diets. Upright bars denote the mean $\pm$ SE (n = 4). In figure (5A), arrows MT, VH, and VW represent intestinal muscle thickness, villus height, and villus width respectively. Upright bars labeled with different letters denote significant differences (p < .05) among groups.

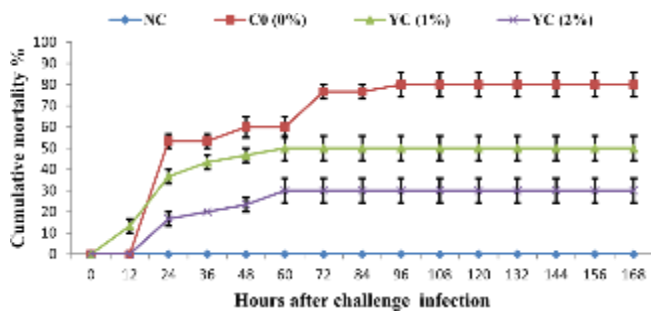


Fig. 6. Cumulative mortality of *L. vannamei* fed YC after 168 h of *V. harveyi* infections. Every plotted value denote mean $\pm$ SE of ten shrimp per three replicates (n = 3).

ability to convert superoxide anions to hydrogen peroxide and oxygen which form the first line of antioxidant enzymatic defense [50]. CAT being an antioxidant enzyme has the ability to catalyze the conversion of hydrogen peroxide molecules into water and oxygen during immune responses in aquatic animals [51]. Shrimp serum SOD and CAT activities were highly elevated in treated groups compared to the untreated group in this study. Similar to our study, improvement in SOD and CAT activities have been reported in shrimps fed with yeast, *Rhodospiridium paludamentum* [52]. Furthermore, Yuan et al. [11], reported enhanced SOD and CAT in yeast hydrolysates diets fed to grass carp (*Cyprinus carpio*). Yeast cell contains vitamins [53] which may have beneficial effects on improving the antioxidant immune system of aquatic animals [36]. In cell culture studies, B-complex vitamins reduce oxidative stress caused by H_2O_2 in monocyte cells [54]. AST and ALT are soluble enzymes found in the cytoplasm of liver cells, and their high activities in serum reflect liver damage [55]. It was observed that YC had no adverse effect on

system to override pathogenic attack [58]. PO, LZM, ACP, and AKP are key immune enzymes that form part of the innate immune responses in shrimps. ACP is a key lysosomal enzyme that plays a critical role in immunity and has been used as a marker of macrophage activation in animal models [59,60] while AKP hydrolyzes phosphate conjugates extracellular enzyme in many organic compounds (carbohydrates, lipids, and proteins) [60,61]. LZM plays defensive roles against several types of bacterial infections through cleaving N-acetylmuramic acid and N-acetylglucosamine bonds in the cell walls of bacteria, thus damage the cell wall and kills bacteria [62]. PO induces antimicrobial substance into the serum to enhance phagocytosis in the shrimp hemocytes. Various studies on aquatic animals have demonstrated that yeast products have positive effects on innate immune responses. In Ussuri catfish (*Pseudobagrus ussuriensis*), dietary YC supplement at 10% improved plasma ACP, AKP, and serum LZM indicating improved innate immune response [63]. Tukmechi and Bandboni [64] reported that supplementation of two combined yeast products in rainbow trout (*Oncorhynchus mykiss*) diets enhanced innate immune responses. With Pacific whiteleg shrimp, improved innate immune response was reported when proPO gene was expressed in shrimps fed with yeast hydrolysates [1]. In the present work, shrimps fed diets containing YC (1%) and YC (2%) modulated ACP, AKP, LZM, and PO activities before and after shrimps were challenged with *V. harveyi*. YC (1%) and YC (2%) diets improving innate immune responses in shrimps may be due to the presence of β -glucan [65]. Yeast cell β -glucan contains binding receptors that wave on the cell surface to phagocyte pathogens. The binding effects increase the activities of phagocyte cells in overriding, destroying and digesting bacteria [66]. β -glucan extracted from yeast cell wall in feed, enhanced innate immune response of Atlantic salmon challenge with several pathogens [67].

Shrimp intestine is an important organ for nutrient breakdown and absorption, contributing to protection against pathogenic infections, thus plays crucial roles in growth and also improve the immunity of the host [68].

shrimp which could be seen in the serum ALT and AST levels. This result corresponds to the YC diet fed in grass carp [56], which could be that active metabolism had occurred in the liver. The present study demonstrated that the YC could enhance the antioxidant immune system in the shrimps as well as protect liver tissues.

Actually, like other crustaceans, Pacific whiteleg shrimp lack an adaptive immune system [57] to resist infections, thus depends on the innate immune

Microbiota diversity and richness shape metabolism and protection activities in shrimps to outweigh pathogenic infections. Irrespective of the diets used in this study, the most abundant intestinal bacterial communities in *L. vannamei* at the phyla level were Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. Earlier findings by Ref. [56] on grass carp reported Fusobacteria, Bacteroidetes, Firmicutes, and Proteobacteria as the most abundant phyla intestinal bacterial populations in grass carp fed with YC for 10 weeks. The discrepancies of

Fusobacteria dominating grass carp intestinal bacterial might be due to differences in fish species. Proteobacteria is the most predominant intestinal bacterial currently been found in *L. vannamei* intestine [69]. The richness variation of Actinobacteria, Bacteroidetes, and Firmicutes in the intestine of *L. vannamei* intestine could be influenced by the culture environment and available nutrient composition in the diet [69]. The relative abundances of these three major intestinal bacteria in this work suggested how well shrimps were adapted to the culture environment and YC diets. These bacteria, Bacteroidetes, Firmicutes, and Actinobacteria, have been well known to play a major role in the physiology of the host health through metabolic activities [70] such as fermentation of non-digestible carbohydrates which in turn produces short chain fatty acids (SCFAs) [71]. Butyrate is a key product from Firmicutes which stimulate the health of the intestinal mucosa [72]. The phylum Actinobacteria is a gram-positive bacteria having probiotic effect of producing phenol oxidase which secretes antibacterial, antioxidant, and mucin, contributing to intestinal health of the host [73,74]. Actinobacteria have been increased in YC (1%) and YC (2%) diet groups than the YC (0%), signifying that YC could improve immune responses in shrimps. *Vibrio* is a common pathogenic bacterium in *L. vannamei* [75,76]. *Pseudoalteromonas* produce several anti-microbial compounds [77] which are involved in the production of marine bioactive and hydrolytic enzymes through antifouling, antibacterial, and anti-biofilm activities in marine animal and its environment [78]. Recognizing its anti-infective roles and probiotic characteristics, strains of these bacteria have been used as probiotics to reduce pathogenic infection of *Vibrio* species in shrimp aquaculture. In the treated groups, YC (2%) group remarkably reduced colonization of *Vibrio* species in *L. vannamei* intestine. However, the YC (1%) group increased the abundance of *Vibrio* species which warrants further investigations.

As an important tissue in digestion processes, intestinal morphology and structures are essential for nutrient assimilation and the maintenance of normal intestinal functions [79–81]. The functioning of the intestinal wall depends on villi length as it helps in better nutrient absorption and also improve growth performances [79,82]. Currently, YC diets fed to *L. vannamei* significantly enhanced villus height and villus width which translated into improved growth. According to Yuan et al. [4], yeast hydrolysates fed to juvenile Jian carp increased villus height and enhanced growth performance in the fish. Furthermore, dietary MOS increased intestinal villus length in *L. vannamei* [19]; this indicates that dietary MOS provided nutrient which enlarged the intestinal villus structures. The growth in intestinal villus surface area in the present study may be attributed to MOS which is a component in YC.

Infectious disease outbreaks have become a core problem and the most preventive factor in shrimp production [83]. *Vibrio* species are well identified as causative agents for infectious disease outbreak in shrimp culture, causing serious economic losses to farmers under intensive culture through mortalities [84–86]. Feeding shrimps with diet and later challenging them with bacteria pathogens have been a useful method in assessing shrimps resistance to disease infections. In the present study, shrimp fed YC diets showed significant defense against *V. harveyi* infection, particularly YC (2%) group. These results are consistent with that of [87] who fed YC to shrimps at 1% diet. Their results also showed improved resistance of shrimps against *V. harveyi* as compared to the control. Increased resistance of shrimps against *V. harveyi* suggests YC could stimulate and extend the immune response of shrimp against pathogenic infections.

In summary, the present study indicates that YC product in *L. vannamei* diet can significantly improve growth performance, and serum immune responses against *V. harveyi* attack, as well as positively

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influence intestinal bacterial composition and morphology, thus YC can be used as a feed supplement in *L. vannamei* diet.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Effects of reducing dietary fishmeal with yeast supplementations on *Litopenaeus vannamei* growth, immune response and disease resistance against *Vibrio harveyi*

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ABSTRACT

The aim of this experiment was to investigate the effects of reducing dietary fishmeal (FM) with yeast culture (SYC) supplementation on growth, immune response, intestinal microbiota, intestinal morphology, and disease resistance of *Litopenaeus vannamei*. A total of 480 shrimps with an average initial body weight of 0.35 ± 0.002 g were randomly distributed into twelve tanks. Three isonitrogenous (40.00 crude protein) and isolipidic (8.00 crude lipids) diets with yeast culture supplementing fishmeal were formulated. The groups were divided into two (2) namely control group and experimental groups. The formulations of the groups were control (0 %, without yeast culture) and the experiment groups (SYC) [(1 % of yeast culture), and (2 % of yeast culture)]. Each diet was delivered in four replicate per treatment group. The results indicate significant improvement on the growth indices (specific growth rate, weight gain rate, survival rate and lower feed conversion ratio) with yeast culture treatment group after 56 days feeding trials ($P < 0.05$). Total hemolymph protein, superoxide dismutase, catalase, alkaline phosphatase, acid phosphatase, lysozyme and phenoxidase were enhanced but low aspartate aminotransferase, alanine aminotransferase, and glucose were observed in shrimp fed yeast culture diets ($P < 0.05$). The SYC groups showed insignificant differences in hemolymph cholesterol and triglyceride. Proteobacteria, Bacteroidetes, and Actinobacteria were the dominant bacteria found in all the SYC groups. At the genus level, *Vibrio* was significantly decreased ($P < 0.05$) in 2 % yeast culture diets supplemented group whereas the beneficial bacteria *Pseudoalteromonas* was significantly enhanced. Moreover, intestinal villus length and width in shrimps fed yeast culture diets were improved ($P < 0.05$). Dietary yeast culture supplementation can improve growth, intestinal health, immune response, and resistance against *Vibrio harveyi* infections in *L. vannamei*.

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1. Introduction

Global aquaculture production is increasing with continuous demand for aqua-feeds production expansion. Commercial production of aqua-feeds from 1995 to 2008 increased from 7.6 million metric tons to 29.2 million metric tons with an expectation of reaching 71.0 million metric tons in 2025 (Tacon et al., 2011). The increasing production of aqua-feeds faces challenges such as high cost and low supply of feed material component (Deng et al., 2013a, b). Fishmeal is an excellent protein source for the growth and health of shrimp; however, prices have inflated due to increase demand which had led to extensive utilization of plant protein (Bulbul et al., 2016). Diet with low fishmeal but high plant protein content frequently affect growth performance, metabolism, and health status in farmed shrimp (Ding et al., 2015) due to imbalance nutrient, high anti-nutritional factors, and fiber content that affect feed intakes, palatability, and digestibility (Cummins et al., 2013; Jannathulla et al., 2017; Taher et al., 2017). In shrimp, strategies used to develop feed

supplement with low-fishmeal diet enhance immunity and disease resistance are important to curb disease outbreaks. For instance, nucleotide (90 mg kg^{-1}) supplemented in low fishmeal diet (18 %) was reported to improve immune response, gut health and disease resistance of shrimp (Guo et al., 2016).

Yeasts are single-cell protein, either alive to feed food organisms or after processing is commonly use as feed ingredients in aquaculture (Jin et al., 2018). Yeasts contain rich source of protein (45–55 % crude protein) and vitamin B-complex (Zhang et al., 2018). As feed supplement, yeasts have been used to compensate amino acids and vitamins in diet formulated with cereal grains (Olvera-Novoa et al., 2002). Furthermore, yeast can be easily produced from carbon- rich substrate byproducts such as molasses, citrus pulp, fruit waste (*Candida utilis*) and paper industrial wastes on large scale production. It has been considered as a cheap source of dietary feed supplement (Olvera-Novoa et al., 2002). Some studies have indicated that yeast culture is capable of improving health of shrimp by influencing the microbial ecosystem in the digestive tract; this improves digestion and assimilation of nutrients which

enhance immunity and growth performance (Burgents et al., 2004; Jin et al., 2018; Li et al., 2009). However, yeast strain and process method may vary in nutrient composition in the yeast product (Guo et al., 2019). Brewers' yeasts (*Saccharomyces cerevisiae*) are natural product of the brewing industry, which at its conventional product form contains 40–47 % crude protein (Guo et al., 2019). Natural fermentation helps in degradation of cell wall in the yeast and cereal grains, this releases nutrients (protein, vitamin, lipid) as well as immunostimulatory compounds (β -glucans, mannan-oligosaccharides and polyamines), which could enhance growth performance, immune response and metabolic activity in shrimps. It is confirmed that yeast culture can also reduce population of pathogenic bacteria settlement on shrimp gut, thus helps in participating in active structuring and shaping of gut wall to improve feed intakes and increases nutrient digestibility (Deng et al., 2013a, b; Marques et al., 2006; Patra and Mohamed, 2003).

Pacific white shrimp (*Litopenaeus vannamei*) remains the dominant shrimp aquaculture practice in China and also the world owing to the advantage of having high growth performance, taste of flesh and good consumer preference (Chen et al., 2019a, b, c; Hu et al., 2009). *L. vannamei* production in China reached 16.7 million tons in 2017 (Xiong et al., 2018) and many of its culture practices depend on more than 36 % crude proteins which is provided by fishmeal (Guo et al., 2016; Smith et al., 1985). The unreliable supply and increase in price of fishmeal has led to the utilization of plant protein. However, poor feed utilization, growth performance, immune stimulation, damage of the intestine structure and changes in the gut microbiota are associated with high plant protein diets (Guo et al., 2016). Previous studies observed the significance of yeast hydrolysates in improving the growth performance, antioxidant responses, the innate immunity, diseases resistance and intestinal diversity in shrimp (Jin et al., 2018; Yuan et al., 2017; Zhou et al., 2018). Much is unknown about the effects of yeast culture products derived from brewer's yeast, whether it could improve *L. vannamei* growth performance and its health. Therefore, the objective of the present study was to evaluate the effects of supplementing high quality protein yeast culture in low fishmeal diet on growth performance, hemolymph immune response, intestinal health, and disease resistance of *L. vannamei*.

2. Materials and methods

2.1. Yeast product and diet preparation

Yeast culture was produced at Enhakor Biotechnology Company in Beijing, China. Briefly, in obtaining yeast culture powder, *Saccharomyces cerevisiae* was aerobically fermented in molasses based medium. Cereal grain was added into a content to form slurry and fermented anaerobically to enhance breakage of cell wall. After drying, the mixture was milled to desirable final particle size. The nutrient composition in the yeast culture powder was listed in Table 1.

The main source of protein in the basal diet was brown fishmeal and the other two diets contained increasing concentrations of yeast culture supplement at 1 % and 2 % of the fishmeal. The three diets were referred to as Control (0 % of no yeast culture), SYC (1 % of yeast culture) and SYC (2 % of yeast culture). The diet was prepared by thoroughly

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Table 1

Nutrient content in yeast culture powder (% dry weight).

Chemical composition	Yeast culture
Crude Protein	55.00
Crude Lipid	0.53
Moisture	3.97
Essential amino acids (%)	
Histidine	1.27
Arginine	3.97
Isoleucine	2.10
Leucine	3.25
Lysine	3.29
Methionine	0.74
Threonine	1.69
Phenylalanine	2.31
Cystine	0.42

Valine	2.47
Σ EAA	21.51
Non-Essential Amino Acids Alanine	
	2.97
Aspartic acid	4.65
Glutamic acid	8.91
Glycine	2.14
Proline	1.93
Serine	1.62
Tyrosine	1.17
Σ NEAA	23.40

blending core ingredients with minerals and vitamins. Lipid source was added to the mixtures and mixed by hand. They were then mixed with water to form dough before processing in meat mincer machine to obtain spaghetti like pellets. Pellets were dried until moisture content decreased to about 10 %. Having cut into approximately 2.00 mm length, they were packaged into seal bags and freeze dried at -20 °C until used. The three experimental diets formulated contained 40 % crude protein and 8 % lipid crude (Table 2).

2.2. Rearing condition

Zhanjiang Allied Pacific Aquaculture Limited supplied juveniles of *L. vannamei* used in this experiment. The shrimps were acclimatized in in-door concrete tanks (4.5 m (L) $\times$ 3.45 m (W) $\times$ 1.8 m (H)) at the Marine Biological Research Base of Guangdong Ocean University for 28 days and fed with commercial diet containing 45 % crude protein and 10 % crude lipids. To begin with the experiment, shrimps were weighed after 12 h of starvation, and those with approximately 0.35 ± 0.002 wt were divided into twelve fiberglass tanks (0.3 m³) containing 200 L sieved seawater. In all, 40 shrimps were stocked into a tank and fed four times daily in four replicates per treatment at 8–10 % of their average body weight. Feed intakes were monitored to evaluate feed conversion ratio, and one third of the seawater in each tank was replaced every 24 h with continuous oxygenation via air stones. Water quality parameters such as temperature during experimental period ranged from 26.5 °C to 30.5 °C, dissolved oxygen 6.0 mg L⁻¹ to 6.5 mg L⁻¹, and pH 7.9 to 8.1.

2.3. Sample collection

At the end of the 56 days feeding trials, hemolymph samples were taken from pericardial cavity of eight shrimps into 1.5 mL Eppendorf tubes, where they were centrifuged at 4000 rpm/min for 10 min at 4 °C; supernatants were collected and stored at -20 °C then used for enzymatic analyses. Shrimps were counterweighed, weighed and length measured from each tank to determine growth performance. The whole body of two shrimps was sampled and freeze dried at -20 °C for proximate chemical composition. Three shrimp was pooled, mid-guts (0.6–1.0 cm) was dissected and fixed into 10 % paraformaldehyde for histological analysis. Another three mid-guts were immediately collected, loaded into liquid nitrogen for microbiota analysis.

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Table 2

Ingredients and nutritional composition in experimental diets (% dry weight).

Ingredient (%)	Control (0 %)	SYC (1 %)	SYC (2 %)
Fish meal (60 % protein) ^a	20.00	19.18	18.38
Yeast culture (55 % protein)	0.00	1.00	2.00
Peanut meal ^b	18.00	18.00	18.00
Soybean meal ^b	18.00	18.00	18.00
Shrimp shell powder ^c	7.00	7.00	7.00
High-gluten flour ^d	20.00	20.00	20.00
Fish oil ^e	1.80	1.89	1.97
Soybean oil ^f	1.80	1.80	1.80
Phospholipid oil ^g	1.80	1.80	1.80
Vitamin mixture ^h	0.20	0.20	0.20
Mineral element mixture ^c	0.50	0.50	0.50
Attractant ^c	0.10	0.10	0.10
Antioxidants ethoxyquin ^c	0.03	0.03	0.03
Choline chloride 99 % ^d	0.50	0.50	0.50
92 % monobasic calcium phosphate ^d	1.00	1.00	1.00

Vc polyphosphate 35 % ^c	0.09	0.09	0.09
Antimony trioxide ^c	0.50	0.50	0.50
CMCC ^d	8.86	8.41	8.15
Total	100.00	100.00	100.00
Nutrient levels Dry matter	89.16	89.12	89.21
Crude protein	40.18	40.25	40.22
Crude lipid	8.00	8.20	8.05
Ash	8.94	8.89	8.81

^a Ingredients purchased from Zhanjiang HaiBao Feed Factory, Zhanjiang, Guangdong, China. ^b Vitamin premix supplied the following per kg of the diet: vitamin A, 22,500 IU; vitamin D3, 6000 IU; vitamin E, 200 mg; vitamin K3, 40 mg; vitamin B1, 30 mg; vitamin B2, 45 mg; vitamin B6, 35 mg; vitamin B12, 0.25 mg; calcium pantothenate, 150 mg; niacin, 225 mg; folic acid, 12.5 mg; biotin, 0.5 mg; inositol, 500 mg (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China).

^c Mineral premix provided the following per kilogram of diet: Fe, 60 mg; Zn, 24 mg; Mn, 16 mg; Cu, 1.4 mg; Co, 0.2 mg; Se, 0.1 mg; I, 0.2 mg (Obtained from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China). ^d Obtained from Shanghai Macklin Biochemical Co. Ltd., 1288 Guangdong Rd., Shanghai, China.

^e Obtained from Shantou Xilong Chemical Factory, Guangdong, China.

2.4. Bacterial challenge test

2.4.1. Bacterial preparation and preliminary test

Vibrio harveyi was used to conduct shrimp disease resistance test. This microorganism were cultured in Luria-Bertani (LB) media at 37 °C for 20 h and centrifuged at 8000 rpm/min for 10 min at 4 °C. The bacteria cells were washed twice with aseptic phosphate buffer saline (PBS) at pH of 7.2 to get rid of culture media. They were serially diluted into four different concentrations (10⁶, 10⁷, 10⁸, and 10⁹ CFU/mL) where 100 µl of *V. harveyi* was intramuscularly inoculated at third abdominal segment of the shrimp, ten shrimps in a tank with two replicate per *V. harveyi* concentration. 7 days mortalities of shrimps was observed and suitable median lethal dose 50 (LD50) was achieved with 10⁸ CFU/mL concentrations.

2.4.2. Experimental challenge test

The experimental challenge test was conducted with 10 shrimp per fiberglass tanks containing 50 L of sieved seawater in five repeat per treatment group. Each shrimp in four repeat tanks per treatment was intramuscularly inoculated with *V. harveyi* containing 10⁸ CFU/mL cells suspended in 100 µl PBS, whereas the negative control group was injected using PBS with the same volume as bacterial infected group. Shrimps were fed thrice with their respective experimental diets during the infection period. At the 12 h after infection, hemolymph samples were collected from different challenge groups, these were used to examine innate immune response in shrimp. Twelve hours interval mortalities were observed for 7 days. Cumulative mortalities curves

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were calculated using the formula presented below.

Cumulative mortality, % = $\frac{\text{Total mortality in each treatment after challenge}}{\text{Total number of shrimp challenged for same treatment}} \times 100$

2.5. Chemical composition analysis

Chemical analyses of the yeast culture, diets and shrimps whole body were assessed using methods described by AOAC International (2012). Crude protein (N x 6.25) was determined by Kjeldahl method. Crude lipid was assessed by ether extraction using Soxhlet system. Dry matter was evaluated by oven drying samples at 105 °C until constant weight was obtained. Ash was evaluated by inclination of 2 g samples into muffle furnace at 550 °C for 3 h. Amino acid analysis of yeast culture powder was measured after acid hydrolysis in 6 M HCl for 24 h at 110 °C separated by

ion-exchange chromatography and reacted with ninhydrin post column derivatization using Hitachi L-8800 Amino Acid Analyzer.

2.6. Intestinal microbiota analysis

Total genomic DNA of microbes in shrimp mid-gut was extracted using E.Z.N.A. stool DNA Kit (OMEGA, US) following the manufacturer's protocol. Amplification and sequencing of the V3-V4 hypervariable region of the bacterial 16S DNA gene was performed using barcoded primers 515 F (GTGCCAGCMGCCGCGGTAA) 806R (GGACTACHVGGGTWTCTAAT). Polymerase chain reactions were carried out in triplicate 25 µL mixture containing 2.5 µL of TransStart Buffer, 2 µL of dNTPs, 1 µL of each primer, and 20 ng of template DNA. Using Illumina MiSeq platform, 16S rRNA tag-encoded high throughput sequencing were performed at Novogene Company (Beijing, China). Sequencing reads were assigned to individual sample with their unique barcode before analyzing with QIIME software package (Quantitative Insights Into Microbial Ecology) and UPARSE pipeline (Quast et al., 2013). Reads were clean and grouped into Operational Taxonomic Units (OTUs) at uniqueness threshold of 97 %. Alpha diversity was applied in analyzing complexity of species diversity for samples through 4 indices, including Chao1, Shannon, Simpson, and ACE using QIIME. The shared and unique species among groups were showed on Venn diagram.

2.7. Histological analysis of shrimp intestine

Dietary effects of yeast culture diet on shrimp intestinal morphology was determined as described by Zhang et al. (2009). Briefly, mid-guts were fixed in Bouin's solution, dehydrated with graded concentrations of alcohol, cleared in toluene, equilibrated in xylene, inserted in paraffin to form wax blocks and sectioned at 5 µm. Samples after being stained with hematoxylin-eosin was captured using microscope (Olympus, model BX51, Serial number: 9K18395, Tokyo, Japan) and electronic measurement on intestinal villus height (VH), villus width (VW) and muscle thickness (MT) at 100× magnifications were taken using software Image-Pro Plus 6.3 (Media Cybernetics, Inc., Rockville, USA). Villus height was determined by taken measurement from the tip of the villus to the villus-crypt junction. Villus width was evaluated by taking measurement at middle portion of the villus. Muscle thickness was at the base of crypt to the base of the muscularis mucosae.

2.8. Hemolymph biochemical analysis

The hemolymph collected from shrimp were used to analyze parameters such as Glucose (Glu), Triglyceride (TG), Cholesterol (CHO), Catalase (CAT), Superoxide dismutase (SOD), Acid phosphatase (ACP), Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT), 3

Alkaline phosphatase (ALP), Lysozyme (LZM) and Phenoxidase (PO). These parameters were determined using commercial kits (Nanjing Jiancheng Biological Engineering Institute, China) following the company's protocol. A unit of SOD activity was described as the quantity of enzyme required to yield 50 % inhibition of the nitroblue tetrazolium reduction (NBT) rate measured at 550 nm using ELISA microplate reader and is expressed as unit/millilitre (U/mL). A unit of CAT activity was defined as the amount of enzyme needed to decrease absorbance of 0.01/min at 405 nm in a microplate reader and is expressed as unit/ millilitre (U/mL). AST and ALT activities were determined following the calorimetric method of Reitman and Frankel's (Reitman and Frankel, 1957) decided by standard curve acquired by contrasting assay between experimental method and Carmen's unit (1 Carmen's unit = 0.482 IU/L, 25 °C) at an absorbance peak of 510 nm. ACP and AKP activities of the hemolymph were determined by spectrophotometer at 520 nm. A unit of AKP activity was defined as the volume of an enzyme that reacted with the matrix to produced 1 mg phenol in 30 min at 37 °C. A unit of ACP activity was defined as the amount of enzyme that reacted with the matrix and produced 1 mg phenol in 30 min at 37 °C. A unit of LZM activity was defined as the sum of the enzyme required to cause decrease absorbance rate of 0.001 min⁻¹ mL⁻¹. The PO activity

was well-defined as the amount of enzyme required to produce an increase in absorbance of 0.001 min^{-1} . TP was quantified as described by Bradford (1976) using bovine hemolymph albumin as a standard.

3. Statistical analysis

SPSS for Windows, version 22 (SPSS Inc. Chicago, IL, USA) was used for statistical analysis. Data was compared by One-Way Analysis of Variance followed by Tukey Range Test where statistical significant differences were established at the $P < 0.05$ level.

4. Results

4.1. Growth performance, survival, and feed efficiency

At the end of the feeding trials, growth performance and feed utilization of shrimp increase with the SYC inclusion levels in the diets ($P < 0.05$). FBW, WGR, and SGR parameters significantly increased in shrimp fed diet SYC (1 %) and SYC (2 %) as compared to the control group ($P < 0.05$) (Table 3). PER and CK were substantially improved in SYC (2 %) supplementation groups whereas the FCR and HSI were significantly lower in SYC (2 %) group ($P < 0.05$) ($P < 0.05$). The different inclusion rates of SYC significantly increased SR than control treatments ($P < 0.05$). The SYC (2 %) treatment group had the highest SR than the SYC (1 %) group ($P > 0.05$).

4.2. Shrimp whole body composition

The whole body composition parameters of shrimp fed SYC supplementation groups were significantly affected with the exception of crude lipid content ($P > 0.05$). Dietary SYC (1 %) and SYC (2 %) groups' results were significantly higher in terms of dry matter and crude protein in the shrimp whole body, as compared to control group ($P < 0.05$). Ash content was only improved in the shrimp fed SYC (2 %) diet group ($P < 0.05$) (Table 4).

4.3. Hemolymph biochemical parameters

The effects of dietary SYC supplementation on hemolymph biochemical parameters are displayed in Table 5. Although MDA, and AST exhibited a decreasing tendency among the SYC groups, there were no significant differences in the MDA and AST between SYC (1 %) and SYC (2 %) groups ($P > 0.05$). The CHO and TG content were increased among SYC treatment groups, however no significant difference was found when compared to control group ($P > 0.05$). TP concentration in

Table 3

Growth performance, feed utilization and morphological indices of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks.

Treatment	Control (0 %)	SYC (1 %)	SYC (2 %)
IBW (g/shrimp)	0.35 ± 0.002^a	0.35 ± 0.000^a	0.35 ± 0.001^a
FBW (g/shrimp)	10.28 ± 0.98^a	11.52 ± 0.14^b	11.73 ± 0.28^b
WGR (%)	2909.26 ± 24.24^a	3182.70 ± 40.44^b	3243.19 ± 81.40^b
SGR (%day ⁻¹)	6.03 ± 0.03^a	6.23 ± 0.03^b	6.26 ± 0.04^b
FCR	1.28 ± 0.01^b	1.18 ± 0.02^a	1.15 ± 0.16^a
PER (%)	233.63 ± 0.55^a	237.17 ± 0.14^b	237.92 ± 0.04^b
SR%	84.37 ± 2.37^a	94.37 ± 2.13^b	97.50 ± 1.44^b
CK (g/cm ³)	0.72 ± 0.01^a	0.73 ± 0.00^{ab}	0.74 ± 0.00^b
HSI (%)	6.63 ± 0.23^b	5.99 ± 0.21^{ab}	5.52 ± 0.14^a

The data was showed as in mean $\pm$ SE. The data in the same row with different superscripts are significantly different (Tukey, $P < 0.05$; $n = 4$).

Initial mean body weight (IBW, g/shrimp).

Final mean body weight (FBW, g/shrimp).

Weight gain (WG, %) = $100 \times (\text{final mean weight} - \text{initial mean weight}) / \text{initial mean weight}$.

Specific growth rate (SGR, %day⁻¹) = $100 \times (\ln W_t - \ln W_i) / t$. Feed conversion rate (FCR) = feed consumed (g, dry weight) / weight gain (g, wet weight).

Survival rate (SR, %) = $100 \times (\text{final amount of shrimp}) / (\text{initial amount of shrimp})$.

Condition factor (CF, g/cm³) = $100 \times \text{body weight (g)} / \text{body length (cm)}^3$.

Hepatopancreas somatic index (HSI, %) = $100 \times \text{liver weight (g)} / \text{body weight (g)}$.

Table 4

Proximate composition in whole body of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks (% dry weight).

Treatment	Control (0 %)	SYC (1 %)	SYC (2 %)
Dry matter	24.01 ± 0.27^a	24.98 ± 0.40^b	24.45 ± 0.34^b
Crude protein	15.66 ± 0.16^a	16.63 ± 0.15^b	17.01 ± 0.18^b
Crude lipid	2.03 ± 0.12^a	2.00 ± 0.21^a	2.14 ± 0.10^a
Ash	2.33 ± 0.07^a	2.69 ± 0.10^a	3.20 ± 0.12^b

The data are presented as the mean $\pm$ S.E. The data in the same row with different superscripts are significantly different (Tukey, $P < 0.05$; $n = 4$).

Table 5

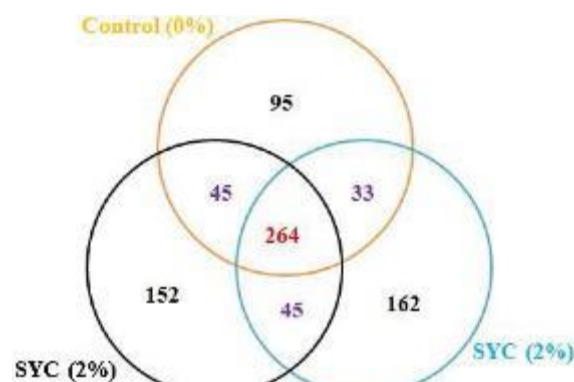
Hemolymph biochemical parameters of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks.

Treatment	Control (0 %)	SYC (1 %)	SYC (2 %)
TP (ugml ⁻¹)	23.50 ± 0.39^a	25.84 ± 0.24^b	27.80 ± 0.45^c
GLU (mmolL ⁻¹)	2.08 ± 0.10^b	1.78 ± 0.08^b	1.49 ± 0.07^a
TG (mmolL ⁻¹)	0.56 ± 0.01^a	0.55 ± 0.02^a	0.57 ± 0.00^a
CHO (mmolL ⁻¹)	0.93 ± 0.09^a	0.99 ± 0.06^a	1.02 ± 0.04^a
MDA (mmolL ⁻¹)	3.11 ± 0.07^b	2.71 ± 0.03^a	2.55 ± 0.10^a
CAT (Uml ⁻¹)	3.94 ± 0.09^a	4.38 ± 0.20^b	4.62 ± 0.08^b
SOD (Uml ⁻¹)	926.6 ± 18.84^a	988.50 ± 5.12^b	1065.24 ± 14.72^c
ALT (UL ⁻¹)	3.12 ± 0.15^b	2.54 ± 0.06^a	2.87 ± 0.12^{ab}
AST (UL ⁻¹)	4.92 ± 0.02^b	4.62 ± 0.05^a	4.51 ± 0.04^a

The data was showed as in mean $\pm$ SE. The value in the same row with different superscripts are significantly different (Tukey, $P < 0.05$; $n = 4$). Where; TP (Total protein), GLU (glucose), TG (Triglyceride), CHO (Cholesterol); CAT (Catalase); SOD, (Superoxide dismutase); AST (Aspartate aminotransferase); ALT (Alanine aminotransferase) and MDA (Malondialdehyde).

hemolymph of the shrimp was significantly increased as yeast culture inclusion increased from control (0 %) to SYC (2 %) ($P < 0.05$). The increasing SYC supplementation showed a decreasing trend of shrimp hemolymph GLU action. Lower level of GLU content was observed in SYC (2 %) compared to the other group ($P > 0.05$). Statistical differences showed that SOD was first enhanced in SYC (1 %) group and then further increased with SYC (2 %) supplementation compared to control group ($P < 0.05$), whereas CAT content in SYC groups differ with

4



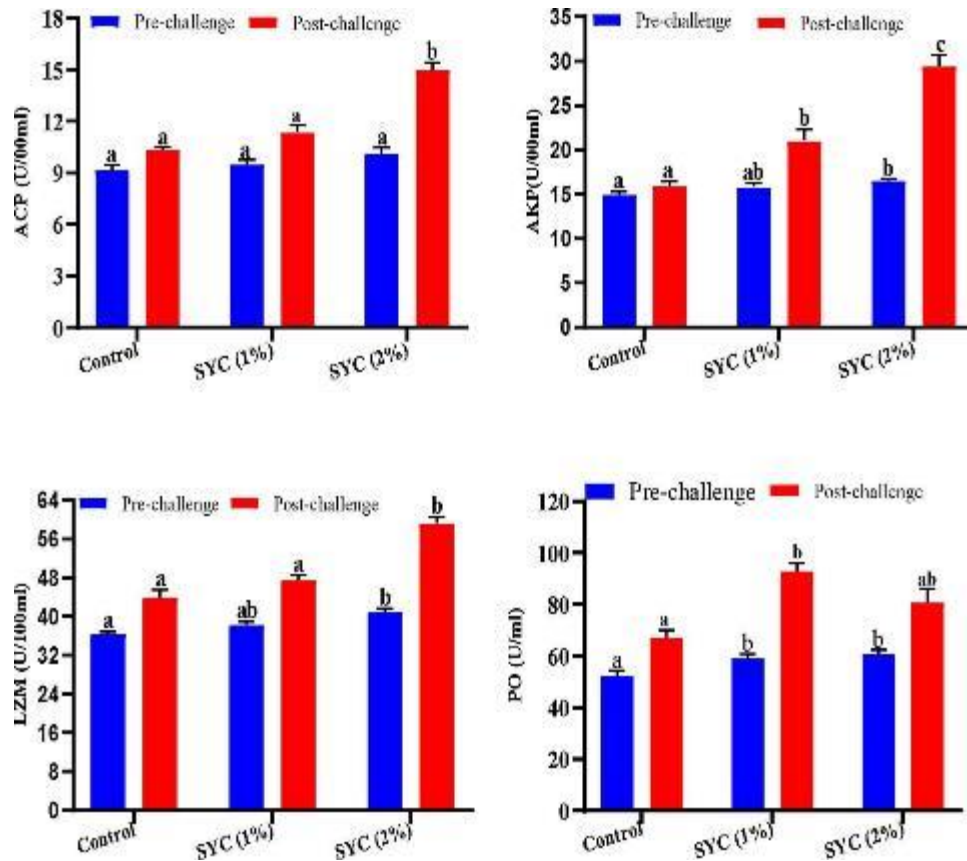


Fig. 1. Yeast culture diets effects on hemolymph innate immune activity of *L. vannamei* pre-challenge and 12 h post-challenge with *V. harveyi*. The data were showed as in mean ± SE (n = 4). The graphs with the same color having different superscript showed significantly different (P < 0.05).

Fig. 2. Venn diagram showing the OTU distribution of *L. vannamei* intestinal microbiota fed with different diets.

different inclusion levels (P < 0.05). The highest CAT content was observed in the SYC (2 %) group without significant differences being observed between SYC (1 %) and SYC (2 %) groups (P > 0.05). ALT in hemolymph was significantly elevated in the control group compared to SYC treatments with the SYC (1 %) experiencing lowest ALT activities (P < 0.05).

4.4. Hemolymph innate immune indexes

The hemolymph innate immune activities (ACP, AKP, LZM and PO) before challenge and after 12 h challenge test are presented in Fig. 1. Before challenge, significant levels of LZM and AKP were maintained in the SYC groups (P < 0.05) while the ACP levels slightly increased in the SYC groups than the control group (P > 0.05). The PO activities were slightly enhanced in shrimp fed SYC diets before challenge (P < 0.05). After 12 h *V. harveyi* injection, the innate immune parameters (LZM, ACP, and AKP) were significantly increased in the SYC (2 %) group (P < 0.05). However, the PO significantly increased in the SYC (1 %) after 12 hours *V. harveyi* injection (P < 0.05). Before and after challenge, the SYC groups indicated significant differences in the innate immune parameters (P < 0.05).

4.5. Microbial composition

The Chao and Ace represent the quantity of bacteria community species without respect to abundance of each species. The Shannon and Simpson indexes are influenced by species richness and species evenness which account for the bacteria community in the treatment group. Ace, Chao and Simpson indexes were significant higher in the SYC groups as compared to control group (P < 0.05). Shannon index showed a decreased trend in the SYC groups' supplementation in the intestinal bacteria of shrimp (P > 0.05). The number of

unique OUT was presented in the Venn diagram (Fig. 2). The mean OUT in the control, SYC (1 %) and SYC (2 %) group were 95, 152 and 632 respectively. The intestinal bacteria richness in SYC groups was higher than the control (Table 6).

4.5.1. The intestinal bacteria composition and the abundance in shrimp

The top ten highest relatively abundant bacteria at phyla level are presented in Table 6a. The most abundant phyla bacteria observed were Proteobacteria, Bacteroidetes, and Actinobacteria in control, SYC (1 %) and SYC (2 %) groups. Proteobacteria abundance in the treatment groups was much more than the untreated group with no significance being found among the treatment groups

(P > 0.05); whereas abundance of Bacteroidetes and Actinobacteria in the control group were

Table 6
Intestinal bacteria diversity indexes and estimate richness of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks.

Index	Control (0 %)	SYC (1 %)	SYC (2 %)
Raw Reads	82771.33 ± 829.00 ^a	86480.25 ± 262.28 ^b	87505.00 ± 336.14 ^b
Clean Reads	80101.03 ± 39.4 ^a	80178.00 ± 20.18 ^a	80324.50 ± 33.07 ^a
Tag	80067.33 ± 8.19 ^a	80168.00 ± 10.44 ^b	80264.33 ± 28.41 ^c
OTU	302.00 ± 5.58 ^a	408.33 ± 17.6 ^b	541.25 ± 16.32 ^c
Chao	227.84 ± 9.46 ^a	368.02 ± 6.40 ^b	451.02 ± 23.03 ^c
Ace	239.54 ± 16.48 ^a	382.13 ± 4.39 ^b	456.48 ± 15.30 ^c
Shannon	0.88 ± 0.00 ^b	0.82 ± 0.01 ^a	0.83 ± 0.00 ^a
Simpson	3.89 ± 0.23 ^a	4.15 ± 0.15 ^b	5.01 ± 0.32 ^c

The data was showed as in mean ± SE. Means in the same row with different superscripts indicated significant difference (Tukey, P < 0.05; n = 4).

Table 6a

Relative abundance (%) of phyla intestinal bacteria of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks.

Phyla	Control (0 %)	SYC (1 %)	SYC (2 %)
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Proteobacteria	67.74 ± 38.20 ^a	69.12 ± 3.55 ^a	69.43 ± 1.01 ^a
Bacteroidetes	14.73 ± 2.10 ^a	14.89 ± 2.48 ^a	17.12 ± 0.05 ^b
Actinobacteria	7.67 ± 0.03 ^a	10.73 ± 0.31 ^{ab}	12.56 ± 1.24 ^b
Firmicutes	0.25 ± 0.01 ^a	0.26 ± 0.02 ^a	0.29 ± 0.05 ^a
Tenericutes	0.15 ± 0.02 ^a	2.17 ± 0.32 ^b	0.17 ± 0.04 ^a
Gemmatimonadetes	0.13 ± 0.00 ^a	0.13 ± 0.13 ^a	0.15 ± 0.03 ^a
Chloroflexi	0.30 ± 0.00 ^a	0.30 ± 0.00 ^a	4.69 ± 0.00 ^b
Acidobacteria	0.30 ± 0.03 ^a	1.02 ± 0.60 ^b	0.49 ± 0.03 ^{ab}
Verrucomicrobia	0.25 ± 0.00 ^b	0.16 ± 0.01 ^a	0.12 ± 0.00 ^a
Cyanobacteria	0.38 ± 0.02 ^a	0.36 ± 0.05 ^a	0.54 ± 0.00 ^b

The data was showed as in mean ± SE. Means in the same row with different superscripts indicated significant difference (Tukey, $P < 0.05$; $n = 4$).

significantly lower than that of SYC (2 %) but not in SYC (1 %) group ($P < 0.05$).

Vibrio belonging to the phylum Proteobacteria was the most abundant microbe observed at genus level (Table 6b). *Vibrio* in the control group was significantly higher than the SYC groups representing 9.53 %, 5.54 % and 3.10 % as in the control, SYC (1 %) and SYC (2 %) respectively ($P < 0.05$). There was a significant abundance of *Pseudoalteromonas* and *Sphingomonas* in SYC groups as compared to the control group ($P < 0.05$). The abundance of *Pseudoalteromonas* and *Sphingomonas* in the control, SYC (1 %) and SYC (2 %) account for 4.51 %, 5.90 % and 7.51 % as well as 0.16 %, 0.26 % and 0.46 % respectively.

4.6. Intestinal histological analysis

The effects of the experimental diet on *L. vannamei* intestinal morphology are presented in Fig. 3. Shrimp fed with the control diet have

Table 6b

Relative abundance (%) of genera intestinal bacteria of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks.

Genera	Control (0 %)	SYC (1 %)	SYC (2 %)
<i>Vibrio</i>	9.53 ± 0.44 ^c	5.54 ± 0.50 ^b	3.10 ± 0.24 ^a
<i>Motilimonas</i>	5.37 ± 0.69 ^a	9.21 ± 0.61 ^{ab}	7.44 ± 0.38 ^b
<i>Pseudoalteromonas</i>	4.51 ± 0.24 ^a	5.90 ± 0.17 ^b	7.36 ± 0.50 ^c
<i>Candidatus-Bacilloplasma</i>	0.23 ± 0.02 ^a	0.23 ± 0.02 ^a	0.15 ± 0.01 ^b
<i>Anaerostipes</i>	0.25 ± 0.01 ^a	0.26 ± 0.01 ^a	0.32 ± 0.02 ^a
<i>Celeribacter</i>	0.36 ± 0.02 ^a	0.63 ± 0.04 ^b	0.30 ± 0.02 ^b
<i>Sphingomonas</i>	0.16 ± 0.02 ^b	0.26 ± 0.02 ^b	0.49 ± 0.05 ^c
Unidentified-Prevotellaceae	0.15 ± 0.00 ^b	0.10 ± 0.00 ^a	0.18 ± 0.01 ^b
<i>Faecalibacterium</i>	0.16 ± 0.00 ^a	0.17 ± 0.00 ^a	0.13 ± 0.00 ^a
<i>Blautia</i>	0.27 ± 0.01 ^a	0.24 ± 0.02 ^a	0.26 ± 0.01 ^a

The data was showed as in mean ± S.E. Means in the same row with different superscripts indicated significant difference (Tukey, $P < 0.05$; $n = 4$).

morphological changes. The mucosal folds separation from intestinal wall was seen in shrimps fed the control diets whereas fusion of mucosal folds was observed in shrimps fed with SYC (1 %) and SYC (2 %) diets.

The measurement of intestinal morphometric parameters are represented in Fig. 4. There was significant increase in VH and WH with SYC supplementation ($P < 0.05$). The shrimp fed SYC (2 %) showed significant higher VH over SYC (1 %) ($P < 0.05$), while there were no improvement in VW observed between SYC (1 %) and SYC (2 %) treatment groups ($P > 0.05$).

The results of MT displayed no significant changes in all the experimental groups ($P > 0.05$).

4.7. Pathogenic challenge test

The cumulative mortality rate of *L. vannamei* challenged with *V. harveyi* for 168 h was presented in Fig. 5. Feeding shrimp at different levels of SYC supplementation in diets showed significant improved resistance to *V. harveyi* infection ($P < 0.05$). All shrimps fed with SYC treatment indicated significant low cumulative mortality rates as compared to the control diet ($P < 0.05$). The 168 h percentage cumulative mortality rates in Control (0 %), SYC (1 %) and SYC (2 %) groups were 60 %, 40.0 %, and 16.7 %, respectively.

5. Discussion

In the present study, a yeast culture product was used to replace small amounts of fishmeal in the experimental diets of *L. vannamei*. At the end of feeding trials, growth parameters (FBW, WGR, and SGR) of the shrimp fed SYC supplementation diets were significantly increased than the control group. In addition, the growth indices slightly increased in SYC (2 %) compared to SYC (1 %) indicating SYC (2 %) improve better growth performance. Earlier studies indicated that, yeast product enhanced the growth performances and immune function in *L. vannamei* which conform to the present study (Deng et al., 2013a, b; Qiu and Davis, 2017; Yang et al., 2009). Furthermore, shrimp feed utilization performances (FCR and PER) were improved by SYC treatment groups in the current study. FCR decreased while PER increased in SYC (2 %) in the present study. The shrimps feed utilization on yeast diet was improved on the commercial scale production of shrimp (Deng et al., 2013a, b). This may be attributed to energy produced by glucanase which is involved the breakdown of glucan in the intestine by secreting more digestive enzymes to aid in protein deposition; thereby influencing the growth performance and feed utilization of shrimps (Chen et al., 2019a, b, c). Besides, the presence of manna oligosaccharides help in growth of intestinal microvilli structures which improve the absorption areas in shrimp; this enhance nutrient utilization and growth performance of shrimp (Zhang et al., 2012). Another reason maybe that SYC diets activates digestive enzyme activities which influence the nutrient digestibility in the shrimps (Ayiku et al., 2020). The survival rate of the shrimp was improved in the dietary SYC groups indicating an improvement in the wellbeing of the SYC groups. HSI of SYC (2 %) was lower compared to the control and SYC (1 %) groups in the current study. The low level of HSI may be related to the shrimps enhanced metabolism because of an improvement of protein synthesis in the shrimp body and enhanced the immunity as a result of glycogen consumption and lipid of hepatocytes (Chen et al., 2019a, b, c). Therefore, the findings of the present study indicated an improvement in the growth performance and feed utilization in the SYC groups than the control group.

The increase in ash and crude protein in a whole body composition indicate improve nutrient utilization of shrimp. Dietary SYC (2 %) group in this study was observed to improve crude protein and ash content in shrimps whereas crude lipid content was not enhanced. Similarly, Chinese mitten crab was fed with yeast diet, crude protein of the body tissue was elevated, which increased its growth performances (Zhang et al., 2019) while another study reported no significant change

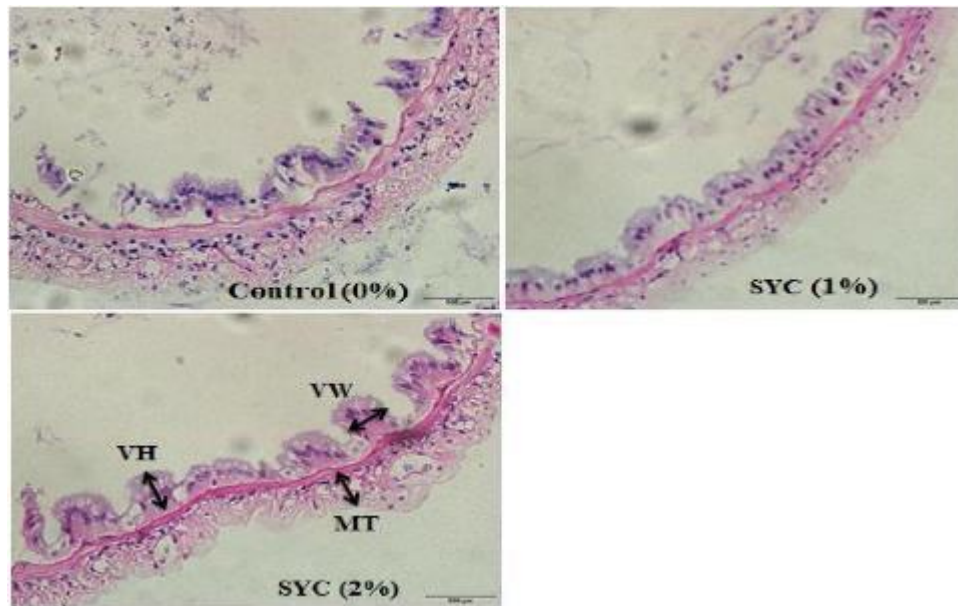


Fig. 3. Intestinal histology of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks. Shrimp intestine transverse photomicrograph segments were presented as: VH - Villus Height, VW- Villus Width and MT - Muscles Thickness.

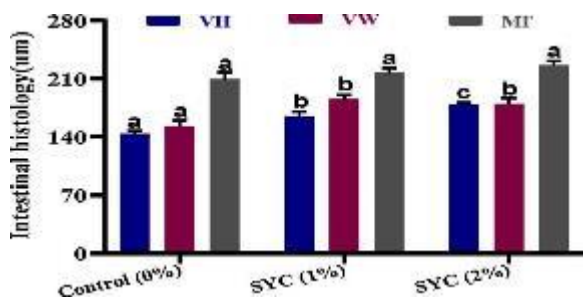


Fig. 4. Intestinal morphology measurements of *L. vannamei* fed with different levels of yeast culture diet for 8 weeks. Graph showed the mean $\pm$ SE (Tukey, $P < 0.05$; $n = 4$). The graphs with the same color having different superscript showed significant difference. Where VH -Villus Height, VW-Villus Width, and MT-Muscles Thickness.

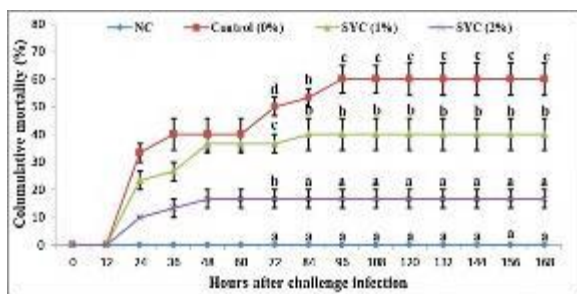


Fig. 5. Cumulative mortality of *L. vannamei* post-challenge with *V. harveyi* for 168 h. Every plotted value denoted as in mean $\pm$ SE of ten shrimp per three replicates (Tukey, $P < 0.05$; $n = 3$).

in ash, crude lipid and crude protein in the body proximate composition of shrimp (Jin et al., 2018). The present study did not focus on digestibility. However, we can speculate that the findings of the current study imply that SYC (2 %) enhanced growth performance than other groups.

Hemolymph lipids, proteins, and glucose may provide insight into metabolism. Research studies reported that, TP content is very essential for reflecting immune responses (Hossain et al., 2017; Xiong et al., 2018). The present study depicts that SYC groups significantly increased the TP content

et al., 2018). CHO is an important constituent of lipoprotein, that helps in lipid absorption and transport in shrimps (Kumar et al., 2018). It is involved in processing steroid hormones which function in reproduction and moulting (Kumar et al., 2018). CHO supports more structural integrity to cell membranes against environmental distortion, such as thermal and salinity stress (Kumar et al., 2018; Yang et al., 2016). Additionally, dietary CHO increases the utilization efficacy of the vital pigment in shrimp (Kumar et al., 2018; Niu et al., 2014). For instance, CHO accounts for approximately 90 % of muscle (Kumar et al., 2018; Tsape et al., 2010). In the present study, yeast culture increases CHO level, especially in SYC (2 %) group. This suggests that the increased CHO in the SYC (2 %) group indicate muscle build-up in the shrimp. TG are the main form in which shellfish's store fatty acids (Huang et al., 2020). The essential nutrients contribution by TG provide energy which facilitates cell division in the shrimp tissues (Chen et al., 2019a, b, c; Xiong et al., 2018). In the present study, the hemolymph TG concentration in the shrimps fed SYC treatments were enhanced than the control group. However, high concentration of TG was observed in the shrimp fed SYC (2 %) than SYC (1 %), indicating that dietary yeast culture diets can support cell division in shrimp to promote growth, reproduction and moulting (Kumar et al., 2018). Yeast cells can utilize a wide range of carbon sources in the presence of GLU to suppresses molecular activities involved in the use of alternate carbon sources as well as it represses respiration and gluconeogenesis (Kayikci and Nielsen, 2015). Through gluconeogenesis pathway, the cell can sense extracellular GLU levels and use this to regulate GLU uptake, thereby triggering GLU repression (Kayikci and Nielsen, 2015). Yeast culture represses/suppresses the GLU through gluconeogenesis; however the present study showed a slight decrease in GLU of the SYC (2 %) than the other groups. This implies that, the SYC (2 %) utilized the GLU as a form of energy or fuel to maintain energy homeostasis. In addition, GLU level in SYC (2 %) can be influenced by nutritional and hormonal factors which are very important for maintaining GLU homeostasis; primarily dependent on the regulation of activity and expression of key enzymes involved in the glycolysis and gluconeogenesis pathways (Pilkis and Graner, 1992). The GLU level in the SYC (2 %) depicts glycogenolytic activity thereby enhancing protein/amino acids synthesis.

AST and ALT are hepatopancreas – specific enzymes that promotes gluconeogenesis from amino acids (Sakya et al., 2020). They are found within some tissues such as hepatopancreas and muscle cells (Kim et al., 2008). When studying aquatic organisms, it is very important to establish whether

(Table 5). Similarly, β -glucan and mannan oligosaccharides improved the TP concentration and innate immune responses in the aquatic animal (Abu-Elala

experimental procedures such as handling, sampling, and other physical stressors may affect physiological responses (Iwama et al., 2004). The releases of these

AST and ALT enzymes are as result of hepatocellular injuries which increases the levels of these enzymatic activities in the hemolymph (Ebrahimi et al., 2017; Kim et al., 2008). The current data indicate increased levels of AST and ALT in the control group than the SYC groups. This may be as result of physical stressors which causes hepatocellular injuries in the control group. Also, the levels of these enzymes in the hemolymph indicate the extent to which hepatopancreas cells are damaged (Sakyi et al., 2020). Contrarily, the current study indicates a significant decreased in AST and ALT in the SYC groups which implies yeast culture improved hepatopancreas from cellular injury (Table 5). Similarly, in a previous study, reduced levels of ALT and AST were found in *L. vannamei* fed yeast culture diet groups (Ayiku et al., 2020). We can speculate that, SYC groups reduce the hepatopancreas damages and improved physiological responses than the control group.

Oxidative stress occurs when there is an imbalance between the generation and elimination of reactive oxygen species (ROS) and could increase shrimps vulnerability to disease conditions (Sheikhzadeh et al., 2012; Yuan et al., 2019). Antioxidant enzymes including SOD and CAT are responsible for protecting the host against ROS that induce oxidative stress. In the present study, increased SYC supplementation groups (SYC, 2 %) significantly improve the activities of SOD and CAT which was similar with the results on *L. vannamei* (Yang et al., 2010) and Chinese mitten crab (Zhang et al., 2019) (Table 5). MDA in the present study decreased in the SYC groups. MDA reflects diminishing lipid peroxidation which may be as a result of generation of excess reactive oxygen species (Zhao et al., 2014). Our findings suggest that increased in antioxidant activities (SOD and CAT) enhanced the antioxidative capability and the gut innate immunity (Zhang et al., 2019). Currently, there is limited information on how antioxidative status is being influenced by yeast culture, its constituent, and its specific mechanisms. We can conclude that SYC (2 %) influence positive antioxidant activities than the other groups.

The present study examined the innate immune parameters (LZM, ACP, AKP, and PO) in the hemolymph of shrimp. Before challenge, significant levels of ACP and AKP were maintained in the SYC groups while the LZM levels slightly increased in the SYC groups than the control group. After 12 h injection, the innate immune parameters (LZM, ACP, and AKP) were highly increased in the SYC (2 %) group. These immune parameters aided in improving innate immune systems of marine shellfish against pathogenic infection and exhibited phagocytic activities in removing foreign bodies (Liu et al., 2019; Wang et al., 2019; Xing et al., 2002; Zhao et al., 2012). Additionally, the enzymatic activities of ACP and AKP are produced by phagocytes and are innate immune promoters. Furthermore, the yeast cell wall possesses a structure known as β -glucan, which has particular receptors on phagocytic cells and also bind the receptor molecules on the surface of distributing and tissue phagocytes (Abu-Elala et al., 2013). The binding elevate the activities of the phagocytes in engulfing, killing, and breakdown bacteria (Abu-Elala et al., 2013). The activities of phagocytes play a vital role in antibacterial defenses. For instance, existing research prove β glucan supplemented diets improve immune response in *L. vannamei* (Zhao et al., 2012). Therefore, the SYC groups exhibited strong immunity and disease resistance than the control group. PO is an important component of innate immune defense for crustaceans which includes shrimps (Li et al., 2009). The enzyme, PO normally activates melanin synthesis pathway; this produce cytotoxic byproducts that kills microorganisms (Mucklow et al., 2004; Palmer et al., 2011). In the present study, the PO activities were slightly enhanced in shrimp fed SYC diets before challenge. After 12 h of *V. harveyi* injection, shrimps fed SYC diets significantly increased the activities of PO. The activities of PO and its immunocompetence have several direct benefits on the shrimp body to identify and defend against foreign pathogens through cell-cell communication (Jin et al., 2018; Johansson and Soderhall, 1989). Shrimp with increased levels of PO activity are less vulnerable to microorganism infection (Zhang et al., 2012). Besides, the increase PO activities in SYC diets group after *V. harveyi* challenge can be attributed to immune stimulatory effects of glucan in shrimp (Chiu Yang, 2014; Sakai, 1999). Furthermore, β -glucan improved the cytotoxic activities of macrophages, lymphocytes and natural killer cells in aquatic animal immune system (Chang et al., 2013a, b; Chiu Yang, 2014; Engstad et al., 1992) and promoted proPO in the lymphatic fluids of crustaceans, leading to a defensive bactericidal effect and phagocytosis, in addition to promotion of hemolymph coagulation (Cerenius and Söderhäll, 2011; Chiu Yang, 2014; Soderhall, 1981). Increased PO activity in the present study also agree with

Chang et al. (2013a, b), who reported that yeast culture enhanced innate immune responses of shrimps against infections. Hence, the yeast culture diets helped in the immune responses of shrimp after challenge.

The treatment significantly affected intestinal bacteria composition using dietary yeast culture supplemented diet fed to shrimp. The SYC (2 %) group had higher α -diversity indices (OTUs, ACE, Chao, and Shannon indices) compared to the control group. At the phylum level, SYC (2 %) group had high abundance of Proteobacteria followed by Bacteroidetes and Actinobacteria than the control group. The abundance of Bacteroidetes was observed which improve the nutrient uptake and digestion of shrimp (Amoah et al., 2019; Hou et al., 2017). Previous studies reported that Actinobacteria (Actinomycetes) enhance the production of cellulose degrading bacterial (Liu et al., 2018; Tremaroli and Bäckhed, 2012; Ye et al., 2014), which maintain intestinal homeostasis in the SYC groups (Binda et al., 2018). Increased Bacteroidetes and Actinobacteria can improve the intestinal microflora composition to enhance the health of shrimp. Proteobacteria are usually dominant bacteria phylum in aquatic environment and in the intestine of the aquatic animals. The current study identified genus of Proteobacteria, *Vibrio*, causative agents of Vibriosis in marine environments (Jiang et al., 2020) which cause tissue pathology to different degree or death. The SYC (2 %) group significantly decreased the abundance of *Vibrio* in the intestine of the shrimp than the control group. Our findings suggest that the SYC group contain some chemical substances such as β -glucan, chitin, and mannan-oligosaccharides which prevented the *Vibrio* colonization (Zhou et al., 2018). Other research studies reported that these substances are able to improve the mechanism of the immune system or have suppressive effects on pathogens and diseases (Dalmo and Bøggwald, 2008; Flint et al., 2008; Liu et al., 2014; Ringø et al., 2012; Wu et al., 2018). In addition, there was an increase in the abundance of *Pseudoalteromonas* genus which serve as probiotics because they produce digestive enzymes such as protease, amylase, phospholipases and extracellular materials which help in preventing settlement and metamorphosis of pathogenic bacteria (Amoah et al., 2019). We can conclude that SYC (2 %) group influenced the intestinal microbiota and improve immunity than the control group.

Intestine of an animal plays a major function in nutrient digestion and absorption as well as providing immune barrier against disease infection (Zhu et al., 2012). Intestinal morphology and histological evaluation are known to be critical which can be influenced by dietary nutrient absorption and also showed immune blockages against pathogenic infection. The exposure of intestinal villi surface has direct correlation with nutrient absorption (Wang et al., 2017). The longer and wider villi exhibit higher absorption of nutrient, increased nutrient uptake and may improve growth performances. Contrarily, impairment such as decreased villi length and width affects nutrient utilization which result in poor growth performances of shrimp and increase diseases infection (Farhangi, 2001). Although, there was no change observed in muscle thickness, significant differences were observed in 8 relation to villus height and width of shrimp fed with SYC groups in the present study. Previous studies demonstrated that mannan oligosaccharides as a component in yeast culture have the capacity to increase villus length in *L. vannamei* and European lobster (Daniels et al., 2010; Zhang et al., 2012). The results of our study suggest that yeast culture can increase villus height and length, therefore enhancing intestinal surface area for more nutrient absorption. In fact, the present data demonstrates SYC (2 %) treatment groups could stimulate the growth of villus length and width in the intestine and increased growth performance of shrimp. However, there are limited studies on the effect of yeast culture on intestinal morphological development in shrimp. To increase our understanding, further studies are required in this regard.

In aquatic animals, survival rate after challenge with bacteria is usually used in determining its immune responses to resist diseases (Deng et al., 2006). The non-specific immune system of the shrimp plays a major role in shielding shrimp from bacterial infection. In the present studies, increase survival rates was observed in the shrimp fed SYC groups after being challenge with *V. harveyi*. However, the SYC (2 %) had the highest survival rate followed by SYC (1 %) and control group (0 %). Studies have reported that inclusion of yeast culture fed diets to shrimp after challenged with *V. harveyi* and *V. alginolyticus* increased survival rate (Burgents et al., 2004; Chang et al., 2013a, b). The abundance of these chemical substances such as β -glucan, chitin, and mannan-

oligosaccharides in the SYC groups exhibit immunostimulatory effects (Hassan, 2011) after pathogenic bacteria challenge which increased their survival. Additionally, post-challenged shrimp after being fed with β -glucan supplement had decreased cumulative mortalities than the untreated group (Chang et al., 2011; Ma et al., 2019). Therefore, the influence of yeast culture on bacterial disease resistance suggests administration of 2 % yeast culture may effectively improve shrimp resistance to *V. harveyi* infection.

The present study demonstrated that dietary yeast culture supplementation can be beneficial for increasing growth performance, antioxidant activities, immune enzyme activities, intestinal health and disease resistance of *L. vannamei*. Also, SYC groups improved the physiological and metabolic responses. Based on the above results, SYC (2 %) yeast culture supplementations in practical diet are recommended for *L. vannamei*. Furthermore, the SYC groups influenced the intestinal microbiota which enhanced the immune system of the *L. vannamei*.

6. Author contributions

Liu Hong-yu conceived and designed the experiment. Stephen Ayiku and Shen Jianfei carried out field experiment. Stephen Ayiku carried out the laboratory work. Bei-ping Tan, Xiao-hui Dong drafted and proof read the manuscript.

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Declaration of Competing Interest

All the authors declare no conflict of interests.

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ABSTRACT

The aim of the present study was to investigate effects of dietary yeast culture on immune response related to TLR2-MyD88-NF- κ B signaling pathway, antioxidant capability and disease resistance against *Aeromonas hydrophila* for Ussuri catfish (*Pseudobagrus ussuriensis*). A total of 240 Ussuri catfish (mean weight of 7.39 ± 0.32 g) were randomly distributed into four groups that fed diets containing 0 (Y0), 10 (Y1), 20 (Y2) and 30 (Y3) g kg⁻¹ yeast culture for 8 weeks. The results indicated that dietary 10 g kg⁻¹ yeast culture supplementation significantly down-regulated mRNA levels of TLR2, MyD88, NF- κ B p65, IL-1 β and IL-8 in the liver tissue compared with the control group ($P < 0.05$). Simultaneously, serum lysozyme (LZM) activity, respiratory burst activity (RBA) of phagocytes, plasma alkaline phosphatase (AKP) activity and immunoglobulin M (IgM) content were significantly improved in fish fed Y1 diet ($P < 0.05$). Fish fed Y1 diet had significantly higher serum alternative complement pathway activity (ACH50) and plasma complement 3 (C3) content than the Y3 group ($P < 0.05$). However, no significant differences were observed in plasma acid phosphatase (ACP) activity and complement 4 (C4) content among the groups ($P > 0.05$). Fish cumulative mortality rate (CMR) in the Y1 and Y2 groups were significantly lower than that in Y0 and Y3 groups ($P < 0.05$), and the lowest CMR was observed in the Y1 group after challenge by *A. hydrophila*. The highest hepatic superoxide dismutase and glutathione peroxidase activities, total antioxidant capacity and the lowest malondialdehyde content were found in Y1 group, but no significant difference was found in hepatic catalase activity among the groups ($P > 0.05$). These results demonstrate that dietary 10 g kg⁻¹ yeast culture could effectively improve the immunity, antioxidant capability and disease resistance against *A. hydrophila* for Ussuri catfish and could down-regulate the mRNA expression levels of pro-inflammatory cytokines modulated by TLR2-MyD88-NF- κ B signaling pathway.

1. Introduction

Aquaculture is the fastest growing food industry in the world and this sector now supplies nearly half of the total fish used for human consumption [1]. However, in intensive fish culture, various disease occurrences are the major concern for farmers. In the past few years, the application of antibiotics and chemical disinfectants is a common measure to control and prevent diseases in aquaculture [2]. Nevertheless, it is well-known fact that these substances lead to enhanced microbial resistance and cause residual effects in the environment and fish [2–4], which were not encouraged to use in disease management of aquatic animals in recent years. The World Health Organization has announced that it is urgently regarding the substitution of antibiotics by safer substances for the control of infectious diseases in farm animals [5]. Therefore, development of safe feed additives is the necessary approach for the purpose of obtaining the high quality and safety aquatic products.

The aquatic feed additives generally include probiotics, prebiotics, immunostimulants, vitamins, and nucleotides. Recently, the use of natural immunostimulants in fish culture for the prevention of diseases has been the major concern for farmers and researchers. Natural immunostimulants are biocompatible, biodegradable and safe for environment and human health. Bakers' yeast, *Saccharomyces cerevisiae* as one of the natural immunostimulants has been widely used in mammals and fish [6–11], and has been recognized to have important effects on immunostimulant functions [12]. The yeast culture (YC) is a complicated yeast fermentation product composed of the yeast and its growth media. In the production of yeast cultures, yeast cells are only used for the production of their metabolites. Recently, YC is widely used in animal feed including aquafeed due to the fact that it contains protein, lipid, B-vitamins, which may possibly serve as an alternative protein source to fish meal [13–15]. Moreover, YC has been also found to be a good immune enhancer for some aquatic animals because it contains yeast cell walls (β -glucans and mannan-oligosaccharides), peptides, nucleotides, cell solubles, and oligosaccharides [16]. These immunostimulants have been used in aquatic animal culture such as

Macrobrachium rosenbergii [17], European sea bass (*Dicentrarchus labrax*) [18] and rohu (*Labeo rohita* L.) [19], altering the immune response and inducing protection against a wide range of diseases [20]. In the light of these observations, it could be hypothesized that YC might be a potential immune modulator for fish. However, the findings mentioned above mainly revealed the influence of different yeast products on performance and resistance to bacterial infection for fish species, the problem about immunomodulatory mechanism of YC has not been studied presently.

Ussuri catfish (*Pseudobagrus ussuriensis*) is an important indigenous species in China and East Asia. It lives at the bottom of freshwater, the most suitable growth temperature of 25–28 °C and the pH range of 7.0–8.5. At present, the farming production of this fish has spread all over China, and it is well popular with Chinese consumers due to its high nutritional value, resistance to stress and availability of reproduction technology. However, with the development of intensive aquaculture, this fish is facing the treat of disease in the process of aquaculture recently. To data, some preliminary studies have been conducted on the nutrition of this fish in our laboratory [21–24], but little information is available on its basal immune response. Fish immunity is strongly associated with cytokines, such as pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), interleukin-8 (IL-8), and the anti-inflammatory cytokine IL-10 [25,26], which were regulated by nuclear transcription factor kappa β (NF- κ B) signaling pathway. TLRs serve as major pattern recognition receptors (PRRs) that recognize and bind conserved pathogen-associated molecular patterns (PAMPs). After recognizing PAMPs, TLRs interact with their corresponding adaptor molecules such as myeloid differentiation factor 88 (MyD88) to elicit downstream signaling events, resulting in NF- κ B activation and the induction of several types of cytokines [27]. Many studies have been clarified that TLR2 plays an important role in innate immune responses of fish [28–30]. However, there is no published information regarding the effects of YC on immune response related to TLR2-MyD88-NF- κ B signaling pathway for Ussuri catfish. Therefore, the purpose of this study was to evaluate the effects of YC on immune response

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related to hepatic TLR2-MyD88-NF- κ B signaling pathway, antioxidant capability and disease resistance against *A. hydrophila* of this fish.

2. Materials and methods

2.1. Ethical considerations

All animal care and handling procedures in this study were conducted under the Guidance of the Care and Use of Laboratory Animals in China and were approved by the Animal Care Committee of Northeast Agricultural University of China.

2.2. Experimental diets

Dietary formulation and approximate composition are shown in Table 1. Four isonitrogenous (crude protein, 450 g kg⁻¹) and isolipidic (crude lipid, 77 g kg⁻¹) experimental diets were formulated, which

Table 1

Formulation and proximate composition of the experimental diets (g kg⁻¹ dry matter).

Ingredients	Diet Y0	Diet Y1	Diet Y2	Diet Y3
Fish meal ^a	280	270	260	250
Soybean meal ^a	300	300	300	300
Cottonseed meal ^a	110	110	110	110
Corn gluten meal ^a	70	70	70	70
Wheat meal	164	164	164	164
Soybean lecithin	10	10	10	10
Soybean oil	33	33	33	33
Vitamin premix ^b	5	5	5	5
Mineral premix ^c	5	5	5	5
Choline	3	3	3	3
Ca(H ₂ PO ₄) ₂	20	20	20	20
Yeast culture ^d	0	10	20	30
Proximate composition (g kg ⁻¹ dry matter)	932.7			
Dry matter		926.0	928.0	931.0
Crude protein	458.3	453.3	455.5	450.4
Crude lipid	77.7	78.0	76.4	76.0
Gross energy (kJ g ⁻¹)	184.9	185.7	186.0	180.0
Ash	121.3	120.1	115.3	118.4
Crude fiber	31.5	31.6	31.4	31.5
Nitrogen free extract	243.9	243	249.4	254.7

^a Fish meal: crude protein: 645.0 g kg⁻¹, crude lipid: 85.0 g kg⁻¹; soybean meal: crude protein: 467.9 g kg⁻¹, crude lipid: 31.4 g kg⁻¹; cottonseed meal: crude protein: 482.0 g kg⁻¹, crude lipid: 14.7 g kg⁻¹; corn gluten meal: crude protein: 602.0 g kg⁻¹, crude lipid: 14.7 g kg⁻¹; These ingredients were supplied by Huada feed Co., Ltd. (Harbin, China).

^b Vitamin premix (IU or mg kg⁻¹ dry diet): retinol (V_A) 3000 IU; cholecalciferol (V_D) 1500 IU; tocopherol (V_E) 40 mg; menadione (V_K) 4.5 mg; thiamin (V_{B1}) 8 mg; riboflavin (V_{B2}) 8.5 mg; pyridoxine (V_{B6}) 6.5 mg; cyanocobalamin (V_{B12}) 0.02 mg; nicotinic acid 45 mg; nicotinamide 45 mg; D-Ca pantothenate 17 mg; inositol 40 mg; biotin 0.15 mg; folic acid 1.3 mg; ascorbic acid 110 mg. ^c Trace mineral mixture use providing the following concentration (mg kg⁻¹ dry diet): copper 6.5 mg; iron 45 mg; selenium 0.35 mg; zinc 70 mg; manganese 8.5 mg; magnesium 100 mg; cobalt 1 mg; iodine 1.2 mg. ^d Yeast culture: crude protein: 487.0 g kg⁻¹, crude lipid: 42.1 g kg⁻¹, supplied by Beijing Enhalar International Tech Co., Ltd. (Beijing, China).

contained graded YC levels from 0 g kg⁻¹ to 30 g kg⁻¹ (referred as Y0, Y1, Y2 and Y3, respectively). The Y0 diet was used as the control diet. The ingredients for each experimental diet were finely ground through a 320- μ m mesh and fully mixed, then the oil was added to the mixture as needed, next the mixture was dissolved by adding deionized water (100 ml kg⁻¹ diet) to produce dough. The dough was then extruded by a laboratory extruder (QL, Henan, China) of diameter 1.5 mm at room temperature. The resultant moist pellets were dried in a convection oven at 45 °C for approximately 6 h, and then allowed to cool overnight at room temperature. Finally, the sinking diets were stored at -20 °C until used.

2.3. Feeding trial procedures

Experimental fish were obtained from Fisheries Research Institute of Harbin Academy of Agricultural Sciences (Harbin, China). Prior to the initiation of experiment, all fish were fed the diet Y0 for 2 weeks to acclimate to the experimental diets and experimental conditions. After fasting for 24 h, fish of homogeneous size (initial average weight of 7.39 $\pm$ 0.32 g) were randomly allotted to 12 aquaria (1.0 $\times$ 0.5 $\times$ 0.8 m, water depth 50–60 cm) with 20 fish to each aquarium. Each experimental diet was randomly assigned to triplicate aquaria. Fish were hand-fed to apparent satiation twice daily (08:00 and 16:00). During the experimental period, the flow rate of water was maintained at 2.5 L min⁻¹ and each aquarium was provided with continuous aeration to maintain the dissolved oxygen level above

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8 mg L⁻¹, the temperature was 24 $\pm$ 1 °C, pH 7.5–8.0, ammonia-N was < 0.1 mg L⁻¹, the photoperiod was set at 12-h light and 12-h dark. The experiment lasted for 8 weeks.

2.4. Analysis and measurement

2.4.1. Sample collection

At the end of the 8-week trial and after an overnight fast, the experimental fish were anesthetized with eugenol (1: 12 000) (Shanghai Reagent Corporation, Shanghai, China) before sampling. Serum samples were collected from the caudal vein of three fish per group using 1 ml syringe and withdrawn into Eppendorf tubes without anticoagulant. Plasma samples were collected from the caudal vein of three fish per group with a heparinized syringe and transferred into a heparinized tube. Blood samples in Eppendorf tubes were allowed to clot for 4 h at 4 °C. Following centrifugation (3000 g for 10 min), serum/plasma was stored at -80 °C for blood biochemical parameter. Another six fish per group were randomly obtained and the liver was immediately frozen in liquid nitrogen and stored frozen at -80 °C for antioxidant related parameter and immune-related gene expression analyses.

2.4.2. AKP, ACP, LYM activities and C3, C4 and IgM levels measurement

The plasma AKP, ACP and serum LYM activities were determined by commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, China) as described by Yin et al. [31]. A LYM activity unit is defined as the amount of enzyme producing a decrease in absorbance of 0.001 min⁻¹ at 530 nm. AKP activity unit is defined as 15 min per 100 ml plasma at 37 °C and substrate effects, produce 1 mg of phenol by a unit of enzyme activity. ACP activity unit is defined as per 100 ml of plasma at 37 °C with the substrate for 60 min, resulting in 1 mg phenol as a unit of enzyme activity.

The plasma C3 and C4 levels were measured as described by Xu et al. [32] following the introduction of the kits (Nanjing Jiancheng Bioengineering Institute, China). The plasma IgM level was determined by the kit of enzyme linked immunosorbent assay (ELISA) as described by Yu et al. [5].

2.4.3. Hepatic antioxidant status analysis

The liver tissues were obtained by homogenization of frozen tissue in ice-cold 0.86% (w/v) NaCl solution in tissue homogenizer and centrifuged at 3000 g 4 °C for 10 min. The supernatants were used as enzyme source for measuring enzymatic activities and hepatic malondialdehyde (MDA) content. Activities of hepatic total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and hepatic MDA content were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) as described by Bu et al. [23]. The protein content in the liver sample was also determined using a total protein quantification kit (Nanjing Jiancheng Bioengineering Institute, China) with bovine serum albumin as the standard.

2.4.4. Respiratory burst activity

Respiratory burst activity of phagocytes was measured by the nitroblue tetrazolium (NBT) assay described by Anderson and Siwicki [33]. Briefly, 0.1 ml of blood was taken in Eppendorf tube which 0.1 ml of 0.2% NBT (Sigma, USA) solution was added. The mixture was incubated for 30 min at 25 °C. From the resultant suspension, 50 μ l was taken, added to 1.0 ml N, N-dimethyl formamide (DMF, Beijing Borunlaite science & technology Co., Ltd, China) in

a glass tube and centrifuged at 3000 g for 5 min. The optical density (OD) of the supernatant was measured using a microplate reader at 540 nm.

2.4.5. Alternative complement pathway activity

The alternative complement pathway activity was determined according to Ai et al. [34]. Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 ml were dispensed into test tubes, and the total volume was brought up to 0.25 ml with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetra acetic acid (EGTA) and Mg^{2+} . Subsequently, 0.1 ml of rabbit red blood cells (RaRBC) was added to each tube. After incubation for 90 min at 20 °C, 3.15 ml of 0.9% NaCl was added to the test tubes. Samples were centrifuged at 1600 g for 5 min at 4 °C to eliminate unlysed RaRBC. The optical density of supernatant was measured at 414 nm. A lysis curve was prepared to determine the volume of serum that yielded 50% hemolysis, and the value of ACH50 units/ml was obtained for each group.

2.4.6. Real-time polymerase chain reaction (PCR) analysis

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were similar to those previously described in another study conducted in our laboratory [23]. The total RNA was extracted from liver using TransZol Up Plus Kit (TransGen Biotech, China) according to the manufacturer's instructions followed by DNase I treatment. The agarose gel (1.2%) electrophoresis and spectrophotometric (A260: 280 nm ratio) analysis were used to assess RNA quality and quantity, respectively. Subsequently, the first strand cDNA synthesis was performed using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China). Reaction conditions were recommended by the manufacturer. Real-time PCR assays were performed on Applied Biosystems® 7500 (USA) in a final volume of 20 µl containing 10 µl 2 × TransStart® Top Green qPCR SuperMix (TransGen Biotech, China), 1 µl of diluted cDNA, 0.4 µl of each primer, 0.4 µl of 50 × ROX Reference Dye II, and 7.8 µl of RNase free water. There were no significant differences in transcript levels of β actin gene among dietary treatments and was used as internal control. The real-time PCR began with 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, 30 s at 60 °C. The melting curve analysis showed only one peak for each PCR product. The gene expression levels were calculated by $2^{-\Delta\Delta CT}$ method [35]. The genes evaluated in the present study were TLR2, MyD88, NF- κ B p65, IL-1 β , IL-8, IL-10. Primers sequences for different genes are shown in Table 2.

2.4.7. Challenge test

A. hydrophila was provided by Chinese Academy of Fishery Sciences (CAFS, China). Bacteria were grown in tryptic soy broth (Sigma, USA) medium and incubated at 27 °C for 24 h. The 96 h LD₅₀ was determined by intraperitoneal injection using graded doses of *A. hydrophila* (10⁵, 10⁶ and 10⁷, 10⁸ CFU per fish): results showed that the 96 h LD₅₀ was 10⁷ CFU per fish.

At the end of the feeding trial, 30 fish from each dietary treatment (10 fish from each aquarium) were randomly assigned to three new

Table 2
Primers used in this study.

Primer names	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
TLR2-F	TTGTACAGCTGGATGAGTTG	206	54
TLR2-R	TGTCGTCAGTGAAATGTCTC		
MyD88-F	TCAGACAGCTGGAGCAGACA	93	59
MyD88-R	CGCTGGTGATGGTCCAAACA		
NF- κ B p65-F	AAGAACCAGCCATACAAGCCACAC	83	60
NF- κ B p65-R	TCAGGCAGGTCCGCTTCGTAG		
IL-1 β -F	CCTGAACACCTTCGAGTCGG	102	58

IL-1 β -R	AGGTGGCTGGTTGCTGATT		
IL-8-F	ATCGAAGGAAAAGCAGAGCG	111	57
IL-8-R	CTTTGCACAGGAGCCACTTG		
IL-10-F	TCATACGCCGTCATCCGAGA	161	58
IL-10-R	CTGACTGCACTGGGCAACAC		
β -actin-F	CCTCCGCTCTGGATTGGCTG	141	60
β -actin-R	TCAAGGGCGACGTAGCAGAG		

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tanks and were challenged by intraperitoneal injection with 0.1 ml of a suspension of *A. hydrophila* (1 × 10⁸ CFU ml⁻¹). Cumulative mortalities were recorded up to 96 h post-injection.

2.5. Calculation and statistical analysis

Cumulative mortality rate = $(N_i - N_f) \times 100 / N_i$, N_i is the initial number of fish in each cage before the immune challenge, while N_f is the final number of fish in each aquarium that survived after the immune challenge ($N_i = 10$).

All data were subjected to analysis of variance using SPSS 20.0 for Windows (SPSS, Inc., USA). Data was subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Data throughout the text are expressed as means ± S.E.M (standard error of the means) with superscript letters indicating differences between groups. A significant level of $P < 0.05$ was employed in all cases.

3. Results

3.1. Non-specific immunity parameters

Serum LZM activity and RBA of phagocytes were significantly increased in fish fed Y1 diet compared with the other groups ($P < 0.05$) (Fig. 1A and Fig. 1B). Fish fed Y1 diet had significantly higher alternative complement pathway activity (ACH50) than fish fed Y3 diet ($P < 0.05$) (Fig. 1C). However, no significant difference was observed in ACH50 among fish fed Y0, Y1 and Y2 diets ($P > 0.05$). The significantly increased plasma AKP activity and IgM content were observed in Ussuri catfish fed Y1 diet compared with fish fed Y0 diet ($P < 0.05$) (Fig. 2A and Fig. 2E). The highest plasma C3 content was observed in fish fed Y1 diet, but no significant difference was observed among fish fed Y0, Y1 and Y2 diets ($P > 0.05$) (Fig. 2C). Moreover, no significant differences in plasma ACP activity and C4 content were found among the groups ($P > 0.05$) (Fig. 2B and D).

3.2. Cumulative mortality rate after *A. hydrophila* challenge

Mortality was recorded daily after the fish were challenged with *A. hydrophila* for 96 h (Fig. 1D). The CMR in the Y1 and Y2 groups were significantly lower than that in the Y0 and Y3 groups ($P < 0.05$), and the lowest CMR was observed in the Y1 group.

3.3. Hepatic antioxidant status analysis

The assessment results of the fish hepatic antioxidative indexes are presented in Table 3. Fish fed Y1 diet showed higher hepatic TAC and SOD activities and lower MDA content than those of the Y0 group ($P < 0.05$). Hepatic GPx activity in Y1 group was significantly higher than that in Y3 group ($P < 0.05$). No significant difference was observed in hepatic CAT activity among the groups ($P > 0.05$).

3.4. Immune-related gene expression analysis

As shown in Fig. 3A and Fig. 3B, the hepatic TLR2 mRNA level was down-regulated in fish fed Y1 diet compared with that of the control group ($P < 0.05$).

Fish fed Y1 diet had significantly lower hepatic mRNA levels of MyD88 and NF- κ B p65 than those of the Y0 and Y3 groups ($P < 0.05$). Moreover, Fish fed Y1 diet had significantly lower hepatic mRNA levels of IL-1 β and IL-8 than those of the control group ($P < 0.05$). However, no significant difference was observed in hepatic IL-10 mRNA level among the groups ($P > 0.05$).

4. Discussion

Currently, yeast culture has been found to be a good immunostimulants for some aquatic animals due to the fact that it contains yeast cell walls (β -glucans and mannan-oligosaccharides), peptides, nucleotides, cell solubles, and vitamins, etc. [16]. Yeast cell walls are constructed almost entirely of two classes of polysaccharides, mannose polymers covalently linked to peptides (or mannoprotein) and glucose polymers (or glucans). The glucans and mannoproteins occur in

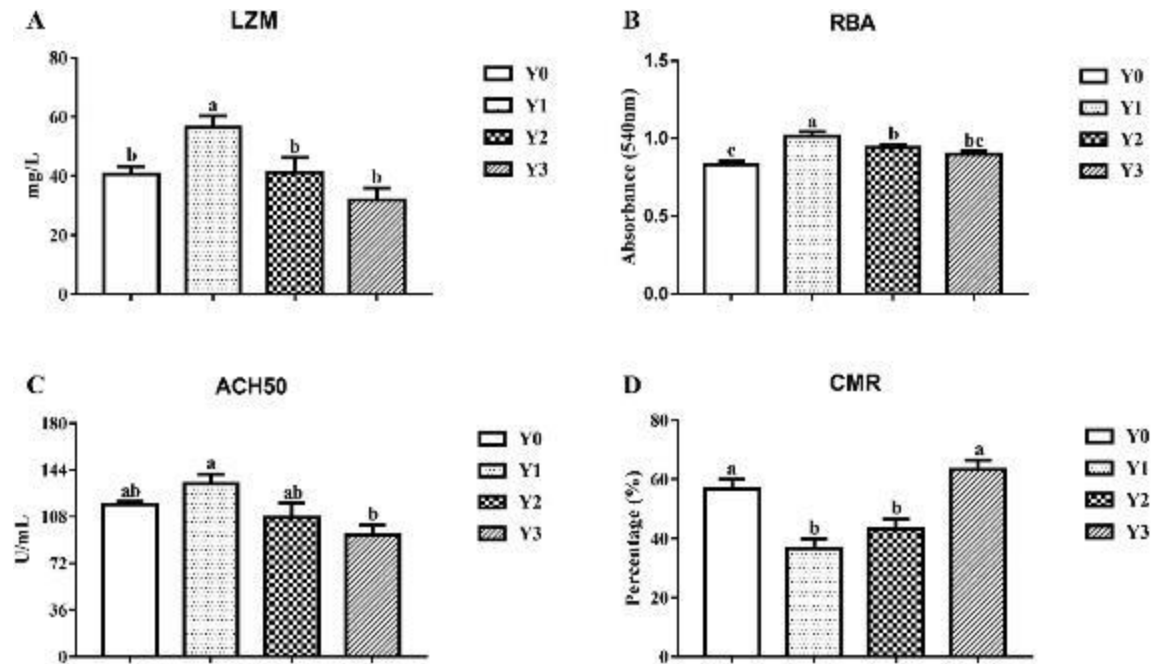


Fig. 1. The effects of dietary YC on the serum LZM activity, RBA of phagocytes, plasma alternative complement pathway activity and disease resistance for Ussuri catfish. Serum lysozyme (LZM) activity (Fig. 1A), Respiratory burst activity (RBA) of phagocytes (Fig. 1B), Alternative complement pathway, which is presented as ACH50 (Figs. 1C) and 96-h cumulative mortality rate (CMR, Fig. 1D) are presented when fish was fed different diets. Values are the means $\pm$ S.E.M of three replicates. Different letters in each figure presents significant difference among dietary treatments ($P < 0.05$). S.E.M: standard error of the means.

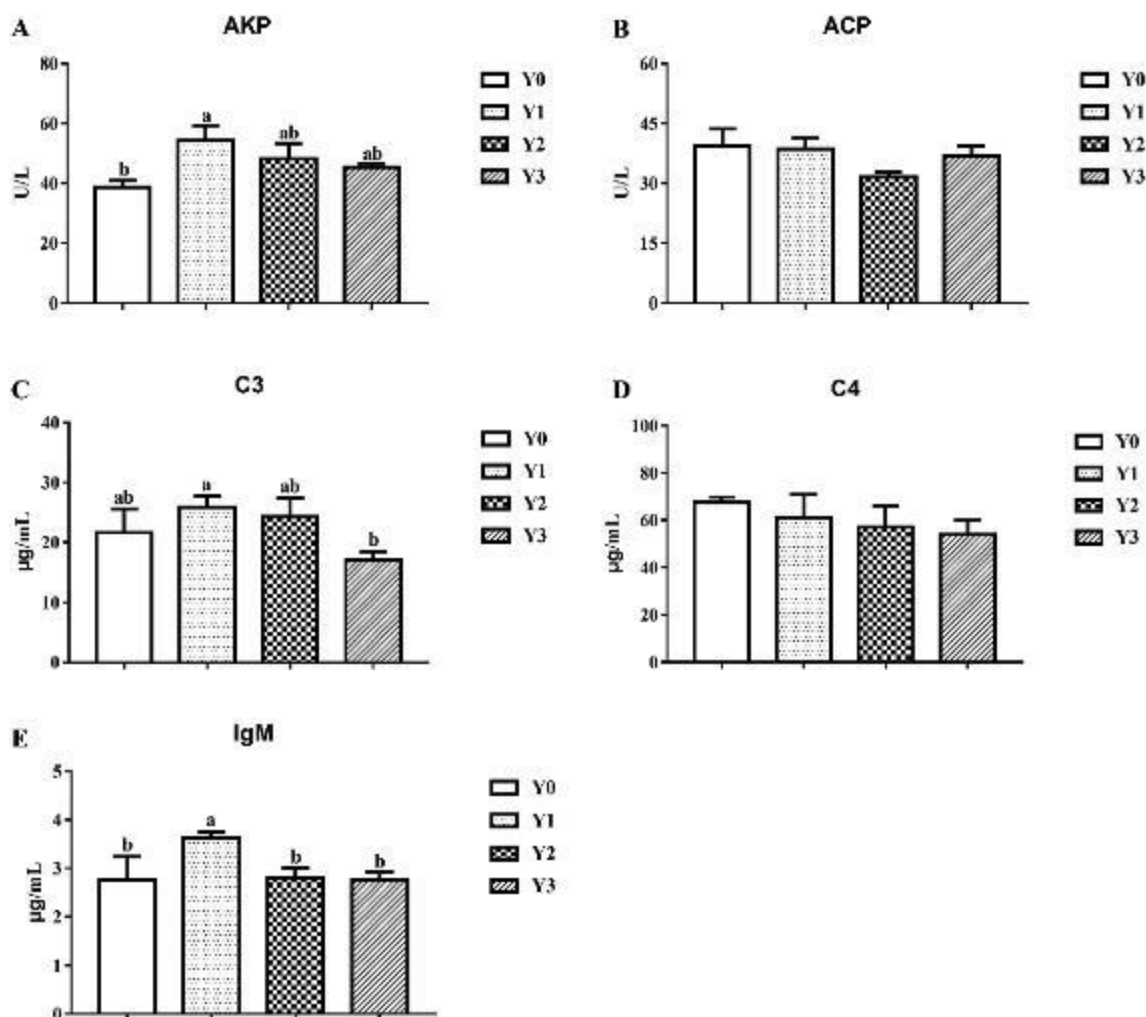


Fig. 2. The effects of dietary YC on the plasma AKP and ACP activities and C3, C4 and IgM contents of Ussuri catfish. Plasma alkaline phosphatase (AKP) activity (Fig. 2A), acid phosphatase (ACP) activity (Fig. 2B), complement 3 (C3) content (Fig. 2C), complement 4 (C4) content (Fig. 2D) and immunoglobulin M (IgM) content (Fig. 2E) are presented when fish was fed different diets. Values are the means $\pm$ S.E.M of three replicates. Different letters in each figure presents significant difference among dietary treatments ($P < 0.05$). S.E.M: standard error of the means.

Table 3
Effect of dietary YC on antioxidant capacity parameters in liver of Ussuri catfish.

	Experimental diets			
	Y0	Y1	Y2	Y3
CAT (U mg ⁻¹)	4.96 $\pm$ 0.45	5.24 $\pm$ 0.23	5.18 $\pm$ 0.70	4.70 $\pm$ 0.04
SOD (U mg ⁻¹)	91.65 $\pm$ 1.75 ^b	103.11 $\pm$ 2.58 ^a	95.06 $\pm$ 0.40 ^{ab}	91.54 $\pm$ 2.77 ^b
GPx (U mg ⁻¹)	99.02 $\pm$ 5.77 ^{ab}	120.98 $\pm$ 10.56 ^a	95.06 $\pm$ 15.34 ^{ab}	83.70 $\pm$ 9.42 ^b
TAC (U mg ⁻¹)	0.39 $\pm$ 0.07 ^b	0.59 $\pm$ 0.02 ^a	0.36 $\pm$ 0.07 ^b	0.33 $\pm$ 0.04 ^b
MDA (nmol mg ⁻¹)	4.01 $\pm$ 0.32 ^a	2.86 $\pm$ 0.18 ^b	3.01 $\pm$ 0.06 ^b	4.19 $\pm$ 0.33 ^a

Data represented as mean $\pm$ S.E.M of triplicate tanks.

Values in the same row with different superscripts are significantly different ($P < 0.05$).

CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; TAC, total antioxidant capacity; MDA, malondialdehyde. S.E.M: standard error of the means.

roughly equal amounts in the wall. A third sugar polymer of N-acetylglucosamine, chitin, is also an important component of fungal walls, but is present only in minor amounts (about 1%) in yeasts [36]. β glucans, mannoproteins and chitin have generally been described as powerful immunostimulants in fish and mammals. Thus, the use of yeast cultures which include different immunostimulants (β -glucans, mannoproteins, chitin and nucleotides) could produce a more general immune response. To the best of our knowledge, this is the first attempt to investigate the effects of yeast culture as an immunostimulant for the Ussuri catfish (*Pseudobagrus ussuriensis*).

In the present study, fish fed diet containing 10 g kg⁻¹ yeast culture had significantly higher RBA than the other groups. The respiratory burst of phagocytes, generates reactive oxygen species, is a crucial step in non-specific immunity and antioxidant responses, and it is a kind of kill mechanism from immune cell, this process is believed to be important in eliminating potential pathogens following phagocytosis [37,38]. Thus, the increase of RBA in the present study demonstrated that the immunity of Ussuri catfish could be enhanced by dietary

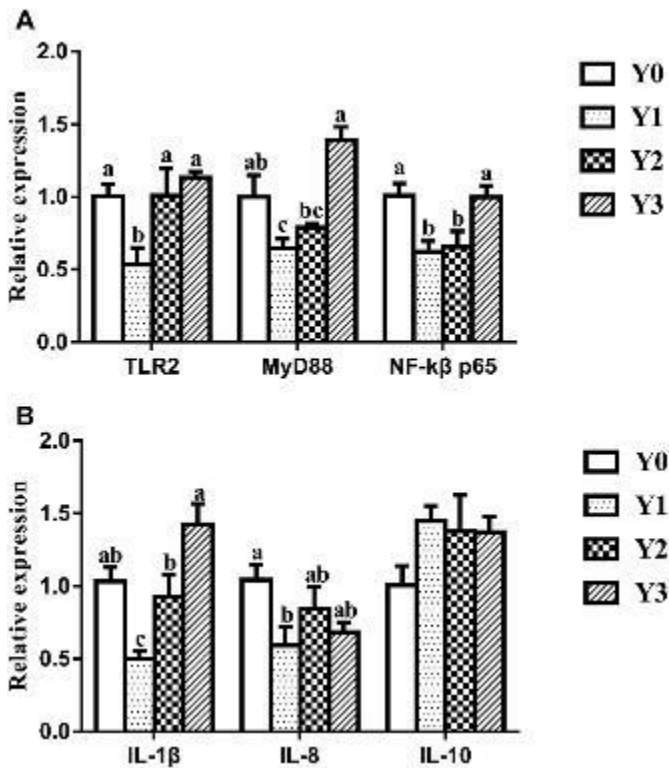


Fig. 3. The effects of dietary YC on the immune related genes expression in liver of Ussuri catfish. The immune related genes expression of TLR2, MyD88, NF-κB p65, IL-1β, IL-8 and IL-10 are determined in the liver of Ussuri catfish fed different diets (Fig. 3A and B). Values are means ± S.E.M of three replicates. Different letters in each figure presents significant difference among dietary treatments ($P < 0.05$). TLR2: toll-like receptor 2; MyD88: myeloid differentiation factor 88; NF-κB p65: nuclear transcription factor kappa β p65; IL-1β: interleukin-1β; IL-8: interleukin 8; IL-10: interleukin 10; S.E.M: standard error of the means.

10 g kg⁻¹ YC supplementation. Moreover, lysozyme, as an important hydrolytic enzyme of non-specific immune system, can breaks β-1,4 glycosidic bonds between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls and is known to attack mainly Gram-positive bacteria [39]. Alkaline phosphatase is an important lysosomal enzyme with potential protective role in fish [40], which is responsible for the hydrolysis of bacterial lipopolysaccharides [41]. In the present study, significantly increased serum lysozyme activity and plasma alkaline phosphatase activity were observed in fish fed Y1 diet, indicating that dietary 10 g kg⁻¹ YC supplementation might improve innate immune defense ability of Ussuri catfish to a certain extent. Likewise, IgM is the most widely studied immunoglobulin in fish, which could be a good biomarker for evaluating the immune status of fish [5,42,43]. In the current study, the highest plasma IgM content was observed in Y1 group, also suggesting that dietary 10 g kg⁻¹ YC supplementation might enhance the immunity of Ussuri catfish. Similar to our results, the inclusion of yeast in diets were reported to improve the innate immunity of some fish species, such as carp (*Cyprinus carpio* var. Jian) [44], gilthead seabream (*Sparus aurata* L.) [45], and Atlantic salmon (*Salmo salar*) [46]. These results all supported the fact that yeast products including yeast culture had strong immunostimulant properties. According to previous studies, the immunostimulatory nature of yeast may be attributed to functional components β-glucan and mannan-oligosaccharide [44,47]. In fact, mannan-oligosaccharide can reproduce probiotic bacteria and restrain the adherence and colonization of pathogen in gut [48], thereby resulting in the enhanced immunity of body [44]. Besides, previous studies have reported an immunostimulatory effect of β-glucan resulting in both increased innate

macrophages by β-glucan [52]. Moreover, the intake of chitin also increases the head-kidney leucocyte immune response [53]. Not only sugars but also nucleic acids, especially yeast RNA, could act as immune activators [45].

Previous studies demonstrated that yeast culture also has a positive effect on the disease resistance of aquatic animals. Burgents et al. [54] reported that dietary administration of yeast culture could protect shrimp against a decline in resistance to bacterial disease. Similarly, Yu et al. [5] demonstrated that dietary 500 mg kg⁻¹ yeast cell wall could enhance the immune response and cumulative survival after challenge with *Aeromonas veronii* for Japanese seabass (*Lateolabrax japonicus*). Previous study also found that diet supplemented with live yeast (*Debaryomyces hansenii*) significantly enhanced resistance of juvenile Leopard grouper (*Mycteroperca rosacea*) against *Amyloodinium ocellatum* [44]. The current study found that the cumulative mortality rate of fish was significantly decreased when 10 g kg⁻¹ and 20 g kg⁻¹ YC were supplemented in the diet of Ussuri catfish. It could be hypothesized that the reduced cumulative mortality rate may be due to the activation of immune defence of fish by adequate yeast culture supplementation in diets, that is to say, dietary 10 g kg⁻¹ YC supplementation was very helpful in improving immunity and protection against infection for the Ussuri catfish.

Generally, metabolic processes of body produce a series of reactive oxygen species (ROS) (e.g., superoxide anion and hydrogen peroxide) which are effective antimicrobial substances. However, oxidative stress occurs when the balance between antioxidants and ROS are disrupted due to depletion of antioxidants or excessive accumulation of ROS, or both [46]. The antioxidant system can effectively eliminate ROS in order to maintain a stable internal environment [55], which constitute the first line of enzymatic defense mechanism against free radicals to maintain the complex immune system of fish. Superoxide dismutase can clear the internal ROS to avoid the occurrence of fatty acid oxidation and consequently protect organisms from oxidative damage, while TAC activity can reflect the oxidation resistance capability of fish [56]. In the present study, fish fed Y1 diet had significantly higher hepatic TAC and SOD activities compared with the fish fed the Y0 diet, suggesting that dietary 10 g kg⁻¹ YC supplementation could enhance antioxidant capacity of Ussuri catfish. The present study also found that the hepatic MDA content was significantly reduced for fish fed diet with 10 g kg⁻¹ YC supplementation, showing that dietary 10 g kg⁻¹ yeast culture inhibited oxidative damage in the liver of Ussuri catfish. This may be explained by the fact that MDA was used as a biomarker for lipid peroxidation and protein oxidation, which has strong biotoxicity and will cause body damage [57]. Similar results were observed in the previous study conducted by Reyes-Cerpa et al. [46], who explained that yeast products are mainly composed of a high content of astaxanthin and β-glucan, which have been reported to play a protective role against oxidative damage preserving the cellular integrity [58].

The recognition of microbial pathogens mediated by pattern recognition receptors (PRRs) is vital to the initiation of innate immune responses [59]. TLRs are transmembrane proteins containing an ectodomain composed of multiple leucine-rich region motifs, a transmembrane region, and an intracellular Toll/interleukin-1 receptor domain, which serve as major PRRs that detect pathogen-associated molecular patterns. TLR2 is one type of PRRs, which has been confirmed to have a strongly associated with immunity of fish [29,60]. In the present study, fish fed diet containing 10 g kg⁻¹ YC had significantly lower hepatic TLR2 mRNA level compared with the other groups. Previous study found that peroxides in body could induce the production of the endogenous TLR ligands, which can activate TLRs [61]. In this study, it was reasonable to hypothesize that the enhanced oxidation resistance in Y1 group could reduce the production of peroxides, and then decreased the number of the endogenous ligands of TLR2, which indirectly down716

regulated the hepatic TLR2 mRNA level. Besides, the enhanced immunity of Ussuri catfish fed diet containing 10 g kg⁻¹ YC may contribute to preventing from the invasion of pathogens, which might decrease the binding of pathogens and TLR2 protein, and then reduced the TLR2 relative gene expression level in liver tissue of Ussuri catfish. MyD88 is the corresponding adaptor molecules of TLR2, which can elicit downstream signaling events, resulting in NF-κB

and adaptive responses as well as increased resistance to experimental infections for many fish species [49–51]. The immunostimulatory mechanisms involved are known to be related to the activation of fish

activation and the induction of several types of cytokines [27]. Interestingly, in the present study, the lowest hepatic MyD88 and NF- κ B p65 mRNA levels were found in Y1 group, indicating that dietary yeast culture supplementation may enhance the immunity of Ussuri catfish through TLR2MyD88-NF- κ B signaling pathway. Cytokines play an important role in the immune system to accommodate various challenges in fish, which are generally good markers to evaluate the immunity of fish [62–64]. Zhang et al. [26] found that the enhanced immunity of *Cyprinus carpio* Huanghe var was close related to the suppressed the mRNA levels of pro-inflammatory cytokines such as IL-1 β and IL-8. Similarly, the present results revealed that the lowest IL-1 β and IL-8 mRNA levels were observed in fish fed Y1 diet. These results may be ascribed to the regulation of NF- κ B signaling pathway. Previous studies have demonstrated that the pro-inflammatory cytokines (e.g., IL-1 β , IL-8) gene expression was positively related to NF- κ B mRNA levels [26,65]. Thus, the lower pro-inflammatory cytokines (IL-1 β and IL-8) mRNA levels may be attributed to the reduced hepatic NF- κ B p65 transcriptional level of Ussuri catfish, which suggests possible anti-inflammatory actions of yeast culture. However, it's necessary to point out that the relationship between dietary yeast culture and TLR2-MyD88-NF- κ B signaling pathway or cytokines gene expression is still unclear in fish and needs further investigation.

In conclusion, the present study suggests the potential benefit of yeast culture as an immunostimulant supplement in aquafeed. The results demonstrated that dietary 10 g kg⁻¹ yeast culture could effectively improve the immunity by down-regulating the expression of pro-inflammatory cytokines attributed to the decreased TLR2, MyD88 and NF- κ B p65 mRNA levels, simultaneously, could enhance the antioxidant capacity and resistance to *Aeromonas hydrophila* for Ussuri catfish.

Conflicts of interest

No potential conflict of interest.

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Full length article

Effects of dietary yeast culture on growth performance, immune response and disease resistance of gibel carp (*Carassius auratus gibelio* CAS III)

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ABSTRACT

A 50-day feeding trial was carried out to evaluate the partial replacement of fishmeal by yeast culture (YC) on growth performance, immune response and resistance against *Aeromonas hydrophila* in gibel carp CAS III (*Carassius auratus gibelio*). Four isonitrogenous and isoenergetic practical diets including a basal diet (the control diet containing 10% fish meal, D0) and three yeast culture diets (substituting 20%, 40%, 60% of the fishmeal in the basal diet, D20, D40 and D60, respectively) were formulated. Each diet was randomly allocated to quadruplicate fish groups (average initial body weight: 28.70 ± 0.03 g) reared in a recirculating system. After the growth trial, bacterial challenge test was conducted. The results showed that no noteworthy variations in feed intake, growth performance and morphology indices were found among groups ($P > 0.05$). YC Supplemented diet exerted little significant influence on plasma parameters including triglyceride, glucose, creatinine, total protein and urea nitrogen compared with the control group ($P > 0.05$). No obvious variations were found in activities of plasma lysozyme, IgM, MPO and SOD before challenge test among dietary treatments ($P > 0.05$), whereas considerable higher value of the foresaid indicators was discovered in D40 after bacteria challenge ($P < 0.05$). Transcriptional levels of Toll like receptor 2 (TLR2), myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain-containing adaptor protein (TIRAP) and interleukin-1 β (IL-1 β) in spleen after challenge were significantly up-regulated in D40 compared with D0 ($P < 0.05$). Cumulative survival rate in D40 and D60 were significantly higher than those in D0 and D20 ($P < 0.05$). Taken together, yeast culture could be a suitable fishmeal alternative in diets of gibel carp and dietary inclusion of 4 gYC per 100 g diet enhanced the immunity and disease resistance of gibel carp partly via TLR2 pathway.

1. Introduction

Fishmeal has been extensively considered to be the preferred protein ingredient in aquafeed [1,2]. However, the shortage of fishmeal due to the declination of fisheries resources and due to the increase of fishmeal demands had become a challenge in global aquafeed industry [3]. The use of fishmeal alternatives was therefore given high priority. Among these, single cell proteins (SCPs) are generally defined as live or killed microorganisms, protein extract such as algae or beneficial bacteria and yeast [4]. SCPs had high nutritional and functional values, balanced amino acid profile, B vitamins, pigments and immunostimulatory substances, such as yeast cell wall, nucleic acids (RNA, DNA) [5,6]. Yeast contains relatively high protein content (45–55%) and can be cheaply and profusely produced [7,8]. Yeast had been reported to partially replace dietary fishmeal in many cultured aquatic species [9–11]. Furthermore, yeast culture enhanced the immunity and disease resistance in Pacific white shrimp (*Litopenaeus vannamei*) [12].

Yeast culture is a complicated compound, which not only had high protein and amino acid values, but also possessed immunomodulatory constituents such as β -glucan, nucleotide, and mannose oligosaccharide (MOS) [13]. Selvaraj [14] reported a significant higher survival and improved immune response post bacterial challenge in carp (*Cyprinus carpio*) injected intraperitoneally with 500 μ g yeast-based β -glucan suspended in 0.1 ml phosphate buffered saline (PBS, pH 7.2) per fish compared with the control group. It was also documented that when exposed to 0.5% yeast oligonucleotide in diet, notably enhanced survival rate and higher neutrophil oxidative radical production post bacterial challenge (*Streptococcus iniae*) were found in hybrid striped bass (*Morone chrysops* $\times$ *Morone saxatilis*) compared with fish fed the basal diet [15]. Staykov et al. [16] had proven that the dietary administration of 2000 ppm mannose oligosaccharide significantly decreased mortality rate and improve the immune status of rainbow trout (*Oncorhynchus mykiss*) cultivated in net cage (42 days) as well as in raceway (90 days). However, it is still far from understanding immunomodulating effect of dietary yeast culture in fish.

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Based on in vitro studies in mammals, β -glucans effects mainly were performed through dectin-1 receptors, Toll-like receptors (TLRs) and complement receptor (CR3), and subsequently activate a set of immune cells including macrophages, neutrophils, monocytes, natural killer cell and dendritic cells resulting in diverse immune responses [17]. However, the presence of sequences in genomes, which can be considered clear orthologs to specific sequence of dectin-1 receptor, seems to be limited mammalian genomes [18,19]. In addition, clear ortholog could not be identified in the search of dectin-1 in the genome of common carp [20]. Furthermore, it has been proven that common carp (*Cyprinus carpio*) macrophage could recognize β -glucans via multiple pattern recognition receptors including TLRs [21]. TLR2 play a crucial role in recognition of β -glucans at least in murine and human dendritic cells [22]. TLR2 pathway genes were therefore emphasized in the current study. In the TLR2 signaling pathway, myeloid differentiation factor 88 (MyD88) and Toll/IL-1 receptor domain-containing adaptor protein (TIRAP), two of Toll/IL-1 receptor domain-containing adaptors, play a critical role [23,24]. TIRAP activates the downstream MyD88 signaling pathway [25]. MyD88 and interleukin-1 receptor-associated kinase (IRAK) 4 share a death domain and conveying the contact between the two molecules. Subsequently, IRAK4 phosphorylates the downstream kinase IRAK1, recruiting tumor necrosis factor receptor-associated factor 6 (TRAF6) [26]. Eventually resulting in the stimulation of the transcription factor nuclear factor κ B (NF- κ B), which cooperates with other transcription factors to induce the expression of a wide variety of target genes including proinflammatory cytokines [27]. Therefore, we assume that the effect of yeast culture on the immune status of fish is mediated partly through TLR2 pathway.

Gibel carp (*Carassius auratus gibelio*) is a very popular cultivated species in China and annual production of this species was approximate three million tones [28]. Gibel carp culture has also the problems of fishmeal alternatives and immune suppression because of its intensive farming. The objectives of this study were to investigate the effect of dietary inclusion of yeast culture on the growth performance, immune responses and disease resistance of gibel carp, as well as on the transcriptional levels of TLR2 pathway genes.

2. Materials and methods

2.1. Diet preparation

Four experimental diets were formulated as isonitrogenous (30% crude protein) and isoenergetic (19.7 MJ kg⁻¹) with the substitution of fishmeal by YC at 0%, 20%, 40% or 60% levels (designated as D0, D20, D40 or D60, respectively) on a dry matter basis and met nutrient and energy requirements of carp [29]. The YC derived from *Saccharomyces cerevisiae* was purchased from Enhalar Biotechnology Company (Beijing, China). The content of nutrients in YC powder were listed in Table 1. The control diet used fishmeal, soybean meal, double-low rapeseed meal and cottonseed meal as protein sources and fish oil/soybean oil (1:1) as lipid sources. All ingredients were completely mixed with appropriate water and extruded into pellets of 2.4 mm diameter by a laboratory granulator (SLP – 45, Fishery Mechanical Facility Research Institute, Shanghai, China). Four diets were oven dried at 50 °C to a

Table 1
The contents of nutrients in yeast culture powder (g kg⁻¹ dry matter).

Chemical composition	yeast culture
Crude protein	677.25
Crude lipid	31.81
β -glucan ¹¹	4.20
Mannose oligosaccharide ^a	4.70
Inosine monophosphate ^b	0.06
Uridine monophosphate ^b	0.02
Cytidine monophosphate ^b	0.04

¹¹ White fish meal was purchased from American Seafood Company, Seattle, Washington, USA, its composition was as follows: Moisture, 5.84%; Crude protein, 64.65%; Crude lipid, 9.14%; Gross energy, 20.59 MJ kg⁻¹. ^a Purchased from Enhalar Biotechnology Company, Beijing, China. ^b Purchased from Coland Feed Co. Ltd., Wuhan, Hubei, China. ^c Composed of 50% choline chloride and 50% silicon dioxide.

Essential amino acid ^c Threonine	26.1
Valine	37.5
Methionine	16.8
Isoleucine	27.9
Leucine	46.2
Phenylalanine	26.5
Lysine	55.2
Histidine	14.7
Arginine	36.4
Cystine	8.8
Non-essential amino acid ^c Aspartic acid	46.4
Serine	36.4
Glutamic acid	78.3
Glycine	39.1
Alanine	27.8
Tyrosine	12.8
Proline	46.2

^a Measured by Instrumental Analysis Center of Jiangnan University, Wuxi, Jiangsu, China.

^b Measured by Pony Testing International Group, Beijing, China.

^c Measured by Hai Rui Zheng Detection Technology Co. Ltd., Zhengzhou, Henan, China.

Table 2
Formulation and chemical composition of experimental diets (% dry matter).

Ingredients	D0 (control)	D20	D40	D60
Fishmeal ^a	10	8	6	4
Yeast culture ^b	0	2	4	6
Soybean meal ^c	25	25	25	25
Rapeseed meal	15	15	15	15
Cottonseed meal	10	10	10	10
Corn starch	10	10	10	10
α -starch	15	15	15	15
Fish oil	3.15	3.15	3.15	3.15
Soybean oil	3.15	3.15	3.15	3.15
Cellulose	3.59	3.59	3.59	3.59
Ca(H ₂ PO ₄) ₂ ·H ₂ O	2	2	2	2
Carboxymethylcellulose sodium	2	2	2	2
Choline chloride ^d	0.11	0.11	0.11	0.11
Vitamin and mineral premix ^b	1	1	1	1

Proximately chemical composition

Moisture	17.81	17.71	18.41	16.62
Crude protein	30.5	30.39	29.45	30.37
Ash	9.66	9.42	9.24	8.62
Crude lipid	6.59	6.26	5.98	6.07
Gross energy (MJ·kg ⁻¹)	19.75	19.67	19.79	19.81

Table 3
Amino acid composition of experimental diets.

moisture content of approximately 17%, subsequently sieved through 16 mesh sifter removing broken particle and finally stored in separate sealed plastic bags at 4 °C. The formula, proximate chemical and amino 401

Amino acids (% dry matter)	D0	D20	D40	D60
Essential amino acid Thr				
	1.11	1.10	1.10	1.11
Val	1.29	1.31	1.31	1.36
Met	0.38	0.26	0.25	0.25
Ile	1.11	1.12	1.11	1.15
Leu	2.00	2.02	1.98	2.02
Phe	1.27	1.26	1.25	1.30
His	0.73	0.72	0.71	0.70
Lys	1.73	1.74	1.71	1.74
Arg	1.95	1.96	1.93	2.01
ΣEAA	11.59	11.48	11.34	11.64
Non-essential amino acid Asp				
	2.63	2.64	2.60	2.61
Ser	1.29	1.32	1.34	1.40
Glu	5.09	5.09	5.03	5.13
Gly	1.40	1.41	1.37	1.38
Ala	1.28	1.27	1.24	1.23
Tyr	0.64	0.61	0.61	0.64
Pro	1.15	1.15	1.14	1.23
ΣAA	25.08	24.97	24.67	25.28

acid compositions were presented in Table 2 and Table 3, respectively.

2.2. Experimental fish and feeding regime

About 1000 fish were obtained from the Haid Group hatchery (Guangzhou, Guangdong province, China) and were transported to the Institute of Hydrobiology, Chinese Academy of Sciences in aerated plastic bags. Afterwards, the fish were bathed in 3% saline for 20 min. The healthy and unwounded fish were transferred into a cylindrical fiberglass tank of 1500 L supplied with circulated, aerated and filtrated freshwater. During the acclimation period, the fish were manually fed with basal diet to apparent satiety twice a day at 9:00 and 15:00 for a fortnight. A total of 33% of the tank water was exchanged once a day prior to the first feeding.

Fish were fasted for a day prior to the beginning of feeding trial. Apparent healthy fish (initial mean body weight of 28.70 ± 0.03 g) were randomly selected and distributed into 16 conical fiberglass of 150 L at a density of 23 fish per tank in quadruplicate. Each tank of the recirculating rearing system was equipped with continuously aerated and dechlorinated tap water. The experiment lasted for 50 days.

The fish were hand-fed to apparent satiation two times daily at 9:00 and 15:00. During the experiment, water temperature ranged from 24.0°C to 25.0°C , dissolved oxygen was $6.0\text{--}6.7$ mg/L and PH was $7.1\text{--}7.4$, daily. Ammonia-N was monitored weekly and was kept under 0.1 mg/L. Illumination intensity was $4.31\text{--}4.68$ $\mu\text{mol}/\text{m}^2/\text{s}$ at the middle depth of the tank and photoperiod was 12 h light/12 h dark.

2.3. Sample collection

At the termination of the trial, tested fish were starved for 24 h, were rapidly netted, were anesthetized by MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA, USA; 50 mg/L) and were batch-weighted. Three fish per replicate were randomly selected to measure the fork length and body weight individually to examine the condition factor (CF). Then the fish were dissected and the weight of isolated viscera and hepatic tissues were determined for viscera somatic index (VSI) and hepatosomatic index (HSI). Blood samples were rapidly withdrawn using heparinized syringes from caudal blood vessels of another four-anesthetized fish per replicate. Sample were centrifuged (3000 g, 15 min, 4°C), plasma was separated (200 μL) and were stored at -80°C for analyses of glucose, urea nitrogen, total protein, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST),

total cholesterol, triglyceride, IgM, lysozyme, myeloperoxidase (MPO) and superoxide dismutase (SOD).

2.4. Bacterial challenge

In the pretest of bacterial challenge, a single colony of *Aeromonas hydrophila*, originally isolated from infected carp, multiplied on brain heart infusion (BHI) agar plate and was picked and incubated in BHI medium at 30°C for 6 h and was centrifuged at 3500 g for 10 min. The precipitate was washed three times with aseptic PBS ($\text{pH} 7.2\text{--}7.4$) to remove color of culture medium, was re-suspended in the identical buffer and adjusted to four doses of 1.0×10^6 CFU mL^{-1} , 1.0×10^7 CFU mL^{-1} , 2.0×10^7 CFU mL^{-1} and 1.0×10^8 CFU mL^{-1} . Then Two fish per replicate were randomly picked out and mixed in a basin. Then, they were divided equally into four parts and injected intraperitoneally with different doses of *A. hydrophila* in 200 μL volume PBS. The 108 h mortalities of the four treatments were 0%, 50%, 75% and 100%, respectively. Hence the dose of 1.0×10^7 CFU mL^{-1} was adopted in the present study.

16 tanks of carp ($n = 13$ per tank) were injected intraperitoneally with median lethal dose of *A. hydrophila* containing 2×10^6 cells suspended in 200 μL . Three groups of 5 fish each tank were injected intraperitoneally with 200 μL PBS per fish as the control group. Injections were executed under anesthesia with MS222.

Twelve hours after challenge, 3 fish per tank were anesthetized with MS222 and plasma samples were collected to analyze plasma IgM, lysozyme, SOD and MPO. Afterwards spleen of the three fish were also sampled. The cumulative survival rate was observed for 108 h after bacteria challenge. When calculating and statistically analyzing the survival percentage of fish post challenge, the sampled fish were excluded.

2.5. Biochemical assays

Proximate composition analyses of the ingredients and diets were undertaken in duplicate using the AOAC methods [30]. Dry matter was determined by oven drying at 105°C until constant. Crude protein content ($N \times 6.25$) was examined by Kjeldahl method after acid digestion using the 2300 Kjetec Analyzer Unit (FOSS Tecator, Haganas, Sweden). Crude lipid content was evaluated by ether extraction with the Soxtec system (Soxtec System HT6, Tecator, Haganas, Sweden) with diethyl ether as extraction lipid. Crude ash content was assessed by incineration in a muffle furnace at 550°C for 12 h. Gross energy was estimated by Oxygen bomb heat meter (Calorimeter, Parr instrument Company Moline, USA). Amino acid except tryptophan analysis of YC, experimental diets were measured after acid hydrolysis in 6 M HCl for 24 h at 110°C , separated by ion-exchange chromatography and reacted with ninhydrin post column derivatization using Hitachi L-8800 Amino Acid Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

The plasma glucose, urea nitrogen, total protein, creatinine, total cholesterol, triglyceride contents, ALT, AST, SOD and MPO activities were tested using the commercial kits according to the manufacturer instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The plasma IgM was appraised on the basis of specification in the fish ELISA kit (Cusabio Biotech Co., Ltd, Wuhan, China). The plasma lysozyme level was measured by turbidimetric assay with slight modification as previously established [31,32].

2.6. Quantitative real time PCR analysis

Total RNAs of spleen were extracted by the trizol method. The concentration and purity of total RNAs were evaluated at 260 and 280 nm. One percent agarose gel electrophoresis was used to assess the quality. The isolated RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) using a M-MLV First-Strand Synthesis Kit (Invitrogen, Shanghai, China) according to the manufacturer's instruction, and the cDNA was used in subsequently for polymerase chain

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Table 4

Sequences of primers applied for quantitative real-time PCR analysis in gibel carp.

Gen bank accession no	Primer name	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp. (°C)	PCR efficiency
GAPDH (AM701793)	GAPDH-F	TCACAGACCACAGTCCATGC	175	60	1.96
	GAPDH-R	GCCCAGTCACGTTCTGTCTA			
TLR2 (KC816575.1)	TLR2-F	ACGTTTCTGCAAGCTACGGA	171	60	2.06
	TLR2-R	CGGGCTTCTGCTCCTTTTCT			
MyD88 (KC816578.1)	MYD88-F	ACAGACAGAGCAAAAGTGACCA	151	53.9	1.98
	MYD88-R	CAACCCTAGTCCTCCTTCCG			
TIRAP (MG659314.1)	TIRAP-F	GGCGCTGTGTCCACTGAGCT	119	60	2.02
	TIRAP-R	GACAGGACCTGGTGCATCTG			
IL-1 β (AB757758.1)	IL-1 β -F	TTTGTAAGATGCGCTGCTC	133	54	2.04
	IL-1 β -R	CCAATCTCGACCTTCTGGTG			
TNF- α 1 (KF500408.1)	TNF- α 1-F	CGTACTCTGATTCCTATGGC	199	54	2.05
	TNF- α 1-R	GCTTTCGCTGTTGCCTTTCT			

Note: F: Forward primer, R: Reverse primer.

Table 5
Growth performance and morphological parameters of gibel

	Experimental diets			
	D0	D20	D40	D60
Initial weight (g/fish)	28.73 $\pm$ 0.04	28.69 $\pm$ 0.03	28.67 $\pm$ 0.05	28.74 $\pm$ 0.02
Final weight (g/fish)	47.13 $\pm$ 0.88	46.06 $\pm$ 1.21	47.00 $\pm$ 0.44	46.65 $\pm$ 1.39
FR (%BW/d) ^a	3.11 $\pm$ 0.04	3.21 $\pm$ 0.05	3.07 $\pm$ 0.10	3.14 $\pm$ 0.10
SGR (%/d) ^b	1.15 $\pm$ 0.04	1.10 $\pm$ 0.07	1.15 $\pm$ 0.02	1.12 $\pm$ 0.07
CF (g/cm ³) ^c	3.19 $\pm$ 0.04	3.14 $\pm$ 0.02	3.25 $\pm$ 0.12	3.29 $\pm$ 0.07
HSI (%) ^d	7.55 $\pm$ 0.25	7.16 $\pm$ 0.18	6.75 $\pm$ 0.37	6.76 $\pm$ 0.60
VSI (%) ^e	16.01 $\pm$ 0.52	16.40 $\pm$ 0.20	14.77 $\pm$ 0.36	15.52 $\pm$ 1.16
SR (%) ^f	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00

Values in the same row with different letters indicate difference at $P < 0.05$ (means $\pm$ SEM $n = 4$). ^aFR: Feeding rate (% BW/d) = $100 \times (\text{feed intake in dry matter}) / (\text{days} \times (\text{initial body weight} + \text{fi}))$; ^bSGR: Specific growth rate (%/d) = $100 \times (\text{Ln}(\text{final body weight}) - \text{Ln}(\text{initial body weight})) / \text{days}$; ^cCF: Condition factor (g/cm³) = $100 \times (\text{body weight}) / (\text{body length})^3$; ^dHSI: Hepatosomatic index (%) = $100 \times (\text{liver weight} / \text{whole body weight})$; ^eVSI: Viscera somatic index (%) = $100 \times (\text{viscera weight} / \text{whole body weight})$; ^fSurvival rates (SR, %) = $(\text{final fish number} / \text{initial fish number}) \times 100$.

Table 6
The effect of replacement of fishmeal by yeast culture on blood physiology in gibel carp.

Plasma indices	Experimental diets			
	D0 (Control)	D20	D40	D60

Glucose (mmol/L)	3.72 ± 0.34	4.03 ± 0.62	4.66 ± 0.34
Urea nitrogen (mmol/L)	1.92 ± 0.10	1.47 ± 0.10	1.65 ± 0.10
Creatinine (μmol/L)	73.82 ± 13.65	77.03 ± 9.02	85.7 ± 13.65
Total protein (g/L)	31.21 ± 0.92	31.73 ± 1.32	32.9 ± 0.92
AST (IU/L)	15.01 ± 0.74 ^a	13.85 ± 0.86 ^a	18.4 ± 0.74 ^a
ALT (IU/L)	3.28 ± 0.51 ^a	2.81 ± 0.80 ^a	5.34 ± 0.51 ^a
Total cholesterol (mmol/L)	11.64 ± 1.68	11.72 ± 0.68	13.7 ± 1.68
Triglyceride (mmol/L)	4.82 ± 0.18	5.03 ± 0.19	5.16 ± 0.18

Values in the same row with different letters indicate difference at $P < 0.05$ (means ± SEM, $n = 8$).

reaction (PCR). The PCR primers were designed based on highly conserved regions of cDNA sequences of these genes in GenBank or transcriptome analysis (previous lab work), and were synthesized by Tsingke Company (Wuhan, Hubei, China). The PCR amplification conditions consisted of a denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s with different annealing temperatures and extension times corresponding to the primers and expected fragment sizes (Table 4). The PCR products were fractionated using 1% agarose gel electrophoresis, then the expected band was observed under the UV light.

Primers for quantitative real-time PCR (qPCR) were designed based on the obtained fragment cDNA sequences (Table 4). Before the quantitative test, a mixture of test templates was used to determine the amplification efficiency of the reference gene and the target genes by serial dilution. The qPCR

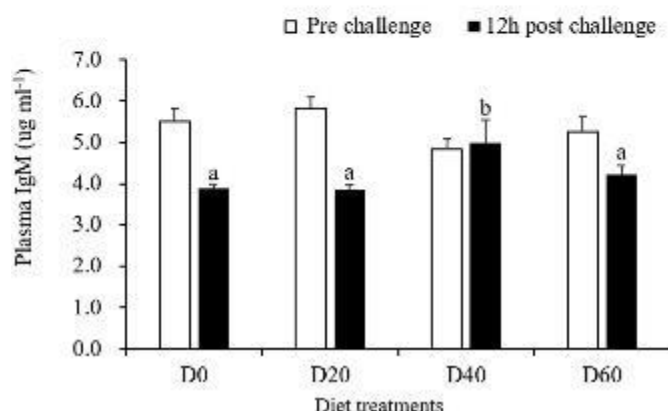
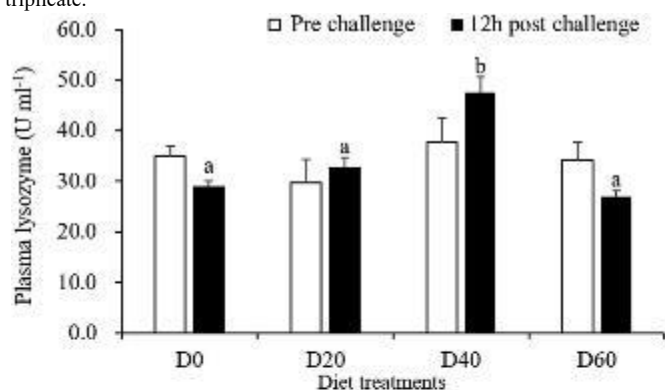
Fig. 1. Plasma lysozyme activity and IgM level in gibel carp fed with yeast culture administrated diets (2%, 4%, 6%) at the end of feeding trial (prechallenge) or 12 h post challenge with *Aeromonas hydrophila*. Data are indicated as mean ± SEM ($n = 8$). Columns with different lowercases mean significant variation among treatments post bacterial challenge ($P < 0.05$). Columns with * mean remarkable changes pre and 12 h post bacterial challenge at the same group ($P < 0.05$).

2.7. Statistical analysis

One-way ANOVA (SPSS 18.0, SPSS Inc., Chicago, IL, USA) was used to measure the effect of dietary yeast culture on growth performance, hematological parameters, immune response and resistance against *Aeromonas hydrophila* in gibel carp CAS III (*Carassius auratus gibelio*). When ANOVA

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amplifications were carried out in duplicate in a final volume of 20 μl containing 1 μl cDNA, 1000 nM each primer and 10 μl iQ™ SYBR® Green Supermix (Bio-Rad, USA). A negative control was done with the template replaced by sterile double distilled water. The qPCR was initiated at 95 °C for 2 min, followed by 35 cycles of a three-step amplification program (15 s at 95 °C, 18 s at the specific primer annealing temperature and 12 s at 72 °C). After amplification, a melt curve of 0.5 °C increments from 65 °C to 95 °C was performed to confirm the amplification of a single product. GAPDH gene was used as the internal reference. Transcriptional levels were calculated according to [33]. Eight samples were used for each treatment and each sample was measured in triplicate.



identified overall differences were significant ($P < 0.05$), Duncan's multiple range tests were used to test the difference between treatments. The results are presented as means ± standard error (S.E.).

3. Results

During the experimental period, no mortality of gibel cap was observed. There were no significant differences of feeding rate (FR) and specific growth rate (SGR) in gibel carp between YC groups and control group (Table 5). Similarly, no remarkable variations ($P > 0.05$) of condition factor (CF), hepatosomatic index (HSI) and viscera somatic index (VSI) of gibel carp were found in YC groups and the control (D0) group.

The effect of replacement of fishmeal by YC on blood physiology of gibel carp were presented in Table 6. The levels of plasma glucose were insignificantly increased with the increase of dietary YC. No significant differences ($P > 0.05$) were observed in urea nitrogen, creatinine, total protein, total cholesterol and triglyceride irrespective of dietary treatments. The highest value of plasma creatinine, total protein, total cholesterol and triglyceride were found in D40 group. The activities of

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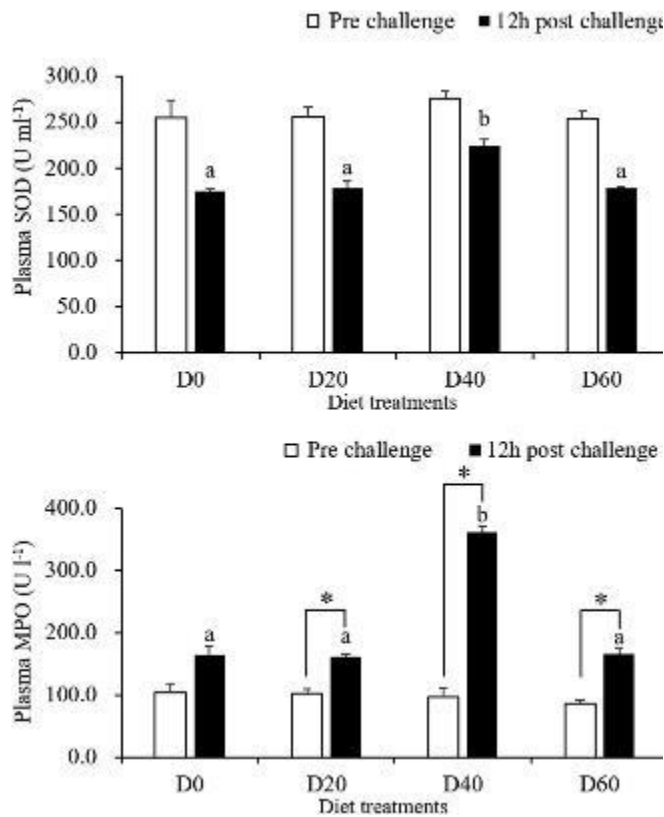


Fig. 2. Plasma SOD activity and MPO activity in gibel carp fed with yeast culture administrated diets (2%, 4%, 6%) at the end of feeding trial (prechallenge) or 12 h post challenge with *Aeromonas hydrophila*. Data are indicated as mean $\pm$ SEM (n = 8). Columns with different lowercases mean significant variation among treatments post bacterial challenge ($P < 0.05$). Columns with * mean remarkable changes pre and 12 h post bacterial challenge at the same group ($P < 0.05$).

plasma AST and ALT in fish fed D60 diet were obviously higher than that of control ($P < 0.05$).

Before challenge, plasma lysozyme, IgM, MPO and SOD of gibel carp were insignificantly changed among all groups. At 12 h following bacterial challenge, plasma lysozyme, IgM, MPO and SOD were significantly improved ($P < 0.05$) in gibel carp fed D40 diet than those of others (Fig. 1 and Fig. 2). Moreover, plasma MPO activities of gibel carp in YC groups were significantly increased ($P < 0.05$) after bacterial challenge than before challenge, but not in the control group (Fig. 2).

At 12 h post bacteria challenge test, gibel carp of D40 group exhibited significant upregulations ($P < 0.05$) of TLR2, MyD88, TRAP and IL-1 β genes, but a significant downregulation ($P < 0.05$) of TNF α 1 gene in spleen tissues compared with those of the fish from the control group (Fig. 3).

During the *A. hydrophila* challenge, no dead fish were found in the PBS-injection group. Fish in the experimental groups started to die after 16 h post bacteria challenge. At the end of the challenge trial, the cumulative survival rate of gibel carp in D40 and D60 groups were significantly higher ($P < 0.05$) than those of groups D0 and D20 (Fig. 4). The mean cumulative survival rate in D0, D20, D40 and D60 groups were 19.7%, 14.9%, 67.4% and 50.0%, respectively.

4. Discussion

In the current study, the results of immuno-hematological parameters (lysozyme activity, IgM level, MPO activity and SOD activity) as well as dramatically improved cumulative survival rate after bacterial challenge in 40% replacement group indicated that supplemented YC in 40%

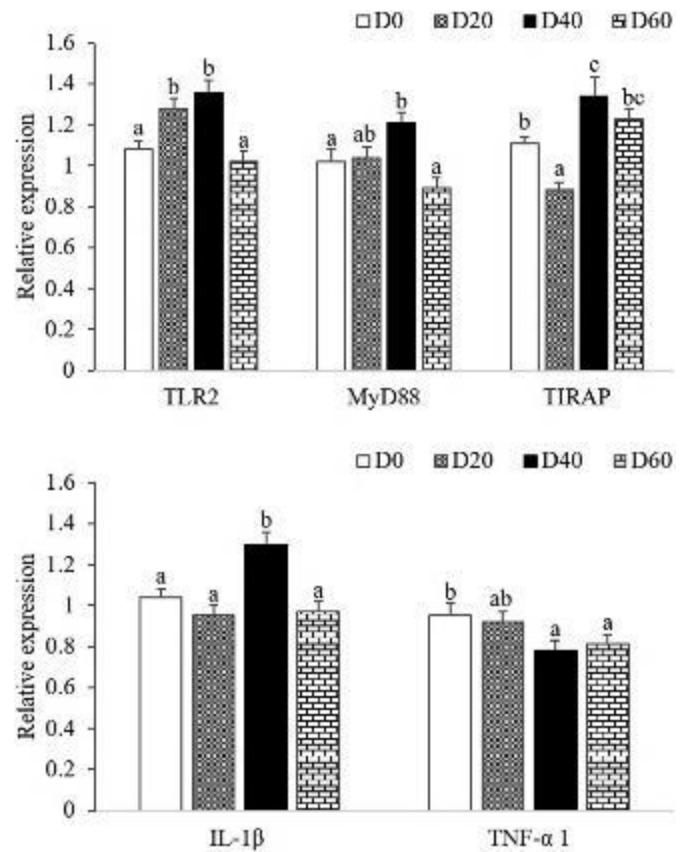


Fig. 3. The transcriptional level of immune-related genes of toll-like receptor 2 (TLR2), myeloid differentiation primary response gene 88 (MyD88), toll/interleukin-1 receptor domain-containing adapter protein (TRAP), interleukin1 β (IL-1 β) and tumor necrosis factor α 1 (TNF- α 1) in spleen of gibel carp fed with yeast culture administrated diets (2%, 4%, 6%) at 12 h post challenge with *Aeromonas hydrophila*. Relative expression levels were normalized to the housekeeping gene (GAPDH) and fold changes were calculated against the respective control group (control value = 1). Values represent the mean $\pm$ SEM. Significant differences between each yeast culture supplemented diet and control diet were determined by one-way ANOVA ($P < 0.05$) (n = 8).

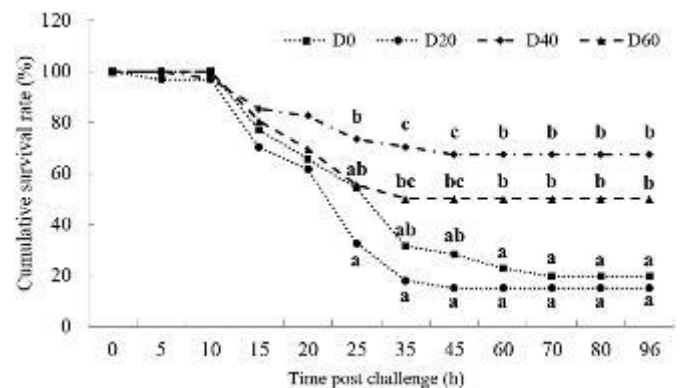


Fig. 4. Effects of dietary administration of yeast culture on post-challenge survival of gibel carp after infection with *A. hydrophila*. The cumulative survival rate of gibel carp were recorded and analyzed. The equation is expressed as survival rate (%) = (final number of fish survivor/initial number of inoculated fish) $\times$ 100. Values of four groups with different lowercase letters at a specific timepoint indicate difference at $P < 0.05$ (n = 4).

diet significantly enhanced immunity and disease resistance of gibel carp particularly after challenge. This might be due to the presence of immune-enhancing substances (yeast β -glucan, MOS and nucleotide) in

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yeast culture. Lysozyme is an important parameter of innate immunity of fish [34] and IgM plays a critical role in the responding to antigenic challenge in aquatic animal [35]. Abundant myeloperoxidase (MPO) was

existed in neutrophils which possessed bactericidal activity [36]. Antioxidant enzymes, such as superoxide dismutase (SOD) play a pivotal role in elimination of free radicals [37]. It was well established that rainbow trout fed the starter diet containing 0.25 g kg⁻¹ yeast culture had a significant lower mortality either during first 4 weeks or during 4–8 weeks post initial feeding [38]. Yeast β -glucan administration at 0.1% in Asian catfish (*Clarias batrachus*) feed, significantly raised the levels of serum MPO, lysozyme and protection against *Aeromonas hydrophila* challenge, regardless of feeding periods (1, 2 and 3 weeks) [39]. In vitro, activity of lysozyme produced by Atlantic salmon (*Salmo salar* L.) head kidney macrophages was markedly elevated in the presence of yeast β -glucan at day 6 in culture compared with that of nontreated controls [40]. Additionally, Guo et al. [41] have reported that activities of lysozyme and SOD were significantly improved by dietary nucleotides in shrimp *Litopenaeus vannamei* after 10-week feeding trial in comparison with nucleotide-free group. It has been reviewed that dietary mannan oligosaccharides supplementation stimulated humoral and cellular innate immune responses and improved disease resistance of fish due to its positive effects including activation and facilitation of antigen processing and regulation of intestinal microbiota [42].

In the present study, genes in TLR2 signaling pathway and some cytokines in spleen have been assessed. We found that transcriptional level of TLR2 in spleen of gibel carp after bacteria challenge was significantly up-regulated when 40% fishmeal was replaced by yeast culture. Douxfils [43] had proved that spleen was a higher responsive organ than gill and head kidney in the contribution of the immune reinforcement by dietary immunostimulants. Toll-like receptor are key components of the innate immune system, hence crucially participated in pathogen defense [44]. TLR2 had been demonstrated to be a key receptor for fungal glucan [45]. In the downstream of TLR2 signaling, TIRAP, MyD88 were also significantly up-regulated in this group. These were agreed with the other present results that IgM and lysozyme, MPO and SOD activities and survival rate post bacterial challenge were the highest in D40 group. IL-1 β , one of the pro-inflammatory cytokines, is a crucial part in host response to microbial invasion, tissue injury and immunological reactions [46]. In this study, the expression of IL-1 β in spleen was remarkably up-regulated in D40 group. Previous study also showed that supplementation of immunomodulator (β -glucan) at the level of 0.1% in gilthead seabream (*Sparus aurata*) significantly upregulated IL-1 β in head kidney after 4 weeks feeding [47]. TNF- α plays a vital role in proinflammatory aspects in fish and is mediated through the activation of endothelial cell [48]. Intraperitoneal injection of 5 mg/ml β -glucan in zebrafish for 6 days did not affect the expression of IL-1 β and TNF- α at 2 h, 4 h and 6 h post-challenge with *A. hydrophila* compared with the fish received PBS [49]. In the current study, however, the lowest gene expression of TNF- α 1 in spleen was found when 4% yeast culture was supplemented in gibel carp diet. In agreement with our findings, Sener et al. (2005) showed lessened TNF- α levels after supplementation of yeast β -glucan on animal model of sepsis [50]. Combined the transcriptional variations of two pro-inflammatory cytokines (TNF- α 1 and IL-1 β) in spleen, it was implied that the protective ability of yeast culture may be partly owing to modulation of the cytokine profile. The present results indicated that the immune response and disease resistance of gibel carp were associated with the regulation of TLR2 signaling pathway activated by dietary yeast culture.

The present study indicated that when inclusion level of dietary fishmeal was 10%, final body weight and SGR of fish fed YC replacement diets showed no significant difference compared to the control group. There was no significant difference in growth performance of shrimp (*Litopenaeus vannamei*) fed the basal diet (containing 25% fishmeal) and the experimental diets in which 15%, 30% and 45% fishmeal were replaced by yeast extract [10]. In sea bass (*Dicentrarchus labrax*), no negative impacts in growth rate was reported, when 50% of fishmeal 405

protein was replaced by brewers' yeast (*Saccharomyces cerevisiae*) [51]. It has been also reported that yeast extract replaced 15%, 30%, 45%, 60% and 100% of dietary fish meal for shrimp diet, the growth parameters among groups were highly correlated with the essential amino acids in diet [10]. In Table 3, the

amino acid composition of YC supplemented groups displayed relatively high similarity compared with those of control group, except the dietary methionine content. Several studies demonstrated that supplementation of methionine in diet could improve fish growth [52,53]. Some studies, however, have indicated that methionine supplementation in sea bass (*Dicentrarchus labrax*) and rainbow trout (*Salmo gairdnerii* Richardson) diets did not exert positive effects on growth performance [54,55]. Similarly, additional YC in diet had no difference in plasma glucose, urea nitrogen and creatinine, total cholesterol and triglyceride in gibel carp. The hematological parameters had been used as effective indicators to monitor health condition as well as physiological status in aquatic animals [56]. In the light of these observation, YC as a candidate ingredient replaced fishmeal in gibel carp exerted no adverse influence on physiological status in the current research. It was reported that aspartate aminotransferase (AST) and blood alanine aminotransferase (ALT) were applied as diagnostic marker for liver functions [57]. In our study, the activities of AST and ALT were significantly enhanced as 60% fishmeal was replaced by dietary YC. It seemed probably that more active metabolism in liver occurred in fish fed diets containing high level of YC. Growth and hematological data in our study suggested that dietary supplementation of yeast culture (2%–6%) in diet extended no detrimental effect on growth performance, health status and physiological condition in gibel carp.

In conclusion, when dietary fishmeal content was 10% on dry matter basis, up to 40% fishmeal could be replaced by yeast culture in gibel carp diet without adverse effects on growth performance. Based on evidently improving plasma IgM content, activities of lysozyme, SOD, MPO, relative expression of immune-related gene in the TLR2 pathway and survival rate post bacteria challenge, yeast culture is a promising substitute for fishmeal and 4 g YC per 100 g diet is recommended to be added in feed for gibel carp.

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