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Effects of *Lactococcus lactis* from *Cyprinus carpio* L. as probiotics on growth performance, innate immune response and disease resistance against *Aeromonas hydrophila*

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ABSTRACT

In the present study, we reported 18 LAB strains isolated from the intestinal contents of *Cyprinus carpio*, and their probiotic properties both *in vitro* and *in vivo*. The results showed that 9 of them had higher *in vitro* immunomodulatory properties, effectively survived under acidic (pH 2.5) and bile salt (ranging from 0.1% to 0.5%) conditions, and inhibited the growth of 4 pathogens. Among them, *Lactococcus lactis* Q-8, *Lactococcus lactis* Q-9, and *Lactococcus lactis* Z-2 showed the strongest adhesion abilities and inhibition of pathogen adhesion to mucin. When the fish consumed diets containing these 3 strains (5×10^8 CFU/g) for 8 weeks, the weight gain (WG) and specific growth rate (SGR) had significantly ($P < 0.05$) increased, especially with *L. lactis* Q-8, which had a WG of 231.45%, and SGR of 2.22%. Survival rate in each LAB supplementation group was also significantly higher than that in control group during the feeding period ($P < 0.05$). For the cytokines expression levels in serum, different expression patterns were also observed. Before the infection with *Aeromonas hydrophila*, *L. lactis* supplementation significant up-regulated protein levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12) compared with negative (CK1) group, while these cytokines were significantly lower than those in positive (CK2) group after infection. However, whether infected or not, the expression of anti-inflammatory cytokines (IL-10, TGF-β) were significantly increased in *L. lactis* Q-8, *L. lactis* Q-9, and *L. lactis* Z-2 treatment groups. In conclusion, these 3 *L. lactis* strains screened from common carp were effective in improving growth, innate immunity and disease resistance. Based on the physiological characteristics in our study, they might be used as potential probiotics in aquaculture.

1. Introduction

In recent years, aquaculture has become one of the fastest growing industries [1]. Among them, *Cyprinus carpio* is an important economic freshwater fish that is cultivated on a large scale in China [1]. However, diseases of common carp have become a limiting factor for production in China and elsewhere. Pathogen invasion exacerbated by poor aquaculture practices results in seriously increasing the mortality of farmed aquatic animals [2], and cause huge economic losses. Disease outbreaks are typically treated with chemotherapeutics and antibiotics, however, often has undesirable side effects, such as antibiotic contamination of the environment and fish, and led to increases in antibiotic resistances, thereby reduced efficiency of antibiotics for diseases [3,4]. Therefore, it is essential to develop strategies that are beneficial for improving the immune response and preventing bacterial diseases of common carp while maintaining stringent food safety standards. More recently, the administration of probiotics, prebiotics and immunostimulants to fish seems to be a very promising control measure for the fish farms [4,5].

In aquaculture, an increasing number of studies have focused on probiotics [5–7], which have been proved to be effective in disease control and promoting growth through enhancing immune response [6,7], supplying nutrients and enzymatic contributions [8] and modifying the host-associated microbiota [9], etc. Among the available probiotics, lactic acid bacteria, such as *Lactococcus* spp., have been widely studied for their probiotic properties in aquaculture [10,11]. Dong et al. demonstrated that *L. lactis* 16-7, which was isolated from crucian carp, could activate the non-specific immune system of crucian carp and reduce intestinal mucosal barrier damage and inflammation induced by *A. hydrophila* [12]. *L. lactis* WFLU12 has been shown to

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confer protection against *Streptococcus parauberis* in olive flounder through competitive exclusion and increased innate immune responses [13]. In addition, this promising probiotic strain significantly promoted fish growth along with better feed conversion and specific growth rate by influencing the metabolism of olive flounder [14]. Although many studies have used probiotics in animals grown in aquaculture, most have not yet been studied in depth. To our knowledge, there is no report about the use of *Lactococcus* spp. in common carp.

In fish, probiotics are strongly associated with their immunity [15] which mainly depends on their immune response, and cytokines as one of the indicators are often detected in this process. However, due to the lack of corresponding antibodies, most studies on the expression levels of inflammatory cytokines in aquaculture are limited to mRNA levels. For instance, to investigate the effects of *L. delbrueckii* on immune response of *C. carpio*, 1×10^7 CFU/g *L. delbrueckii* supplementation downregulated mRNA levels of *TNF-α*, *IL-8*, *IL-1β*, and up-regulated *IL-10* and *TGF-β* mRNA levels in the intestine [16]. However, in another study, a significant increase in expression of *TNF-α* and *IL-10* was observed on probiotic stimulation [17]. In addition, the expression of pro-inflammatory cytokines *IL-4*, *IL-12* and *IFN-γ* in the liver of Nile tilapia were significantly up-regulated after infection with *A. hydrophila* [18]. Overall, little research has been done on the expression of inflammatory cytokines at protein level [19]. This restriction limits further research on the immune response of aquaculture animals.

Therefore, we isolated LAB from the intestinal contents of common carp, screened functional *Lactococcus* spp. through different indicators, and investigated the effects of adding *L. lactis* in the feed on the growth performance and disease resistance against *A. hydrophila* of common carp. In addition, we prepared polyclonal antibodies against cytokines and detected the expression of immune-related genes in serum at protein level.

2. Materials and methods

2.1. Isolation and identification of lactic acid bacteria from common carp

Lactic acid bacteria were isolated from intestinal contents of nine healthy *Cyprinus carpio* L., according to the method described by Sha et al. [20] and Al-Madboly et al. [21]. The bacterium was identified by partial sequencing of the 16S rDNA gene as previously described [22]. For routine use, the strains were cultured at 37 °C without shaking for 20 h in MRS broth.

2.2. Test pathogens

The antimicrobial activity of LAB was studied against four virulent indicator strains, including *Escherichia coli* K88, *Staphylococcus aureus* ATCC 29213, *Salmonella Enteritidis* ATCC 13076 and *Aeromonas hydrophila* Ah 01. The strains were cultured at 37 °C with shaking at 180 rpm for 16 h in Luria–Bertani (LB) medium for routine use.

2.3. Production of polyclonal antibodies against cytokines

Polyclonal antibodies against cytokines (antigens) were obtained by immunizing female New Zealand white rabbits (2–2.5 kg body weight) with TNF-α, IL-1β, IL-6, IL-10, IL-12 and TGF-β as previously described [23,24]. The primers were designed using Primer 5.0 software based on the available gene sequences in GenBank (Table 1). All primers were synthesized by Shanghai Biotech Engineering Co., Ltd.

2.4. Detection of immunomodulatory activities of LAB strains in vitro

2.4.1. Head kidney cells culture and treatment

Head kidney monocytes/macrophages cells from freshly euthanized common carp were isolated following the method as previously described [25]. Then, head kidney cells were cultured in triplicate (2×10^6 cells/mL of RPMI-1640 medium without penicillin or streptomycin) in 24-well tissue culture plates. LAB cells were centrifuged at 8000 ***g*** for 5 min and the pellets were resuspended in RPMI-1640 medium and added in 24-well plates at a final concentration 5×10^8 CFU/mL. *A. hydrophila* (5×10^8 CFU/mL) was used as a positive control. The production of TNF-α and IL-12 in the culture supernatants were detected after 24 h.

Table 1
Nucleotide sequences of the primers used in prokaryotic expression.

Primers	Primer sequences (5′-3′)	GenBank accession no.
TNF-α F	CGGGATCCAACAAGTCTCAGAACAATCAG	AJ311800.2
TNF-α R	CCAAGCTTTGCCGTCAGTAGGAATG	
IL-1β F	CGGGATCCTGTACCCAGTTAGATGGTTCT	AB010701.1
IL-1β R	CCAAGCTTTTGGCATCTGGTTCATTT	
IL-6 F	CGGGATCCACAGTGATGGAGCAGCA	AY102632.1
IL-6 R	CCAAGCTTAAGAACCATTTTTGGTACTT	
IL-10 F	CGGGATCCGAGGGCTTTCCAGTGAG	AB110780.1
IL-10 R	CCAAGCTTGAGGATATCAAGCTCTCCC	
IL-12 F	CGGGATCCATCAAGAATACTCCTGAGAAAG	AJ621425.1
IL-12 R	CCAAGCTTTCAGGTTGATGCGCTC	
TGF-β F	CGGGATCCAACCGCATAATCTCCCAG	AF136947.1
TGF-β R	CCAAGCTTGAAATGTCTTCTGAGGATCA	

Notes: the underlined parts were the restriction sites of *Bam*H I and *Hin*d III, respectively.

2.4.2. Detection of TNF-α and IL-12 expression

To detect the immunomodulatory activities of LAB strains, the inflammatory cytokines TNF-α and IL-12 contents of the culture supernatants were measured by ELISA method with their corresponding polyclonal antibodies prepared in our lab [24]. For detailed information on the detection of TNF-α and IL-12 expression, according to the method described by Feng et al. [23].

2.5. Low pH and bile resistance of the LAB strains

Overnight cultured LAB strains were centrifuged, the pellets were washed three times and re-suspended in PBS (10^8 CFU/mL). As for low pH and bile resistances, methods described by Feng et al. [23] were used, and the assays were performed in triplicate and repeated three times.

2.6. Antimicrobial activities of LAB

Antibacterial activities of LAB strains were studied using an “agar well diffusion method”, as described by Feng et al. [23]. LAB strains with inhibition zones < 11, 11–16, 17–22 and > 23 mm were classified as strains of no -; mild +; strong ++; and very strong +++ inhibition, respectively [26]. The assays were performed in triplicate and repeated three times.

2.7. Adhesion to gut mucus of common carp

As an *in vitro* model to address the interaction between probiotics and host, mucus samples were isolated from the intestines of three common carp as described by Zhang et al. [27]. The protein concentration of the gut mucus was adjusted to 0.5 mg/ml as determined by the Protein Assay kit (BioRad). Mucus adhesion assays and LAB labeling were conducted according to previous reports [23,28]. Subsequent processing, detection and calculation referred to Feng et al. [23]. Three independent experiments were performed for each LAB strain.

2.8. Inhibition of A. hydrophila adhesion to gut mucus by LAB strains

Each LAB strain (5×10^7 CFU per well) was added to mucin in a

fresh tissue culture medium without Penicillin-Streptomycin and incubated at 37 °C for 2 h in a 5% CO_2/95% air atmosphere incubator before incubation with 100 μL of FITC labeled *A. hydrophila* (5×10^8 CFU/mL), which was labeled as previously described [26]. Subsequent processing, detection and calculation referred to Feng et al. [23]. Three independent experiments were performed for each LAB strain.

2.9. Preparation of probiotic diets

Preparation of experimental diets following the method previously described with slight modifications [20]. Briefly, Strains Q-8, Q-9 and Z-2, which selected by their strong immunomodulatory activities and adhesion capacities, and identified as *L. lactis* by 16s sequencing, were grown at 37 °C for 20 h in MRS broth (OXOID). The cultures were centrifuged at 8000 ***g*** for 10 min. The pellets were washed three times with sterile distilled water, re-suspended in PBS, and sprayed on commercial feed at a concentration of 5×10^8 CFU/g of feed, as the previous study of LAB strains used in common carp culturing [16,29,30]. Three supplemental diets were prepared: a basal diet with 5×10^8 CFU/g of *L. lactis* Q-8, a basal diet with 5×10^8 CFU/g of *L. lactis* Q-9, and a basal diet with both 5×10^8 CFU/g of *L. lactis* Z-2 (Table 2). The diets were air-dried at room temperature and stored in individual airtight plastic containers at 4 °C until used. The LAB activities in the prepared feed were measured once a day. In addition, the leaching rate of LAB in the feed was also examined as previously described [20], and the feed was prepared every 6 days.

2.10. Fish and rearing conditions

Cyprinus carpio L. were obtained from the Aquaculture Breeding Base of Henan Aquatic Technology Extension Station, Henan Province, China. Healthy fish were acclimatized in 500-L tanks at 26–27 °C for one week in laboratory conditions and fed the basal diet. Thereafter, the fish, which were normal eating, disease-free and non-injured, were routinely examined physically and microbiologically to ensure the absence of bacterial diseases or any abnormal clinical signs, and 450 common carp of similar size (33.07 ± 0.55 g) were randomly allocated to fifteen 30-L tanks (thirty fish per tank and triplicate tanks per treatment). Each aquarium was equipped with a filter connected to an air stone for aeration. Fish were fed to apparent satiation four times daily (08:30, 11:30, 14:30 and 17:30) for 8 weeks as described in Table 2. During the trial, about 30% of the culture water was replaced with fresh, dechlorinated water of similar temperature once a day and kept oxygen–saturated by aeration. The tanks were maintained under a natural light/dark regime. During the experimental period, the monitored water quality parameters (mean ± S.D.) were as follows: water temperature 26.0 ± 0.5 °C, pH 6.5–7.1, dissolved oxygen > 6.1 ± 0.5 mg/L, NH_4^+-N < 0.5 mg/L and NO_2–N < 0.05 mg/L.

After starting the trials, the common carp in each group with continuous aeration were fed daily at a 3% feed rate of body weight. The methods and conditions of rearing were the same as for acclimatization.

Table 2
The treatments of the experiment for comparison of *L. lactis* Q-8, Q-9 and Z-2 as dietary supplements in common carp.

Treatments	Diet
[a]CK1	Basal
[b]CK2	Basal
L. lactis Q-8	Basal contained 5×10^8 CFU *L. lactis* Q-8/g feed
L. lactis Q-9	Basal contained 5×10^8 CFU *L. lactis* Q-9/g feed
L. lactis Z-2	Basal contained 5×10^8 CFU *L. lactis* Z-2/g feed

[a] As a control group in the feeding experiment, while as a negative control group in the subsequent challenge test.
[b] As a positive control group in the challenge test.

The feed rate was adjusted according to the body weight at the end of the second week.

2.11. Growth performance and sampling

After 8 weeks of treatment, fish were fasted for 24 h. Six fish per tank were randomly selected and anaesthetized, blood was collected from the caudal vasculature by puncturing with a syringe, and then centrifuged (4 °C, 3000 rpm, 10 min) to obtain the serum. The cytokines content in the serum was measured to evaluate the immunomodulatory effects of *L. lactis* Q-8, Q-9 and Z-2 on common carp. Survival rate % (SR), Weight gain (WG), and Specific growth rate (SGR) were calculated as described previously [9,31].

2.12. Challenge with A. hydrophila

After eight weeks, eighteen fish from each replicate (except the CK1 group) were injected intraperitoneally with 100 μL of *A. hydrophila* (Ah 01) (LD50 = 5×10^6 CFU/mL), which was provided by our lab, and cultured as previously described. The cultures were centrifuged (Thermo, USA) at 2000 ***g*** for 5 min. The pellets were washed twice with PBS. The number of the bacterial cells in the suspensions was determined by turbidimetry. In the negative control group (CK1), fish was injected with 100 μL of sterilized PBS. The culture conditions were the same as previously mentioned. Blood was taken from the caudal vein at 6 h, 12 h, 24 h and 48 h after *A. hydrophila* infection. After centrifuging, the cytokine content in the serum were measured to evaluate the immunoprotective effects of *L. lactis* Q-8, Q-9 and Z-2 on common carp.

2.13. Cytokines assays

ELISA was performed with the cytokine polyclonal antibodies (provided by our lab) in a Microplate Reader (PerkinElmer, USA), according to the previous description. Briefly, 96-well Immuno-Maxisorp plates (Nunc) were coated with blood serum (1:1) in coating buffer (0.05 M carbonate buffer, pH 9.6) overnight at 4 °C. The plates were blocked and washed. The polyclonal antibodies against TNF-α, IL-1β, IL-6, IL-12, IL-10 and TGF-β (1:2000) were added to the plates, and the other treatments were exactly the same as Feng et al. [23].

2.14. Calculations and statistical analysis

$$\text{Weight gain (WG)} = (Wt - Wo) \times 100/Wo$$

$$\text{Specific growth rate (SGR)} = (\text{Ln } Wt - \text{Ln } Wo) \times 100/\text{t}$$

$$\text{Survival rate (SR)} = Nt \times 100/No$$

Where *Wt* and *Wo* were final and initial mean individual weight of common carp in each replicate, and t was duration of experimental time-56 days; *Nt* and *No* are final and initial number of living common carp [9,31].

Results are expressed as the mean values ± standard deviation (SD). Differences between treatments were determined using a one-way analysis of variance with the statistical software package SPSS Version 17.0. Significant differences were accepted at $P < 0.05$.

3. Results

3.1. TNF-α and IL-12 production by head kidney cells in response to recall antigen stimulation

Immunomodulatory activity of LAB strains *in vitro* were shown as Fig. 1. For TNF-a, compared with the positive control group, the LAB strains significantly increased the production of TNF-a after 24 h ($P < 0.05$), except for Q-15, Q-17, Q-18 and Z-1, especially Q-8 (32.72 pg/mL), Q-9 (30.02 pg/mL), Q-5 (29.63 pg/mL), Q-6 (28.60 pg/

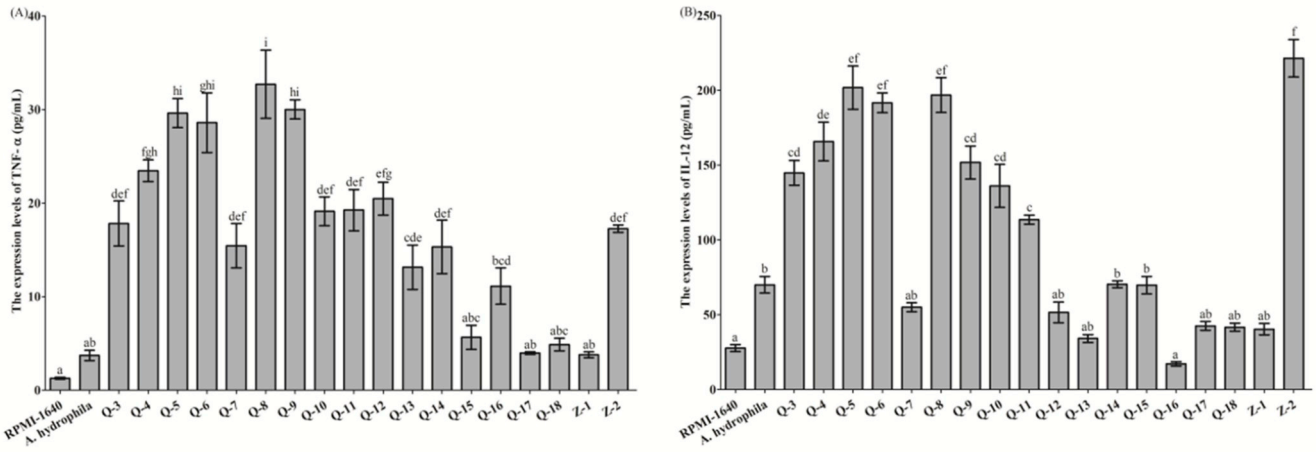

Fig. 1. (A) TNF-α, (B) IL-12 production by head kidney cells after incubation with different LAB strains. The data are expressed as the means ± SDs (n = 6). The error bars represent the standard deviations, and values marked with different letters are significantly different ($P < 0.05$), the same below.

mL), Q-4 (23.47 pg/mL), Q-11 (19.25 pg/mL), Q-10 (19.13 pg/mL), Q-3 (17.82 pg/mL) and Z-2 (17.26 pg/mL) (Fig. 1A).

However, half of the LAB strains could not significantly induce IL-12 production. The strains that significantly increased the production of IL-12 ($P < 0.05$) include Z-2 (221.36 pg/mL), Q-5 (201.75 pg/mL), Q-8 (196.78 pg/mL), Q-6 (191.56 pg/mL), Q-4 (165.67 pg/mL), Q-9 (151.69 pg/mL), Q-3 (144.76 pg/mL), Q-10 (136.07 pg/mL) and Q-11 (113.52 pg/mL) (Fig. 1B). These 9 strains are also the highest yield of TNF-α. Therefore, they were retained for further analysis, and the other 9 strains of LAB were not further studied.

3.2. Resistance to low pH and bile, antimicrobial activity

Resistance to gastric (low pH) and intestinal (high bile concentration) conditions are frequently tested *in vitro* to evaluate probiotic candidates [32]. When incubated in simulated gastric juice at low pH for 3 h, all selected 9 strains were able to survive with negligibly reduced viable counts (Table 3). In addition, when incubated at different bile concentrations, all 9 LAB strains showed high tolerance since there were no significant differences of survive abilities during the increase of bile concentrations from 0.1% to 0.5% (Table 3).

The results of bacteriostatic activities of LAB were shown in Table 4, the above-mentioned 9 LAB strains showed strong inhibitory effects on *E. coli* ATCC K88, *S. aureus* ATCC 29213, *S. Enteritidis* ATCC 13076 and *A. hydrophila* Ah 01. Especially strains Q-5, Q-8 and Z-2 exhibited very strong against *A. hydrophila* Ah 01 (Table 4).

Table 3
Resistance to acid and bile of the 9 LAB strains.

LAB strains	Bacterial counts (log CFU/mL)				
	Control 0 h	Low pH 3 h, pH 2.5	0.1% bile 3 h	0.25% bile 3 h	0.5% bile 3 h
Q-3	7.92 (0.31)	7.82 (0.37)	7.91 (0.35)	7.82 (0.45)	7.81 (0.46)
Q-4	8.11 (0.26)	8.02 (0.38)	8.11 (0.35)	8.02 (0.50)	8.00 (0.51)
Q-5	8.05 (0.41)	7.96 (0.32)	8.04 (0.33)	7.95 (0.19)	7.94 (0.43)
Q-6	8.12 (0.36)	80.2 (0.31)	8.12 (0.32)	8.02 (0.18)	8.01 (0.42)
Q-8	8.15 (0.31)	8.06 (0.35)	8.12 (0.34)	8.05 (0.32)	8.02 (0.72)
Q-9	8.18 (0.25)	8.09 (0.38)	8.17 (0.32)	8.09 (0.52)	8.04 (0.65)
Q-10	8.13 (0.37)	8.04 (0.35)	8.12 (0.35)	8.02 (0.14)	7.98 (0.33)
Q-11	8.07 (0.32)	7.98 (0.31)	8.06 (0.32)	7.98 (0.18)	7.92 (0.42)
Z-2	8.28 (0.21)	8.19 (0.35)	8.27 (0.35)	8.18 (0.14)	8.14 (0.33)

Values are means from three experiments.
* Considered significantly different in comparison with those at 0 h ($P < 0.05$).

Table 4
Antimicrobial activities of LAB against pathogenic bacteria *in vitro*.

LAB strains	*E. coli* (EC) ATCC K88	*S. aureus* ATCC 29213	*S. Enteritidis* ATCC 13076	*A. hydrophila* Ah 01
Q-3	+ +*	+ +	+ +	+ +
Q-4	+ +	+ +	+ +	+ +
Q-5	+ +	+ +	+ +	+ + +
Q-6	+ +	+ +	+ +	+ +
Q-8	+ +	+ +	+ +	+ + +
Q-9	+ +	+ +	+ +	+ +
Q-10	+ +	+ +	+ +	+ +
Q-11	+ +	+ +	+ +	+ +
Z-2	+ +	+ +	+ +	+ + +

*LAB strains with inhibition zones 11–16, 17–22 and > 23 mm were classified as strains of mild (+), strong (+ +), or very strong (+ + +) inhibition, respectively.

3.3. Adhesions to intestinal mucus of common carp

Adhesion to intestinal epithelial cells or mucin is often used to select and evaluate probiotics [33]. In this study, fluorescein FITC labeling method was used to examine the adhesion of LAB to intestinal mucin (Fig. 2). The results showed that all 9 tested strains could adhere to mucin, and the adhesion ability varied from 1.16% to 4.96%. Among them, Z-2, Q-9 and Q-8 had the strongest adhesion (from 4.96% to 4.14%), while Q-11 was the least effective one to adhere to mucin (1.16 ± 0.04%).

3.4. Inhibition of pathogen adhesion to mucin by LAB

The competitive inhibition of LAB strains on adhesion to mucin of *A. hydrophila* was shown in Fig. 3. The adhesion inhibition of strain Z-2 was the strongest, reaching 74.34%. Followed by strains Q-9 and Q-8, with inhibition rates of 66.13% and 62.14% respectively (Fig. 3). The results showed that the inhibitory effect of LAB on the adhesion of pathogenic bacteria to mucin was positively correlated with their own adhesion abilities (Figs. 2 and 3).

3.5. Growth and survival performance

The growth and survival performance of common carp during the experiment was shown in Table 5. Final weight of common carp fed diet supplemented with *L. lactis* Q-8 and *L. lactis* Z-2 were significantly higher than the CK group ($P < 0.05$). No significant difference of final

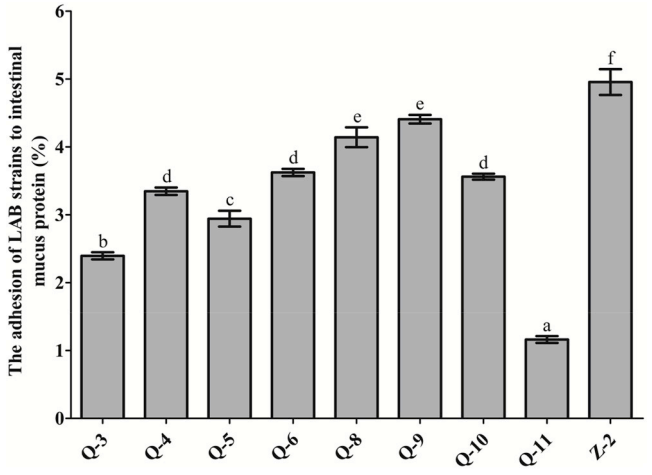

Fig. 2. Adhesions of the selected LAB strains to mucin. The data are expressed as the means ± SDs (n = 3).

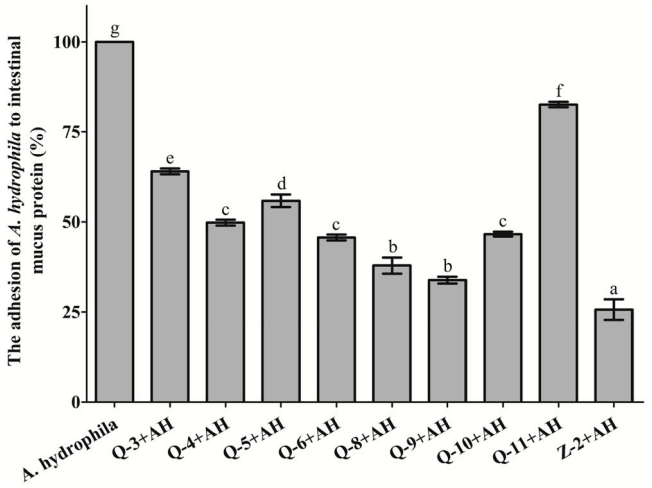

Fig. 3. Effects of LAB culture on the adhesion of mucin by *A. hydrophila*. The data are expressed as the means ± SDs (n = 3).

weight was observed among the *L. lactis* Q-9 and CK groups ($P > 0.05$). The WG, SGR, and SR of *L. lactis* Q-8, *L. lactis* Q-9, and *L. lactis* Z-2 were all significantly higher than the CK group ($P < 0.05$). In addition, compared with the *L. lactis* Q-9 and *L. lactis* Z-2 groups, the WG and SGR were significantly higher in the *L. lactis* Q-8 group ($P < 0.05$), but no significant difference in the SR was observed between *L. lactis* Q-8, *L. lactis* Q-9, and *L. lactis* Z-2 groups ($P > 0.05$).

3.6. Assay of the cytokines TNF-α, IL-1β, IL-6, IL-12, IL-10 and TGF-β in common carp serum

It is well known that cytokines play a key role in immunological processes. Therefore, the levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12 and anti-inflammatory cytokines IL-10 and TGF-β in serum were evaluated to further examine the immunomodulatory and immunoprotective activities of *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2. As shown in Fig. 4, these 3 LAB strains significantly stimulated the secretion of TNF-α, IL-1β and IL-6 ($P < 0.05$), among which the activity of *L. lactis* Z-2 was the strongest, and the secretion of TNF-α, IL-1β and IL-6 were approximately 1.89, 1.84 and 1.57 times as much as that of the CK1 group (Fig. 4A). In addition, only *L. lactis* Z-2 significantly increased the expression of IL-12 ($P < 0.05$, Fig. 4A). For anti-inflammatory cytokines, *L. lactis* Z-2 could significantly increase the expression of IL-10 ($P < 0.05$), but interestingly, *L. lactis* Q-8 and *L. lactis* Q-9 could only significantly increase the expression of TGF-β ($P < 0.05$, Fig. 4B). The results showed that these 3 LAB strains exhibited different immunomodulatory patterns.

Similar to elucidate the immunoregulatory effects of LAB strains, the levels of cytokines TNF-α, IL-1β, IL-6, IL-12, IL-10 and TGF-β in serum of common carp, which infected with *A. hydrophila*, were evaluated to further examine the immunoprotective effects of *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 on host immune system. Results were shown in Fig. 5, compared with CK2 group, the expression of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-12 were significantly decreased in all 3 LAB strains at any time point ($P < 0.05$, Fig. 5A, B, C, D). Especially at 48 h, the expression of TNF-α, IL-1β and IL-12 in *L. lactis* Z-2 treatment group had no significant difference compared with CK1 group ($P > 0.05$, Fig. 5A, B, D). Similar results showed that there was no significant difference in the expression of TNF-α between *L. lactis* Q-8 and CK1 group ($P > 0.05$, Fig. 5A). For *L. lactis* Q-9 group, the overall abilities to reduce the expression of pro-inflammatory cytokines was weaker than that of *L. lactis* Z-2 and *L. lactis* Q-8 treatment groups. For anti-inflammatory cytokines, all of these 3 LAB strains could significantly increase the synthesis of IL-10 and TGF-β, and showed a trend of increasing first and then decreasing, and the expression of IL-10 by L. *lactis* Q-8 and *L. lactis* Q-9 had no significant difference compared with the CK2 group at 48 h ($P > 0.05$, Fig. 5E). In addition, it can be seen from the results that the effect of *L. lactis* Z-2 on promoting the expression of anti-inflammatory cytokines was more effective than that of *L. lactis* Q-8 and *L. lactis* Q-9 (Fig. 5E and F), which was also consistent with the results of pro-inflammatory cytokines.

4. Discussion

Although the roles of probiotics in improving fish growth performance, feed utilization, immune response and regulating intestinal microflora have been well documented in the past decades [29,34–36], there is no report has been directed towards the possible effects resulting from the administration of *Lactococcus* species to common carp.

Table 5
Effects of *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 supplementation on survival and growth of common carp.*.

Index	Treatment			
	CK	*L. lactis* Q-8	*L. lactis* Q-9	*L. lactis* Z-2
Initial weight/g	33.49 ± 0.14	33.03 ± 0.50	33.15 ± 0.35	32.95 ± 0.08
Final weight/g	101.57 ± 2.28^{A}	114.43 ± 2.15^{C}	106.16 ± 2.25^{AB}	107.05 ± 1.81^{B}
WG/%	203.24 ± 5.97^{A}	246.49 ± 5.08^{C}	220.24 ± 5.41^{B}	224.92 ± 5.81^{B}
SGR %/d	1.98 ± 0.04^{A}	2.22 ± 0.03^{C}	2.08 ± 0.03^{B}	2.10 ± 0.03^{B}
SR %	82.22 ± 1.57^{A}	90.00 ± 2.72^{B}	87.78 ± 1.57^{B}	88.89 ± 1.57^{B}

*In the same line, means with different letters are significantly different ($P < 0.05$), means with the same letters are not significantly different ($P > 0.05$). Data represent means ± SD (n = 6).

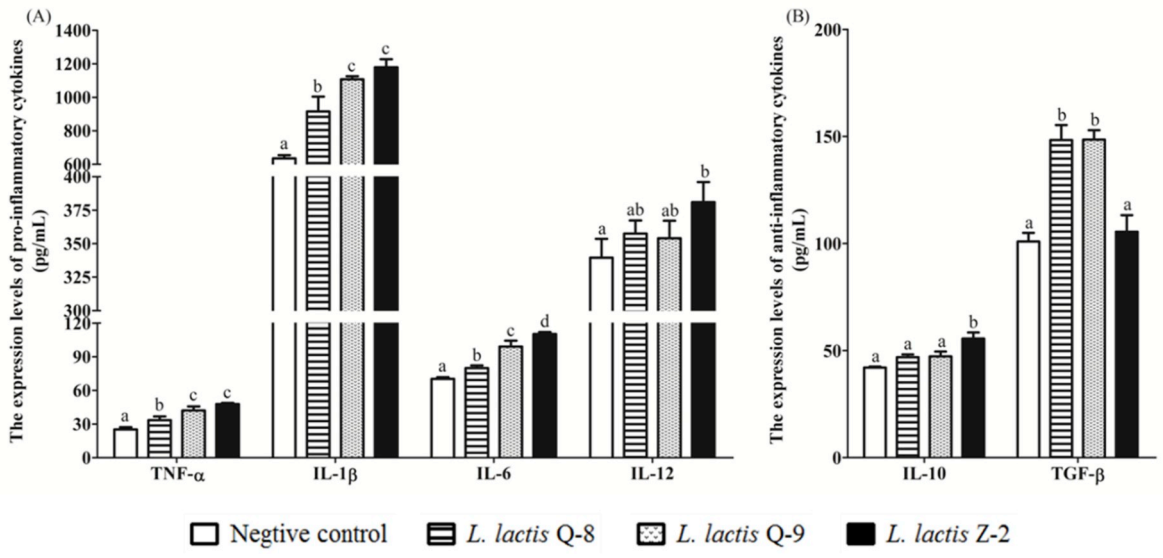

Fig. 4. Immunomodulatory effects of the selected LAB strains on cytokine levels in serum. (A) Pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-12; (B) anti-inflammatory cytokines IL-10 and TGF-β. The data are expressed as the means ± SDs (n = 6).

In addition, there is a great deal of evidence to show that probiotic bacteria from an autochthonous source have a greater chance of colonizing the host gut and hence confer a health benefit on the host [37,38]. Therefore, the present study was conducted to isolate and screen functional *Lactococcus* from the intestinal contents of common carp, and evaluate the effects of dietary supplementation of *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 on the growth performance, immunity and disease resistance of common carp.

Immunemodulating effects of LAB *in vitro* have received increasing attention as potential selection parameters for probiotics recently [23,39]. In the present study, 18 LAB strains derived from the intestinal contents were isolated. By the detection of immune performance, these 18 LAB strains had different *in vitro* immunomodulatory to induce the expression of TNF-α and IL-12 in head kidney cells of common carp (Fig. 1). The results were in agreement with the previous studies on Japanese pufferfish (Takifugu rubripes) [40]; Labeo rohita [39]; and Broiler Chicks [23]. Therefore, the abilities of LAB to induce TNF-α and IL-12 production were used to examine the immune-enhancing activities of LAB will help to obtain functional probiotics [23,41]. LAB can survive effectively in gastrointestinal conditions and adhere to the intestinal tract are prerequisites to play their probiotics roles [42,43]. As one of the metabolites of LAB, lactic acid is found to be an effective out membrane-disintegrating agent which can induce lipopolysaccharide (LPS) release and damage the out membrane of gram-negative bacteria [44]. So through further screening, the results showed that the isolated LAB strains could effectively survive under gastrointestinal conditions (Table 3), had different adhesion (Fig. 2) and anti-pathogen adhesion abilities (Fig. 3), and had strong antibacterial activities (Table 4).

Similar with the previous studies on common carp [16,29,30], humpback grouper (Cromileptes altivelis) [45], Nile tilapia (Oreochromis niloticus) [46] and rainbow trout (Oncorhynchus mykiss, Walbaum 1792) [47], the concentration of the selected *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 strains used in this study were 5×10^8 CFU/g of feed. In addition, following the method previously described [20], these LAB strains were sprayed on commercial feed, the cell viability and leaching ratio of *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 in diets were evaluated every day. We found that the cell viability at day 7 was still close to the initial level of CFU added to the feed, and the leaching ratio of diets was 6‰ (data not shown). These findings demonstrate that the method for preparing diets was feasible and that the feed could be prepared every 6 days.

In general, the survival rate and growth performance were intuitive indices to evaluate the effects of potential probiotics on culturing of aquatic animals, and studies on *L. lactis* as an aquaculture feed additive have reported on its capacity to improve the growth and health of aquatic animals [11,35]. The enhanced growth performance of fish fed dietary probiotics might be attributed to elevated health status, digestibility, and stimulation enzymatic secretion, immune defense, and improved intestine microbial balance, which in turn improves nutrient absorption and utilization [48,49]. In *Cromileptes altivelis*, for example, Sun et al. had reported that *L. lactis* HNL12 improves the growth rate, stimulates macrophage activation, secretes extracellular enzymes, and improves immunity and disease resistance against *V. harveyi* [45]. Beck et al. found that olive flounder fed a probiotic mixture of *L. plantarum* FGL0001 and *L. lactis* BFE920 demonstrated improvements in host innate immunity, weight gain, and survival rate following an *S. iniae* challenge compared to fish fed a control diet [50]. In addition, *L. lactis* RQ516 was beneficial for tilapia in terms of increasing growth performance and the concentrations of serum protein and globulin and enhancing immune responses [51]. The application of *L. lactis* in common carp has achieved relatively positive results. In our study, WG, SGR, and SR of fish fed with 5×10^8 CFU/g *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 were significantly higher than the control group. Similarly, Kim et al. had reported 1.25×10^8 CFU/g *L. lactis* BFE920 were effective for olive flounder [10]. While Zhang et al. found that the optimal level of *L. delbrueckii* at 1×10^6–1×10^7 CFU/g diet could be a more effective in *Cyprinus carpio* Huanghe var [16], and the four LAB strains (*Lactobacillus plantarum* LL11, *Weissella confuse* LS13, *Lactococcus lactis* LH8 and *Enterococcus faecalis* LC3) indicated positive effects on growth performance and immune response for *A. japonicus* at 1×10^9 CFU/g diet [31]. It is likely that the optimal supplementary dose of different LAB stains using in teleosts is a little different.

Due to the non-specific immunity first plays a defensive role when the pathogen invades the body, so enhanced immune defence is one of the promising effect in the teleost fish that lacking of adaptive immunity [31,45]. Previous studies have shown that *Lactococcus* spp. play a role in activating the innate immune system to stimulate production of cytokines, which can enhance or inhibit production of other cytokines and then regulate the immune response [45,52]. In the present study, the common carp fed a diet with added *L. lactis* showed significant inductions of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12 and anti-inflammatory cytokines IL-10 and TGF-β expression in serum, suggesting that a diet supplemented with Q-8, Q-9 and Z-2 could induce non-specific immunity, which promoted the fish's immune

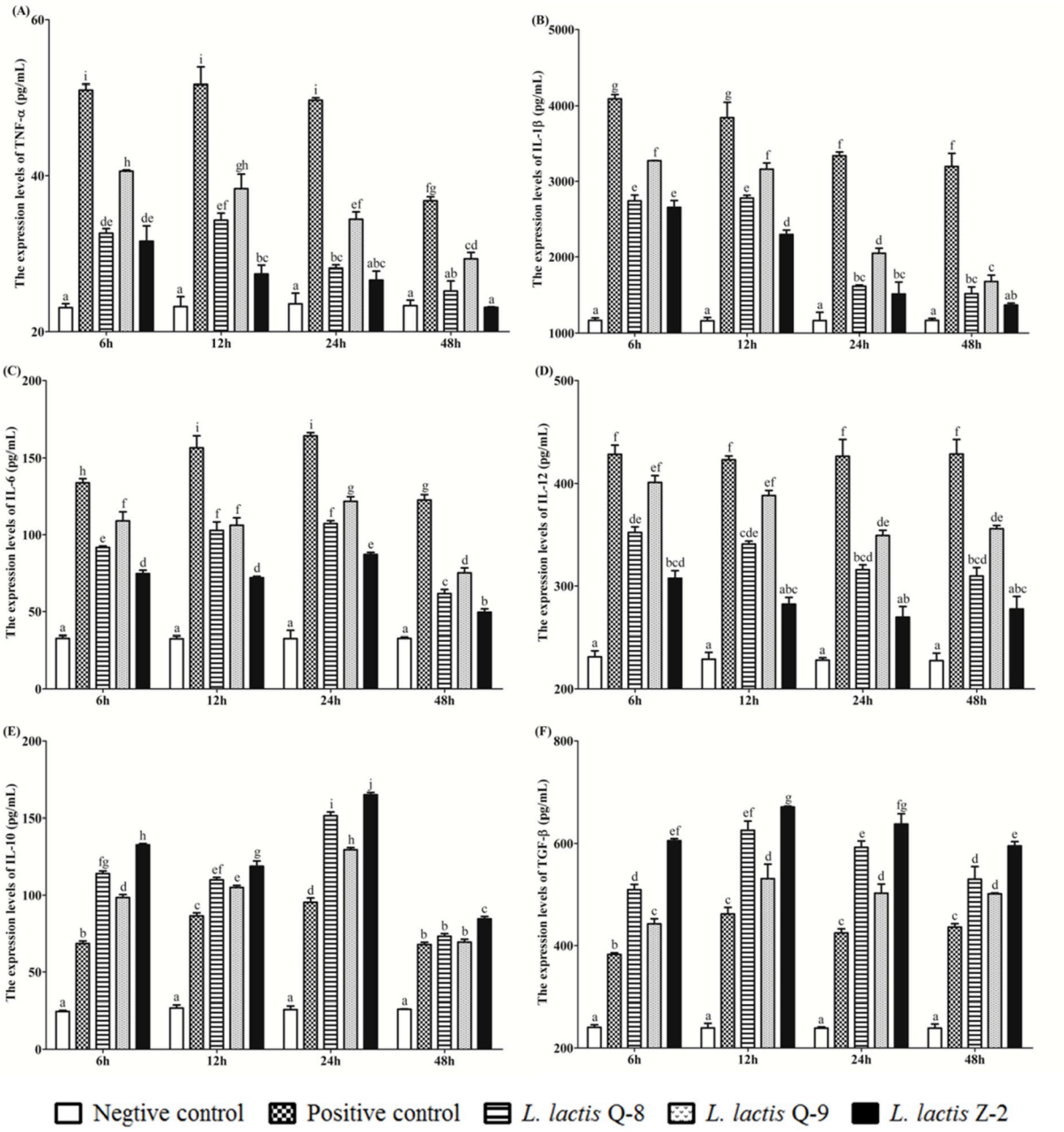

Fig. 5. Immunoprotective effects of the selected LAB strains on cytokine secretion in serum. (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-12, (E) IL-10 and (F) TGF-β. The data are expressed as the means ± SDs (n = 6).

system against invading pathogens. Similarly, Zhang et al. showed that the mRNA levels of *IL-10* and *TGF-β* were significantly up-regulated by feeding with *L. delbrueckii* [16]. In *Nile tilapia*, transcript levels of TNF-α and IL-1β of fish fed *L. rhamnosus* JCM1136 and *L. lactis* JCM5805 were significantly increased [9]. the fish that received the diet supplemented with *L. lactis* 16-7 exhibited a significantly enhanced the expression levels of immune-related genes (INF-γ, IL-1β, IL-11, TNF-α), indicating that *L. lactis* 16-7 could activate the non-specific immune system of crucian carp [12].

Once the innate immune system is activated, the status of activation is controlled and maintained at a constant level (homeostasis) in healthy conditions [53]. In the present study, dietary administration of 5×10^8 CFU/g *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 significantly increased the survival rate of common carp and against *A. hydrophila* by down-expression the levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-12 and up-expression the anti-inflammatory cytokine IL-10 and TGF-β in serum. In agreement with our findings, Sun et al. found that feeding with *L. lactis* HNL12 could significantly enhance the survival rate of humpback grouper against *V. harveyi* [45]. Feng et al. demonstrated that the LAB treated group improved the resistance ability against *Salmonella* in poultry by reducing the levels of pro-inflammatory cytokines IL-1β, IL-6 and IL-12 and increasing the anti-inflammatory cytokine IL-10 in serum [23]. The tested LAB reduced acute inflammation under *E. coli* infection by decreasing IL-8, IL-113, and IL-6 expression in cattle [54]. In aquaculture, however, the effects of dietary supplementation of probiotics on cytokines were studied extensively with inconsistent results and expression levels. For instance, Qin et al. demonstrated that the *L. casei* BL23 treated group could significantly enhance the survival rate of zebrafish against *A. hydrophila* by increasing the levels of pro-inflammatory cytokines *TNF-α*, *IL-1β* and anti-inflammatory cytokine *IL-10* at mRNA levels [55]. Dietary marine *L. plantarum* AH 78 could enhance the cellular and humoral immune

responses of juvenile Nile tilapia infected with *A. hydrophila* by up-regulating the expression of cytokines *IL-4*, *IL-12* and *IFN-γ* [18]. These inconsistent results may be related with the dosage, usage mode, time of applying the probiotics, the health status of animals and the water environment factors. Future work should be performed to obtain information about the detailed mechanisms by adding probiotics.

In summary, *L. lactis* Q-8, *L. lactis* Q-9 and *L. lactis* Z-2 showed the abilities to enhance the growth performance, stimulate macrophage activation and secretes cytokines, and improve immunity and disease resistance against *A. hydrophila* in common carp. Due to the limited information of *Lactococcus* spp. usage in common carp, whether feeding *Lactococcus* spp. alter the balance of intestinal microbes, thereby affect the host growth and immune response remains unknown. Therefore, the detailed mechanisms of growth promotion and immune enhancement in common carp are needed to further investigate.

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