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Effects of single or conjoint administration of lactic acid bacteria as potential probiotics on the growth, immune responses, and disease resistance of *Carassius auratus*

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Abstract
Probiotics have been documented to promote growth, immunity, and disease resistance in farmed fish. In the present study, we aimed to evaluate single or conjoint effects of some lactic acid bacteria (LAB), namely, *Lactococcus lactis*, *Lactobacillus rhamnosus*, and *Enterococcus faecalis* on the growth performance, immune response, and disease resistance of *Carassius auratus*. Fish were fed a basal diet supplemented with *L. lactis* (group B), *L. rhamnosus* (group C), *E. faecalis* (group D), *L. lactis*+*L. rhamnosus* (group E), *L. rhamnosus*+*E. faecalis* (group F), *L. lactis*+*E. faecalis* (group G), and *L. lactis*+*L. rhamnosus*+*E. faecalis* (group H) at 5.0×10^8 CFU/g diet for 34 days. After feeding, the final body weight (FBW), weight gain (WG), specific growth rate (SGR), and survival had significantly increased ($p<0.05$) for group E. The results indicated that single or conjoint administration of LAB induced high levels of IgM, LZM, AKP, and SOD activity in serum, which may effectively induce humoral immunity, and group E induced even higher levels. At the same time, when compared to the basal diet (group A), the results of qPCR showed that probiotic administration significantly upregulated ($p<0.05$) the expression of IL-10, IL-1β, TNF-α, and IFN-γ in the spleen, kidney, liver, and intestine of *C. auratus*. After challenge with the *Aeromonas hydrophila*, the survival rates in all probiotic-fed groups were significantly higher ($p<0.05$) than those of group A, and the relative protection rates of groups B, C, D, E, F, G, and H were 27%, 30.7%, 40.94%, 42.32%, 39.38%, 33.61%, and 37.5%, respectively. Our data indicated that probiotics could promote growth, enhance immune indicators, and enhance immune capacity. In summary, combined administration of probiotics had a better effect on *C. auratus*. This experiment could provide a reference for the optimal combination of probiotic additives for aquatic animals.

Keywords Compound probiotics · Different combinations · *C. auratus* · Growth · Immunity

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Introduction

In recent years, an increasing number of fishery disease outbreaks have caused major economic losses in the aquaculture industry (Mahmoud et al. 2016; Anjan et al. 2019; Camilo et al. ; Zhang et al. 2017). The improper use of antibiotics is one of the important factors that cause diseases (Kong et al. 2019). Not only can improper use of antibiotics or abuse of antibiotics increase drug residues in water and animal bodies, decrease animal immunity, and increase resistance to pathogenic bacteria, but it can also increase the cost of aquaculture and cause serious environmental problems, which ultimately affects human health (Giri et al. 2014). There is an urgent need to explore safe and high-quality feed additives and potential alternatives to antibiotics. At present, lactic acid bacteria (LAB) have been increasingly widely used as a substitute for veterinary antibiotics (Wang et al. 2019; Zuo et al. 2018; Bo et al. 2015; Marjan et al. 2016). As a feed additive, LAB can colonize in the intestine or reproductive system of animals, produce excellent effects on the animal's body and improve the animal's microecological balance (Wang et al. 2019; Mahmoud et al. 2016). As a probiotic that can replace antibiotics in fish farming, it has the characteristics of no pollution, residue, toxic side effects, or drug resistance (Gao et al. 2018; Batista et al. 2016; Yang et al. 2019). The addition of LAB in the feed not only produces excellent effects on the body of fish but also improves the water environment, prevents the growth of pathogenic bacteria, and improves the growth and immune function of the fish (Gregor et al. 2006; Qi et al. 2009). Therefore, LAB is used more and more widely. In our previous study, strains of three probiotics originally isolated from the intestine of *C. auratus* were noticed to be antagonistic against aquatic pathogens, which might have the better potential to inhibit pathogens and enhance immunity systems.

In recent years, many studies have shown that a certain amount of LAB intake may be beneficial to the health of aquatic animals, such as *Scylla serrate* (Talib et al. 2017), *Cromileptes altivelis* (Sun et al. 2018), *Oreochromisspp* (Yu et al. 2017), *Channa argus* (Kong et al. 2020), etc. Probiotics can be added to feed as a single strain or a mixture of two or more strains. Some previous studies demonstrated that a combined use of probiotics was better than single use. The combined application of *L. lactis* and *L. plantarum* strains can improve the growth, immune response, and disease resistance of *Paralichthys olivaceus* compared to a single probiotic preparation (Bo et al. 2015, 2016). In addition, dietary supplementation with the mixture of *L. rhamnosus* and *L. lactis* could improve growth, immunity, antioxidant, and lipid-lowering effects for *Pagrus major* compared to supplementation with a single strain (Xia et al. 2018). However, some scholars have shown that a single probiotic is more advantageous in some aspects. A previous study demonstrated that feeding *L. lactis* and/or *E. faecalis* improved the growth rate, immune status, and disease resistance of *A. veronii* infected with *Channa argus* (Kong et al. 2020). Single *L. lactis* showed higher survival than did a combination. Different types of LAB and their matching ratios with different types of fish will have different effects. Therefore, the purpose of our research is to explore reasonable ways of adding probiotics. Consequently, the present investigation made an attempt to evaluate the effects of a single or conjoint application of LAB as potential probiotics on growth, blood-immune parameters, immune response, and disease resistance of *C. auratus.*

Materials and methods

Probiotic bacteria and diets preparation

The three probiotics, including *L. lactis*, *L. rhamnosus*, *E. faecalis*, and the pathogen *Aeromonas hydrophila* TPS strain ($LD_{50} = 1 \times 10^6$ CFU/mL), were all isolated from

healthy *Carassius auratus* intestines and provided by our lab. The three probiotics were cultured in Man-Rogosa-Sharpe (MRS) medium (Solarbio, China) for 48 h at 37 °C in an anaerobic environment. The cultures were centrifuged (Beckman Coulter, AK, USA) at 5000 g for 10 min. The pellets were washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 10.1 mM NaH_2P_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, and pH 7.4) and suspended in PBS. The number of bacterial cells in the suspensions was determined by turbidimetry and administered to *C. auratus* in the experimental diets at a concentration of 5.0×10^8 CFU/g with slight modifications. Every 50 mL of LAB is coated with 20 g of feed, mixed with 1.5% sodium alginate solution, and dried in a drying oven at 37 °C for later use. The formulation and chemical composition of the basal diet are indicated in Table 1. The basal diet was used as a control. The addition ratio of LAB and the content of live bacteria in each group of fish food are shown in Table 2, which makes the addition ratio of different lactic acid bacteria in the same group the same, and the number of viable lactic acid bacteria should be the same between different groups.

Experimental design and rearing trial

Healthy *C. auratus* weighing 25.28 ± 0.5 g were obtained from a commercial fish farm (Changchun, China), acclimatized in 300-L tanks with aeration filtered dechlorinated water at 25 ± 1 °C for 2 weeks and fed the basal diet three times daily. After acclimation, *C. auratus* were randomly divided into four treatment groups. The glass tanks were kept under experimental conditions (temperature: 25 ± 1 °C; pH: 7.2 ± 0.1; ammonia nitrogen: < 0.5 mg/L; nitrites: < 0.05 mg/L; dissolved oxygen: 7.0 mg/L) and a specific cycle (12 h light/12 h dark photoperiod). Water pH, nitrite, ammonia, temperature, and dissolved oxygen were monitored weekly and maintained at optimal levels. The fish were fed two times (08:00 and 16:00) a day for 34 days at a rate of 3% of their body weight. Approximately 1/3 to 1/2 of the water in each plastic tank was changed every 2 days.

Table 1 Formula of feed ingredients and proximate composition of basal diet

Feed ingredients	Percentage (%)
Fish meal	10
Soybean meal	40
Cotton rapeseed meal	5
Spirulina meal	15
Rapeseed meal	10
Yeast meal	5
Wheat germ meal	6
Ca $(H_2PO_4)_2$	2
NaCl	0.5
$ZnSO_4$	0.5
Multivitamin	2
Amino acids	2
Multiple oils	2

Table 2 The treatments of the experiment for comparison of LAB as dietary supplements in *C. auratus*

Group	LAB addition ratio			Viable count
	L. rhamnosus	*E. faecalis*	*L. lactis*	
A	0	0	0	0
B	0	0	1	5×10^8 CFU/g
C	1	0	0	5×10^8 CFU/g
D	0	1	0	5×10^8 CFU/g
E	1	0	1	5×10^8 CFU/g
F	1	1	0	5×10^8 CFU/g
G	0	1	1	5×10^8 CFU/g
H	1	1	1	5×10^8 CFU/g

Growth performance and sampling

At 0, 7, 16, 25, and 34 days, fish were fasted for 24 h, anesthetized using 300 mg/L methane-sulfonate-222 (MS-222), counted, and weighed. Three fish per tank were randomly collected. The blood was sampled by caudal venipuncture, centrifuged at 4000 rpm for 15 min to acquire serum, and stored at − 20 °C until analysis. In addition, the spleen, kidney, gill, liver, and intestine were immediately sampled. All tissues were flash-frozen in liquid nitrogen and stored at − 80 °C until analysis. The final body weight (FBW), weight gain (WG), feed efficiency ratio (FER), specific growth rate (SGR), and protein efficiency ratio (PER) were calculated as described previously, using the following formulas:

$$Survival\ rate\ (\%) = (final\ number\ of\ fish/initial\ number\ of\ fish) \times 100.$$

$$Specific\ growth\ rate\ (\%/day) = [(Ln\ final\ body\ weight - Ln\ initial\ body\ weight)/days\ of\ rearing] \times 100.$$

$$Weight\ gain\ (\%) = [(mean\ final\ body\ weight - mean\ initial\ body\ weight)/mean\ initial\ body\ weight] \times 100.$$

$$CF = (Ln\ final\ body\ weight/fish\ length\ composition^3) \times 100.$$

$$VSI = (visceral\ weight/\ln\ initial\ body\ weight) \times 100.$$

Moisture was determined by the constant-temperature drying weight loss method at 105 °C (GB 5009.3—2010). Crude protein was determined by Kjeldahl's method (GB 5009.5—2010). Crude lipids were determined by the Soxhlet ether extraction method (GB 5009.6—2010). Ash was determined by the muffle furnace burning method (GB 5009.4—2010).

Humoral immunity parameters

Immunoglobulin M (IgM), lysozyme (LZM) activity, alkaline phosphatase (AKP), and superoxide dismutase (SOD) content in the serum were measured with commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), as described by Liu et al. (2019) and Gou et al. (2018).

RNA extraction and cDNA synthesis

Total RNA from the spleen, kidney, gill, liver, and intestine was extracted using a High Pure RNA Tissue Kit (Takara, Japan). RNA quality and quantity were analyzed by 1.0% agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific USA). Subsequently, cDNA was synthesized using a PrimeScriptTM RT reagent kit (Perfect Real Time, Takara, Japan) according to the manufacturer's instructions.

Immune-related gene expression

The selected immune-related genes were IL-10, IL-1β, IFN-γ, and TNF-α. qPCR was performed to determine gene expression levels with an SYBR Premix ExTaq™ Π kit (Takara, Japan) and subjected to the Stratagene MxPro system (Stratagene Mx3005 P, USA) in 96-well reaction plates. The GenBank accession numbers for the selected genes and primer sequences are indicated in Table 3. The fluorescent quantitative PCR solution consisted of 12.5 µL SYBR® Premix Ex Taq™ (2×), 1.0 µL forward primer, 1.0 µL reverse primer (10 mM), 1.0 µL cDNA, and 9.5 µL ddH_2O. The reaction conditions were as follows 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, annealing for 30 s, and 60 °C for 30 s. Data were quantified by the $2^{-\Delta\Delta CT}$ method.

Challenge test

After 34 days, 20 fish from each tank were injected intraperitoneally with 100 µL of *A. hydrophila* TPS strain ($LD_{50} = 1 \times 10^6$ CFU/g), which was provided by our lab. Injected fish were monitored for clinical signs, postmortem lesions, and daily mortalities for 14 days to calculate the relative protection rates (RPS).

Statistical analysis

Statistical analysis was performed using SPSS 20.0 for Windows (SPSS Inc.). All results data are presented as the mean ± SD (standard deviation), using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. $p < 0.05$ was considered to be significant.

Table 3 List of immune-related gene primers used for qPCR

Primer name	Sequence (5′–3′) forward	Sequence (3′–5′) reverse	Accession no
IL-10	AACTGATGACCCGAATGGAAAC	CACCTTCTCCCAGTCGTCAAA	HQ259106.1
IL-1β	AACTGATGACCCGAATGGAAAC	CACCTTCTCCCAGTCGTCAAA	AJ249137.1
IFN-γ	AACAGTCGGGTGTCGCAAG	TCAGCAAACATACTCCCCAG	EU909368.1
TNF-α	TTATGTCGGTGCGGCCTTC	AGGTCTTTCCGTTGTCGCTTT	EU069818.1
β-actin	CAAGATGATGGTGTGCCAAGTG	TCTGCTCCGGCACGAAGTA	AB039726.2

Results

Growth performance

The growth efficiency results of *C. auratus* fed the LAB are depicted in Table 4. The results indicated that FBW, BWG, SGR, and other indicators of the probiotics group were higher than those of the control group. Compared with the control group, each index of the compound probiotics groups was significantly improved ($p < 0.05$). The indicators of Group E were the highest and were significantly higher than those of the other probiotic groups. There was no significant difference among the other probiotic groups. The probiotic groups had no significant difference in *C. auratus* WG, BWG, or survival rate ($p > 0.05$).

As shown in Table 5, the content of crude protein in the muscle of the probiotic groups was higher than that of the control group. In addition, group E was significantly higher than the control group ($p < 0.05$). However, there was no significant difference between group E and the other probiotic groups ($p > 0.05$) and the rest of the probiotic groups were not significantly different than the control group ($p > 0.05$). Additionally, no significant difference in crude fat, crude ash, or moisture was observed between the control and treatment groups ($p > 0.05$).

Humoral immunity analysis

Serum AKP, SOD, and LZM activities and IgM concentrations of *Carassius auratus* fed LAB are presented in Fig. 1. As shown in Fig. 1, there was no significant change in IgM levels in group A during the trial period. However, in the treatment groups, serum IgM antibody levels were all increased. In addition, the IgM levels of groups C and G started to rise from day 0, reached a maximum at day 16, and subsequently began to fall, which were significantly different from groups A, B, D, F, and H ($p < 0.05$). The IgM level in group E showed an upward trend from 0 to 34 days and reached a maximum at 34 days, which was significantly higher than that of groups A, B, C, D, F, G, and H ($p < 0.05$).

As shown in Fig. 2, there was no significant change in the LZM level in group A during the trial period. However, the level of serum LZM in the treatment groups increased. The LZM level of group G increased from day 0 and reached a maximum at day 16, which was significantly higher than that of group A ($p < 0.05$) and then decreased slightly. The levels of LZM in groups B, C, D, E, F, and H reached a maximum at 34 days, which was significantly higher than that of group A ($p < 0.05$). Group E reached the highest level at 34 days compared with the other groups. There was no significant difference between group E and groups D and H ($p > 0.05$). However, there was a significant difference from group G ($p < 0.05$).

In Fig. 3, the AKP level in group A did not change significantly during the experiment. However, the serum AKP level of *C. auratus* in the test groups was increased. The serum AKP level in groups B, C, D, E, F, G, and H reached its maximum value at 34 days, which was significantly higher than that in group A ($p < 0.05$). Group E reached the highest value among the groups at 34 days, but there was no significant difference from groups B, C, D, F, G, and H ($p > 0.05$).

The SOD level is shown in Fig. 4. In group A, there was no significant change in SOD level, but the SOD level of *C. auratus* in the test group first increased and then decreased. Groups B, C, and D increased from 0 to 16 days, reached the maximum value, which was

Table 4 Effects of LAB supplementation on the survival and growth of *C. auratus* after 34 days

Group	A	B	C	D	E	F	G	H
IBW/g	24.95 ± 0.15	24.99 ± 0.2	24.72 ± 0.7	25.00 ± 0.28	25.03 ± 0.34	24.84 ± 0.23	24.97 ± 0.15	24.73 ± 0.71
FBW/g	29.56 ± 1.15^{a}	30.65 ± 0.49^{ab}	30.58 ± 0.33^{ab}	31.33 ± 0.35^{ab}	34.75 ± 0.9^{c}	31.82 ± 1.98^{b}	31.23 ± 0.76^{ab}	31.76 ± 0.7^{b}
WG/%	18.46 ± 3.74^{a}	22.71 ± 4.21^{ab}	23.72 ± 0.56^{ab}	25.37 ± 3.34^{ab}	38.79 ± 1.95^{c}	29.44 ± 6.98^{b}	25.05 ± 2.94^{ab}	31.12 ± 1.99^{b}
SGR/% d^{-1}	0.5 ± 0.09^{a}	0.6 ± 0.1^{ab}	0.63 ± 0.01^{ab}	0.66 ± 0.08^{ab}	0.96 ± 0.04^{c}	0.76 ± 0.16^{b}	0.66 ± 0.07^{ab}	0.8 ± 0.04^{b}
CF	2.02 ± 0.73	1.68 ± 0.41	1.99 ± 0.56	2.13 ± 0.46	1.55 ± 0.34	1.52 ± 0.3	1.68 ± 0.37	1.84 ± 0.57
VSI/%	16.45 ± 2.11	16.67 ± 1.78	16.56 ± 4.68	17.73 ± 1.38	16.67 ± 2.35	16.65 ± 2.6	17.33 ± 1.32	15.65 ± 2.31
Survival/%	92.5	95	92.5	92.5	97.5	97.5	92.5	100

Mean values in the same row with different superscript letters are significantly different ($p < 0.05$)

Table 5 Effects of LAB on muscle nutrition of *C. auratus*

Ingredient	A	B	C	D	E	F	G	H
Crude protein/%	17.41 ± 0.16^{a}	17.54 ± 0.1^{ab}	17.64 ± 0.08^{ab}	17.6 ± 0.08^{ab}	17.74 ± 0.12^{b}	17.54 ± 0.06^{ab}	17.59 ± 0.06^{ab}	17.67 ± 0.11^{ab}
Crude fat/%	2.64 ± 0.04	2.46 ± 0.19	2.45 ± 0.13	2.65 ± 0.14	2.56 ± 0.17	2.52 ± 0.14	2.47 ± 0.23	2.54 ± 0.16
Coarse ash/%	1.23 ± 0.02	1.21 ± 0.04	1.24 ± 0.06	1.27 ± 0.04	1.25 ± 0.02	1.21 ± 0.06	1.24 ± 0.06	1.24 ± 0.03
Moisture/%	77.83 ± 0.78	77.42 ± 0.21	77.50 ± 0.16	77.54 ± 0.11	77.47 ± 0.22	77.43 ± 0.06	77.40 ± 0.12	77.42 ± 0.12

Mean values in the same row with different superscript letters are significantly different ($p < 0.05$)

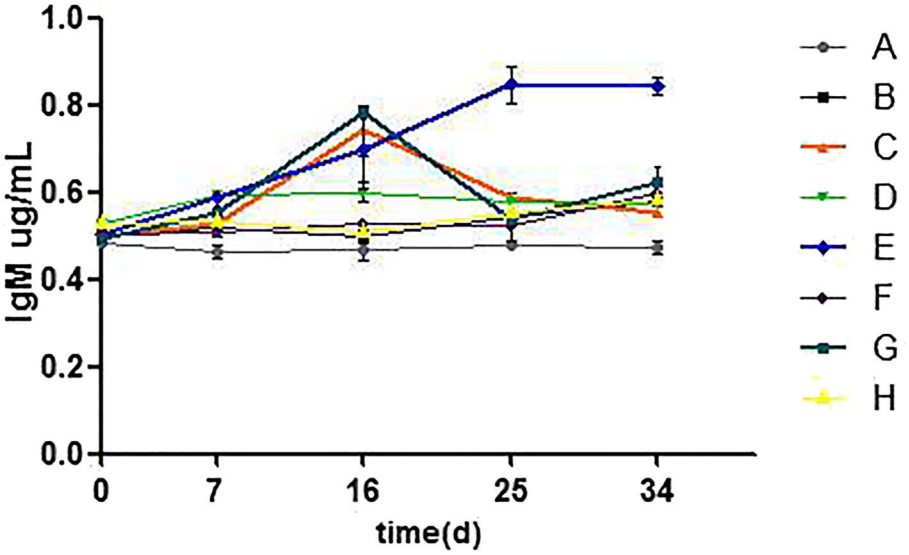

Fig. 1 Antibody level of IgM in serum of *C. auratus*

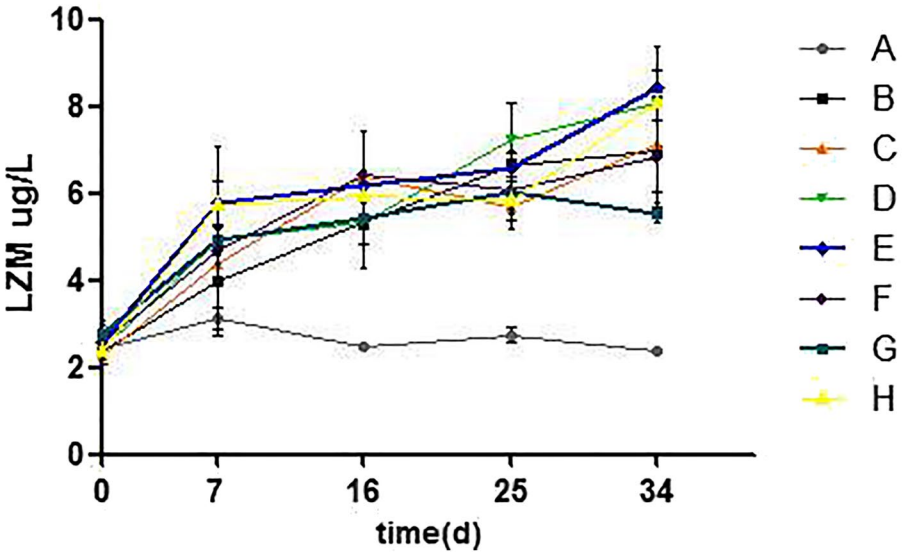

Fig. 2 Antibody level of LZM in serum of *C. auratus*

significantly higher than that in group A ($p < 0.05$), and then decreased. In addition, SOD levels in groups E, G, and H reached their maximum value at 25 days, which was significantly higher than those in group A ($p < 0.05$). There was no significant difference in SOD content among the test groups at 34 days ($p > 0.05$).

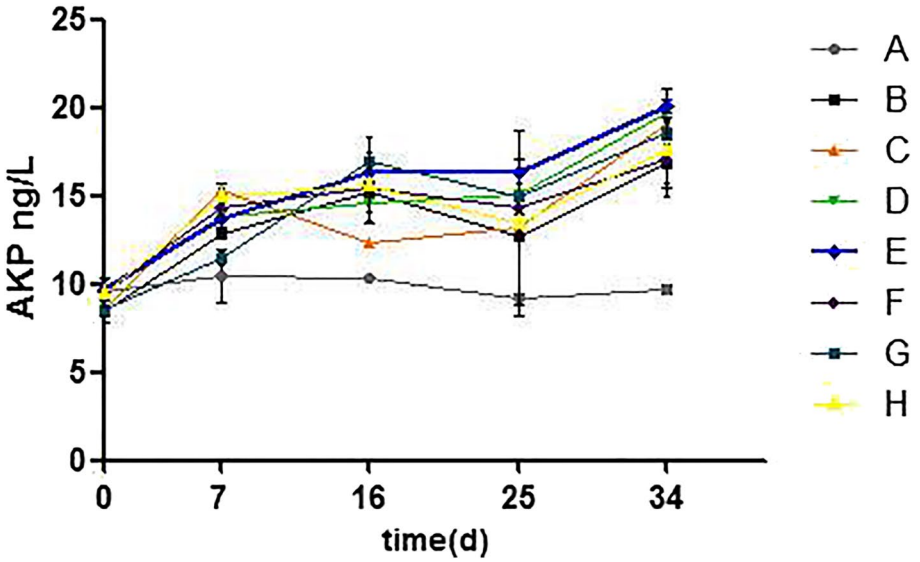

Fig. 3 Antibody level of AKP in serum of *C. auratus*

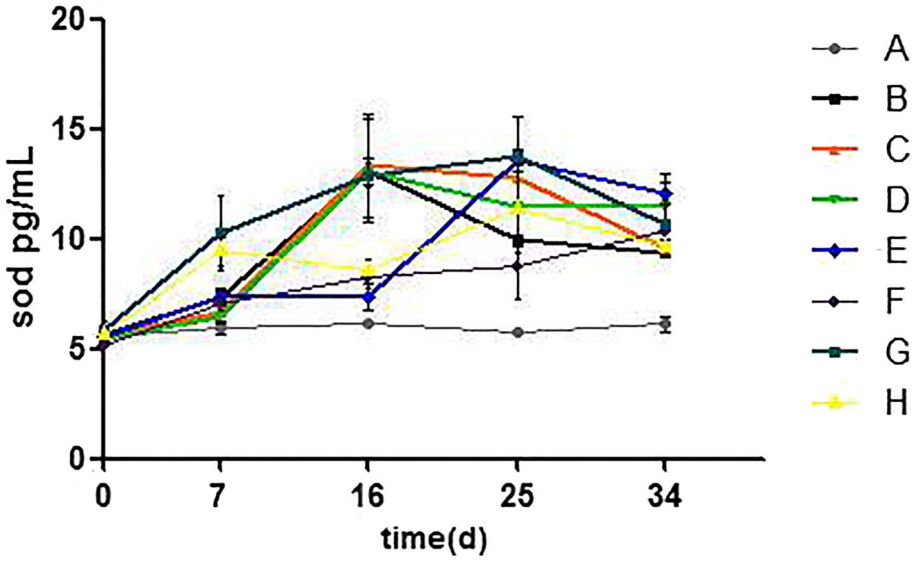

Fig. 4 Antibody level of SOD in serum of *C. auratus*

Cytokine expression analysis

The cytokine expression levels of immune-related genes (IFN-γ, TNF-α, IL-1β, and IL-10) in the spleen, kidney, liver, and intestine are shown in Fig. 5.

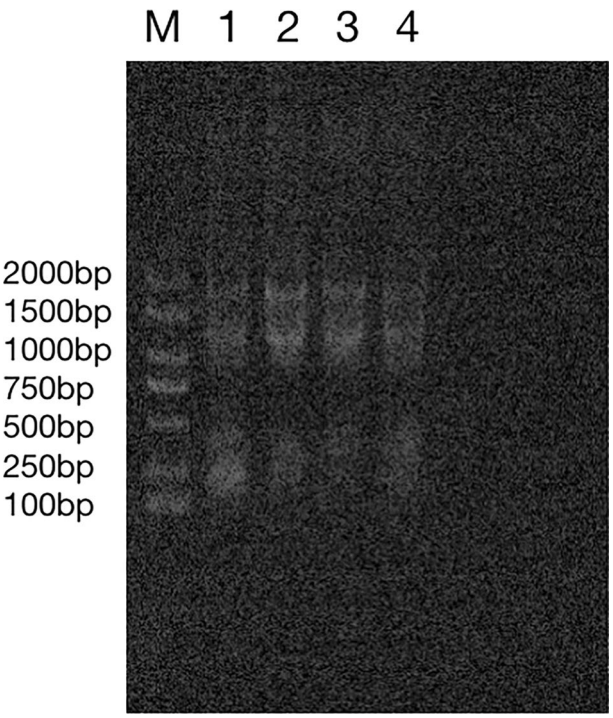

Fig. 5 Total RNA detection in different tissues. M, DL2000 DNA molecular marker; 1, total intestinal RNA; 2, total liver RNA; 3, total spleen RNA; 4, total kidney RNA

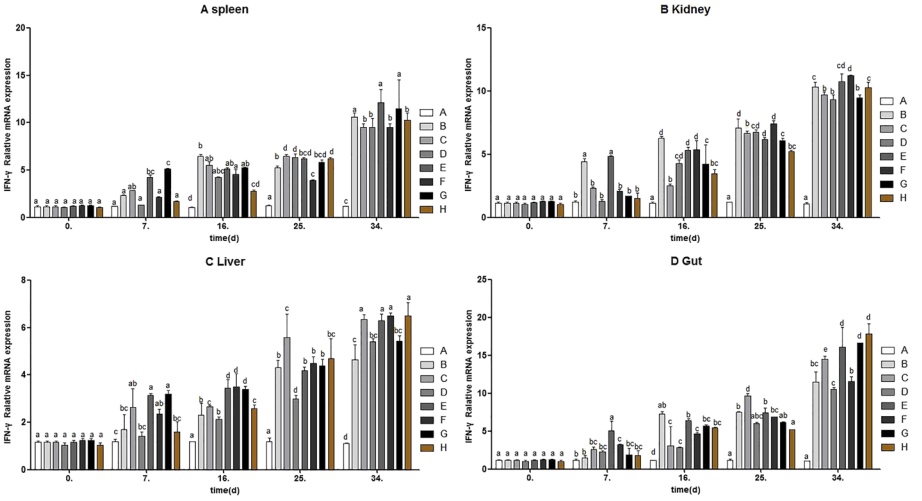

Fig. 6 Relative expression levels of IFN-γ in various tissues. Note: Different superscript letters in the same indicator indicate significant differences ($p < 0.05$); the same or unlabeled letters indicate no significant differences ($p > 0.05$). qPCR analysis of the expression of IFN-γ in spleen (**A**), kidney (**B**), liver (**C**) and gut (**D**) of C. *auratus*

As shown in Fig. 6, except for group A, the other groups had increased expression levels of IFN-γ in various tissues, and the expression level of IFN-γ in various tissues continued to rise, reaching the highest value at 34 days, which was significantly higher than that of

group A ($p < 0.05$). The expression levels of IFN- in the spleen, kidney, and intestine were relatively high. When the experiment was carried out for 34 days, the expression level of IFN-γ in the spleen of the E group was the highest, and it was significantly higher than that of the A, C, D, F, and H groups ($p < 0.05$). At 34 days, the expression level in the kidney of group F was the highest and significantly higher than that of groups A, B, C, D, G, and H ($p < 0.05$) and the expression level in the liver of groups C, E, F, and H was the highest, which was significantly higher than that of groups A, B, D, and G ($p < 0.05$). Group H had the highest expression level in the intestinal tract, which was significantly higher than that of groups A, B, C, D, and F ($p < 0.05$).

As shown in Fig. 7, except for group A, the expression level of TNF-α in each tissue was increased in each group, and the expression level of TNF-α in various tissues showed a continuous upward trend, reaching the highest value at 34 days, which was significantly higher than that in group A ($p < 0.05$). In addition, the expression in the spleen, kidney, and liver was higher. At 34 days, the expression level of TNF-α in the spleen in group E was the highest and was significantly higher than that in groups A, B, C, D, and H ($p < 0.05$). The expression level in the kidney of group E was the highest and was significantly higher than that of groups A, B, C, and F ($p < 0.05$), and the expression level in the liver of group E was the highest and was significantly higher than that of groups A, B, D, F, G, and H ($p < 0.05$). The highest expression level was found in group E, which was significantly higher than that in groups A, B, C, D, F, and H ($p < 0.05$).

As shown in Fig. 8, the expression level of IL-1β in the treatment groups was increased. When the experiment was carried out for 34 days, the expression level of IL-1β in the spleen of groups E and F was the highest and was significantly higher than that of groups A, B, D, and H ($p < 0.05$). At 34 days, the expression level of group D in the kidney was the highest, which was significantly higher than groups A, B, C, E, F, G, and H ($p < 0.05$). However, in the liver, group H was the highest and significantly higher than that of groups A, B, D, E, F, and G ($p < 0.05$). In addition, groups D, E,

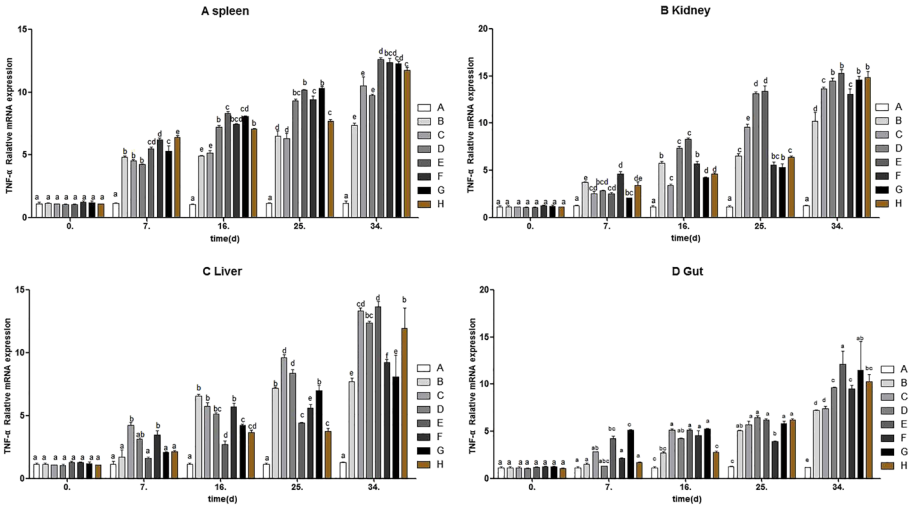

Fig. 7 Relative expression levels of TNF-α in various tissues. Different superscript letters in the same indicator indicate significant differences ($p < 0.05$); the same or unlabeled letters indicate no significant differences ($p > 0.05$). qPCR analysis of the expression of TNF-α in spleen (**A**), kidney (**B**), liver (**C**) and gut (**D**) of C. *auratus*

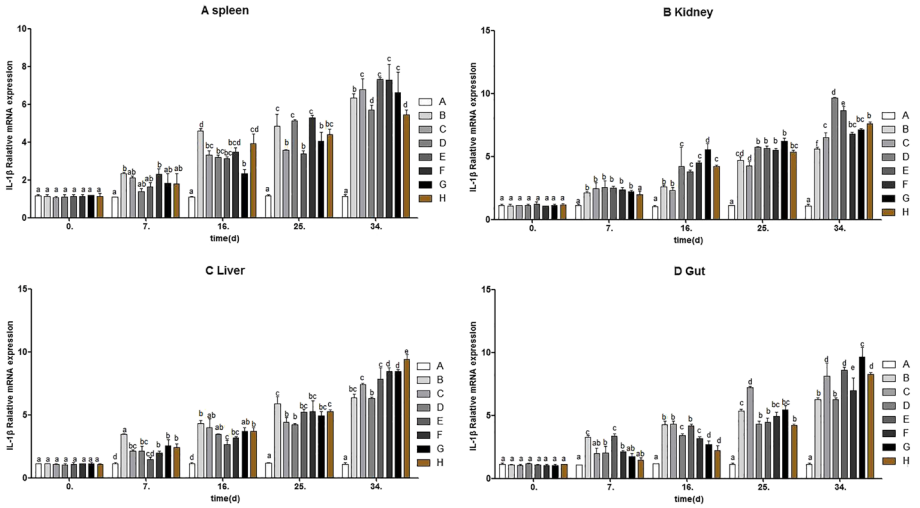

Fig. 8 Relative expression levels of IL-1β in various tissues. Different superscript letters in the same indicator indicate significant differences ($p < 0.05$); the same or unlabeled letters indicate no significant differences ($p > 0.05$). qPCR analysis of the expression of IL-1β in spleen (**A**), kidney (**B**), liver (**C**) and gut (**D**) of C. *auratus*

and G had the highest expression in the intestine, which was significantly higher than that of groups A, B, C, F, and H ($p < 0.05$).

As shown in Fig. 9, the expression level of IL-10 in all tissues was improved in the other groups except the control group. At 25 days, the expression level of IL-10 in the spleen of group F decreased slightly and then increased, but the expression level of IL-10 in the other

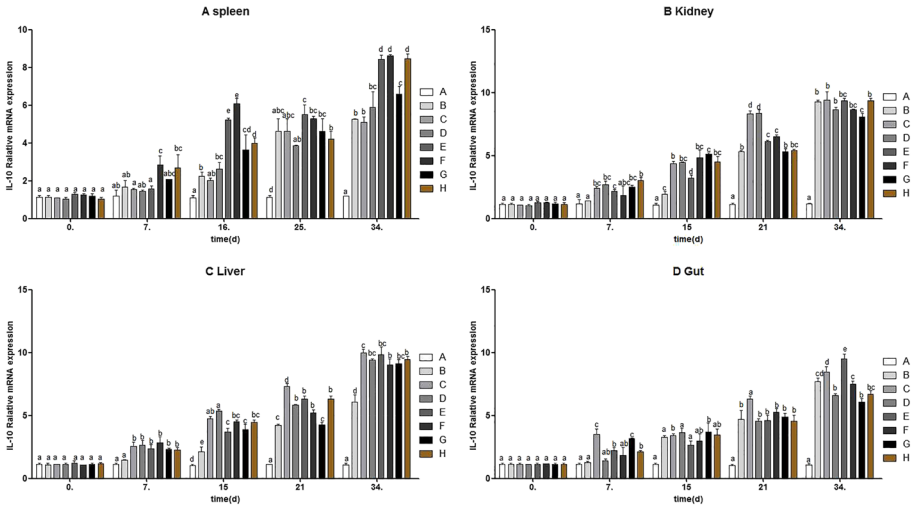

Fig. 9 Relative expression levels of IL-10 in various tissues. Different superscript letters in the same indicator indicate significant differences ($p < 0.05$); the same or unlabeled letters indicate no significant differences ($p > 0.05$). qPCR analysis of the expression of IL-10 in spleen (**A**), kidney (**B**), liver (**C**) and gut (**D**) of C. *auratus*

tissues continued to increase, reaching the highest value at 34 days, which was significantly higher than that in all tissues of group A ($p < 0.05$). In comparison, the expression level of IL-10 was higher in the kidney and liver. At 34 days, the expression level in the spleen in groups E, F, and H was the highest and was significantly higher than that in groups A, B, C, D, and G ($p < 0.05$). Groups C, E, and H showed levels in the kidney that was the highest and were significantly higher than those of the A and G groups ($p < 0.05$). However, groups C and E had the highest expression in the liver, which was significantly higher than that of groups A and B ($p < 0.05$). Group E had the highest intestinal expression and was significantly higher than that of groups A, B, C, D, F, G, and H ($p < 0.05$).

Resistance against A. hydrophila TPS strain

C. auratus was injected with the *A. hydrophila* TPS strain in each group. As shown in Fig. 10, all fish died within 7 days after the challenge with the *A. hydrophila* TPS strain. The relative immune protection rate of the probiotic group was higher than that of group A. Group E had the highest relative immune protection rate. The relative protection rates of groups B, C, D, E, F, G, and H were 27%, 30.7%, 40.94%, 42.32%, 39.38%, 33.61%, and 37.5%, respectively.

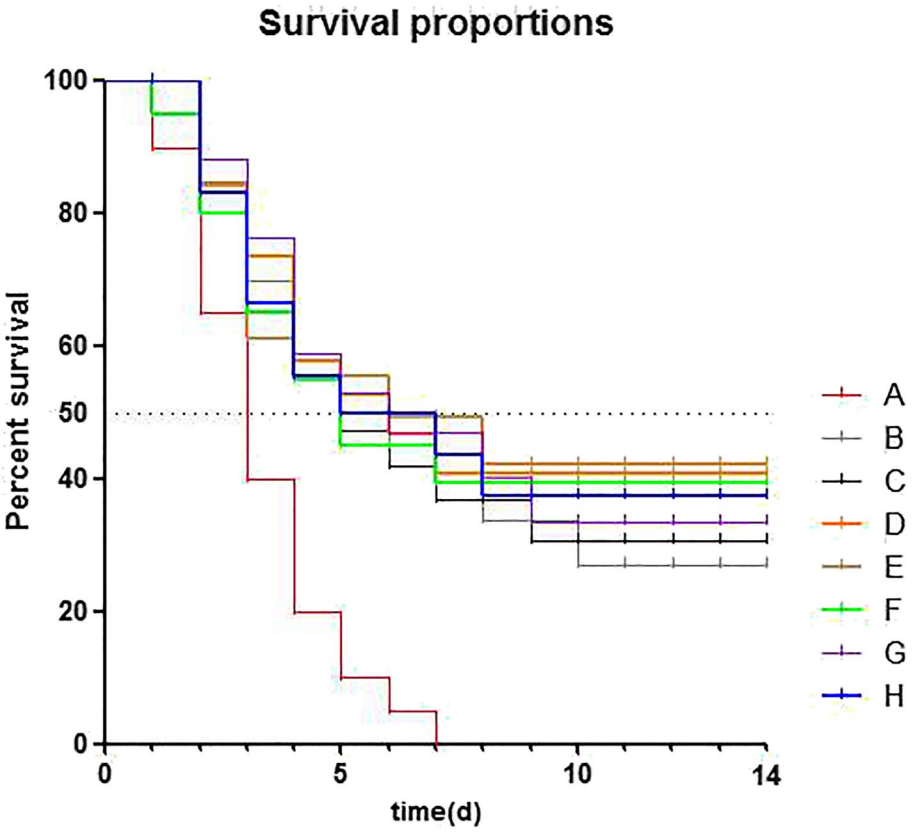

Fig. 10 Attack protection rate of *C. auratus*

Discussion

As a safe and high-quality feed additive and potential alternative to antibiotics, the main benefits of LAB as a probiotic used in aquaculture are to improve growth performance, immunity, antioxidant capacity, and disease resistance (Hoseinifar et al. 2019; Doan et al. 2016). In addition, there are a large number of microbial flora in fish intestines, which have varying degrees of influence on the growth and health of fish. The composition and structure of fish intestinal microbes are easily affected by nutrients in food and the environment in the intestine. Therefore, a good source of food is essential for the balance of fish intestinal microbes (Llewellyn et al. 2014). In the present study, *C. auratus* fed three different LAB strains singly or conjointly, group E significantly increased in FBW, WG, and SGR than the other probiotic groups, and the control group did at the end of the work (34 days). However, compared with the control group, the other probiotic groups showed a certain improvement, but the difference was not significant. Similarly, preceding studies reported that supplementation with probiotics (LAB) significantly increased the WG and SGR in *Lutjanus peru* (Reyes-Becerril et al. 2014), *Pangasius bocourti* (Doan et al. 2014), Oreochromis niloticus (Mariya et al. 2019), Rutilus frisii kutum (Mirghaed et al. 2018; Tarkhani et al. 2020), etc. A similar observation was shown by Hoseinifar et al. who reported a significant increase in the SGR of *Xiphophorus helleri*–fed *Lactobacillus acidophilus* (*L. acidophilus*) as a probiotic. The results demonstrate the beneficial effects of dietary *L. acidophilus* on mucosal immune parameters, intestinal microbiota, stress resistance, and growth parameters of *Xiphophorus helleri*, and the appropriate inclusion is 6×10^8 CFU/g (Hoseinifar et al. 2015). Therefore, the results indicated that different concentrations of compound probiotics would have different effects on fish growth performance. Combined with this experiment, we found that under the same concentration, different combinations of probiotics had different effects on the growth performance of *C. auratus*. However, compared with single probiotics, the production performance of composite probiotics was improved to a certain extent. There was no significant difference between the compound probiotic groups and the single probiotic groups except Group E, which may be caused by the symbiotic, antagonistic, or competitive coexistence between different strains in the intestinal tract at the same concentration. Moreover, the physiological characteristics of different strains were different, and their metabolites had different effects on fish tissues and organs. Environmental factors, individual differences, and differences in the optimal concentration of strains were also possible reasons for the results of this experiment. Similarly, the diet supplemented with 1.0×10^8 CFU/g *L. plantarum*, alone or in combination for 28 days, showed a significant increase in SGR compared with that of the control group. Analysis of the possible reason for the results of this experiment is that the growth of the fish body is mainly achieved through the synthesis of proteins in the body, and fish can be used for tissue repair and renewal through the absorption and utilization of proteins and amino acids in food to promote growth. LAB can secrete bacterial toxins, produce organic acids, occupy nutrient-receiving sites, and inhibit the proliferation and growth of harmful bacteria in the intestinal tract to promote the health of the intestinal tract of fish and enhance the effective absorption and utilization of nutrients in the intestinal tract.

Serum immune parameters are valuable tools for monitoring fish health. SOD, AKP and LZM in fish serum are important components in evaluating the immune performance of fish (Magnadottir et al. 2010; Lee et al. 2017). Superoxide dismutase (SOD) is a metal enzyme that widely exists in animals, plants, and microorganisms. Studies have shown that SOD can remove ROS and improve the antioxidant capacity in fish. AKP is an important

nonspecific immune marker enzyme for fish (Magnadottir et al. 2006). It is widely present in the tissues and organs of fish. It plays a role in immune defense and digestion and can regulate metabolism and the phosphorus ratio. APK is also a marker enzyme of cell lysosomes in blood and plays an important role in the digestion and degradation of foreign bodies in lysosomes (Boshra et al. 2006). LZM exists in the skin mucus and blood of fish and can decompose bacterial cell walls and peptidoglycans to achieve immune defense (Saurabh et al. 2008). In this experiment, SOD, AKP, and LZM in *C. auratus* serum of the probiotics group in the test group were all increased, which was significantly higher than that of the control group ($p < 0.05$), while the levels of single probiotics and composite probiotics peaked at different time stages. Similarly, the health-promoting effects of dietary combined probiotics have been reported to stimulate innate immune responses in gilthead *Sparus aurata* (Salinas et al. 2005, 2008), *Epinephelus coioides* (Sun et al. 2012) and *Labeo rohita* (Giri et al. 2014). The results showed that some indices of the composite probiotics were higher than those of the single probiotics group at different time stages. For instance, the serum SOD level of groups E and G was higher than that of the other groups at 25 days. However, the compound probiotics did not show an absolute advantage during the overall test, which might be related to the different experimental animals and the different types and contents of strains. In addition, the immune system of fish contains a complex physiological response, and the role of probiotics in the intestinal tract is often influenced by the body itself, the internal environment, and other factors. The specific immunity of fish is also an important part of the fish immune system, and it is mainly involved in IgM, which can directly act on pathogenic microorganisms (Bilal et al. 2016; Vervarcke et al. 2005; Van-Muiswinkel et al. 1991). Bahi et al. studied the addition of single or compound probiotics to the diet of *Sparus aurata* and found that the probiotic group could upregulate the level of IgM after three weeks, which was similar to the results of this test (Bahi et al. 2017). The result may be that probiotics can stimulate the secretion of IgM antibodies by B lymphocytes in *C. auratus* and increase the level of antibodies in the body, thereby obtaining a higher level of resistance to foreign pathogens. The composite probiotic groups all had peak levels of IgM at different time periods, but the IgM levels of the remaining probiotic groups had relatively slow growth because there are many factors that can affect differences in antibody levels in fish blood, e.g., the level of IL-4 and IL-13 (Yamaguchi et al. 2011), fish body length, age, and weight (Davis et al. 1999), environmental factors, individual differences, and et al.

Cytokines are essential mediators secreted from immune cells that regulate immune responses, repair damaged tissue, and defend against infection. IL-1β and TNF-α are two important pro-inflammatory genes that act as bacterial and viral invasion and are important markers for evaluating fish health. Il-1β is a cytokine produced when an immune response occurs that has pro-inflammatory effects and a wide range of biological activities and is involved in the regulation of a variety of immune active cells (Kono et al. 2013). TNF-α has a proinflammatory effect and is a cytokine that can directly kill tumor cells. The effector cells of nonspecific immunity mainly include monocytes-macrophages, neutrophils, and natural killer cells (NK). Mononuclear macrophages can be activated by IFN-γ and can secrete IFN-γ and other cytokines (Hasan et al. 2018). IL-10 is mainly synthesized by mononuclear macrophages and T helper cells, which are multicellular pluripotent cytokines that regulate cell growth and differentiation, participate in inflammation and immune responses, and are inflammatory and immunosuppressive factors. The spleen, kidney, liver, and intestine are the main immune organs of bony fish. In this experiment, supplementation of single or conjoin administration of probiotics exhibited significant inductions of IFN-γ, TNF-α, Il-1β, and IL-10 in the spleen,

kidney, liver, and intestine, showing that dietary supplementation of single or combined LAB might induce nonspecific immunity, thereby boosting the immune system of fish against invading pathogens. A previous study showed that Biswas et al. also found that *L. paracei* and *L. plantarum* could stimulate the expression of TNF-α and Il-1β to different degrees in *Takifugu rubripes* (Biswas et al. 2013), which is similar to the results of this experiment. Similarly, Panigrahi et al. (2011) indicated that *L. rhamnosus* could induce the upregulation of TNF-α cytokines in the kidney and spleen of rainbow trout, and the above results were like those in this study. Likewise, LAB can significantly increase the levels of the proinflammatory cytokines TNF-α and IL-1β and upregulate the anti-inflammatory cytokine IL-10 by adding 5×10^8 CFU/g three different LAB to the diet (Feng et al. 2019). The results may be due to supplementation of single or conjoin administration of probiotics changed the microbiome in the gut and the interaction between probiotics, which stimulated other signaling pathways. Probiotics live in the fish intestinal tract and secrete related immune-promoting factors, metabolites, and enzymes from cells to stimulate the secretion of IL-10 by mononuclear macrophages or other immune cells in the body and promote and enhance the immune performance of the body. Relevant mechanisms should be further studied and discussed.

The immune-protective effect of probiotics was evaluated through an immune protection test. The results showed that all the patients in the control group died within 7 days after the challenge. The relative protection rates of probiotic groups B, C, D, E, F, G, and H were 27%, 30.7%, 40.94%, 42.32%, 39.38%, 33.61%, and 37.5%, respectively, which showed that the probiotic groups had a certain protective effect on *C. auratus* infection with *A. hydrophila*. The reason may be that the probiotic groups promoted the upregulation of immune factors in the body, enhanced the barrier function of the first immune defense system of fish, such as skin, scales, and mucous membranes, and effectively prevented the invasion of pathogenic microorganisms. The colonization ability of different probiotics in the intestine and the adaptability to the environment in the intestine are different, resulting in different immune protection effects.

In this study, different combinations of probiotics were fed to *C. auratus* to explore the effects of different combinations on the immune performance of *C. auratus*, providing a reference for the development of high-quality probiotic additives in feed. The induction mechanism of probiotics on the immune performance of *C. auratus* is being studied and explored. In addition, adequate theories are still needed for reference. The mechanism of compound probiotics and single probiotics on the immune performance of fish still needs to be further explored. However, the mutual influence between different strains under environmental conditions in the body is also an area that deserves further exploration. At present, the evaluation standards for probiotics are not unified, and the functions of probiotics are mostly different. Although numerous studies have shown that probiotics can improve the immune performance of fish, it is impossible to uniformly evaluate the pros and cons of a certain type of probiotic in aquaculture due to the wide variety of probiotics and different test subjects. Therefore, the establishment of a probiotic evaluation system is of great significance for the application of probiotics in aquaculture.

Conclusion

In conclusion, the present study demonstrates that a single or combined LAB-supplemented diet improves growth performance and humoral immunity, as well as regulates immune-related gene expression and disease resistance against *A. hydrophilia*. Moreover,

the mixture of *L. lactis* + *L. rhamnosus* was more effective than those of the other groups for promoting growth performance and enhancing the immune response and disease resistance of *C. auratus*.

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Author contribution Ruoming Li completed the main experimental work and wrote the paper. Teng Chi and Rui Zhou analyzed the data. Qing Xu contributed significantly to the analysis and manuscript preparation. Juntong Liu helped perform the analysis with constructive discussions. Xiaofeng Shan and Jiayun Yao contributed analysis tools and materials. Wuwen Sun designed the experiments. Guiqin Wang contributed to the conception of the study.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate All the experimental fish handling procedures were used in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Ethics Committee of Jilin Agricultural University.

Human and animal ethics Not applicable.

Consent for publication The author agrees to publication in the journal indicated below and also to publication of the article in English by Springer in Springer's corresponding English-language journal.

Competing interests The authors declare no competing interests.

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