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Dietary *Ginkgo biloba* leaf extract alters immune-related gene expression and disease resistance to *Aeromonas hydrophila* in common carp *Cyprinus carpio*

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ABSTRACT

Ginkgo biloba leaf is widely used in traditional medicine in China. The present study aimed to illustrate the effects of dietary *Ginkgo biloba* leaf extract (GBLE) on growth performance and immune responses in common carp infected by *Aeromonas hydrophila*. Six different diets either not treated (control) or treated with 0.5, 1, 2, 5 and 10 g/kg of GBLE were designed to feed the fishes for 8 weeks. The results indicated that, compared to the control groups, 10 g/kg dietary GBLE significantly increased body growth and feed utilization. In GBLE dietary groups, red blood cell levels, white blood cells, hematocrit, hemoglobin, total protein, albumin and globulin were significantly increased relative to the control groups. Dietary supplementation with 5 g/kg GBLE increased the phagocytic ratio, and phagocytic indexes increased in the 2, 5 and 10 g/kg groups relative to the control groups. Moreover, 2, 5 and 10 g/kg GBLE diets increased O_2^- production compared to the control groups. Additionally, GBLE diets stimulated lysozyme activity (in 10 g/kg group) and inhibited bactericidal activity (in 0.5, 2, 5 and 10 g/kg group). Quantitative real-time PCR showed that IL1β, IL8, TNF-α, IL10, TGFβ, and inducible enzyme genes were prone to decrease while SAA, hepcidin and GPX1 were increased due to the GBLE diet in the intestine. In the head-kidney, the GBLE treatment decreased IL1β, IL8, TNF-α, IL10, TGFβ, INOS and arginase gene expressions, whereas SOD upregulation was found in the GBLE condition. The mRNA expressions of IL1β, IL8, TNF-α, IL10 and INOS were decreased, but SAA, hepcidin, GPX1 and SOD mRNA levels were increased in the spleen in the GBLE diet compared to the control. Additionally, diet supplemented with GBLE improved the survival rate infected with *A. hydrophila*. Our observations suggest that GBLE effectively enhanced growth performance, modulated immune-related gene expression. It improved survival rate of common carp after *A. hydrophila* infection and the optimum concentration we recommend is 10 g/kg of GBLE.

1. Introduction

Aquaculture provides protein sources for the food industry, which is fast growing in developing countries [1]. With the development of aquaculture, production risks such as the outbreak of epidemic diseases and environmental destruction are increasing [2]. Moreover, fish cultured in high density are more susceptible to pathogens [3,4]. In this circumstance, drugs and antibiotics are used to treat infectious diseases [5,6]. However, these drugs could contaminate the water, and drug residue can pass through the food chain, which negatively affect human health [6]. The consequences hinder the sustainable development of aquaculture. Thus, finding new strategies to deal with pathogenic infections is urgently needed.

Herbal medicinal products that are safer and more environmentally friendly provide a potential alternative therapy to chemical drugs and antibiotics [7]. Recently, traditional Chinese medicines, herbal extracted compounds, and commercial plant-derived products have been used and are an effective and cheap approach for aquaculture [8]. As immunostimulants, herbal medicinal products have been studied in humans, mice and other model animals, and they have been used as feed additives and chemotherapeutics for fishes [9]. Essential oils of clove, basil and ginger improved resistance to *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*) [10]. Dietary supplementation with guava leaves improved the postchallenge survival rate of *Labeo rohita* to *A. hydrophila* infection [11]. In carp, *Astragalus* root (*Radix astragalin* seu Hedysari) and Chinese *Angelica* root (*R. Angelicae sinensis*) elevate the function of nonspecific immunity [12]. These findings suggested the improvement of anti-disease function by herbal medicinal products.

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Ginkgo biloba is a type of large tree that has existed for at least 200 million years on earth [13]. As an herbal medicine, *Ginkgo biloba* has been commonly known in traditional Chinese medicine to relieve coughand acts as an antibacterial agent. It helps individuals with mood and memory and treats cardiovascular disease [14,15]. The polyphenols isolated from *Ginkgo biloba* leaves inhibited *Salmonella enterica*, *Staphylococcus aureus* and *Aspergillus niger* [16]. In *Ginkgo biloba* leaf extract (GBLE), the flavone glycoside, terpene lactones and other active substrates have anti-oxidant functions and anti-inflammatory effects [17]. *Ginkgo biloba* extract regulated innate immunity of human leukocytes [18] and improved immune function in weaned piglets [19]. In addition, the *Ginkgo biloba* extract treatment can potentially extend the shelf-life of silver pomfret (*Pampus argenteus*) during storage in ice [20]. Moreover, the aqueous extracts of *Ginkgo biloba* leaves inhibited lipid oxidation and apoptosis of fish erythrocytes [21]. In hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀), dietary *Ginkgo biloba* extract stimulated immune-related gene expression and decreased apoptosis-related gene expression [22]. These clues indicate that dietary *Ginkgo biloba* extract may be a potential feed additive for fighting against bacterial infections in aquaculture.

Common carp (*Cyprinus carpio*) is widely distributed around the world and has been reared in Asia for several centuries [23]. This fish species represents a Cyprinid fish model to investigate the effects of different dietary formulas and the immune response to pathogens. *Aeromonas hydrophila* is a Gram-negative bacterium that is one of the most common bacterial pathogens in freshwater [24]. Infection by *A. hydrophila* is associated with hemorrhagic septicemia [25]. In the present study, the effect of dietary GBLE on *A. hydrophilia* infection in common carp is reported. Herein, we investigated the effects of dietary GBLE on growth performance, plasma biochemical parameters, oxidative stress indexes and the gene expression of immune responses in common carp. In addition, we determined the influence of dietary GBLE on resistance to *A. hydrophila*.

2. Materials and methods

2.1. Feed preparation

To determine the effects of GBLE on resistance of fish to *A. hydrophila*, six different diets were designed as the basal diet supplemented with 0, 0.5, 1, 2, 5 and 10 g GBLE per kilogram feed (g/kg GBLE) according to a previous study [22]. The formula for each group is shown in Table 1. GBLE contained 24% ginkgo flavonoids and 6% terpene lactones was purchased from Mei Herb Biotech Co., Ltd., Dongguan, China. The ingredients were mixed by a laboratory food mixer, and the diets were formed into 2-mm pellets in a drying cabinet at 40 °C. The dried diets were stored at −20 °C.

2.2. Fish and experimental conditions

The experimental fish were collected from a local market in Changsha, Hunan province, China. During the experimental trial, the fish were maintained in 1000 L acrylic tanks with running water (5 L/min). Juvenile common carp (7.84 ± 0.35 g) were randomly selected and cultured in 18 tanks (20 fish in each tank) for the trial. Three replicates were assigned to each dietary group. Before the trial, the fish were fed with the basal diet for 10 days to be adapted to the experimental conditions. The experiment trial was performed for 8 weeks. The fish were fed with diet twice (3% of body weight)a day at 8:30 and 17:30. During the trial, the conditions were as follows: temperature of the water ranged from 23.5 to 27.5 °C; pH ranged from 7.2 to 7.5; NH_3–N ranged from 0.11 to 0.15; dissolved oxygen ranged from 5.7 mg/L to 6.8 mg/L; the photoperiods followed a natural daylight cycle.

2.3. Growth measurements

Eight fish individuals from each group were measured to determine growth performance. The weight (Wt) gain, specific growth rate (SGR), and feed conversion ratio (FCR) were calculated according to Tukmechi [26]:

Wt gain% = [(final Wt – initial Wt)/initial Wt] × 100%

SGR = [(ln final Wt-ln initial Wt)/t] × 100

FCR = feed given (dry weight) / weight gain (wet gain) of fish

"t" represents the times (days) during the feeding experiment

2.4. Blood and serum collection

Blood from tested fish were collected from the caudal vein. In each group, eight fish were included after 8 weeks of the feeding trial experiment. The cells were stained by Giemsa staining and counted based

Table 1
Composition and nutrient levels of experimental diets.

Ingredients (g)	0.00 g/kg	0.50 g/kg	1.00 g/kg	2.00 g/kg	5.00 g/kg	10.00 g/kg
Fish meal	90	90	90	90	90	90
Soybean meal	280	280	280	280	280	280
Rapeseed meal	250	250	250	250	250	250
Wheat middlings	210	210	209	208	205	200
Rice bran	110	110	110	110	110	110
Fish oil	10	10	10	10	10	10
Soybean oil	10	10	10	10	10	10
Choline chloride	10	10	10	10	10	10
Mineral premix[a]	20	20	20	20	20	20
Vitamin premix[b]	10	10	10	10	10	10
Ginkgo biloba leaf extract	0	0.5	1	2	5	10
Analyzed proximate composition %						
Crude protein	46.69	46.76	46.59	46.87	46.91	46.53
Crude lipid	14.13	14.01	14.17	14.17	14.11	14.21
Ash	10.21	10.19	10.33	10.21	10.41	10.52
Gross energy (MJ/kg)	14.8	14.88	14.82	14.87	14.91	14.9

[a] Mineral premix (mg/kg): KCl, 200 mg; KI, 60 mg; $CoCl_2{\cdot}6H_2O$, 7 mg; $CuSO_4{\cdot}5H_2O$, 14 mg; $FeSO_4{\cdot}H_2O$, 400 mg; $ZnSO_4{\cdot}H_2O$, 200 mg; $MnSO_4{\cdot}H_2O$, 80 mg; $Na_2SeO_3{\cdot}5H_2O$, 65 mg; $MgSO_4{\cdot}7H_2O$, 3000 mg; $Ca(H_2PO_4)2{\cdot}H_2O$, 20 g; NaCl, 136 mg.

[b] Vitamin premix (mg/kg): Vitamin A, 25 mg; Vitamin D3, 5 mg; Vitamin E, 40 mg; Vitamin C, 500 mg; Vitamin B1, 12 mg; Vitamin B6, 6 mg; Vitamin B12, 0.05 mg; Vitamin K3, 5 mg; riboflavin, 5 mg; inositol, 100 mg; pantothenic acid, 30 mg; niacin, 35 mg; folic acid, 2 mg; biotin, 0.06 mg; ethoxyquin, 150 mg; wheat middlings, 14.09 g.

Table 2
Primers used for qPCR.

	Forward (5′ to 3′)	Reverse (5′ to 3′)
IL1β	CAGAGCAACAAACTAAGTGACGAG	ACCATCTAACTGGGTACAAGCAAG
IL8	GTCTTAGAGGACTGGGTGTA	ACAGTGTGAGCTTGGAGGGA
TNFα	GGTGATGGTGTCGAGGAGGAA	TGGAAAGACACCTGGCTGTA
IL10	CGCCAGCATAAAGAACTCGT	TGCCAAATACTGCTCGATGT
TGFβ	CGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA
INOS	AACAGGTCTGAAAGGGAATCCA	CATTATCTCTCATGTCCAGAGTCTCTTCT
COX2	CCCCAGTCCGTGTCCTAGTA	GTCCGTAAAAGACCCCTGGG
Arginase-2	GGAGACCTGGCCTTCAAGCATCT	CTGATTGGCACGTCCAACT
SAA	AACTGAGCGAACCTGAGAGC	AAGGCTTTCCGGTCTGTAGC
Hepcidin	ACATGCGTCTGCTTCCTCC	CTGGTTCTCCTGTGGTGCTT
GPX1	ACCTGTCCGCGAAACTATTG	GAGCTCGTTCATCTGGGTGT
SOD	GGAATACTCGGTCATTGG	ACTGAGTGATGCCTATAAC
β-actin	AGACATCAGGGTGTCATGGTTGGT	CTCAAACATGATCTGTGTCAT

Table 3
Effects of dietary GBLE on growth performance and feed utilization in common carp.

	0.00 g/kg	0.50 g/kg	1.00 g/kg	2.00 g/kg	5.00 g/kg	10.00 g/kg
Initial weight	7.62 ± 0.28	7.51 ± 0.33	7.42 ± 0.29	7.56 ± 0.24	7.62 ± 0.24	7.45 ± 0.39
Final weight	43.77 ± 1.69	44.44 ± 1.11	44.03 ± 1.30	44.58 ± 1.73	43.82 ± 1.15	46.80 ± 2.61
Wt gain	475.26 ± 32.34^{a}	492.92 ± 35.00^{ab}	493.82 ± 28.15^{ab}	490.61 ± 33.84^{ab}	475.32 ± 16.41^{a}	528.90 ± 38.48^{b}
SGR	3.12 ± 0.10^{a}	3.18 ± 0.11^{ab}	3.18 ± 0.09^{ab}	3.17 ± 0.10^{ab}	3.12 ± 0.05^{a}	3.28 ± 0.11^{b}
FCR	1.21 ± 0.06^{b}	1.18 ± 0.04^{ab}	1.19 ± 0.04^{b}	1.18 ± 0.06^{ab}	1.21 ± 0.03^{b}	1.11 ± 0.07^{a}

Wt = weight gain; SGR = specific growth rate; FCR = feed conversion ratio. The different superscript at each row indicated significant differences among the groups ($P < 0.05$).

Table 4
Effects of dietary GBLE on blood biochemical parameters in common carp.

	0.00 g/kg	0.50 g/kg	1.00 g/kg	2.00 g/kg	5.00 g/kg	10.00 g/kg
Red blood cells (10^6/μl)	1.42 + 0.36^{a}	1.52 + 0.30^{ab}	1.55 + 0.29^{ab}	1.86 + 0.26^{bc}	1.47 + 0.18^{ab}	2.12 + 0.31^{c}
White blood cells (10^4/μl)	4.36 + 0.28^{a}	4.6 + 0.32^{ab}	4.32 + 0.26^{a}	4.35 + 0.30^{a}	4.49 + 0.40^{ab}	4.81 + 0.13^{b}
Hematocrit (%)	23.02 + 1.18^{a}	22.57 + 1.17^{a}	24.05 + 1.95^{ab}	23.5 + 1.70^{a}	23.72 + 2.09^{a}	26.14 + 1.20^{b}
Hemoglobin (g/l)	31.91 + 1.67^{a}	34.61 + 2.20^{ab}	35.03 + 2.66^{ab}	35.3 + 1.58^{b}	35.4 + 2.82^{b}	36.49 + 1.29^{b}
total protein (g/dl)	9.26 + 0.96^{a}	11.07 + 2.49^{ab}	11.07 + 1.48^{ab}	10.87 + 1.48^{ab}	13.52 + 2.65^{b}	14.13 + 3.19^{b}
Albumin (g/dl)	0.36 + 0.02^{a}	0.37 + 0.01^{ab}	0.36 + 0.02^{a}	0.39 + 0.06^{ab}	0.41 + 0.06^{ab}	0.43 + 0.05^{b}
Globulin (g/dl)	0.31 + 0.04^{a}	0.36 + 0.03^{ab}	0.39 + 0.07^{a}	0.36 + 0.07^{ab}	0.37 + 0.08^{ab}	0.4 + 0.04^{b}
Albumin:Globulin	1.2 + 0.14	1.03 + 0.10	0.94 + 0.13	1.1 + 0.26	1.12 + 0.26	1.08 + 0.18

The different superscript at each row indicated significant differences among the groups ($P < 0.05$).

on morphology. Diluted blood was used to count erythrocytes (1:1000 dilution by PBS) and leucocytes (1:100 dilution by PBS). The serum was collected by centrifugation at 3000*g* for 10 min at 4 °C. The total protein, albumin and globulin in serum were determined by commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

2.5. Phagocytic activity

The phagocytic activity of head-kidney leucocytes from eight fish in each group after the feeding trial was determined by a method described previously [27]. Briefly, the leucocytes from the head-kidney were suspended in 100 μL (1×10^7 cell/mL) and incubated at 25 °C for 30 min on sterile slides. Yeast was suspended in 100 μL (10^8 cell/mL, Baker's yeast, Sigma, USA) and added to the monolayer cells on the slide. After incubation at room temperature for 45 min at 25 °C, the unattached cells were washed with PBS buffer. Giemsa staining was performed to calculate the number of phagocytic cells, phagocytic activity and phagocytic index.

2.6. Respiratory burst activity

The respiratory burst activity analysis of the phagocytes was performed according to a previous method [28,29]. Eight fish in each group were included in this analysis. First, 100 mL of cells were suspended, and the cells were stained by 100 μL of 0.3% nitroblue tetrazolium (Shanghai Sangong Biotech Co., Shanghai, China) and 100 μL Phorbol 12-myristate 13-acetate (Shanghai Sangong Biotech Co., Shanghai, China) for 30 min. After terminating the staining using 100% methanol and washing with 70% methanol for three times, 120 μL of 2 M KOH and 140 μL dimethyl sulfoxide was added (Shanghai Sangong Biotech Co., Shanghai, China). The respiratory burst activity was determined at 630 nm on a spectrophotometer (BioRad, USA).

2.7. Lysozyme activity

The serum lysozyme activity was determined by spectrophotometer with a lysozyme-detection kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The data were shown as units of lysozyme per mL. The control was a suspension of *Micrococcus Lysodeikticus*. One unit (U) was the activity reducing absorbance by 0.001 per min at 530 nm compared to control.

2.8. Quantitative real-time PCR (qPCR)

The gene expression profiles of innate immune related genes in common carp in head kidney, spleen and liver were analyzed (n = 6 per group). Total RNA was extracted from the homogenized tissues using

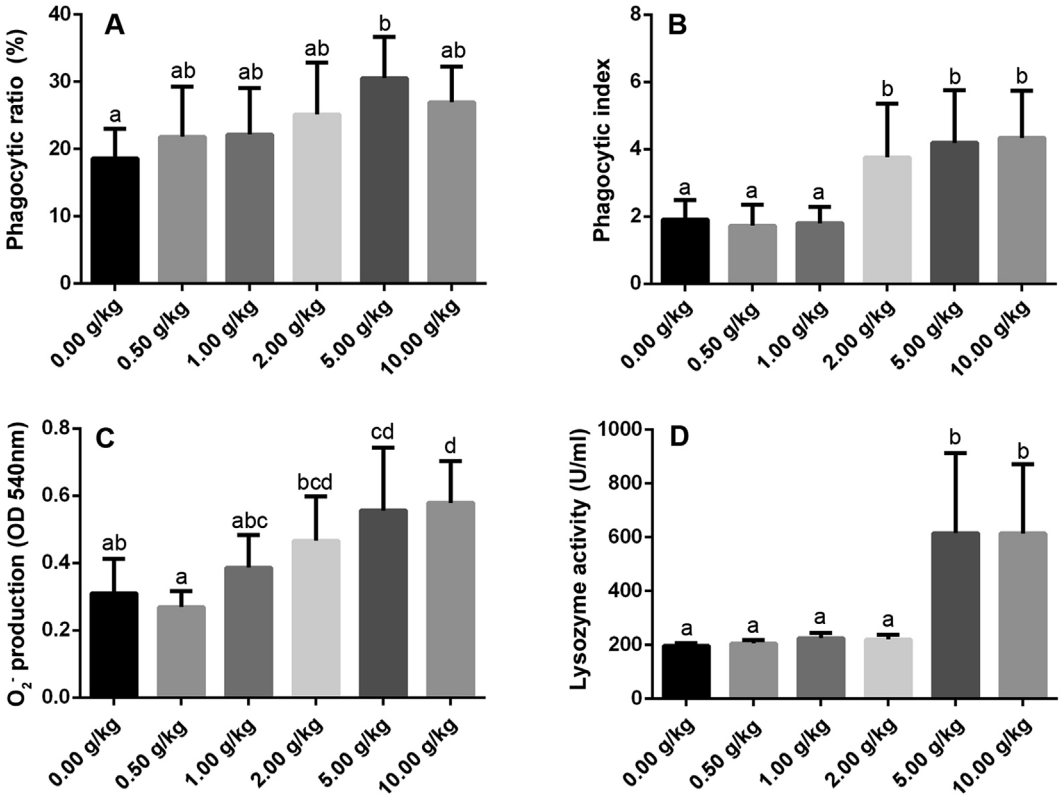

Fig. 1. Effects of different amount of dietary GBLE on the phagocytic ratio (A), phagocytic index (B), O_2^- production (C) and lysozyme activity (D). Values are means ± SEM of eight replications. Different superscripts on each bar showed significantly different ($P < 0.05$).

1 mL Trizol reagent (Life Technologies, Carlsbad, CA, USA). The purification and concentration of total RNA were assayed by a spectrophotometer (Beckman DU 730, Fullerton, CA, USA). The RNA integrity was determined by 1.2% agarose gel. In total, 1 μg total RNA was used as template for synthesis of first-strand cDNA by PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) following the manufacturer's instructions.

The primers are shown in Table 2. The β-actin gene was used as the internal standard. qPCR was performed on an ABI 7500 real-time PCR machine (Applied Biosystems, USA) using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China) according to the manufacturer's instructions. The reaction systems for qPCR were 20 μL in total, containing 3 μL diluted cDNA (1:40), 0.5 μL forward and reverse primers (10 μM) and 6 μL H_2O. The amplification was performed at 94 °C for 3 min, followed by 40cycles at 94 °C for 15 s and 94 °C for 30 s. All of the samples were assayed with three replicates. After the amplification, dissociation curve analysis (melting curve) was used to verify single product. The relative expression was evaluated using the $2^{-\Delta\Delta CT}$ method [30].

2.9. Challenge experiment with A. hydrophilia

The *A. hydrophila* was purchased from Guangdong Institute of Microbiology (Guangzhou, China) and cultured in LB medium. Previous report showed that the 48 h median lethal dose (LD50) was 4×10^7 CFU/fish [31]. We used lower concentration (3.1×10^7 cfu/mL) of *A. hydrophila* according to a previous study to trace a 20 days trail according to a previous study [32]. The fish were injected with 3.1×10^6 cfu *A. hydrophila* in 100 μL PBS. Control fish were injected with 100 μL PBS. Thirty fish in each group were traced, and the mortality was recorded over a period of 20 days for Kaplan–Meier survivorship curve analysis.

2.10. Statistical analyses

All of the results were presented as the means ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to identify significance. When the P value was 0.05 or less, the comparison between the groups was considered significantly different. The statistical analyses were performed using SPSS v16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Growth performance and body indicators

As shown in Table 3, the initial body weight showed no significant difference among the groups. At the end of the trial, significantly higher final weight, Wt gain, SGR and FCR were found in the 10 g/kg dietary GBLE group ($P < 0.05$), while other groups had no difference compared to the control of 0 g/kg ($P > 0.05$) (Table 3).

3.2. Blood biochemical profile

The red blood cell levels significantly increased ($P < 0.05$) in the 2 and 10 g/kg groups at 8 weeks compared to the control (0 g/kg group) (Table 4). The white blood cells and hematocrit significantly increased ($P < 0.05$) in the 10 g/kg group compared to the control. The hemoglobin increased in the 2, 5, and 10 g/kg groups significantly ($P < 0.05$) compared to control. In the 5 and 10 g/kg GBLE groups, the total protein levels were significantly upregulated ($P < 0.05$). In the 10 g/kg group, both albumin and globulin significantly increased compared to the other groups ($P < 0.05$). The albumin:globulin ratio did not significantly change among the experimental groups (Table 4).

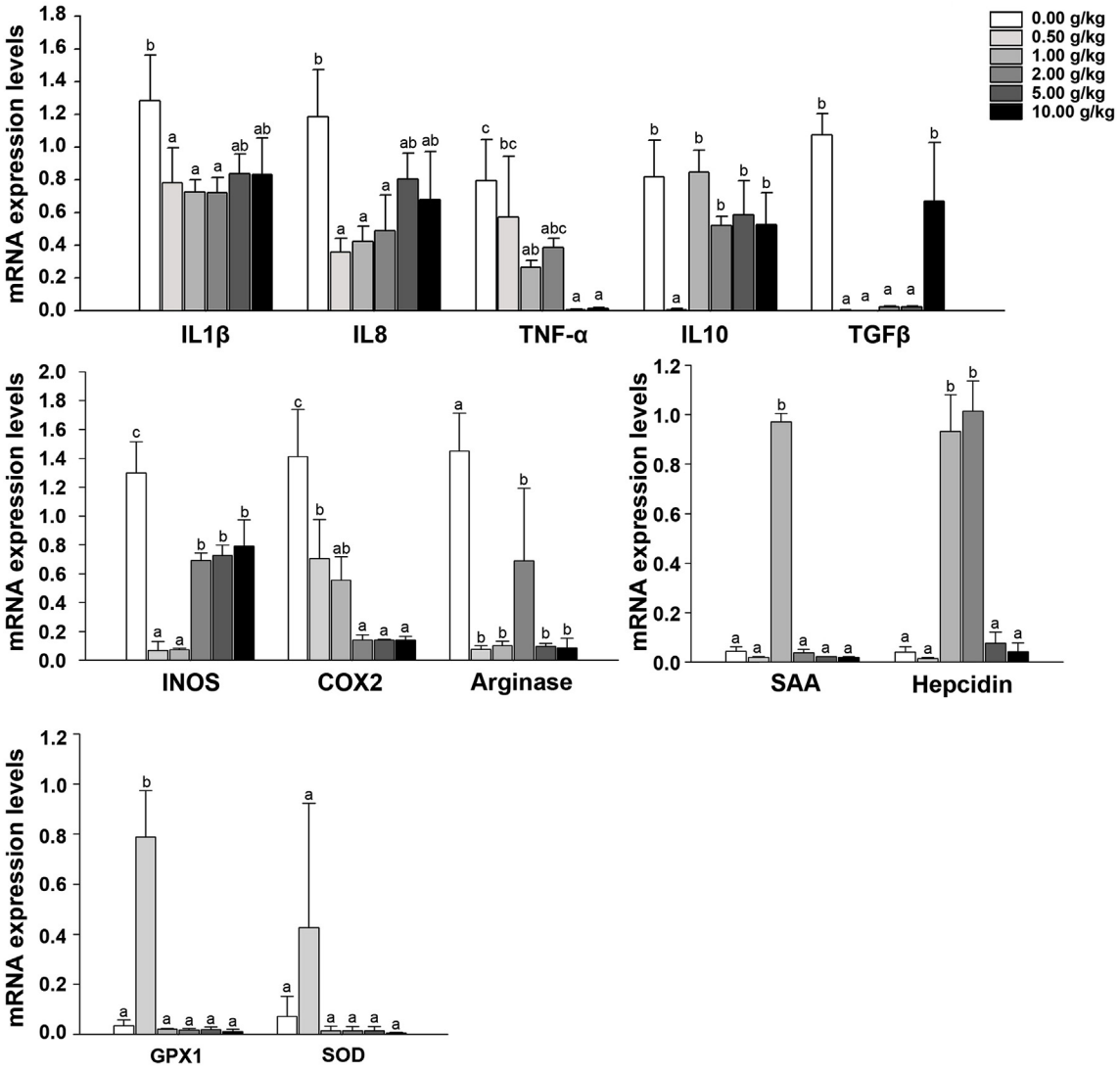

Fig. 2. The mRNA transcription profiles of gene expression in intestine of common carp after 8 weeks fed with different amount of dietary GBLE. Values are means ± SEM of eight replications. Different superscripts on each bar showed significantly different ($P < 0.05$).

3.3. Phagocytic activity

The phagocytic activity of the macrophages from the head-kidney was assayed in all of the groups by determining the phagocytic ratio and phagocytic index. Only the 5 g/kg group showed a significant increase in the phagocytic ratio ($P < 0.05$) (Fig. 1A). The phagocytic indexes increased in the 2, 5 and 10 g/kg groups compared to the control ($P < 0.05$) (Fig. 1B).

3.4. Superoxide anion production

We used superoxide anion production to reflect the respiratory burst activity after 8 weeks of GBLE feeding. The 2, 5 and 10 g/kg groups showed significant increases ($P < 0.05$) of O_2^- production compared to the 0, 0.5 and 1 g/kg groups. No significant differences were observed among the 0, 0.5 and 1 g/kg groups (Fig. 1C).

3.5. Serum lysozyme activity

The 5 and 10 g/kg groups showed significantly enhanced ($P < 0.05$) the lysozyme activity in common carp after 8 weeks of GBLE feeding whereas 0.5, 1 and 2 g/kg did not change the lysozyme activity compared to the control (Fig. 1D).

3.6. Gene expression

After 8 weeks of GBLE feeding, the mRNA expression of cytokine genes (IL1β, IL8, TNFα, IL10, TGFβ), inducible enzyme genes (INOS, COX2, arginase), acute phase protein SAA gene, antimicrobial peptide hepcidin gene and anti-oxidant enzyme genes (GPX1, SOD) in the intestine, head-kidney and spleen were analyzed using qPCR. The results of mRNA analysis showed that cytokine genes tended to decrease with GBLE feeding (Fig. 2- 4).

In the intestine, the IL1β and IL8 mRNA expression were significantly decreased ($P < 0.05$) in the 0.5, 1 and 2 g/kg groups. The expression of TNF-α was significantly downregulated ($P < 0.05$) in the 1, 5 and 10 g/kg groups in the intestine. Only the 0.5 g/kg group had decreased ($P < 0.05$) IL10 intestinal mRNA expression. For intestinal TGFβ, the 0.5, 1, 2 and 5 g/kg groups had significantly decreased ($P < 0.05$) mRNA expression. All of the GBLE treatments downregulated ($P < 0.05$) the expression of inducible enzyme genes significantly in the intestine. The intestinal SAA (1 g/kg group) and hepcidin (1 and 2 g/kg groups) were upregulated ($P < 0.05$) by GBLE treatment. Only the 1.00 g/kg group had increased ($P < 0.05$) GPX1 gene expression. GBLE treatment had no effects on SOD mRNA expression in the intestine (Fig. 2).

In the head-kidney, IL1β, IL8, and TNF-α were significantly

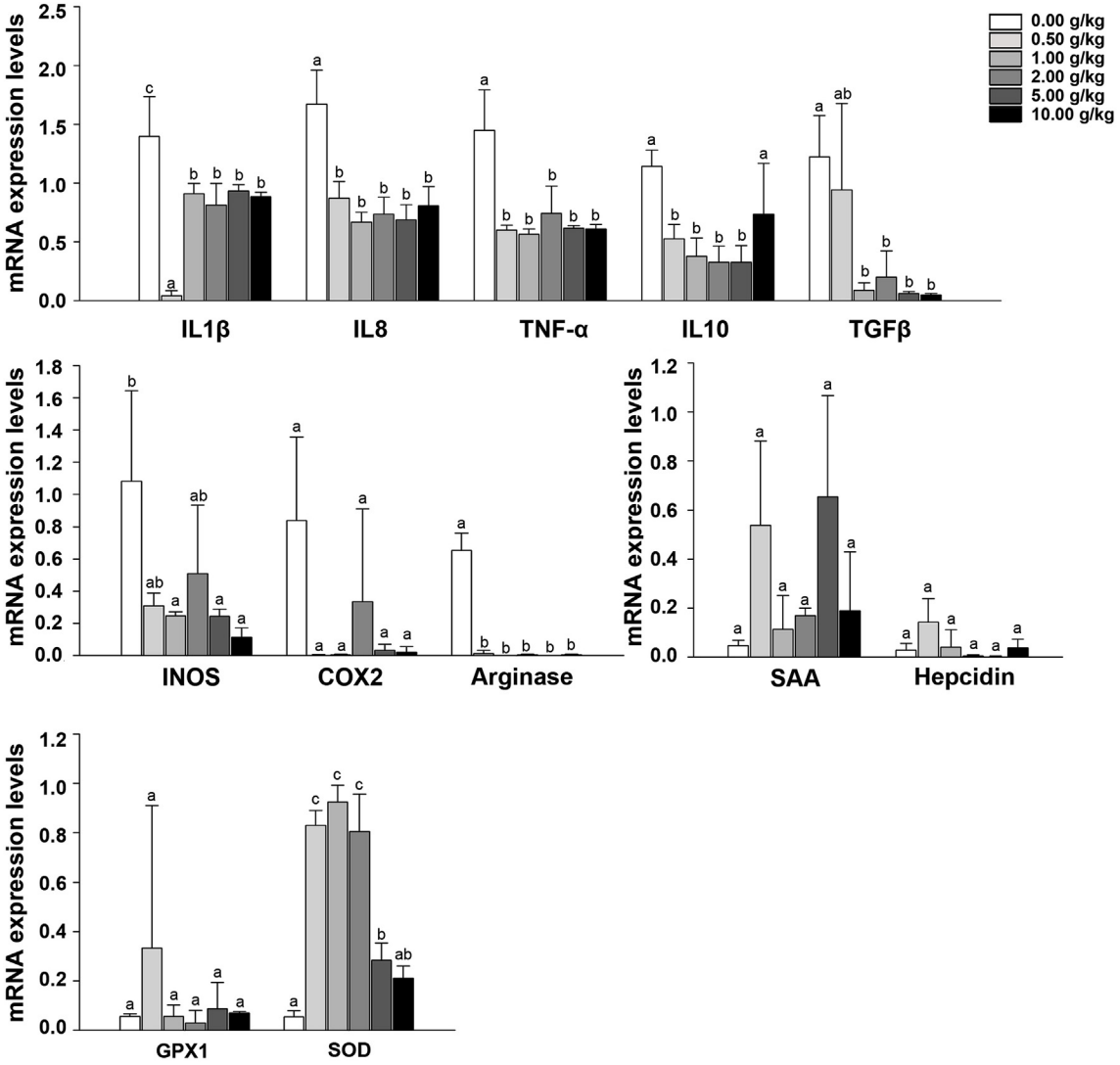

Fig. 3. The mRNA transcription profiles of gene expression in head-kidney of common carp after 8 weeks fed with different amount of dietary GBLE. Values are means ± SEM of eight replications. Different superscripts on each bar showed significantly different ($P < 0.05$).

decreased ($P < 0.05$) in all of the GBLE treatment groups compared to the control group. The 0.5, 1, 2 and 5 g/kg groups had significantly lower ($P < 0.05$) IL10 expression compared to the control. Feeding with 1, 2 and 5 g/kg GBLE decreased ($P < 0.05$) TGFβ significantly in the head-kidney. The mRNA expressions of INOS were downregulated ($P < 0.05$) in the 1, 5 and 10 g/kg groups. No effects of GBLE treatment on COX2 was found in the head-kidney. All of the treatment groups had significantly lower expression ($P < 0.05$) of arginase compared to the control. The expressions of SAA, hepcidin, and GPX1 in the head-kidney were not affected by GBLE. Higher expression ($P < 0.05$) of SOD was found in the 0.5, 1, 2 and 5 g/kg groups than the control group (Fig. 3).

Expressions of IL1β in the spleen were significantly decreased ($P < 0.05$) in the 0.5, 1, 2 and 5 g/kg groups. The 1, 2, 5 and 10 g/kg GBLE treatment had decreased ($P < 0.05$) IL8 expression compared to the control group. All groups, except the 2 g/kg group, had significantly lower ($P < 0.05$) TNF-α expression in the spleen. Only the 1 g/kg group had no significant difference from the control whereas the other groups had significantly decreased IL10 expression ($P < 0.05$). The INOS mRNA levels were significantly downregulated ($P < 0.05$) in the 0.5, 2, 5, and 10 g/kg groups while COX2 and arginase was not affected by GBLE treatment in the spleen. SAA mRNA expressions were increased ($P < 0.05$) in all of the GBLE treatment groups. The 1, 5 and 10 g/kg groups showed higher expression ($P < 0.05$) of hepcidin in the spleen. GPX1 expressions were higher ($P < 0.05$) in the 1, 2, and 10 g/kg groups. Higher expression ($P < 0.05$) of SOD was found in the 1, 5 and 10 g/kg groups than the control group (Fig. 4).

3.7. Disease resistance

Significant effects of GBLE treatment on the survival of fish after injection with *A. hydrophila* were observed. The 0.5, 1, 2, 5 and 10 g/kg GBLE groups showed significant increases in the survival rate (%) compared to the control in common carp (Fig. 5).

4. Discussion

Extracts from plants have been widely studied for their potential effects on promoting growth and resistance against bacterial disease in cultured fish. Previous studies demonstrated that dietary supplementation with GBLE promoted the growth of weaned piglets and hybrid grouper [19,22]. In the present study, diet supplemented with 10 g/kg GBLE significantly increased SGR and decreased FCR in common carp. These consistent results showed that GBLE diets stimulate growth performance in animals, suggesting that GBLE could be a potential feed additive in the aquaculture industry.

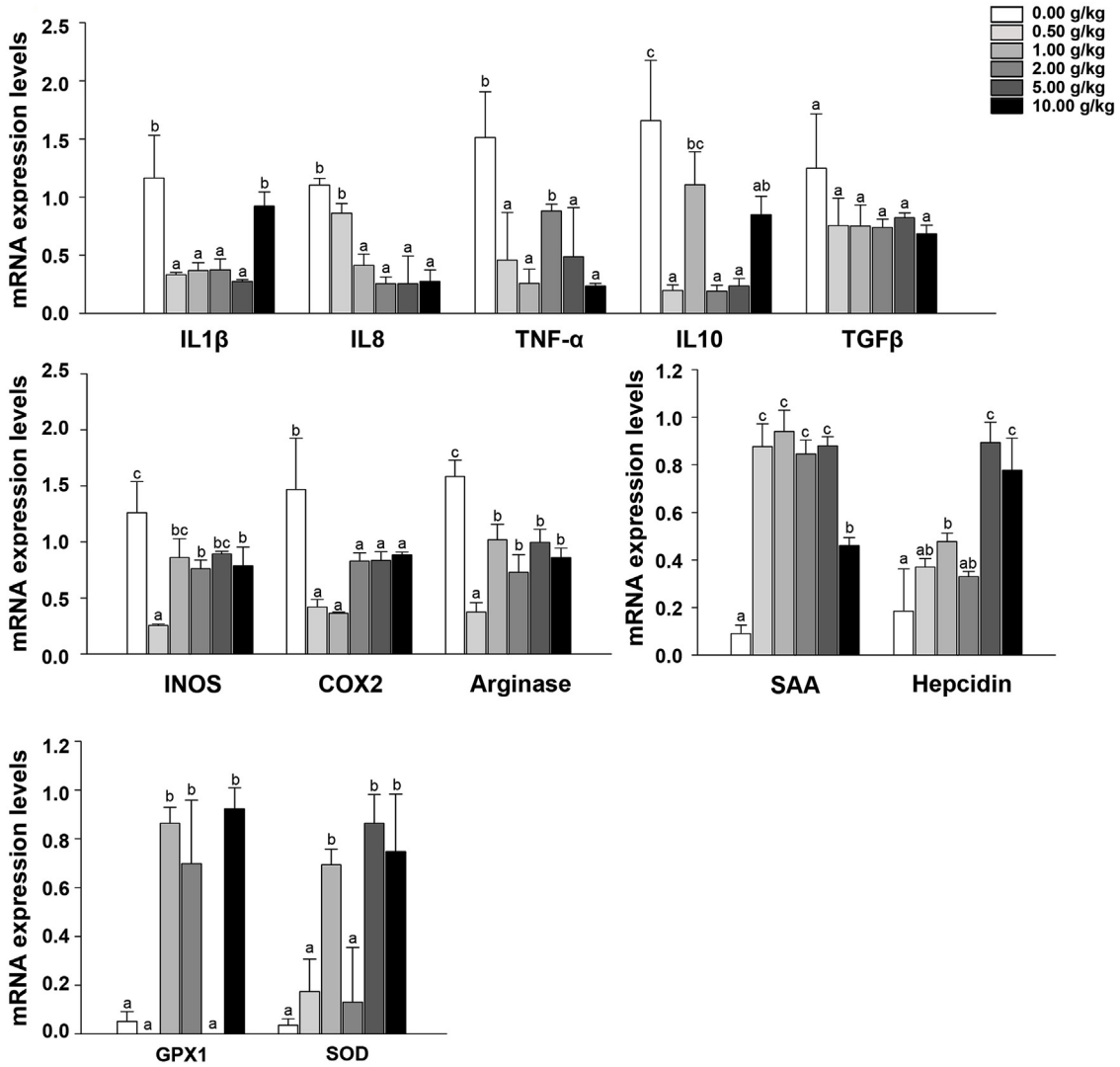

Fig. 4. The mRNA transcription profiles of gene expression in spleen of common carp after 8 weeks fed with different amount of dietary GBLE. Values are means ± SEM of eight replications. Different superscripts on each bar showed significantly different ($P < 0.05$).

The red blood cells, white blood cells, hematocrit and hemoglobin levels were significantly increased in the 10 g/kg group in common carp after 8 weeks of 10 g/kg GBLE feeding. Red blood cells are the backbone of oxygen transportation. The increased red blood cells by GBLE showed the stimulation of oxygen transport capacity. Similarly, *Ginkgo biloba* extract increased heart red blood cells in the thrombotic zebrafish model [33]. White blood cells are responsible for resistance against infectious agents induced by microbial and/or chemical factors. The significantly stimulated white blood cells by GBLE diets compared to the control reflected the general immune system statuses in common carp. The dietary astaxanthin in common carp also increased white blood cells and showed high resistance to *A. hydrophila* [32]. Both hematocrit and hemoglobin were significantly increased in the 10 g/kg group. The hematocrit and hemoglobin levels were induced by 4 weeks of dietary astaxanthin (50 and 100 mg/kg) in common carp, showing positive effects against *A. hydrophila* [32]. Total protein and peptides levels, such as albumin and globulin, reflect a strong innate immunity status in fish [34]. Higher total protein was found after GBLE feeding in common carp in the present study. This result is consistent with findings in hybrid grouper with 0.5 g/kg GBLE diets [22]. Similar results were observed in crucian carp where GBLE diets upregulated serum total protein levels [35]. Albumin is a plasma carrier and can bind to several domains as a nonspecific ligand [36]. Activated albumin by *Ginkgo biloba* extract diet showed a stimulated ability against infection. High levels of globulin indicated the stimulated immune functions in the blood. The immune system can be stimulated by additives, such as garlic [37]. Thus, these biochemical serum changes showed a physiological regulation by *Ginkgo biloba* extract diet and potential effects on the immune system in common carp.

Enhanced phagocytic activity and respiratory burst activity were found in fish responses to various pathogens. In the present study, we found that phagocytic indexes increased after feeding with 2, 5 and 10 g/kg GBLE in common carp. The enhanced or elevated phagocytic activities were also observed in fish treated with various medical herbals [38–40]. The phagocytic cells are crucial components of the innate immune system, and phagocytic activity is a primitive defense mechanism. During the respiratory burst process, phagocytes produce toxic forms of oxygen. Thus, O_2^- production was used to assess the respiratory burst. The increased O_2^- production, namely, induced respiratory burst, was also found in fish fed with a combination of herbs in common carp (*Cyprinus carpio*) [12]. In addition, the serum lysozyme activity was upregulated by 8 weeks of feeding with 5 and 10 g/kg GBLE. Serum lysozyme activity participates in nonspecific defense mechanisms. The stimulated serum lysozyme activity showed activation of nonspecific defense mechanisms, similar to previous findings [41–43]. Thus, the present study indicates that GBLE, as a feeding

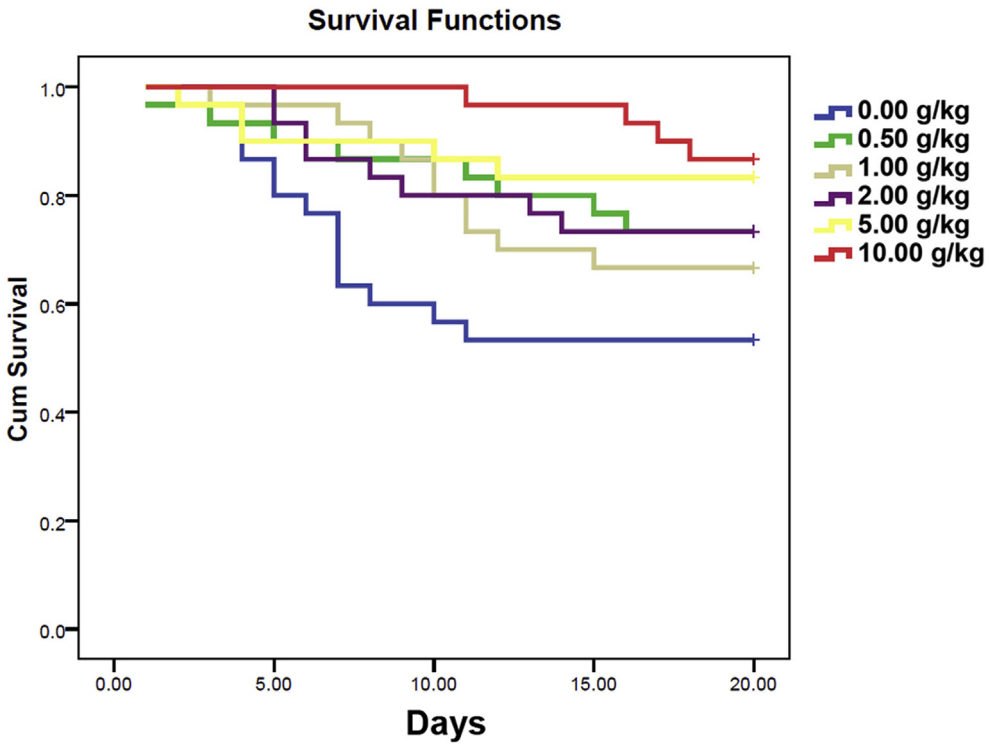

Fig. 5. Kaplan–Meier survivorship curves for common carp after challenge with *A hydrophila* last for 20 days. The fish were fed with GBLE supplemented diets.

additive, could induce antimicrobial mechanisms in common carp, which may regulate phagocytic activity and the production of reactive oxygen species and stimulate serum lysozyme activity.

The results demonstrated that cytokine genes and inducible enzyme genes tended to decrease by feeding with GBLE diets in common carp (Figs. 2–4). Both anti-inflammatory cytokine genes and pro-inflammatory genes were decreased by GBLE diets, guaranteeing a balance of cytokine genes in fish. Either excessive or continuous inflammation is harmful for fishes [44]. Thus, the changes of cytokine genes did not affect the balance in common carp in the present study. The iNOS gene encodes inducible nitric oxide synthase to catalyze nitric oxide production. Additionally, iNOS can be regulated by anti-inflammatory cytokines [45]. Decreased iNOS indicated decreased nitric oxide production. Similarly, The COX2 and arginase expressions were downregulated by GBLE diets. COX2 regulates immunosuppressive TGFβ expression in hepatocytes [46]. However, the present results showed that both COX2 and TGFβ were decreased by adding GBLE. The mechanism of TGFβ inhibition by COX2 was not consistent with the results in hepatocytes, which showed a different mechanism with herbal extracts. Arginase expression is essential for the regulation of the cellular immune response and the inflammatory process. Lower expression of arginase was also found in common carp by *Ginkgo biloba* extract, indicating inhibited amino acid metabolism, similar to the results in human hepatocellular carcinoma cell lines [47]. The SAA gene is an acute phase gene that participates in lipid transportation and metabolism during inflammatory processes. Hepcidin controls iron levels in tissues and affects infection outcomes [48]. In the present study, except in the head-kidney, SAA and hepcidin expression levels were increased in at least one dose of the GBLE group of common carp. These results suggested the stimulation of acute phase protein and antimicrobial activities in common carp by GBLE. The anti-oxidant enzyme genes tended to increase in common carp (Figs. 3 and 4). Similarly, these effects in rainbow trout (*Oncorhynchus mykiss*) by *Rhodomyrtus tomentosa* leaf extract injection were also demonstrated [49], suggesting an improved resistance to inflammation, stress, or infectious diseases in fish.

Developing new immunostimulants to combat pathogenic microorganisms is important for aquaculture. To date, several studies have focused on improving resistance to bacterial infection by medicinal herbal supplementation. The synergistic activity between *Rehmannia glutinosa* extract and *A. hydrophila* on the survival rate was demonstrated in common carp [31]. These results suggested that orally delivering GBLE leads to an increased anti-microbial activity. This finding might be due to the regulation of nonspecific immune parameters, including phagocytic activity, superoxide anion production, and serum lysozyme activity. These process changes may be mediated by immune-related genes. Our research showed that medicinal herbs are potential resources for ameliorating pathogenic infection.

In conclusion, this study indicated that dietary supplementation of GBLE has potential functions improving immune responses by regulating related gene expression in common carp and the optimum concentration we recommend is 10 g/kg of GBLE. Thus, GBLE could be used in the aquatic feed industry to deter *A. hydrophila*. Further studies on practical approaches during culturing periods in the aquaculture industry are needed.

Conflicts of interest

The authors declare no competing interests.

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