



Full length article

Modulation of innate immunity, expression of cytokine genes and disease resistance against *Aeromonas hydrophila* infection in goldfish (*Carassius auratus*) by dietary supplementation with *Exiguobacterium acetylicum* S01

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ABSTRACT

Probiotic strains play an increasing role in the production of healthy animals used as a food source. Elucidating the mechanisms of action that allow probiotic-driven immunomodulation may facilitate different applications such as the prevention of infectious diseases in food organisms. This study elucidates the probiotic effects of *Exiguobacterium acetylicum* S01 on the growth, haematological profile, innate immune capacity, expression of cytokine genes, and resistance to diseases of *Carassius auratus* caused by *Aeromonas hydrophila* infection. Three fish groups were fed with the following diets containing different doses of *E. acetylicum* S01 (CFU g⁻¹): basal diet 0 (BD, without probiotic), 2.5 × 10⁷ (DI) and 2.7 × 10⁹ (DII)–CFU g⁻¹ for 4 weeks. After 4 weeks, the fish were injected intraperitoneally with *A. hydrophila* and the percentage of survival was recorded over 21 days of post-challenge. Results revealed that dietary supplementation of *E. acetylicum* S01 significantly ($P < 0.05$) enhanced the growth, haematological profile and cellular immune responses including respiratory burst, phagocytic activities and antimicrobial enzymes (myeloperoxidase and lysozyme) and total immunoglobulin levels were improved by probiotic feeding at both occasions. Comparatively, expression of c- and g-type lysozyme followed by pro- and anti-inflammatory cytokines (IL-1β, IL-10 and TGFβ) was up-regulated in kidney, head-kidney and spleen. Moreover, fish fed with diet DII had a significantly higher ($P < 0.05$) survival rate (73.2%) after challenging. The survival rate was only 33.2% of the BD group against *A. hydrophila* infection. Our results revealed that *E. acetylicum* S01 delivered probiotic in feed exerts its influence on growth performance and provides disease resistance by stimulating the immune system at the cellular and molecular levels in *C. auratus*.

1. Introduction

The global aquaculture production was considerably increased in the last decade. Fish farming is one of the fastest growing and most promising economic sectors for food production worldwide [1]. According to the Food and Agriculture Organization (FAO) reports, infectious diseases caused by pathogenic bacteria provide a substantial drawback on the considerable industrial development of aquaculture. Among the etiological agents of bacterial fish diseases, *Aeromonas hydrophila* is considered one of the most virulent species, causing substantial mortalities and economic losses to freshwater fish production [2]. Newly emerging multidrug-resistant pathogens are providing a particular threat by causing acute or chronic infectious disease viz.,

hemorrhagic septicemia, exophthalmia and ulcer disease in fish [3]. Such pathogens are also causing gastrointestinal infection in humans [4]. Antibacterial antibiotics are questioned for their effectivity since many are increasingly ineffective due to antibacterial resistance [5]. As a result of extensive research, the dietary supplementation of probiotic (s) appears as a promising alternative to antibacterial antibiotics considering their nutritive value and to overcome the adverse effects of antibiotics and drugs [6].

Bacterial strains belonging to *Bacillus*, *Lactobacillus* and *Saccharomyces* are most widely used in probiotic applications in aquaculture. Their health-promoting characteristics include the maintenance of the gut barrier function, and the innate and adaptive modulation of the host immune system [7–9]. In recent years, remarkable

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progress was made in characterizing fish cytokine genes [10]. Especially, stimulatory and inhibitory molecules are widely studied to assess immune-regulatory mechanisms in various fish [6,11]. These include interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), pro-inflammatory cytokines, and anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor- β (TGF β). These cytokines are produced by immune cells and contribute to the defense mechanisms of the fish upon bacterial infection [6]. The most important function of IL-10 is reducing excessive inflammatory responses [12]. Lysozymes (c- and g-type) are important innate immune factors in the fish immune system, which plays a vital role in resistance against the invasion of bacterial pathogens by the action of antimicrobial activity [13]. Nevertheless, studying the modulation of immune responses and characterization of transcription levels in cytokine genes in response to probiotic supplementation appears as a promising way to enhance the immunological response in fish. However, the growth performance and immunostimulatory effects of probiotic *Exiguobacterium acetylicum* S01 on the teleost goldfish (*Carassius auratus*) were not studied intensively but require also additional exploration. Our research group has recently isolated and evaluated the probiotic attributes of *E. acetylicum* S01 from soils [14]. The strain *E. acetylicum* S01 was shown to effectively inhibit the growth of the fish pathogen *A. hydrophila* and *Vibrio* species *in-vitro* study [14]. Furthermore, S01 exhibited higher adhesion ability to hydrocarbons and mannose-specific adhesion (msa) gene expression. Strain S01 also provided susceptibility towards a broad spectrum of antibiotics, and a high degree of resistance to low pH (1.0–4.0) and higher (6%) bile salt concentrations under *in-vitro* conditions. An *in-vivo* study further demonstrated that *Artemia* nauplii treated with strain S01 showed higher survival and individual life expectancy [14]. Hence, we ascertain that *E. acetylicum* S01 provides immunostimulation in *Carassius auratus*. Therefore, the objective of the present investigation was to pursue the hypothesis that dietary administration of *E. acetylicum* S01 could stimulate the growth, haematological profile, innate immunity, and expression of cytokines in lymphoid organs (kidney, head-kidney and spleen) of *C. auratus* and to determine its disease resistance ability against *A. hydrophila* infection.

2. Material and methods

2.1. Bacterial strain

The novel pigmented probiotic *E. acetylicum* S01 was previously isolated from indigenous soil sediments of the Sathiyar river basin, Tamil Nadu, India. The identity of this bacterium was genetically confirmed applying 16S ribosomal RNA gene sequencing, and it exhibited desirable functional probiotic attributes as described earlier [14], which was used in this study. This bacterium was grown in nutrient broth (HiMedia; AS061) for 48–72 h at 37 °C. Thereafter, the bacterial cells were harvested using a centrifuge at 6500 \times g for 15 min and washed twice in phosphate buffer saline (1X PBS, pH 7.2) to obtain solid yellowish orange pigmented bacterial biomass that was used as a probiotic diet formulation.

2.2. Diet formulation

Experimental diets were made following the procedure described by Kumar et al. [15] with slight modification. The formulation of the basal diet was shown in Table 1 and proximate composition was analysed using the Association of Official Analytical Chemists method [16] revealing 43.2% crude protein, 52.9% carbohydrate and 24% ash, respectively. The dough was prepared by adding water to the above ingredients. The material was then steam sterilized (autoclaved at 120 °C for 20 min) and pelletized by hand to get pellets of 3 mm size. The pellets were dehydrated in a forced-air oven at 55–60 °C for 48 h when the final moisture content < 15%. After drying, the bacterial biomass was mixed with 2.5% vegetable oil onto the basal diet (BD) at two

Table 1
Formulation of the experimental diet (g%).

Ingredients	Percentage of dry weight (g%)		
	Basal diet	Diet-I	Diet-II
Fishmeal	14	14	14
Ragi flour	15	15	15
Wheat flour	14	14	14
Corn flour	17	17	17
Rice bran	10	10	10
Groundnut oil cake	18	18	18
Tapioca power	5	5	5
Vegetable oil	5	5	5
Vitamin premix ^a	1	1	1
Mineral premix ^b	1	1	1
<i>E. acetylicum</i> CFU g ⁻¹	–	2.5 \times 10 ⁷	2.7 \times 10 ⁹

^a Vitamin (IU or mg per kg premix): Vitamin A, 700000IU; Cobalamin, 200IU; Vitamin D₃, 70000IU; Vitamin E, 250 mg; Nicotinamide, 1 g.

^b Mineral premix (per kg premix): Cobalt, 150 g; Copper, 1200 mg; Iodine, 325 mg; Iron, 1500 mg; Magnesium, 6000 mg; Manganese, 1500 mg; Potassium-100 mg; Sodium, 5.9 mg; Sulphur, 0.72%; Zinc, 9600 mg; Calcium, 25.5%; Phosphorus, 12.75%.

different doses 2.5 \times 10⁷ and 2.7 \times 10⁹ CFU g⁻¹ in the respective test diets (DI and DII) whereas the vegetable oil was only mixed with the BD group. The basal and probiotic test diets were packed in airtight plastic containers and stored at –20 °C until use. The amounts of *E. acetylicum* in their respective diets were determined on day 0, 2 and 4 weeks of storage by the spread plate method on nutrient agar. After 2 weeks, we did not find any viability loss, whereas a viability loss of > 5% was observed after 4 weeks of storage. Therefore, the same diets were provided through the feeding trial.

2.3. Ethical statement and experimental design

Laboratory animal care principles were followed and experimental procedures were conducted according to the guidelines established by the National Institutes of Health [17], and every effort was made to minimize suffering. This study was approved by the institutional ethical committee of Madurai Kamaraj University (Ethical Clearance (EC), Biosafety and Animal Welfare Committee). Healthy goldfish (*C. auratus*) were obtained from the local fish farm, Madurai, Tamilnadu, India. Before experimentation, the goldfish were not been vaccinated nor exposed to fish diseases and were considered free of pathogens by common microbiological and microscopic examinations of skin, gills, kidney tissues and intestines of representative samples. Before probiotic administration, fish were acclimatized for 2 weeks in 150-L rectangular glass aquaria to laboratory conditions at 28 \pm 2 °C. During the acclimatization period, all fish have received a basal diet. After acclimatization, fish were divided randomly into three experimental groups (Control (without probiotics), DI-2.5 \times 10⁷ and DII-2.7 \times 10⁹ CFU g⁻¹) with three replicates in each treatment group. Each group consisted of 30 fish (10 fish \times 3 tanks = 30 fish). The fish were fed with probiotic diets DI-2.5 \times 10⁷ and DII-2.7 \times 10⁹ CFU g⁻¹ of *E. acetylicum* whereas the basal diet (BD) was only provided to the control group. Goldfish received the experimental diet at the rate of 3% of initial body weight twice a day (at 09:00 and 17:00 h) to approximate satiation (*ad libitum*) for 4 weeks post-feeding trial. The water of the tanks was exchanged daily, partially, to remove waste and fecal materials. Water quality of each tank was maintained at an optimal range of physical parameters; temperature (28 °C); pH (7.3–7.9); ammonia-nitrogen (0.1–0.35 mg L⁻¹) and dissolved oxygen (6.3 mg L⁻¹) during the experimental period. The day/night cycle was maintained at a constant change of 12 h light and 12 h dark.

2.4. Effects of *E. acetylicum* S01 on the growth performance of goldfish

The growth performances of goldfish were measured as the difference between the initial weight at the start of the experiment on day 1, and the final weight at the end of the 2 and 4 weeks. All fish from the individual tank ($n = 3/\text{treatment group}$) were weighted at the end of the 2 and 4 weeks, approximately 12 h after the last feeding. The fish were weighted by a digital scale (electronic balance) after they had been anaesthetized. Based on the results of the weight, the daily ration of the fish in the probiotic-supplemented groups and in the control group was determined. At the end of the 2 and 4 weeks of probiotic feeding trial, weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate were calculated according to the following formula: $\text{WG (\%)} = [(\text{FW} - \text{IW})/\text{IW}] \times 100$; $\text{SGR (\%)} = [(\text{Ln FW} - \text{Ln IW})/T] \times 100$; $\text{FCR} = \text{FI}/\text{WG}$; $\text{Survival (\%)} = (\text{final number of fish survived}/\text{initial number of fish stocked}) \times 100$. Where, FW and IW were the final and initial weight of the fish, respectively; T is the feeding duration (days); FI represents the feed intake.

2.5. Blood and tissue sample collection

At the conclusion of the post-feeding trial at 2 and 4 weeks, fish from each tank was randomly selected; the blood and serum samples were examined immunologically for cellular changes related to dietary modifications. Three fish from each of the three replicate tanks were anaesthetized by bath immersion with $50 \mu\text{L L}^{-1}$ clover oil (Himedia; GRM340) for 5 min. After anaesthesia, blood samples were retrieved from the caudal vein using a 1.0 mL hypodermic syringe and 24 gauge needles, which was rinsed with 0.5 M ethylene diamine tetra-acetic acid (EDTA) solution before use. Blood samples were divided into two aliquots; one aliquot was used for haematological assessment (with EDTA) while another aliquot was transferred into a tube without EDTA, placed at room temperature for 30 min and centrifuged at $3500 \times g$ for 10 min at 4°C . Supernatants (plasma and serum) were stored at -80°C until analysis. For immune-related gene expression study, kidney, head-kidney and spleen samples were collected from anaesthetized fish in each time interval based on the anatomy, then immediately stored at -80°C in TRIzol reagent until RNA isolation.

2.6. Haematological parameters

Haematological parameters were studied from freshly collected blood samples that were transferred to a tube containing EDTA for complete blood cell investigation. The total white blood cells (WBC), red blood cells (RBC), and platelet cells (PLT) and the differential white blood cells were enumerated using an automated haematology analyzer (Cobas Micros-18 OT, France) [18]. Haemoglobin levels (Hb) were determined to apply cyanomethemoglobin spectrophotometry method as reported by Kumar and collaborators [15].

2.7. Immune parameters analysis

2.7.1. Respiratory burst activity

A respiratory burst activity assay was used to measure the production of intracellular superoxide anion (O_2^-) by phagocytes as previously described by Stasiack and Bauman [19] with slight modification. Concisely, $100 \mu\text{l}$ of EDTA blood was transferred to 96 well plates and incubated for 1 h to allow the adhesion of phagocytic cells. After adhesion, the supernatants were removed and washed twice with 1X PBS followed by the addition of 0.2% (w/v, 2 mg mL^{-1}) nitroblue tetrazolium (NBT, $100 \mu\text{l}$). To induce respiratory burst $10 \mu\text{l}$ of stimulus (Zymosan, Sigma-Aldrich) were used. Following 1 h incubation, the cells were fixed in 100% methanol, washed twice with 70% methanol, and then air dried. The formazan deposits were solubilized in $120 \mu\text{l}$ 2N potassium hydroxide (KOH) and $140 \mu\text{l}$ dimethyl sulphoxide (DMSO, Sigma). The absorbance of the turquoise-blue coloured solution was

measured in a microplate reader at 620 nm using 2N KOH/DMSO as a blank.

2.7.2. Cellular assay

Phagocytic activity was studied using *Staphylococcus aureus* (ATCC 29213) described by Anderson and Siwicki, [20]. Briefly, an equal volume (0.1 mL) of whole blood cells and *S. aureus* ($1.2 \times 10^7 \text{ CFU mL}^{-1}$) suspension were placed into 96 well plates and incubated at room temperature for 30 min. Thereafter, $10 \mu\text{l}$ of this cell suspension was smeared on glass slides. The smear was air dried, then fixed with ethanol (95%) for 5 min and air dried. The air-dried smears were stained with 7% Giemsa solution (Himedia, TCL083) for 10 min. The number of phagocytic cells per 100 adhered cells was enumerated microscopically. Phagocytic activity (PA) was calculated by applying the formula: $\text{PA} = (\text{phagocytic leucocytes}/\text{total leucocytes}) \times 100$.

2.7.3. Myeloperoxidase (MPO) activity

The production of endogenous peroxidases was estimated using chromogenic substrate 3',5'-tetramethylbenzidine (TMB, Himedia), according to the method of Kumari and Sahoo [21] with slight modification. Here, $15 \mu\text{l}$ of serum were immersed in $135 \mu\text{l}$ of Hank's balanced salt solution (Sigma, RNB0710) in microtitre plates. Thereafter, $25 \mu\text{l}$ of 20 mM 3',5'-tetramethylbenzidine (TMB) and $25 \mu\text{l}$ of 5 mM hydrogen peroxide (freshly prepared) were added. After 5 min the reaction was interrupted by adding $50 \mu\text{l}$ of 4 M sulfuric acid. The supernatants were taken by centrifugation at $6500 \times g$ for 10 min, which was transferred ($150 \mu\text{l}$) to flat bottom 96 well plates. The absorbance was measured at 450 nm in a microplate reader (BioRad, USA). The wells without serum were used as blanks.

2.7.4. Lysozyme activity

Serum lysozyme activity was studied following the protocol provided by Ellis [22]. Concisely, $200 \mu\text{l}$ of *Micrococcus lysodeikticus* (ATCC 4698) (2 mg mL^{-1}) suspension in 0.05 M sodium phosphate buffer, pH 6.2 was taken, to which $50 \mu\text{l}$ of serum sample was added to a microplate. The reaction was done at 25°C and the absorbance was read at 450 nm after 0.5 and 10 min. A unit of lysozyme activity was defined as the enzyme amount reducing the absorbance by $0.001 \text{ min}^{-1} \text{ mL}^{-1}$ serum.

2.7.5. Total immunoglobulin (Ig) in plasma

Immunoglobulin levels were estimated following Kumar et al. [15]. Plasma (0.1 mL) was transferred to a plastic serum vial and the same volume of polyethylene glycol (PEG) (12%) was added and incubated at 37°C for 2 h with constant mixing. The supernatant was taken out by centrifugation at $7500 \times g$ for 10 min. The protein concentration in the supernatant was analysed following the method of Lowry and collaborators [23]. The total immunoglobulin level was determined by subtracting the protein content in the supernatant (with PEG treatment) from the total protein content in the plasma (without PEG treatment). Total Ig levels were expressed as mg mL^{-1} .

2.8. RNA isolation and gene expression analysis

Total RNA was isolated from each tissue sample (50–100 mg) using a TRIzol Reagent (TaKara, Japan) following the manufacturer's protocol. RNA concentrations and purity were determined using a NanoDrop-1000 (Thermo fishers, USA). The RNA was documented by ethidium bromide staining of 28S and 18S ribosomal RNA bands on a 2% agarose gel. Any DNA contamination was removed by applying the RNeasy MinElute cleanup kit (Qiagen). The resulting RNA samples were stored at -80°C until later use. One microgram of RNA was used for cDNA synthesis using a RevertAid cDNA synthesis kit (Thermo Fisher Scientific, USA) following the instructions of the company. The primers were designed based on the known sequence for immune-related genes in *C. auratus*. Primer sequences used in this study were shown in

Table 2
List of primers used for sqRT-PCR amplifications.

Gene	Genebank accession number	Amplicon size (bp)	Primer
IL-1β1	AJ249136.1	268	FWD: 5'-TTCATTTGAAGGCAGTGACG-3' REV: 5'-TAAGCTGTGCCCGTCTCTTT-3'
IL-10	HQ259106.1	268	FWD: 5'-CTTGCCAAAATCCCTTTGAG-3' REV: 5'-AGGGTGAAGTCCATTGTGC-3'
TGFβ	EU086521.1	283	FWD: 5'-ACCATATGCCAAAGCCTCAC-3' REV: 5'-TGATGCCTATACAGCGCAAG-3'
CLYZ	KR092198.1	275	FWD: 5'-GCCGAAATGCCTGAAATAA-3' REV: 5'-GTGGTCTGGCATCGATATT-3'
GLYZ	KM100713.1	300	FWD: 5'-CCGTATCTTCAAGCGAGAGG-3' REV: 5'-CTCCAGGTGTCCCATGATTT-3'
β-actin	AB039726.2	267	FWD: 5'-TGCTGACCGTATGCAGAAAG-3' REV: 5'-TTGAGAGGTTGGGTTGGTC-3'

IL: interleukin; TGF: transforming growth factor; CLYZ: C-type lysozyme; GLYZ: g-type lysozyme; pb: basepair.

Table 2. The semi-quantitative reverse transcriptase-polymerase chain reaction (sqRT-PCR) was performed using the Applied Biosystems (ABI) Veriti Thermal Cycler (ABI, USA). The sqRT-PCR was conducted using reaction mixture (20 μl) containing 10 μl Taq Master Mix (2X; AmpliQon), 2 μl of each primer (Sigma-Aldrich), 4 μl of the template (4 μg) followed by 2 μl of Milli-Q water. The samples made an initial denaturation of 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 45 s at 55 °C and 1 min at 72 °C followed by an elongation of 7 min at 72 °C. All sqRT-PCRs were run for at least three times. β-actin served as a housekeeping gene in order to normalize the expression levels.

2.9. Challenge experiments

Aeromonas hydrophila (ATCC 7966) was purchased from American Type Culture Collection Centre (ATCC) in Virginia, USA and it was grown for 24 h at 37 °C in nutrient broth (Himedia, AS061). In order to confirm, the isolate was verified by biochemical test and it was maintained in the laboratory on nutrient agar slant at 4 °C. The 7 day lethal dose 50 (LD₅₀), determined by intraperitoneal injection of graded doses of *A. hydrophila* (10⁴, 10⁵, 10⁶ and 10⁷ CFU/fish), into 10 fish. The LD₅₀ value of 10⁵ CFU mL⁻¹ was confirmed by our earlier study [14]. Following a period of 4 weeks feeding the respective diets, a subsample of the remaining fish from each tank was experimentally exposed to live *A. hydrophila*. Twelve fish were taken randomly from each treatment; 4 fish/tank were then intraperitoneally injected with 0.2 mL of *A. hydrophila* suspended in 1X PBS with a dose of 1.5 × 10⁵ CFU/fish [14]. A group of twelve fish (n = 12; fed with basal diet during feeding trial) were sham injected with 0.2 mL of 1X PBS as a negative control to get some clues about the effects of post-infection due to cohabitation. The challenged fish were kept under observation for 21 days of post-infection and all fish groups were fed only the BD. Confirmation that infection was actually accomplished, this observation was carried out by re-isolating the *A. hydrophila* from the dead fish intestine using *Aeromonas* isolation medium (Himedia; M884). Relative percentage survival (RPS) was quantified using the following formula: RPS (%) = (No. of surviving fish after challenge/No. of fish injected with *A. hydrophila*) × 100.

2.10. Statistical analysis

All experimental data were expressed as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyse the data. Multiple comparisons were performed with Dunnett's test to analyse the difference between treatments. Comparisons between the two groups were performed with the unpaired parametric student's t-test. All statistical analyses were performed using the Windows-based Graph pad prism statistical software (San Diego, CA, USA). The level of significance was accepted if the null hypothesis was rejected at P ≤ 0.05.

3. Results

3.1. Growth performances

First, we observed the body weight and length of goldfish growth was increased in the probiotic-supplemented diets compared to the basal diet (see supplementary data, Fig S1A). The growth parameters of goldfish fed with different doses of *E. acetylicum* as diet supplements were given in Table 3. Diet supplementation with *E. acetylicum* had significant (P < 0.05) effects on WG and SGR compared to BD at 2 and 4 weeks. The percentage of WG (51.92 ± 4.59%; P = 0.0003) and SGR (102.08 ± 9.74%; P = 0.0004) was significantly higher in the diet DII probiotic level compared to BD and DI at 4 weeks. There was a significant drop in the feed conversion ratio of diet DII compared to BD, whereas no significant difference occurred in the DI group at 2 and 4 weeks. However, the FC ratio was decreased in the probiotic-supplemented diets, DI (2.85 ± 0.10) and DII (1.02 ± 0.97), compared to fish fed with BD (3.12 ± 0.10) at the end of probiotic feeding trial.

3.2. Haematological studies

As for the haematology parameters of healthy goldfish at 2 and 4 weeks, the total RBC, WBC and PLT counts were significantly higher (P < 0.05) in the treatment where fish were fed with diet DII than the

Table 3

Growth performances of *C. auratus* after 2 and 4 weeks of feeding with basal diet and diets supplemented with different doses level of *E. acetylicum* S01.

Sampling point	Growth parameters	BD	DI	DII
0–2 weeks	Initial weight (g)	58.15 ± 1.84	58.13 ± 1.56	58.95 ± 1.38
	Final weight (g)	61.84 ± 1.90	62.04 ± 2.08	71.44 ± 1.62 ^a
	Weight gain (%)	6.34 ± 0.82	6.70 ± 0.82	21.18 ± 0.53 ^c
	SGR (% day)	24.56 ± 3.16	26.03 ± 3.73	83.26 ± 2.44 ^c
	FCR	2.73 ± 0.33	2.81 ± 0.14	0.98 ± 0.06 ^a
	Survival (%)	100	100	100
0–4 weeks	Initial weight (g)	58.15 ± 1.84	58.13 ± 1.56	58.95 ± 1.38
	Final weight (g)	68.01 ± 2.29	69.98 ± 1.61	89.58 ± 3.69 ^a
	Weight gain (%)	16.93 ± 0.41	20.36 ± 0.96	51.92 ± 4.59 ^b
	SGR (% day)	32.84 ± 1.62	39.49 ± 2.79	102.08 ± 9.74 ^b
	FCR	3.12 ± 0.10	2.85 ± 0.10	1.02 ± 0.97 ^b
	Survival (%)	100	100	100

Values are represented as mean ± SD (n = three replicates in treatment). BD – Basal diet, DI – 2.5 × 10⁷ CFU g⁻¹; DII – 2.7 × 10⁹ CFU g⁻¹ ^a***P < 0.001 vs BD, ^b****P < 0.001 vs BD, ^c****P < 0.0001 vs BD.

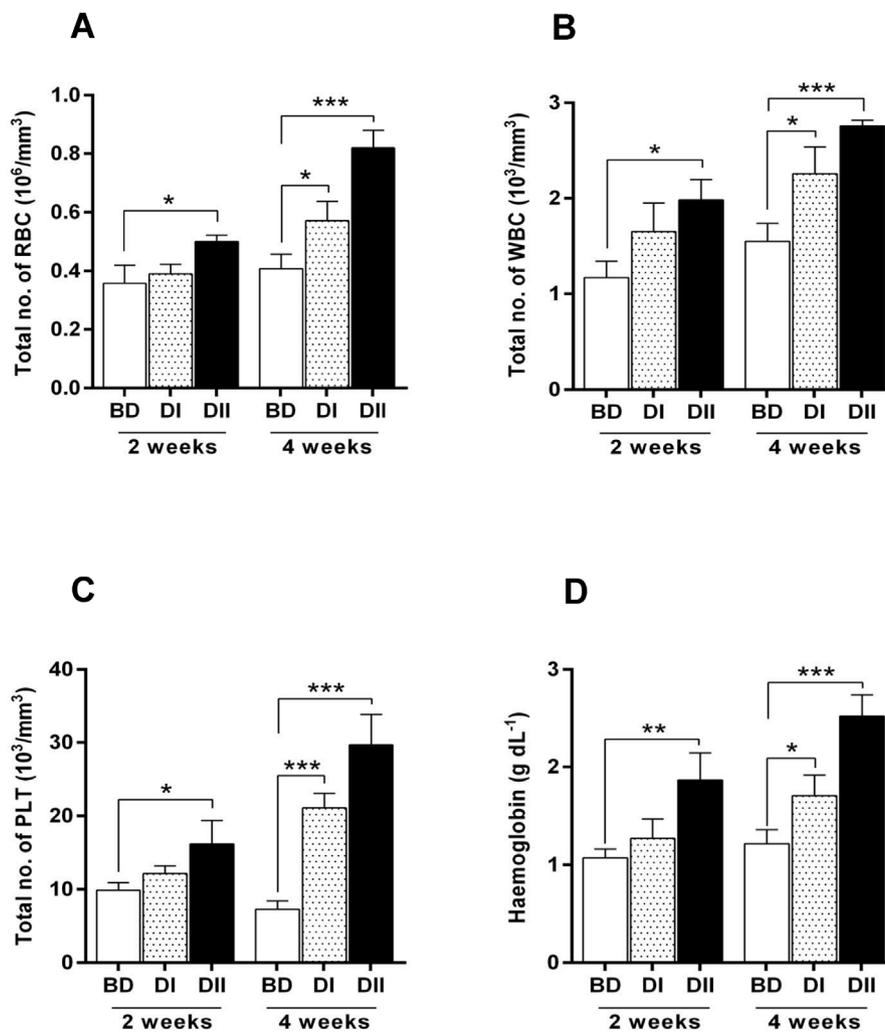


Fig. 1. Haematological parameters of *C. auratus* fed with basal diet (BD) and *E. acetylicum* S01 supplemented diets at 10^7 (DI) and 10^9 (DII) CFU g^{-1} . Each bar represents the mean \pm SD (n = 9). Bar graphs showing red blood cell count (A), white blood cell count (B), platelet count (C), and haemoglobin content (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicates a significant difference between the basal diet in response to *E. acetylicum* supplemented diets.

Table 4

Differential blood cell counts of *C. auratus* fed with basal diet (BD) and *E. acetylicum* S01 containing diets at 10^7 (DI) and 10^9 (DII) CFU g^{-1} for 2 and 4 weeks.

Leukocyte type	BD	DI	DII
2 weeks			
Neutrophil	38.5 \pm 1.96	41.33 \pm 1.74	43.9 \pm 1.99 ^a
Lymphocyte	29.5 \pm 3.21	33.81 \pm 1.23	34.76 \pm 1.84 ^a
Monocyte	17.7 \pm 1.4	17.11 \pm 1.94	17.21 \pm 1.41
Eosinophils	5.6 \pm 0.26	2.9 \pm 1.07 ^a	1.66 \pm 0.44 ^b
Basophils	5.66 \pm 0.98	4.38 \pm 1.10	3.9 \pm 1.58
4 weeks			
Neutrophil	42.43 \pm 1.98	45.0 \pm 2.15	45.56 \pm 0.81
Lymphocyte	30.9 \pm 1.39	34.81 \pm 1.15	36.43 \pm 2.60 ^a
Monocyte	17.73 \pm 3.49	12.15 \pm 1.65	12.73 \pm 1.12
Eosinophils	3.01 \pm 1.24	3.33 \pm 0.77	1.73 \pm 0.90
Basophils	5.76 \pm 1.09	4.55 \pm 1.07	4.2 \pm 0.74

Values are represented as mean \pm SD (n = 9). Mean values are expressed percentage (100%) in different blood counts. BD - Basal diet; DI - 2.5×10^7 CFU g^{-1} ; DII - 2.7×10^9 CFU g^{-1} ^a * $P < 0.05$ vs BD, ^b ** $P < 0.001$ vs BD.

fish fed with the BD group (Fig. 1). We found RBC count showed a significant increase in the group of fish receiving diet DII (0.81 ± 0.05 cells mm^3 ; $P = 0.0008$), whereas the RBC count was only

0.40 ± 0.04 cells mm^3 in the BD group at 4 weeks (Fig. 1A). Likewise, the leukocytes and thrombocyte counts were significantly increased in the diet DII (2.76 ± 0.05 cells mm^3 ; $P = 0.0004$) and (29.66 ± 3.75 cells mm^3 ; $P = 0.0009$) in comparison to the fish group fed with the BD group (1.55 ± 0.16 cells mm^3) and (7.27 ± 1.02 cells mm^3) respectively, at the end of 4 weeks (Fig. 1B and C). Also, the haemoglobin content was significantly higher in the same diet DII (2.52 ± 0.19 g dL^{-1} ; $P = 0.0010$) compared to BD (1.07 ± 0.07 g dL^{-1}) group (Fig. 1D). Consequently, the total number of circulatory leukocyte counts (neutrophil and lymphocyte) were significantly elevated ($P < 0.05$) in the probiotic diet DII compared to the BD group at 2 weeks (Table 4). There was no significant difference in the diet DI fish group. The eosinophil counts were significantly ($P < 0.05$) reduced in the probiotic diet group compared to BD at 2 weeks. Moreover, only the lymphocyte count was significantly elevated in the same dietary group (DII) after 4 weeks compared to the BD group (Table 4).

3.3. Immunological responses

3.3.1. Respiratory burst (RB) activity

The RB activity of blood monocytes was significantly ($P = 0.05$) higher in the diet DII group compared to BD and DI at 2 weeks (Fig. 2A). After 4 weeks, there was a significant up-regulation in the RB

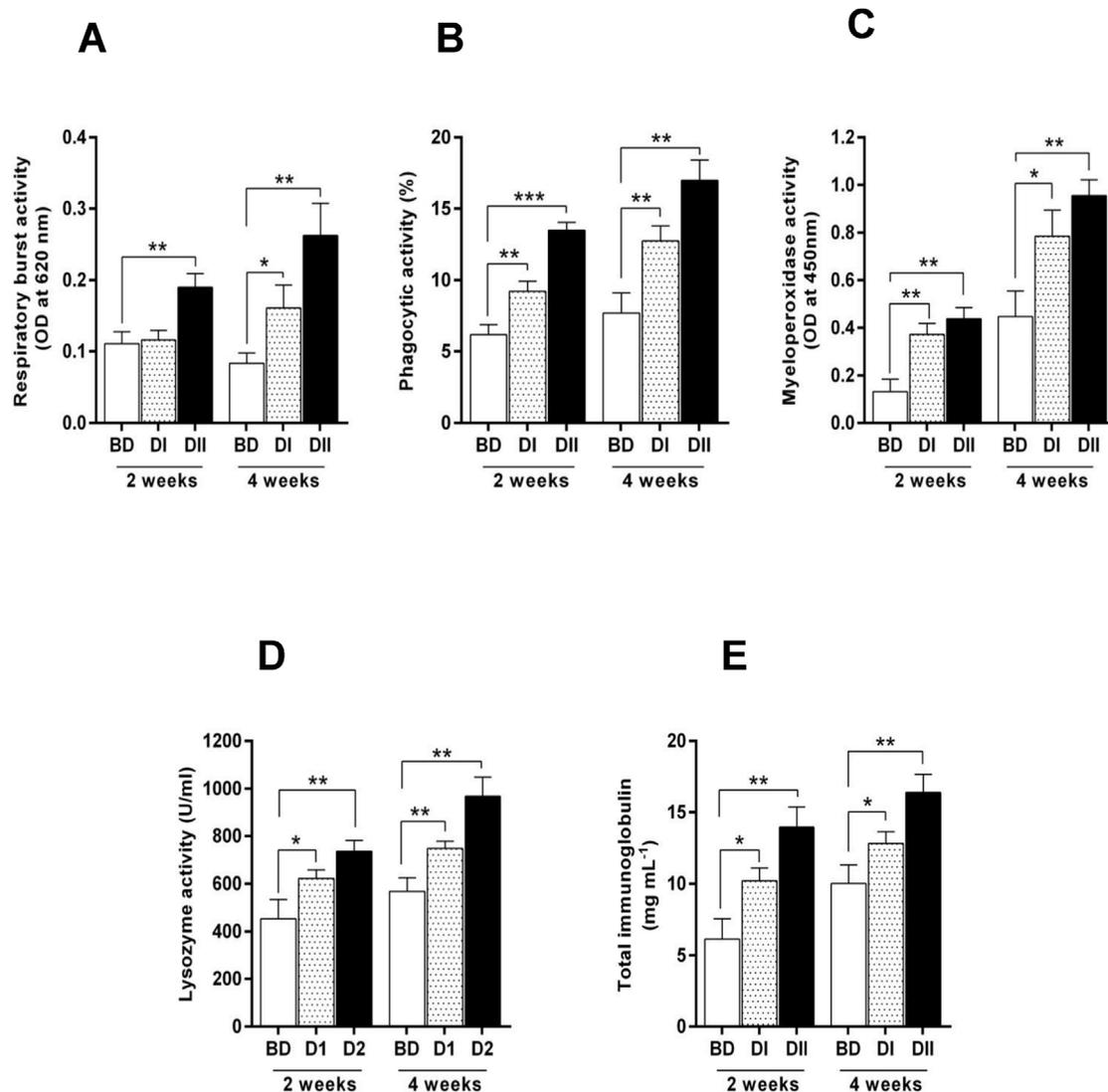


Fig. 2. Immunological assessments of *C. auratus* fed with basal diet (BD) and *E. acetylicum* S01 supplemented diets at 10^7 (DI) and 10^9 (DII) CFU g^{-1} . Each bar represents the mean \pm SD (n = 9). Bar graphs showing respiratory burst activity (A), phagocytic activity (B), myeloperoxidase activity (C), lysozyme activity (D) and total immunoglobulin level (E). *P < 0.05, **P < 0.001, ***P < 0.0001 indicates a significant difference between the basal diet in response to *E. acetylicum* supplemented diets.

activity of fish fed diet supplements provided from *E. acetylicum*; DI (0.16 ± 0.02 ; $P = 0.0186$) and DII (0.26 ± 0.04 ; $P = 0.0028$), whereas the RB activity was low (0.08 ± 0.01) in the BD group (Fig. 2A).

3.3.2. Cellular assay

The phagocytic activity of fish fed with probiotic diets was significantly higher ($P < 0.001$) than that fish were fed with BD at 2 and 4 weeks (Fig. 2B). The fish fed with a diet DII group exhibited the highest phagocytic activity (16.97 ± 1.26 ; $P = 0.0013$) compared to BD fish (7.70 ± 1.26) after 4 weeks.

3.3.3. Myeloperoxidase activity

The MPO activities of goldfish fed with *E. acetylicum* supplemented diets; DI (0.37 ± 0.02 ; $P = 0.0039$) and DII (0.43 ± 0.3 ; $P = 0.0016$) were significantly up-regulated compared to the fish fed with BD (Fig. 2C). After 4 weeks, the MPO activity was significantly higher (0.95 ± 0.05 ; $P = 0.0022$) in the fish fed DII compared to BD fish (Fig. 2C).

3.3.4. Serum lysozyme activity

Goldfish serum showed a significantly increased lysozyme activity ($P < 0.05$) after feeding the fish for 2 weeks with different diets that were supplemented with probiotics compared to the basal diet group (Fig. 2D). A maximum serum lysozyme activity was present in the fish group given diet DII (968.89 U ml^{-1} ; $P = 0.0021$) whereas the lysozyme activity was only (567.55 U ml^{-1}) in the BD group (Fig. 2D).

3.3.5. Total immunoglobulin (Ig) in plasma

After 2 and 4 weeks, the total Ig levels were significantly higher ($P < 0.05$) in diets supplemented with *E. acetylicum* (DI and DII) compared to the BD group (Fig. 2E). At both points in time, the fish group receiving diet DII (10^9 CFU g^{-1}) exhibited the highest Ig levels whereas the Ig level always declined in the BD group (Fig. 2E).

3.4. Expression levels of cytokine and lysozyme genes in the lymphoid organs of goldfish

The pro-inflammatory cytokine IL-1 β expression levels of goldfish fed with different doses of *E. acetylicum* as diet supplements were shown in Fig. 3A (kidney), 3B (head-kidney), and 3C (spleen). The results

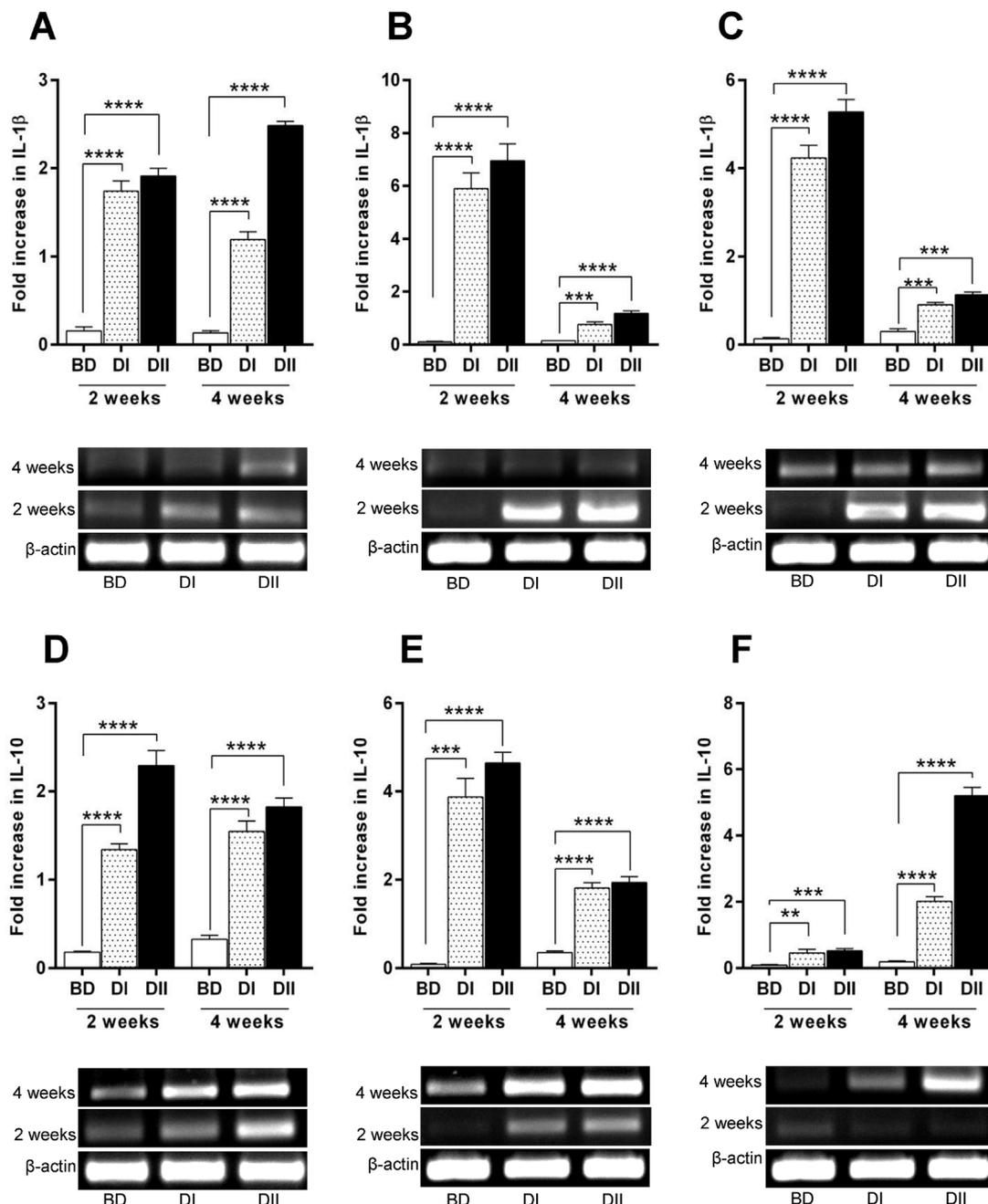


Fig. 3. Cytokine gene expression level in the different tissues of *C. auratus* fed with basal diet (BD) and *E. acetylicum* S01 supplemented diets at 10^7 (DI) and 10^9 (DII) CFU g^{-1} . The sqRT-PCR analysis was performed in triplicates, and each bar represents the mean \pm SD ($n = 9$). Bar graphs showing IL-1 β expression in kidney (A), head-kidney (B), and spleen (C). Bar graphs showing IL-10 expression in kidney (D), head-kidney (E), and spleen (F). Bar graphs showing TGF β expression in kidney (G), head-kidney (H), and spleen (I). ** $P < 0.001$, *** $P < 0.0001$, **** $P < 0.0001$, indicates a significant difference between the basal diet in response to *E. acetylicum* supplemented diets.

demonstrated that supplementation of fish diet with *E. acetylicum* had a significant ($P < 0.05$) up-regulation of IL-1 β expression in various tissues compared with those of fish fed with the BD group on both occasions. There was up-regulation of IL-1 β expression, 2.4-fold, 1.18-fold, and 1.12-fold, in kidney, head-kidney and spleen, respectively, in the DII fed fish when compared to BD fish ($P < 0.001$) at 4 weeks of feeding. Anti-inflammatory cytokine IL-10 levels were also significantly up-regulated, 1.82-fold, 1.93-fold, and 5.21-fold, respectively, in kidney, head-kidney, and spleen in the DII fed fish compared to BD ($P < 0.001$) (Fig. 3D–F) at 4 weeks. In addition, the TGF β expression was significantly up-regulated, 1.37-fold (kidney), 1.60-fold (head-kidney) and 2.54-fold (spleen), in the diet DII group compared with the

BD group ($P = 0.0013$, 0.0004 and 0.0001), respectively (Fig. 3G–I), at 4 weeks. The g-type lysozyme gene expression levels of goldfish fed with different doses of *E. acetylicum* (10^7 and 10^9 CFU g^{-1}) as diet supplements were shown in Fig. 4A (kidney), 4B (head-kidney), and 4C (spleen). The results showed that g-type lysozyme gene expression in kidney, head-kidney and spleen exhibited a significant fold increase ($P < 0.001$) in the fish with *E. acetylicum* supplemented diets (DI and DII) compared to the BD group at 2 weeks (Fig. 4A–C). After 4 weeks, fish fed with *E. acetylicum* supplemented diet the DII group exhibited the highest g-type lysozyme gene expression in kidney (1.45 fold; $P = 0.0001$), head-kidney (1.54-fold; $P = 0.0006$) and spleen (1.97-fold; $P = 0.0001$) when compared to the BD group (Fig. 4A–C). A

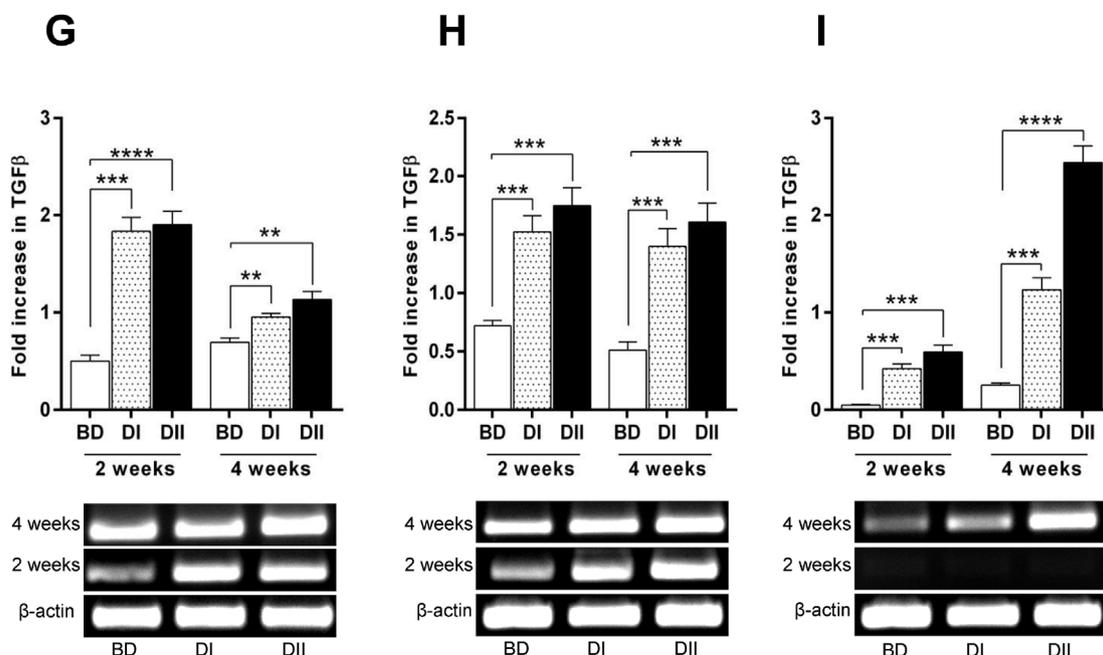


Fig. 3. (continued)

similar expression pattern was observed for c-type lysozyme gene expression; 2.47-fold, 10.35-fold and 7.26-fold up-regulation, respectively, in the kidney, head-kidney and spleen were observed in the diet of the DII group compared with the BD group ($P < 0.0001$; Fig. 4D–F).

3.5. Challenge against *A. hydrophila* infection

Dietary provision of *E. acetylicum* S01 enhanced the resistance of *C. auratus* against *A. hydrophila* infection (Fig. 5). The post-challenge survival rate (73.2%; $P = 0.0132$) was significantly higher in the fish fed with the *E. acetylicum* supplemented DII group (Fig. 5) whereas survival rate was reduced (33.2%) in the BD group. The negative control (PBS) showed no mortality during the experiment. Pathological symptoms of haemorrhagic septicaemia, ulcers, fin rot, tail rot, became apparent in moribund fish that were dying subsequently. We isolated colonies of *A. hydrophila* from the intestines of dead fish.

4. Discussion

Probiotics applications in fish aquaculture were reviewed recently by Nayak [6] and Qi and collaborators [24]. General beneficial effects of probiotic applications in fish aquaculture are the improvement of growth performance [25], prevention of infectious diseases through the stimulation of innate and acquired immunity [6,24], and the reduction of pathogens [24]. In this study, the WG and SGR of *C. auratus* significantly increased by dietary administration of *E. acetylicum* S01 for 4 weeks. Our study demonstrated further that dietary additions of S01 at 10^9 CFU g^{-1} for 4 weeks decreased the FCR of *C. auratus*. These findings correlate well with previous reports, that *Bacillus pumilus* supplemented diets enhanced higher growth and a low FCR in *Epinephelus coioides* (orange-spotted grouper) [26]. Son et al. [27], reported that *L. plantarum* supplemented diet (5.0×10^8 CFU g^{-1}) exhibited a significant increase in the growth performances in *E. coioides*. In addition, the dietary provisions of *Lactobacillus* sp. in the diets showed improved growth parameters in ornamental fishes (*C. auratus* and *Xiphophorus helleri*) [28]. *E. acetylicum* S01 supplemented diet showed growth improvements may be attributed to enhanced digestive activity by stimulating enzymatic activity and the synthesis of vitamins, with the improvement of digestibility and weight gain [29,30]. Glodin [31] reported also that both infection control through antagonistic activity and

increased digestibility of nutrients may be responsible for the considerably improved growth parameters. A possible explanation for the significant reduction in the FCR revealed that the fish utilized dietary nutrients more efficiently when the feeds were supplemented with *E. acetylicum* S01. To the best of our knowledge, this is the first report about *E. acetylicum* used as probiotics in fish aquaculture providing improved growth performance.

Blood cells are important for the host providing cellular defence mechanisms and also secrete humoral defence molecules to prevent the infection by microbes [32]. Red blood cells usually constitute a major part of blood and their number varies with goldfish but they usually range between 1.05 and 3.0×10^6 cells mm^{-3} reported by Kumar et al. [15]. In this study, the RBC count was significantly higher in fish fed with diet DII, when compared to BD for 2 and 4 weeks. This result clearly indicates that the RBC count was within the limit of standard blood counts were described by Kumar et al. [15] and not causing any pathological symptoms inspecting the external morphology of goldfish fed with probiotic diets and basal diet (see supplementary data, Fig S1A). We observed further a higher accumulation of pigments from goldfish tissue in the probiotic-supplemented diets (DI and DII) compared to control (see supplementary Fig S1A and B) at end of the feeding trial after 4 weeks. Our findings supported an earlier study, where the counts of RBC were significantly higher in catfish fed with a *Weissella cibaria* diet [33]. This result suggests that observable increases in the RBC count after dietary supplementation with *E. acetylicum* may increase the availability of nutrients which are required for RBC production such as iron, vitamin A (β -carotene) and vitamin B12 [34].

Similarly, the WBC and PLT counts were higher in the goldfish fed with *E. acetylicum* supplemented diets (DI and DII), throughout the feeding trial. This agrees with WBC and PLT counts of the Pacific salmon *Oncorhynchus mykiss* fed with an *Enterobacter cloacae* and *B. mojavensis* diet at 1×10^8 cells g^{-1} for 60 days [35]. Interestingly, the haemoglobin content also shows an increasing pattern in concert with RBC count, which conforms to the finding by Faramarzi et al. [36], in *O. mykiss* fed with *L. acidophilus* supplemented diet. Therefore, this study collectively suggests that *E. acetylicum* administrated diets enhanced the complete blood counts by the stimulation of the innate immune response in *C. auratus*.

In fish food supplementation with *Bacillus* and *Lactobacillus* strains could increase disease resistance through the stimulation of

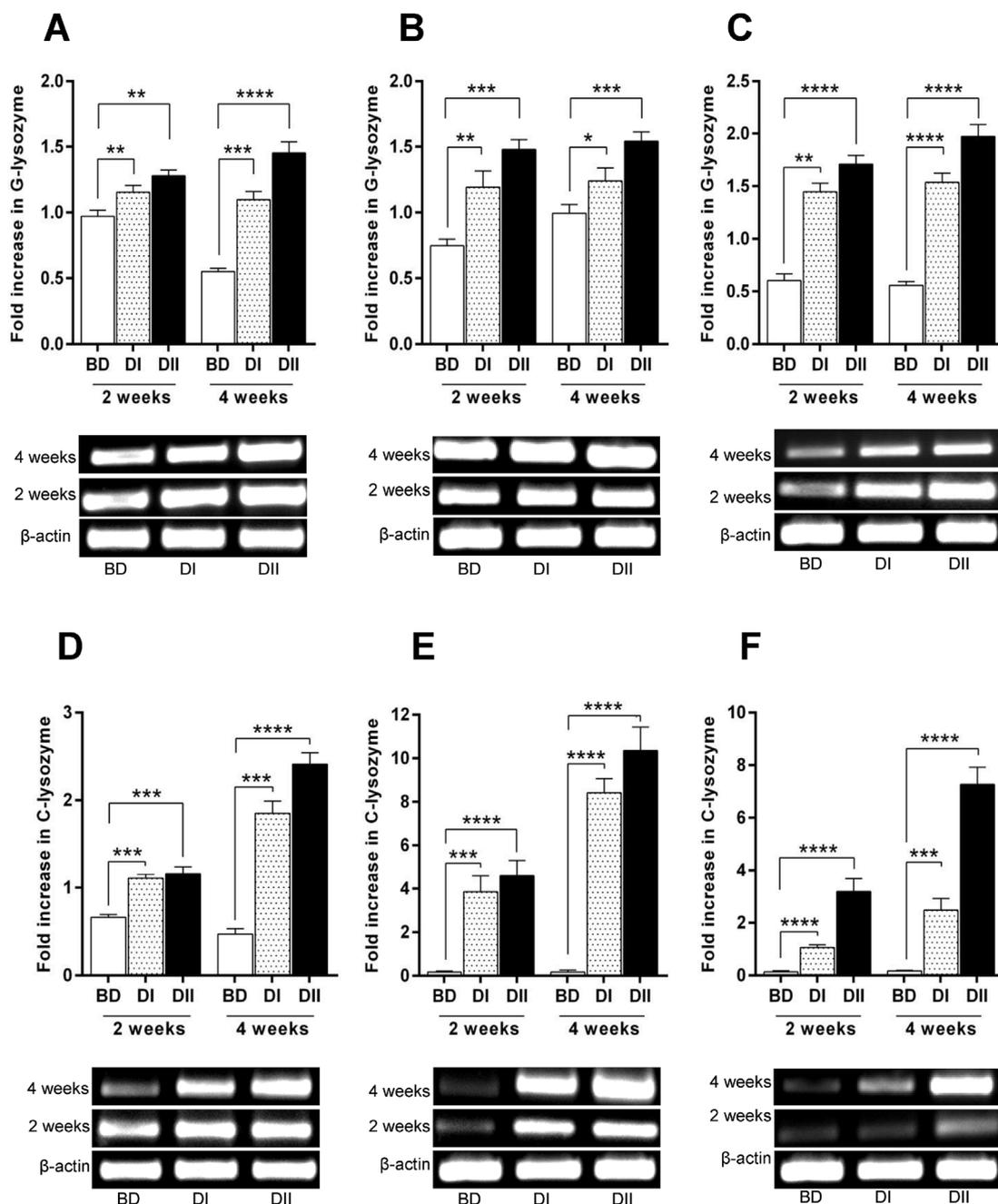


Fig. 4. Lysozyme gene expression level in the different tissues of *C. auratus* fed with basal diet (BD) and *E. acetylicum* S01 supplemented diets at 10^7 (DI) and 10^9 (DII) CFU g^{-1} . The sqRT-PCR analysis was performed in triplicates, and each bar represents the mean \pm SD (n = 9). Bar graphs showing g-type lysozyme gene expression in kidney (A), head-kidney (B), and spleen (C). Bar graphs showing c-type lysozyme gene expression in kidney (D), head-kidney (E), and spleen (F). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, **** $P < 0.0001$, indicates a significant difference between the basal diet in response to *E. acetylicum* supplemented diets.

phagocytotic cells and antimicrobial enzymes [6,9]. Respiratory bursts are achieved by phagocytes accordingly to attack invasive pathogens by eating them up. The accumulation of reactive oxygen intermediates (ROIs) is commonly very toxic to host cells [37]. The injuring effects of ROIs were minimized by various immunostimulants. In the present study, both the respiratory burst and phagocytic activities of blood cells in *C. auratus* fed with *E. acetylicum* supplemented diets (DI and DII) were significantly increased throughout the experimental period. These findings were well correlated with *E. coioides* fed with *L. plantarum* supplemented diets for 4 weeks [27] and *O. mykiss* was fed with an *L. rhamnosus* diet at 10^9 and 10^{11} CFU g^{-1} for 30 days, and at 7.9×10^4 CFU g^{-1} for 2 weeks showed significantly increased

phagocytic activity of head-kidney leucocytes [38], and RB activities of blood cells [39], respectively. Salinas et al. [40], had reported that fish fed with *B. subtilis* supplemented diet exhibited a highest phagocytic activity of blood leucocytes of *Sparus aurata* after 2 weeks. Taken together, both respiratory burst and phagocytic activities revealed that *E. acetylicum* S01 supplemented diet enhanced the cellular immune response to the activation of immune cells.

Myeloperoxidase (MPO) utilizes oxidative radicals to produce hypochlorous acid that carries out their antimicrobial activity. Myeloperoxidase is probably released from azurophilic granules of neutrophils during oxidative bursts [41]. In the present study, significant increases in the MPO activity on fish fed with DI and DII

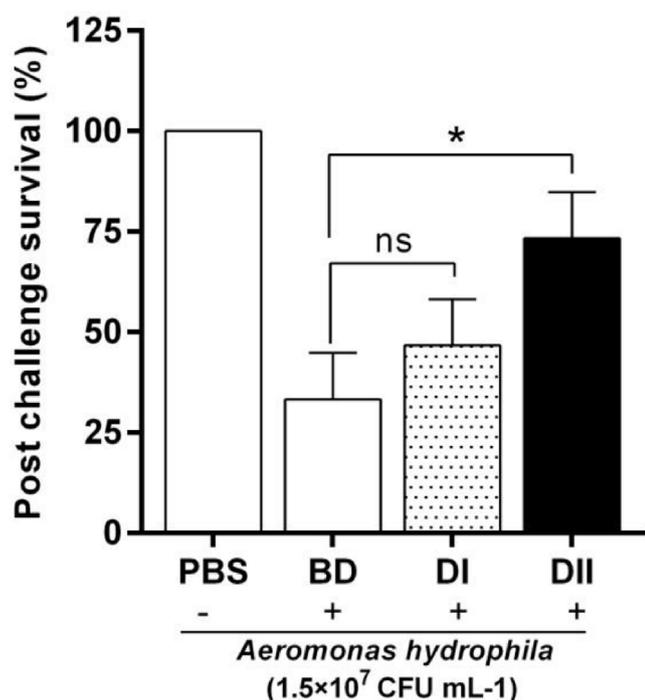


Fig. 5. Effects of dietary supplements of *E. acetylicum* on the post-challenge survival of *C. auratus* after infection with *A. hydrophila*. Data are expressed as mean \pm SD (n = 12). *P < 0.05, indicates a significant difference between the basal diet in response to *E. acetylicum* supplemented diets.

compared to a basal diet for 2 and 4 weeks. This observation was well correlated with the serum peroxidase activity in *Catla catla* fed with a *B. amyloliquefaciens* (1×10^9 CFU g⁻¹) diet for 4 weeks [42], and *S. aurata* fed with *B. subtilis* for 3 weeks [43]. Lysozymes catalyze the hydrolysis of β -(1,4)-glycosidic linkage of n-acetyl-glucosamine and n-acetyl-muramic acid, and this way protect the host against the invasion of bacterial pathogens [44]. In this study, the serum lysozyme activity was significantly higher in *C. auratus* fed with DI and DII compared to BD at 2 and 4 weeks. Our results correlated well with the higher serum lysozyme activity of *E. coioides* fed with an *L. plantarum* enriched diet at 2 and 4 weeks [28]. From the present investigation, our data revealed that *E. acetylicum* supplemented diets in *C. auratus* significantly increased the levels of antimicrobial enzymes (myeloperoxidase and lysozyme) by the stimulation of innate immunity against invading bacterial pathogens.

Immunoglobulin is a well documented immune player offering disease prevention in aquatic animals as well as humans. Immunoglobulin stimulation following dietary supplementation of probiotics was reported in several studies [9,15,39,45]. In the present study, the immunoglobulin levels were significantly higher in probiotic *E. acetylicum* supplemented diets (DI and DII) compared to BD for 2 and 4 weeks. This finding is supported by the earlier study in *O. mykiss* fed with *L. rhamnosus* diet for 20 days and grouper fed with *Bacillus* supplemented diets for 30 days [45,46]. These results suggest that *E. acetylicum* supplemented diets significantly enhances the total immunoglobulin level by inducing humoral immunity.

Immune system activation by probiotics leads to the synthesis of pro- and anti-inflammatory cytokine mediators. Later they dependent on cell-cell communicators that are known as paracrine signals. Interleukin-1 β stimulates immune responses by activating lymphocytes or by inducing the release of other cytokines capable of triggering macrophages, granulocytes, lymphocytes and natural killer cells [10]. The other two molecules considered in this study, IL-10 and TGF β are potent anti-inflammatory cytokines to inhibit the Th1 cytokines (IL-2, INF- γ , and TNF- α). They also deactivate monocyte/macrophage-

derived proinflammatory cytokine synthesis [47,48]. In addition, TGF β plays a crucial role in the remodeling of tissues, in wound repair, and haematopoiesis. TGF β is also expressed by different cells and tissues [11]. In this study, there was a significant up-regulation of IL-1 β , IL-10 and TGF β expression in kidney and head-kidney of fish fed with *E. acetylicum* supplemented diets compared to a basal diet for 2 and 4 weeks. This implies a probiotic action through this mediator of the immune response. We suggest that it is possible that IL-1 β expression is balanced by the up-regulation of anti-inflammatory cytokine gene expression in both organs of *C. auratus* fed with *E. acetylicum* supplemented diets. Although the increased IL-1 β expression shown in *C. auratus* fed with *E. acetylicum* supplemented diets may be responsible for downstream effects such as an increased respiratory burst, myeloperoxidase and phagocytic activity. It is also associated with the promotion of neutrophils and leukocytes counts also increased in the blood of *C. auratus*.

In contrast, IL-1 β gene expression was significantly up-regulated in the spleen, while the IL-10 and TGF β were at lower transcription level in the same organ at 2 weeks. After 4 weeks, the IL-1 β expression was down-regulated by the up-regulation of IL-10 and TGF β . This happens through a negative feedback mechanism that limits the immunologically balanced inflammatory response in the spleen of *C. auratus* fed with *E. acetylicum* supplemented diets (DI and DII) compared to BD. Here, despite the up-regulation of IL-1 β for 2 and 4 weeks, there was no evidence of inflammation and pathogenic effects from phenotypic observation in fish fed with and without probiotic-supplemented dietary groups (see supplementary data, Fig S1A). In agreement with our results, Pirarat and collaborators [49] and Villamil and collaborators [50], reported that tilapia fed with lactic acid bacteria exhibited higher expression of IL-1 β . It is suggested that the induction of proinflammatory cytokines enhance the immune response of the host. In this regard, disease resistance studies in tilapia have shown that probiotics can upregulate the expression of TNF α and IL-1 β . Consequently, the tilapia survival levels were significantly higher when exposed to *A. hydrophila* [51]. Furthermore, interleukins IL-10 and IL-21 were the most efficient cytokines capable of activating human B cells for IgG1 and IgG3 production [52]. TGF β promoted a switching of B cells to IgA synthesis [53]. Though we do not estimate the specific antibody response, the up-regulation of IL-10 and TGF β expressions demonstrated in *C. auratus* may be responsible for downstream effects including a significant increase in the total immunoglobulin level, which was associated with the activation of B cell function, notably with *E. acetylicum* supplemented diets. Anti-inflammatory cytokines commonly have an immune suppressive effect on the host. This could be symptomatic for a tolerance mechanism where the host does not interpret the probiotic as a threat. This is the first report showing that *E. acetylicum* S01 modulates the expression of IL-1 β , IL-10, and TGF β in the major lymphoid organs of *C. auratus*.

The serum lysozyme activity of goldfish fed with *E. acetylicum* supplemented diets were significantly higher and it's associated with g- and c-type lysozyme expression also correspondingly higher in the same dietary groups at various immune organs (kidney, head kidney and spleen) compared to a basal diet. This observation was correlated with only the g-type lysozyme expression was up-regulated in head-kidney of *Gadus morhua* fed with a *Psychrobacter* sp. GP11 supplemented diet [54]. Up-regulation of this lysozyme expression of fish fed with probiotic lactic acid bacteria was previously reported by the intestine and hepatopancreas of *Marsupenaeus japonicas* [55]. Hong et al. [56] suggested that localized cellular immune responses, together with the systemic production of IL-1 β , lysozyme, and COX-2 may be crucial for the rapid elimination of *A. salmonicida*. The up-regulation of the c-type lysozyme was much stronger than that of the g-type lysozyme in three immune organs. To the best of our literature search, dietary supplementation of *E. acetylicum* and other probiotic strains hitherto not been reported the c-type lysozyme gene expression in immune organs of *C. auratus*. Hence, this is the first report showing a significant up-

regulation of g- and c-type lysozyme gene expression in kidney, head-kidney and spleen of *C. auratus* fed with *E. acetylicum* supplemented diets, suggesting that these two lysozymes might play an important role providing disease resistance and also a defense against invading bacterial pathogens.

Immune system modulation is one of the most important benefits of probiotics [6]. Dietary supplementation of *E. acetylicum* at 10^7 and 10^9 CFU g^{-1} for 4 weeks significantly increased the survival of *C. auratus* that were challenged by *A. hydrophila*. The highest survival rate (73.2%) was exhibited in the fish fed with *E. acetylicum* supplemented diet DII compared to a basal diet. This observation was well correlated with the higher survival rate of *E. coioides* fed with *L. plantarum* [27], and *L. rohita* juveniles fed with *L. plantarum* [45], *C. catla* fed with a *B. amyloliquefaciens* supplemented diet, after challenging with *A. hydrophila* [42]. Ramesh et al. [9] had reported that *L. rohita* fed with *B. aerophilus* bacteria supplemented diets for 6 weeks, which enhanced the resistance against the pathogen *A. hydrophila*. Collectively, our results suggest that *E. acetylicum* supplementation could increase the cellular and humoral immune responses at molecular levels in *C. auratus*, resulting in a resistance to *A. hydrophila* infection. Therefore, microbial feed supplements have beneficial effects on host health by the stimulation of immunological responses. They also alter the immune-related gene regulation in the lymphoid organs of *C. auratus*. Thereby, they provide protection against invading pathogenic microorganisms.

In conclusion, this is the first report clearly demonstrating the dietary administration of *E. acetylicum* efficiently colonizing the intestine of *C. auratus* (see supplementary data, Table S1) and improving its growth performance, and stimulation of cellular and humoral immune responses, at the cellular and molecular levels in vital lymphoid organs (kidney, head-kidney and spleen) together with increased resistance against infection by *A. hydrophila*. To elevate the growth and immune resistance ability of *C. auratus*, dietary *E. acetylicum* administration at 10^9 CFU kg^{-1} is an optimal dose. This finding paves the path for future studies on molecular interactions between probiotics, immune-related gene expression in vital lymphoid organs of fish, and the modulation of innate immunity.

Compliance with ethical standards

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical statement

Principles of laboratory animal care were followed and all procedures were conducted according to the guidelines established by the National Institutes of Health (Guidelines for the use of fishes, 2004), and every effort was made to minimize suffering. This study was approved by the institutional ethical committee of Madurai Kamaraj University [Ethical Clearance (EC), Biosafety and Animal Welfare Committee].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2018.10.026>.

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