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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-1β (IL-1β) 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 g YC 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 μg yeast-based β-glucan

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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 exposured 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* × *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 immune-modulating effect of dietary yeast culture in fish.

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 κB (NF-κB), 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 Enhalor 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 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

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[a]	4.20
Mannose oligosaccharide[a]	4.70
Inosine monophosphate[b]	0.06
Uridine monophosphate[b]	0.02
Cytidine monophosphate[b]	0.04
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
$Ca(H_2PO_4)_2{\cdot}H_2O$	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

[a] 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}.
[b] Purchased from Enhalor Biotechnology Company, Beijing, China.
[c] Purchased from Coland Feed Co. Ltd., Wuhan, Hubei, China.
[d] Composed of 50% choline chloride and 50% silicon dioxide.

Table 3
Amino acid composition of experimental diets.

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 6.0–6.7 mg/L and PH was 7.1–7.4, daily. Ammonia-N was monitored weekly and was kept under 0.1 mg/L. Illumination intensity was 4.31–4.68 μmol/m^2/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-weighed. 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-anaesthetized fish per replicate. Sample were centrifuged (3000 g, 15min, 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 10min. The precipitate was washed three times with aseptic PBS (pH7.2–7.4) 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 × 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

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-1β (AB757758.1)	IL-1β-F	TTTGTGAAGATGCGCTGCTC	133	54	2.04
	IL-1β-R	CCAATCTCGACCTTCCTGGTG			
TNF-α1 (KF500408.1)	TNF-α 1-F	CGCTACTCTGATTCCTATGGC	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 carp fed experimental diets.

	Experimental diets			
	D0	D20	D40	D60
Initial weight (g/fish)	28.73 ± 0.04	28.69 ± 0.03	28.67 ± 0.05	28.74 ± 0.02
Final weight (g/fish)	47.13 ± 0.88	46.06 ± 1.21	47.00 ± 0.44	46.65 ± 1.39
FR (%BW/d)[a]	3.11 ± 0.04	3.21 ± 0.05	3.07 ± 0.10	3.14 ± 0.10
SGR (%/d)[b]	1.15 ± 0.04	1.10 ± 0.07	1.15 ± 0.02	1.12 ± 0.07
CF (g/cm^3)[c]	3.19 ± 0.04	3.14 ± 0.02	3.25 ± 0.12	3.29 ± 0.07
HSI (%)[d]	7.55 ± 0.25	7.16 ± 0.18	6.75 ± 0.37	6.76 ± 0.60
VSI (%)[e]	16.01 ± 0.52	16.40 ± 0.20	14.77 ± 0.36	15.52 ± 1.16
SR (%)[f]	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Values in the same row with different letters indicate difference at $P < 0.05$ (means ± SEM n = 4).
[a] FR: Feeding rate (% BW/d) = 100 × (feed intake in dry matter)/(days × (initial body weight + final body weight)/2).
[b] SGR: Specific growth rate (%/d) = 100 × (Ln (final body weight) – Ln (initial body weight))/days.
[c] CF: Condition factor (g/cm^3) = 100 × (body weight)/(body length)^3.
[d] HSI: Hepatosomatic index (%) = 100 × (liver weight/whole body weight).
[e] VSI: Viscera somatic index (%) = 100 × (viscera weight/whole body weight).
[f] Survival rates (SR, %) = (final fish number/initial fish number) × 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 ± 0.19	5.04 ± 0.77
Urea nitrogen (mmol/L)	1.92 ± 0.10	1.47 ± 0.10	1.65 ± 0.71	1.71 ± 0.26
Creatinine (μmol/L)	73.82 ± 13.65	77.03 ± 9.02	85.74 ± 9.66	74.28 ± 4.99
Total protein (g/L)	31.21 ± 0.92	31.73 ± 1.32	32.96 ± 0.49	30.70 ± 1.69
AST (IU/L)	15.01 ± 0.74[a]	13.85 ± 0.86[a]	18.48 ± 1.75[ab]	23.24 ± 3.34[b]
ALT (IU/L)	3.28 ± 0.51[a]	2.81 ± 0.80[a]	5.34 ± 0.72[a]	9.12 ± 1.06[b]
Total cholesterol (mmol/L)	11.64 ± 1.68	11.72 ± 0.68	13.74 ± 0.58	11.56 ± 1.03
Triglyceride (mmol/L)	4.82 ± 0.18	5.03 ± 0.19	5.16 ± 0.45	4.94 ± 0.20

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 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.

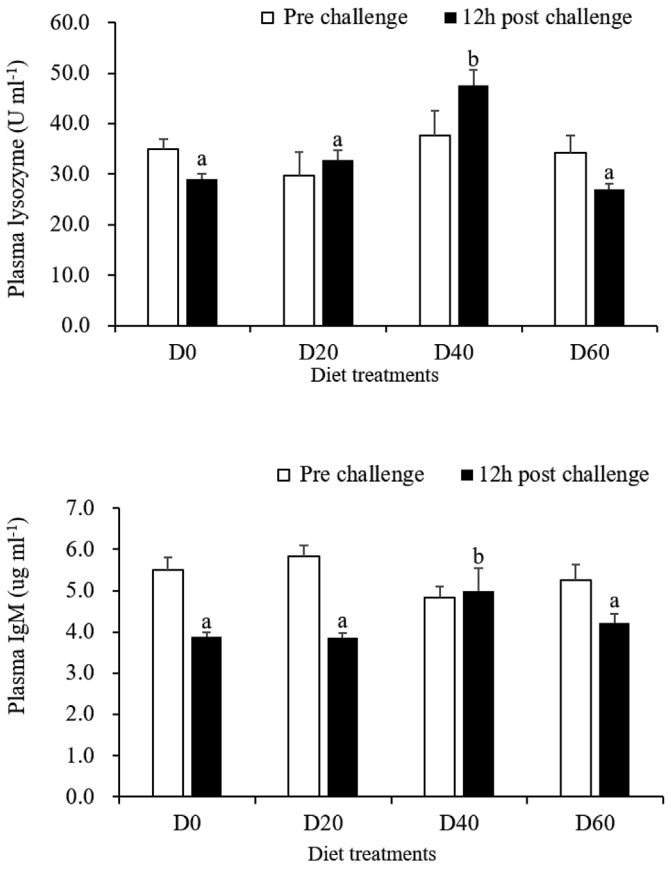

Fig. 1. Plasma lysozyme activity and IgM level in gibel carp fed with yeast culture administrated diets (2%, 4%, 6%) at the end of feeding trial (pre-challenge) or 12 h post challenge with *Aeromonas hydrophila*. Data are indicated as mean ± SEM (n = 8). Columns with different lowercases mean significant 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 ($P < 0.05$).

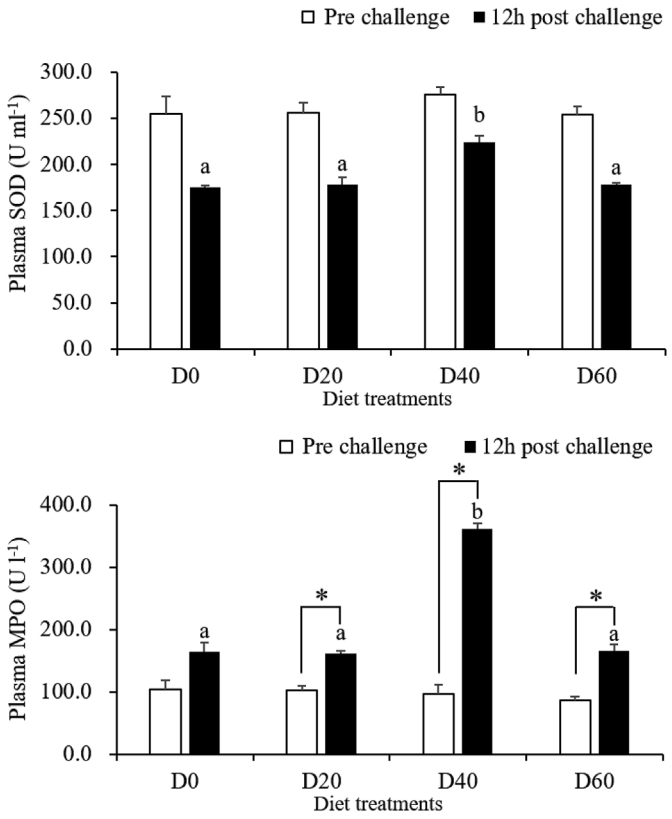

Fig. 2. Plasma SOD activity and MPO activity in gibel carp fed with yeast culture administrated diets (2%, 4%, 6%) at the end of feeding trial (pre-challenge) or 12 h post challenge with *Aeromonas hydrophila*. Data are indicated as mean ± SEM (n = 8). Columns with different lowercases mean significant 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 identified overall differences were significant ($P < 0.05$), Duncan's multiple rage 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 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, TRIAP and IL-1β genes, but a significant downregulation ($P < 0.05$) of TNF-α1 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

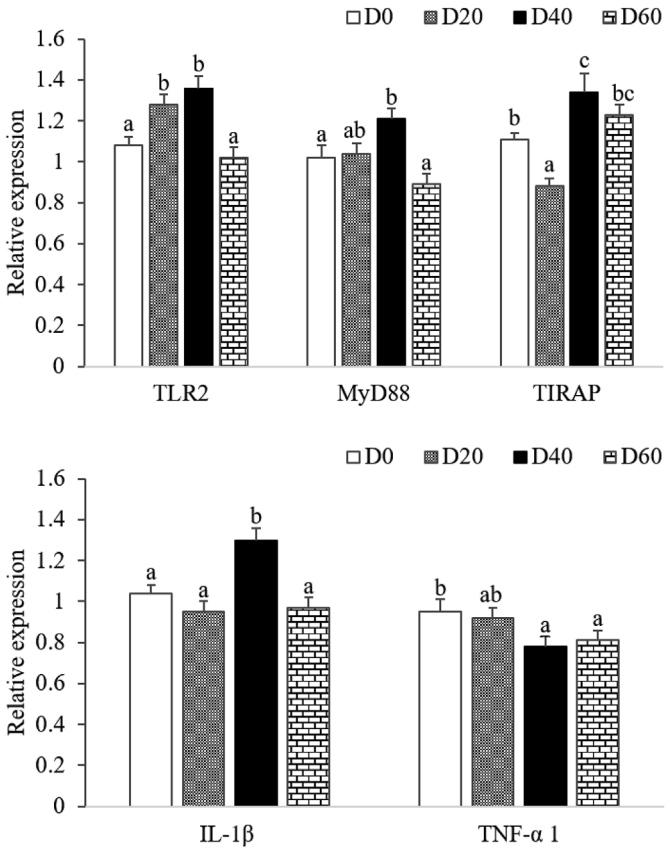

Fig. 3. The transcriptional level of immune-related genes of toll-like receptor 2 (TLR2), myeloid differentiation primary response gene 88 (MyD88), toll/interleukin-1 receptor domain-containing adapter protein (TIRAP), interleukin-1β (IL-1β) and tumor necrosis factor α1 (TNF-α1) in spleen of gibel carp 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 ± SEM. Significant differences between each yeast culture supplemented diet and control diet were determined by one-way ANOVA ($P < 0.05$) (n = 8).

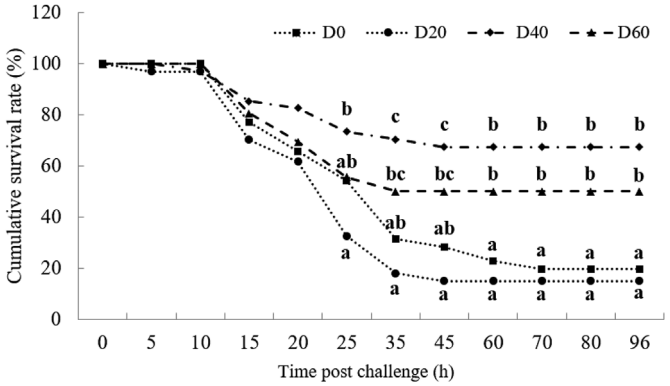

Fig. 4. Effects of dietary administration of yeast culture on post-challenge survival of gibel carp after infection with *A. hydrophila*. The cumulative survival rate of gibel carp were recorded and analyzed. The equation is expressed as survival rate (%) = (final number of fish survivor/initial number of inoculated fish) × 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 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 non-treated 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-1β, 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-1β 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 up-regulated IL-1β 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-1β 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-α1 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-α1 and IL-1β) 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

protein was replaced by brewers' yeast (*Saccharomyces cerevisae*) [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 significant 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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