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Dietary yeast culture modulates immune response related to TLR2-MyD88-NF-kβ signaling pathway, antioxidant capability and disease resistance against *Aeromonas hydrophila* for Ussuri catfish (*Pseudobagrus ussuriensis*)

Xianyong Bu[a,1], Xuqiu Lian[a,1], Yi Wang[a], Chengzeng Luo[a], Shengqiang Tao[a], Yilu Liao[a], Jiaming Yang[b], Aijing Chen[a,*], Yuhong Yang[a,**]

[a] College of Animal Science and Technology, Northeast Agricultural University, Harbin, 150030, PR China
[b] Harbin Jiaming Fisheries Technology Co., Ltd., Harbin, 150030, PR China

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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-kβ 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-kβ p65, IL-1β 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-kβ 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

* Corresponding author.
** Corresponding author.
E-mail addresses: chenaijing168@163.com (A. Chen), yuhongyang68@163.com (Y. Yang).
[1] The first two authors contributed equally to this work.

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environment and human health. Bakers' yeast, *Saccharomyces cerevisae* 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-1β (IL-1β), interleukin-8 (IL-8), and the anti-inflammatory cytokine IL-10 [25,26], which were regulated by nuclear transcription factor kappa β (NF-κβ) 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-κβ 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-κβ signaling pathway for Ussuri catfish. Therefore, the purpose of this study was to evaluate the effects of YC on immune response related to hepatic TLR2-MyD88-NF-κβ 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 handing 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 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-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 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
$Ca(H_2PO_4)_2$	20	20	20	20
Yeast culture[d]	0	10	20	30
Proximate composition (g kg^{-1} dry matter)				
Dry matter	932.7	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; antiscorbic 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).

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 ± 0.32 g) were randomly allotted to 12 aquaria (1.0 × 0.5 × 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

8 mg L^{-1}, the temperature was 24 ± 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, LZM activities and C3, C4 and IgM levels measurement

The plasma AKP, ACP and serum LZM activities were determined by commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, China) as described by Yin et al. [31]. A LZM 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 μl 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-κβ p65, IL-1β, 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 LD_{50} was determined by intraperitoneal injection using graded doses of *A. hydrophila* (10^5, 10^6 and 10^7, 10^8 CFU per fish): results showed that the 96 h LD_{50} was 10^7 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-κβ p65-F	AAGAACCAGCCATACAAGCCACAC	83	60
NF-κβ p65-R	TCAGGCAGGTCCGCTTCGTAG		
IL-1β-F	CCTGAACACCTTCGAGTCGG	102	58
IL-1β-R	AGGTGGCTGGTTTGCTGATT		
IL-8-F	ATCGAAGGAAAAGCAGAGCG	111	57
IL-8-R	CTTTGCACAGGAGCCACTTG		
IL-10-F	TCATACGCCGTCATCCGAGA	161	58
IL-10-R	CTGACTGCACTGGGCAACAC		
β-actin-F	CCTCCGTCTGGATTTGGCTG	141	60
β-actin-R	TCAAGGGCGACGTAGCAGAG		

tanks and were challenged by intraperitoneal injection with 0.1 ml of a suspension of *A. hydrophila* (1×10^8 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-kβ 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-1β 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

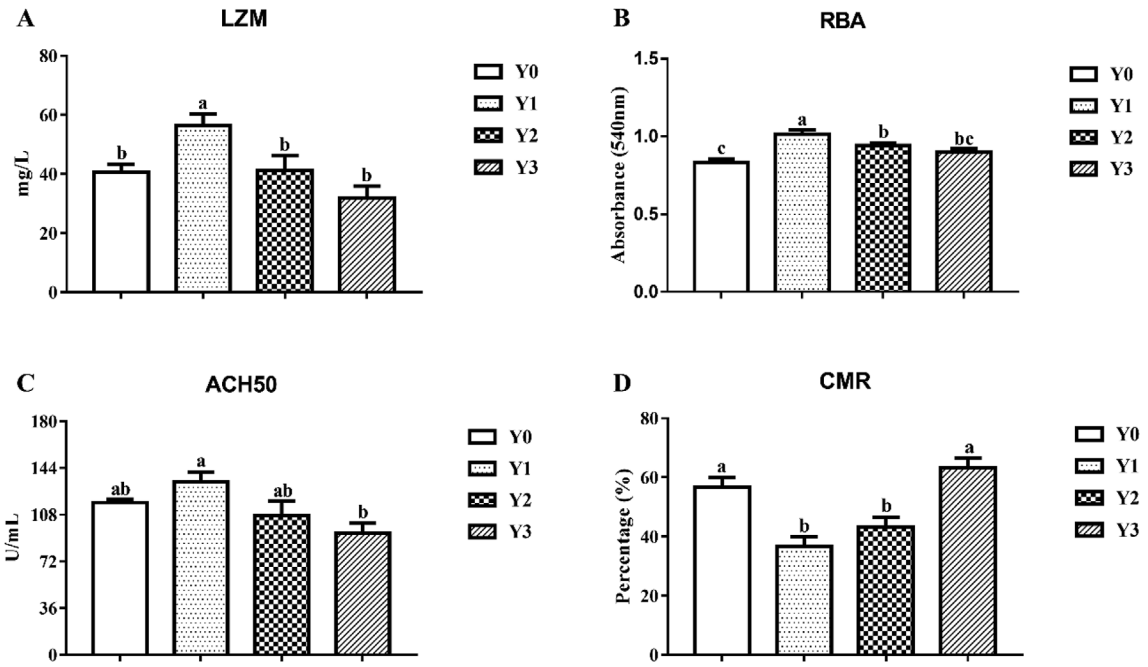

Fig. 1. The effects of dietary YC on the serum LZM activity, RBA of phagocytes, plasma alternative complement pathway activity and disease resistance for Ussuri catfish. Serum lysozyme (LZM) activity (Fig. 1A), Respiratory burst activity (RBA) of phagocytes (Fig. 1B), Alternative complement pathway, 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 ± 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.

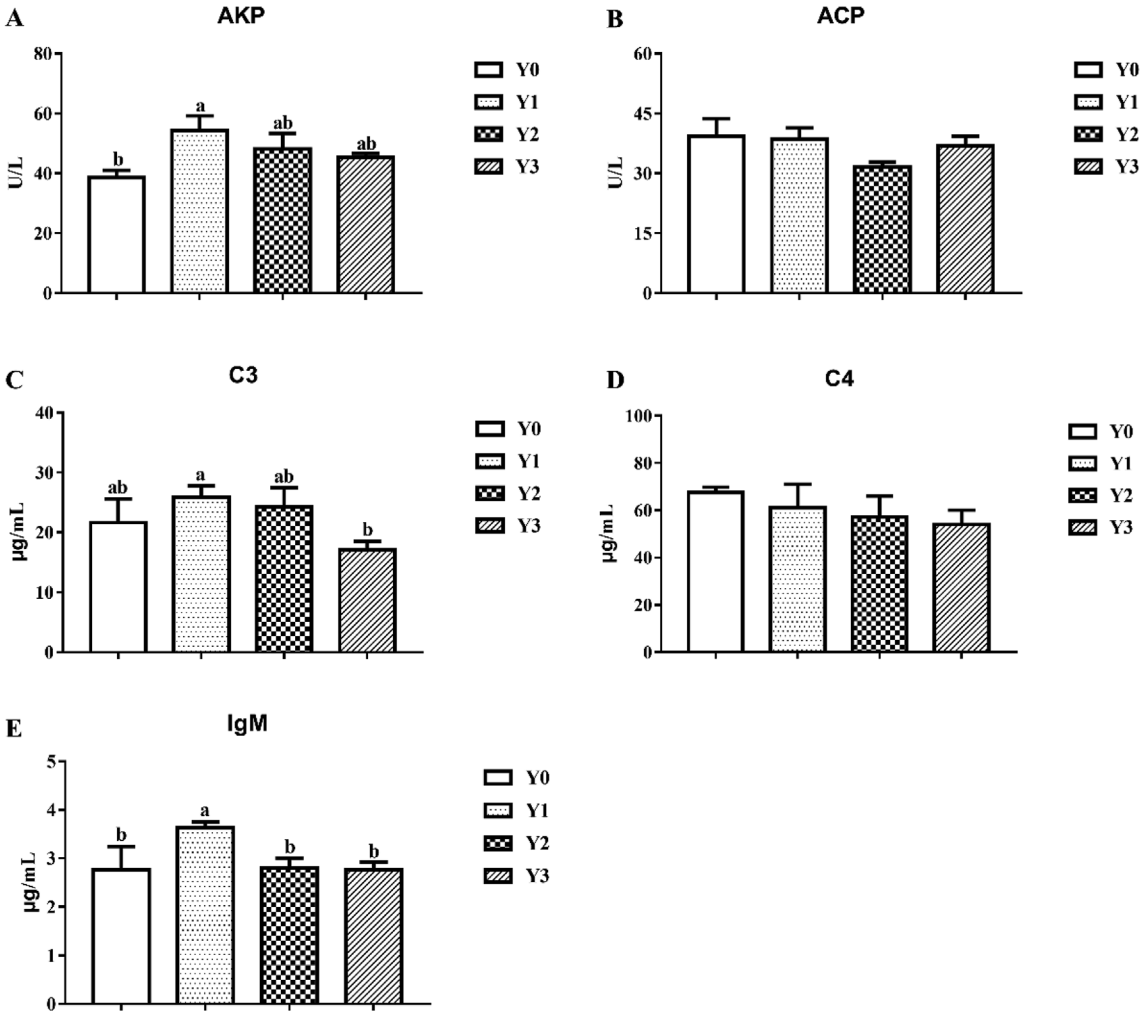

Fig. 2. The effects of dietary YC on the plasma AKP and ACP activities and C3, C4 and IgM contents of Ussuri catfish. Plasma alkaline phosphatase (AKP) activity (Fig. 2A), acid phosphatase (ACP) activity (Fig. 2B), complement 3 (C3) content (Fig. 2C), complement 4 (C4) content (Fig. 2D) and immunoglobulin M (IgM) content (Fig. 2E) are presented when fish was fed different diets. Values are the means ± 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 ± 0.45	5.24 ± 0.23	5.18 ± 0.70	4.70 ± 0.04
SOD (U mg^{-1})	91.65 ± 1.75^{b}	103.11 ± 2.58^{a}	95.06 ± 0.40^{ab}	91.54 ± 2.77^{b}
GPx (U mg^{-1})	99.02 ± 5.77^{ab}	120.98 ± 10.56^{a}	95.06 ± 15.34^{ab}	83.70 ± 9.42^{b}
TAC (U mg^{-1})	0.39 ± 0.07^{b}	0.59 ± 0.02^{a}	0.36 ± 0.07^{b}	0.33 ± 0.04^{b}
MDA (nmol mg^{-1})	4.01 ± 0.32^{a}	2.86 ± 0.18^{b}	3.01 ± 0.06^{b}	4.19 ± 0.33^{a}

Data represented as mean ± 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

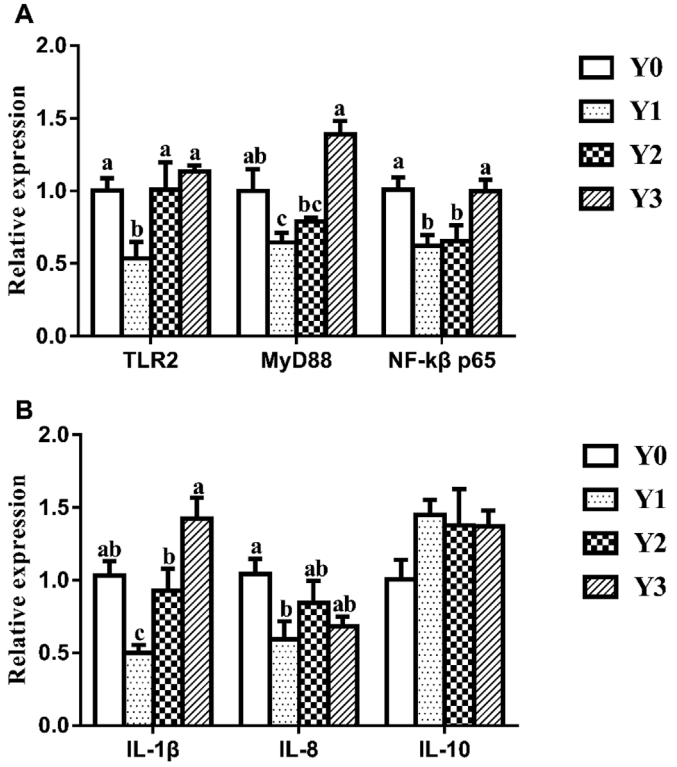

Fig. 3. The effects of dietary YC on the immune related genes expression in liver of Ussuri catfish. The immune related genes expression of TLR2, MyD88, NF-kβ p65, IL-1β, IL-8 and IL-10 are determined in the liver of Ussuri catfish fed different diets (Fig. 3A and B). Values are means ± S.E.M of three 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-kβ 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 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 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 (*Mycteropera 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 down-

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-κβ activation and the induction of several types of cytokines [27]. Interestingly, in the present study, the lowest hepatic MyD88 and NF-κβ p65 mRNA levels were found in Y1 group, indicating that dietary yeast culture supplementation may enhance the immunity of Ussuri catfish through TLR2-MyD88-NF-κβ 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-1β and IL-8. Similarly, the present results revealed that the lowest IL-1β and IL-8 mRNA levels were observed in fish fed Y1 diet. These results may be ascribed to the regulation of NF-κβ signaling pathway. Previous studies have demonstrated that the pro-inflammatory cytokines (e.g., IL-1β, IL-8) gene expression was positively related to NF-κβ mRNA levels [26,65]. Thus, the lower pro-inflammatory cytokines (IL-1β and IL-8) mRNA levels may be attributed to the reduced hepatic NF-κβ 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-κβ 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-kβ 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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