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Potential use of probiotic- and triherbal extract-enriched diets to control *Aeromonas hydrophila* infection in carp

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ABSTRACT: This study reports the effect of probiotic- and triherbal extract-enriched diet on *Cyprinus carpio* (52 ± 2 g, n = 250) inoculated intramuscularly (50 µl) with *Aeromonas hydrophila* (1.8×10^6 CFU ml^{-1}). On Day 6 post-infection, the fish were divided into 4 groups and fed with the basal diet (IU) or with diets supplemented with triherbal extract (H), Sporolac (S) or *Lactobacillus* (L). A fifth group of non-infected fish fed with the basal diet was used as a control. Haematology and immunology parameters were measured in Weeks 1, 2 and 4. In the H, S, and L groups, white blood cell levels were significantly increased ($p < 0.05$) throughout the experimental period. In the H and S groups, red blood cell and haemoglobin levels decreased significantly ($p < 0.05$) in Weeks 1, 2 and 4. In all the diet groups, the haematocrit and mean corpuscular volume did not differ significantly ($p > 0.05$) in Week 1, and these values remained near those of the control in Week 2. The S and L groups did not exhibit significant changes ($p > 0.05$) in glucose, and cholesterol levels in Weeks 2 and 4 compared to controls, and the L group also showed no change in total protein. Respiratory burst activity was significantly increased in Weeks 1 to 4 in the H, S and L groups. Serum-mediated killing of *Escherichia coli* was significantly increased in the L group in Weeks 1 to 4. Mortalities after *A. hydrophila* challenge were higher in the IU (85%) and H (50%) groups than in the S (45%) and L (35%) groups. Our results show that formulated probiotic-enriched diets can speed up the recovery of ulcerative dermatitis induced by *A. hydrophila* in carp.

KEY WORDS: *Aeromonas hydrophila* · *Cyprinus carpio* · Haematology · Triherbal extract · Probiotics

INTRODUCTION

Common carp *Cyprinus carpio* is a species of global importance, not only because of its aesthetic value, but also due to its immense commercial value in the export trade. *Aeromonas hydrophila* is an opportunistic Gram-negative pathogen and causes an ulcerative condition, called motile aeromonad septicaemia (MAS), in wild and cultured fish (Leung et al. 1995). The bacterium causes various disease conditions in freswater fish, such as haemorrhagic septicaemia, infectious abdominal dropsy, and fin and tail rot (Austin & Austin 1993). Diseased fish can be assessed through microbiological, histological and molecular techniques, such as polymerase chain reaction (PCR) analysis and enzyme-linked immunosorbent assay (ELISA). However, these methods are expensive and occasionally time consuming (Chen et al. 2003). Historically, studies have used haematological indices and immunological assays to determine the health status of fish (Harikrishnan et al. 2003, 2009).

In order to improve the efficiency of feed utilization of finfish and shellfish, a number of feed additives, including both prophylactics and growth promoters, have been tested. However, this practice has several negative aspects, such as accumulation of antibiotic residues in the tissues that results in consumer reluctance (Ringø & Birkbeck 1999). Hence, management of fish diseases, except those for which vaccines are available, continues to be a challenging problem (Anderson 1992).

One promising method of controlling infection in aquaculture is strengthening the defence mechanisms

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of fish through prophylactic administration of probiotics and herbs (Gibson et al. 1997, Harikrishnan et al. 2003, 2010, Sharifuzzaman & Austin 2009). Probiotics such as lactic acid bacteria, produce specific compounds such as bacteriocins or bacteriostatic peptides (Klaenhammer 1988) that inhibit the growth of fish pathogens, including *Aeromonas hydrophila*, *Vibrio anguillarum* and *A. salmonicida* (Lewus et al. 1991, Gildberg et al. 1995, Santos et al. 1996), in addition to improving water quality (Skjermo & Vadstein 1999).

In India, as many as 500 medicinal plants have been used to treat various diseases of humans and animals (Vaidyaratnam 1995). The active ingredients of *Azadirachta indica* leaf trigger antibody responses and promote non-specific defence mechanisms in fish (Venkatalakshmi & Michael 2001). Both the aqueous and ethanolic herbal extracts of *A. indica*, *Oscimum sanctum* and *Curcuma longa* can limit the growth of *Aeromonas hydrophila in vitro* and promote innate immunity against this pathogen (Harikrishnan & Balasundaram 2005, Harikrishnan et al. 2009). As a result, *A. indica, O. sanctum* and *C. longa* were selected for this study, all of which are cost effective and locally available. A number of probiotic preparations are commercially available and have been used in fish, shrimp, and molluscan farming as feed additives or incorporated in pond water (Wang et al. 2005). The aim of the present work was to assess the efficiency of dietary administration of 2 types of probiotics and a triherbal extract on haematological changes and innate immunity in common carp infected with *A. hydrophila*.

Table 1. Composition of treatment diets used for common carp. Basal diet (% by weight): fish meal (18%), ground nut oil cake (18%), sesame oil cake (16%), soya flour (16%), rice bran (16%), tapioca flour (16%). Vitamin premix (g 100 g^{-1}): thiamin hydrochloride (0.7), riboflavin (1.2), pyridoxine hydrochloride (0.5), cyanocobalamin (0.05), ascorbic acid (60.0), niacin (4.8), calcium pantothenate (1.2), inositol (25.0), biotin (3.5), folic acid (0.2), p-aminobenzoic acid (0.6), vitamin A acetate (1.0), vitamin D_3 (1.0) and vitamin K_3 (0.6). Mineral premix (g 100 g^{-1}): NaCl (1), $MgSO_4 \cdot 7H_2O$ (15), $NaH_2PO_4 \cdot 2H_2O$ (25), KH_2PO_4 (32), $Ca(H_2PO_4)_2 \cdot 2H_2O$ (20), $FeC_6H_5O_7 \cdot H_2O$ (2.5), $ZnSO_4 \cdot 7H_2O$ (1.2), $MnSO_4 \cdot 5H_2O$ (0.6), $CuSO_4 \cdot 5H_2O$ (0.1), $CoCl_3 \cdot 6H_2O$ (0.01), KIO_3 (0.01) and cellulose (1.6). Vitamin and mineral premixes were mixed in equal proportions before being added to the diet treatment. –: not added

Treatment	Diet component (%)				
	Basal diet	Vitamin and mineral mix	Lacto-bacillus	Sporolac	Herbal extract
Pretreatment/ control	99.5	0.5	–	–	–
Lactobacillus diet	99.4	0.5	0.1	–	–
Sporolac diet	99.4	0.5	–	0.1	–
Triherbal extract diet	99.4	0.5	–	–	0.1

MATERIALS AND METHODS

Fish. Apparently healthy common carp *Cyprinus carpio* (mean weight: 52 ± 2 g), obtained from a local fish farm in Tiruchirapalli, Tamilnadu, India, were transported to the laboratory in plastic bags filled with oxygenated water. The fish were acclimatized in plastic aquaria (60 × 50 × 70 cm) containing 100 l of water for 3 wk under laboratory conditions (14 h light:10 h dark cycle). The observed water quality parameters were: dissolved oxygen concentration of 5.2 to 7.8 mg l^{-1} (Winkler's method), pH 5.5 to 7.1 and temperature of 28 ± 2°C during the whole trial. The fish in the control and infected untreated groups were fed ad libitum once daily with a formulated normal diet (without probiotics or triherbal extract). The treated groups were fed with a normal diet enriched with probiotics or triherbal extract at a rate of 2% of their body weight per day throughout the experiment (Table 1). Once every 5 d, 50% of the water in the aquaria was renewed to remove the uneaten and faecal material.

Aeromonas hydrophila. *A. hydrophila* (MTCC 646) was obtained from the Institute of Microbial Technology, Government of India, Chandigarh, and maintained in the laboratory under standard conditions (Harikrishnan et al. 2003). The identity of *A. hydrophila* was verified using biochemical tests, as described previously (Harikrishnan et al. 2010). Subcultures were maintained on tryptic soy agar (TSA; Himedia) on slopes at 5°C and routinely tested for pathogenesis (Joseph & Carnahan 1994) by inoculation into American eels (Le Sueur) (Davis & Hayasaka 1983). Stock culture in tryptic soy broth (TSB; Himedia) was stored at –70°C in 0.85% NaCl with 20% glycerol (v/v) to provide stable inoculate throughout the experiment (Chabot & Thune 1991). Subcultures were taken from the TSA slope and harvested in TSB. The inoculated broth was incubated for 24 h in a shaker at 25°C, and then centrifuged at 10 000 × *g* for 20 min at 4°C (Harikrishnan et al. 2003). The supernatant was discarded and the bacterial pellet was washed 3 times with phosphate-buffered saline (PBS) at pH 7.2 (Yadav et al. 1992).

Bacterial procurement, growth and harvest. The probiotic bacterial strains of *Lactobacillus rhamnosus* (*Lactobacillus*) and *L. sporogenes* (Sporolac) were obtained from Inter Care, Mehsana, Gujarat and UNI-Sankyo, Ratnagiri, Maharashtra (Ahilan et al. 2004). The bacteria were cultured in MRS broth (De Man et al. 1960) for 48 h at 30°C and subsequently preserved in 50% glycerol at –80°C and kept as stab cultures for

further use. A pure colony was taken for inoculation of seed cultures of 50 ml each and incubated at 30°C for 24 h before mass culture in MRS broth. After 1 d of culture, the bacteria were harvested by centrifuging at 16 500 × *g* for 10 min and washing 3 times with sterile peptone water (0.85% NaCl and 0.1% Polypeptone).

Supplementation of the standard diet with probiotics. The fish feed was prepared in the laboratory using soybean and fish meal as the protein source (Table 1). To enrich the normal diet with the probiotics, the required amount of bacterial suspension was sprayed into the feed slowly, mixing part by part in a drum mixer, after which it was air dried under sterile conditions for 12 h. The viability of the incorporated bacterial cells was assessed by spreading onto triplicate plates of TSA (Becton, Dickinson), MRS agar (MERCK) and BA blood agar (Nissui). The colony count was taken after incubation at 30°C for 48 h. The bacterial count of the feed was taken at this point and twice during the trial, and averaged 2.45×10^9 and 1.07×10^{11} CFU g^{-1} for *Lactobacillus* and Sporolac, respectively. The pellets were dried in an oven at 30°C for 18 h, packed and stored in a freezer at –20°C until use. Before use in the feeding trial, bacterial counts confirmed the final concentration of live *Lactobacillus* and Sporolac in feed pellets to be 2.45×10^9 and 1.07×10^{11} CFU g^{-1}, respectively.

Preparation of herbal extract. Fresh leaves of neem *Azadirachta indica*, tulsi *Oscimum sanctum* and turmeric *Curcuma longa* were collected from the Bharathidasan University campus during May 2007. About 5 kg of each leaf were washed in sterile distilled water. The leaves were separately shade dried for 10 d until weight constancy was achieved. Each sample was finely powdered in an electric blender. *C. longa*, *O. sanctum* and *A. indica* leaf powders were evenly mixed at a ratio of 1:1:1. The triherbal extract was made with 100 g of the mixed powder dissolved or soaked in 1000 ml sterile distilled water in 2000 ml conical flasks. Conical flasks were tightly covered with aluminium foil, kept for 7 d at room temperature and agitated daily. The extract was then filtered through sterile muslin cloth. The filtrate was collected and the solvent was evaporated using a rotary vacuum evaporator (Buchi SMP). The residue obtained after evaporation was mixed with the normal diet at 1000 mg kg^{-1} (w/v) (Harikrishnan et al. 2009).

Experimental design. Healthy common carp *Cyprinus carpio* (52 ± 2 g, n = 250) were inoculated intramuscularly (50 µl) with *Aeromonas hydrophila* (1.8×10^6 CFU ml^{-1}). Fish were anaesthetized with MS-222 (tricaine methanesulphonate, Sigma), 1:4000 in dechlorinated water, for 2 min (Ortuno et al. 2000). On Day 6 post-infection, the following treatments were applied: (1) control, without inoculum fed with normal diet (C, n = 50); (2) inoculated fish, fed with normal diet (IU, n = 50); and inoculated fish treated with diets enriched with (3) triherbal extract (H, n = 50); (4) *Lactobacillus* (L, n = 50); and (5) Sporolac (S, n = 50) in triplicate groups. Fish were fed daily to satiation by hand at 08:00 and 14:00 h.

Blood sampling for haematology and immunology. Sampling was carried out in Weeks 1, 2 and 4. Feeding was ceased for 24 h prior to sampling, after which 6 fish were selected randomly from each group. Individual fish were anaesthetized with MS-222 and approximately 0.5 ml of blood was collected from the caudal vein of each using a 1 ml 27-gauge syringe needle fitted with a vacutainer (Sigma). One half of each blood sample was mixed with anticoagulant then stored at 4°C using EDTA serum tubes (BD vacutainer, Becton Dickinson), while the remainder was used immediately for haematological examination. Samples in serum tubes were placed at room temperature and allowed to clot for 2 h. Sera were separated by centrifugation at 1500 × *g* for 20 min and sera from the same groups were pooled before being stored at –70°C for biochemical and immunological analyses. Red blood cells (RBC: 10^6 mm^{-3}) and white blood cells (WBC: 10^4 mm^{-3}) were counted manually by haemocytometry (Houston 1990) using a Neubauer haemocytometer after diluting blood samples by adding Hayem's solution for RBC and Türk's solution for WBC. Haemoglobin concentration (Hb: g dl^{-1}) was measured spectrophotometrically at 540 nm using the cyanmethemoglobin method (Drobkin 1945). The haematocrit (Ht: %) was measured by the microcentrifuge method, using standard heparinised microhaematocrit capillary tubes (75 mm at 7000 × *g* for 10 min). The mean corpuscular volume (MCV: μm^3), mean corpuscular haemoglobin (MCH: pg) and mean corpuscular haemoglobin concentration (MCHC: g dl^{-1}) of derived erythrocytes were calculated according to Houston (1990). The total protein (TP: mg dl^{-1}), glucose (GLU: mg dl^{-1}) and cholesterol (CHO: mmol dl^{-1}) were determined as described by Hawk et al. (1954).

Preparation of chemiluminescence reagents. Zymosan A (Sigma) was boiled and washed 3 times with Hanks' balanced salt solution (HBSS). A stock solution of 10 mg ml^{-1} was made in the same buffer and stored at –70°C until required. Washed zymosan was opsonised with 25% rainbow trout sera diluted in HBSS, incubated for 25 min at room temperature and washed 3 times with HBSS. A 10 mM stock solution of luminol (5-amino-2,3-dihydro-1,4-pthalazinedione, Sigma) was prepared in 0.2 M sodium borate buffer, pH 9.0, and stored at –70°C until use.

Respiratory burst assay. The respiratory burst (RB) activity was determined according to the method of

Lilius & Waris (1984) and Marnila et al. (1995). The RB activity of phagocytes was measured as zymosan-induced, luminol-enhanced chemiluminescence (CL) emission in the diluted blood of the individual carp. Individual blood samples collected in EDTA tubes were diluted 1:400 in HBSS. The CL measurements were performed using 200 µl diluted blood, 50 µl 10 mM luminol in borate buffer, pH 9.0, and 50 µl opsonised zymosan (10 mg ml^{-1}), in a Wallac 1251 luminometer. The measurements were performed at 20°C. The CL emission was measured for 80 min in order to obtain kinetic curves for each sample.

Complement-mediated killing. Complement-mediated killing was determined according to the method of Nikoskelainen et al. (2002). *Escherichia coli* MC1061 pEGFPLucAmp containing reporter genes for green fluorescent protein (GFP) and luciferase was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; pH 7.0) containing 100 µg ml^{-1} ampicillin at 37°C with agitation, to the mid logarithmic growth phase. The cells were harvested by centrifugation at 2500 × *g* and washed twice with saline. The light absorption of the cell suspension was adjusted to 0.5 ± 0.05 at 600 nm, which equals 4×10^8 CFU ml^{-1}. The serum dilutions were prepared using heat-inactivated foetal calf serum (Fetal Clone[2], HyClone Laboratories) by heating at 56°C for 30 min to keep the protein concentration equal in all samples. The complement reactions were carried out by mixing equal volumes of pooled carp serum (individual sera from the same group at each time point were pooled) and *E. coli* to obtain final serum concentrations of 0, 3, 6, 9, 12, 15, 17, 20, 25, 30, 35, 50, 100, and 150 µl ml^{-1} and a bacterial concentration of 2×10^8 CFU ml^{-1}. Reaction mixtures were incubated for 90 min at 20°C. The reaction was stopped by placing the sample tubes in ice for 10 min. The viability of *E. coli* in the reaction mixtures was determined by placing 100 µl into microtiterplate wells (White Cliniplate, Labsystems) and adding 100 µl 0.5 mM D-luciferin in citrate buffer, pH 5. The luminescence of viable *E. coli* was measured with an Ascent FLII fluoro-luminometer (Labsystems). The luminescence data were converted to % viability, assuming that the bacterial viability was 100% when the active serum concentration was 0 µl ml^{-1}. The % viability was then plotted as a function of the active serum concentration (µl ml^{-1}) in Origin (MicroCal). The unit of complement (µl of the active serum that kills 50% of the bacteria) was obtained from the plot and the CB50 value was calculated as units ml^{-1} of active serum.

Disease resistance. *Aeromonas hydrophila* (MTCC 646) was inoculated into TSB at 28°C. The culture was centrifuged at 800 × *g* for 15 min at 4°C. The packed cells were washed and the required dose was prepared in PBS. On Day 1, fish were challenged with 50 µl of *A. hydrophila* (1.8×10^6 CFU ml^{-1}) intraperitoneally. On Day 6 post-infection, groups of 10 fish, each in triplicate, were adminitered with H, S and L diets. The control and IU fish were administered normal diets. In our previous study, the fish challenge dose was standardized to give 85% mortality in the untreated group (Harikrishnan et al. 2009). Mortality was recorded on daily basis for 30 d. The relative percent survival (RPS) was calculated using the following formula (Amend 1981):

$$RPS = 1 - \frac{(\text{Percent mortality in treated group}) \times 100}{(\text{Percent mortality in control group})}$$

Statistics. All results are presented as the average and standard deviation of 3 independent measurements. The nonparametric ANOVA (SPSS computer programme) was used to determine whether there was a significant difference ($p < 0.05$) in the haematological and immunological parameters between control and tested groups.

RESULTS

Haematological profile

The WBC levels were significantly higher ($p < 0.05$) in all the experimental groups in Weeks 1 to 4 when compared to the control. The RBC values were significantly lower ($p < 0.05$) than the control values in all the experimental groups throughout the experimental period. The Hb concentrations of the IU and H groups were significantly lower than that of the control. There was no change in the Hb level of the infected groups fed with the S and L diets between Weeks 1 and 4. The Ht was significantly lower in IU and H diet groups. However, in the S and L groups, the Ht values did not differ in any sampling week. The MCV was significantly increased in all the experimental groups in Weeks 2 and 4 when compared to the control. However, there was no change in the MCV in the first week. The MCH was significantly increased in Weeks 1 and 2 in all the experimental groups. However, the MCHC values did not show a significant change ($p > 0.05$) in all of the experimental groups compared to control (Fig. 1).

Biochemical profile

In all the diet groups, the TP levels did not change in the first week when compared to the control. In the H and S groups, the TP level was significantly decreased in Week 2, after which it was restored to a level near that of the control in Week 4. In the L group, the TP level did not change from Week 2 to 4. The GLU and

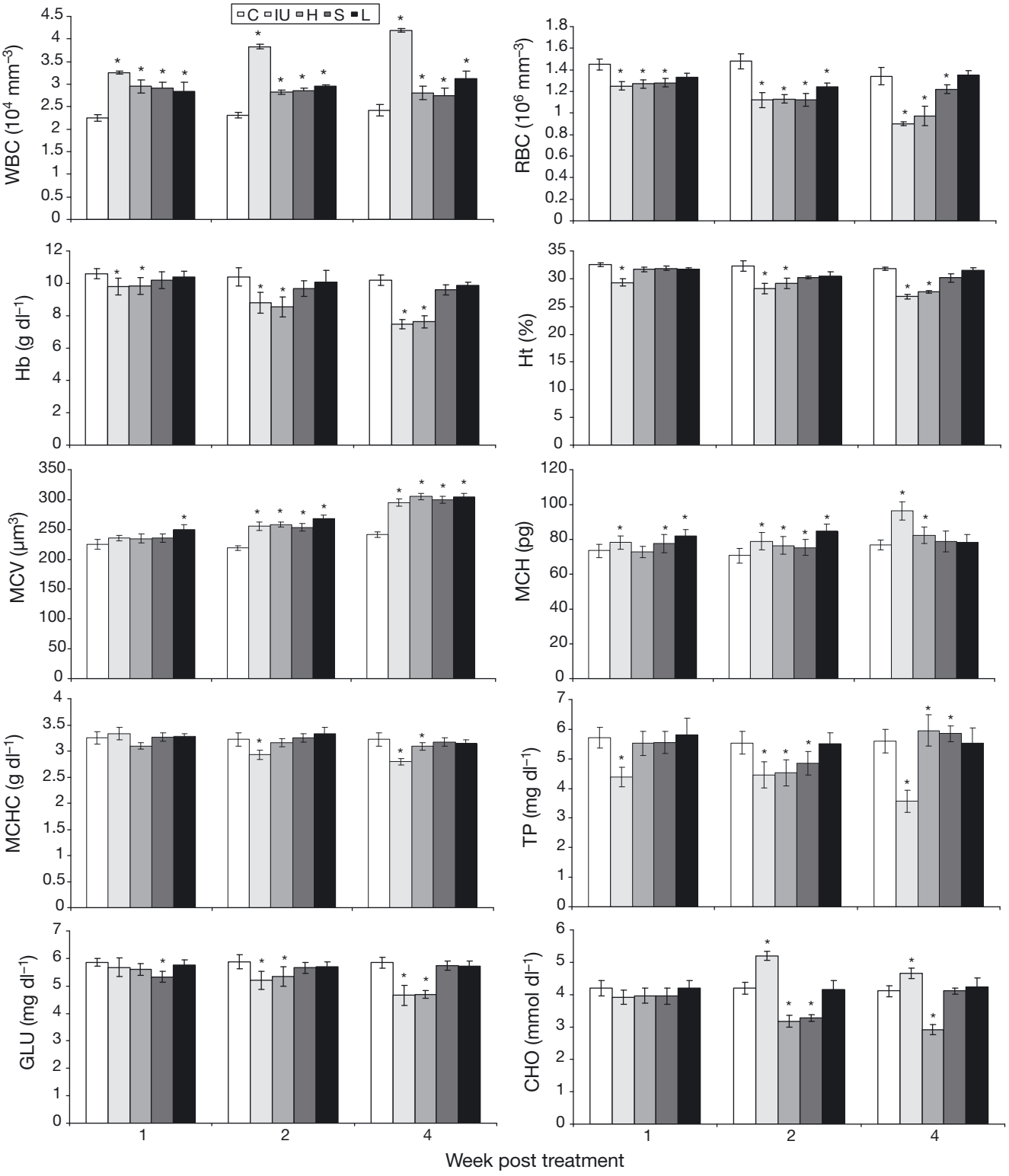

Fig. 1. *Cyprinus carpio* infected with *Aeromonas hydrophila*. Changes in haematological and biochemical profile of different experimental groups (n = 6 in each group). C: Control, fed with normal diet, IU: infected, fed with normal diet, H: infected, fed with triherbal extract-enriched diet, S: infected, fed with Sporolac-enriched diet, L: infected, fed with *Lactobacillus*-enriched diet, WBC: white blood cells, RBC: red blood cells, Hb: haemoglobin, Ht: haematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, TP: total protein, GLU: glucose, CHO: cholesterol. *Statistically significant difference ($p < 0.05$) compared to the control group in the same sampling week

CHO levels of the S and L groups were similar to those of the control group throughout the experimental period. However, in the IU and H groups, the GLU significantly decreased, whereas in the IU group the CHO level was significantly increased in Weeks 2 and 4 (Fig. 1).

Respiratory burst activity

In all the treated groups, respiratory activity did not vary significantly while it increased in the L group in Week 1. In the H, S, and L groups, it increased significantly in Weeks 2 and 4. In the IU group, the respiratory burst activity was significantly decreased in all weeks when compared to the control (Fig. 2).

Serum bactericidal activity

The complement activity of common carp serum against *Escherichia coli* at 20°C was enhanced in the S and L groups in Week 4. Fish in the H group had no increased complement activity in any sampling week when compared to the control. However, the IU group had a significantly lower complement activity (Fig. 3).

Disease resistance

Maximum protection was found in the group fed with the L diet. This group had the lowest mortality (35%) and highest RPS. However, the cumulative mortality was high in fish fed with the H and S diets, being 50% and 40%, respectively, after 30 d. The IU group had the maximum mortality (85%), while there was no mortality in the control group (Fig. 4).

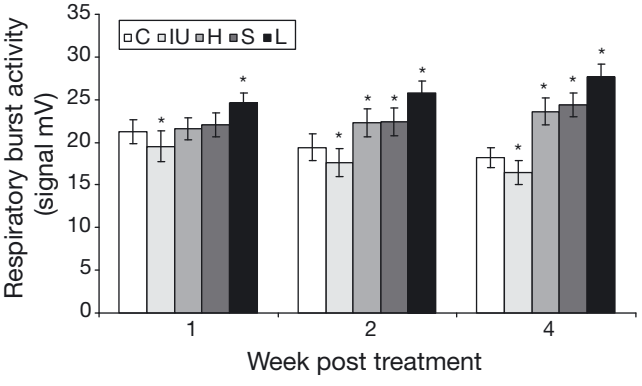

Fig. 2. *Cyprinus carpio* infected with *Aeromonas hydrophila*. Changes in respiratory burst (RB) activity measured in the diluted blood of the fish as zymosan-induced, luminol-enhanced chemiluminescence (CL) emission in control (C), infected, untreated (IU) and infected fish fed with triherbal extract (H)- or probiotic (S or L)-enriched diets. *Statistically significant difference ($p < 0.05$) compared to the control group in the same sampling week

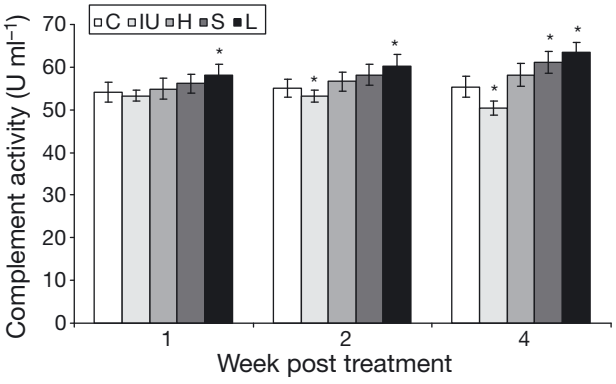

Fig. 3. *Cyprinus carpio* infected with *Aeromonas hydrophila*. Changes in complement activity measured as D-luciferin-enhanced luminescence after 90 min incubation in control (C), infected, untreated (IU) and infected fish fed with triherbal extract (H)- or probiotic (S or L)-enriched diets. *Statistically significant difference ($p < 0.05$) compared to the control group in the same sampling week

DISCUSSION

In this study, the WBC levels of infected untreated carp increased significantly from Week 1 to 4. In the infected groups treated with the Sporolac (S)- and *Lactobacillus* (L)-enriched diets, the WBC levels were significantly increased compared to control values by Week 4, indicating suppression of the growth of the pathogen. However, the RBC levels in all groups except the L group were significantly decreased compared to control in all weeks. Monitoring these values and profiling leucocytes provides information about the general immune status of the fish. In our study, RBC levels, Hb and Ht declined significantly in the infected, untreated group, indicating the destruction of RBCs leading to anaemia (Haney et al. 1992, Denton & Yousef 1975). Such reductions have been confirmed in yellowtail flounder *Limanda ferruginea* (Allen et al. 2003) infected with various pathogens. Ht is the proportion of blood volume occupied by RBCs. In common catfish, Hb content has been shown to decrease due to the swelling of RBCs and poor mobilization of Hb from the spleen and other haematopoietic organs (Scott & Rogers 1981). Low levels of RBC, Hb, Ht, MCV, MCH and MCHC have been reported in rainbow trout (Rehulka 1998) and goldfish (Brenden & Huizinga 1986) in response to *Aeromonas hydrophila* infection

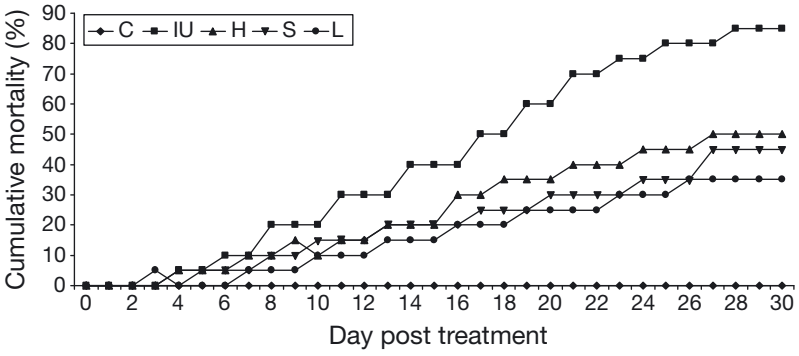

Fig. 4. *Cyprinus carpio* infected with *Aeromonas hydrophila.* Percentage of cumulative mortality in control (C), infected, untreated (IU) and infected fish fed with triherbal extract (H)- or probiotic (S or L)-enriched diets over 30 d

and epizootic ulcerative syndrome (EUS). Hb content is related to the growth and volume of RBCs (Houston 1990).

In our study, RBC and Hb values in fish fed with the L diet were close to those of the control. Groups of lactic acid bacteria exhibit inhibitory activities against Gram-positive and Gram-negative fish pathogens *in vitro* (Jöborn et al. 1997, Nikoskelainen et al. 2003) and *Lactobacillus acidophilus* has been shown to induce inhibition zones in *Aeromonas hydrophila* and *Streptococcus iniae* (Aly et al. 2008). Similar correlations have also been reported with the use of lactic acid bacteria as probiotics in *Vibrio anguillarum* and *A. salmonicida* (Gildberg et al. 1995). The general concept that the use of probiotics in aquaculture may produce various beneficial effects has been proven beyond doubt (Gatesoupe 1999, Balcázar et al. 2006). The aqueous extract of *Azadirachta indica* and other herbals has been shown to restored the altered haematological and biochemical parameters healing the lesions in *Cyprinus carpio* and *Labeo rohita* caused by either *Aeromonas* or *Aphanomyces* infections (Mitra & Varshney 1994, Harikrishnan et al. 2003, 2005).

Our results showed that the total protein and glucose levels in the IU group decreased while the cholesterol level increased throughout the experimental period. Similar trends have been reported in Atlantic salmon infected with *Vibrio* (Waagbo et al. 1988), and in rainbow trout infected with haematopoietic necrosis virus (Amend & Smith 1975). Haney et al. (1992) found that the total plasma protein and glucose levels increased in fish fed with probiotics-enriched diets due to the destruction of RBCs and the resultant release of cell contents into the blood stream.

In the present study, the respiratory burst activity in the probiotics and herbal extract diet groups (H, S and L) increased throughout the experimental period. This shows that the probiotic diet increases respiratory burst activity and complement activity in common carp. It has been shown that certain probiotic bacteria and triherbal extracts are able to stimulate phagocytic activity (Arunachalam et al. 2000, Schiffrin et al. 1997, Harikrishnan et al. 2009) as well as complement receptor expression (Pelto et al. 1998). Respiratory burst activity correlates well with phagocytosis of bacteria in fish (Hardie et al. 1996, Neumann et al. 2001) as well as in humans (Loimaranta et al. 1999). The chemiluminescence-based respiratory burst assay is, therefore, a rapid method for analysing phagocytic activity (Lilius & Waris 1984). The present study demonstrates that oral administration of *Lactobacillus* and Sporolac stimulated respiratory burst activity within 2 wk.

Oral administration of probiotic bacteria at an optimal dose (from 10^4 CFU g^{-1} to 10^8 CFU g^{-1} of feed) has been shown to stimulate respiratory burst activity augmenting the protective mechanism (Nikoskelainen et al. 2001), and to induce cell-mediated immunity by stimulating cytokine production in fish as well as in mammals (Miettinen et al. 1996). In humans, probiotic bacteria are known to stimulate antibody production (Malin et al. 1996). In the present study, the complement bactericidal activity increased significantly in Weeks 2 and 4 in the *Lactobacillus* and Sporolac diet groups. However, the mechanism of the effect of probiotic bacteria on this activity is unclear.

In a previous study, it was demonstrated that oral administration of *Lactobacillus rhamnosus* could reduce mortality of fish challenged with a virulent strain of *Aeromonas salmonicida* (Nikoskelainen et al. 2001). The best protection against *A. salmonicida* was observed when fish were treated with a *Lactobacillus*-enriched diet (mortality 35%). Moreover, in that study, higher protection was observed in fish treated with a Sporolac-enriched diet (mortality 45%) followed by those receiving a triherbal extract-enriched diet (mortality 50%) compared to the infected, untreated group (mortality 85%). A higher dose of *Lactobacillus* (1.9 to 9.7×10^{10} CFU g^{-1}) in feed did not increase the respiratory burst activity. Furthermore, it is known that virulent strains of fish pathogens like *A. salmonicida* can resist both the cellular (Daly et al. 1996, Barnes et al. 1999) and humoral defences (Merino et al. 1994, 1997) of fish but they can be killed by activated macrophages.

We have shown that probiotic treatment is can restore altered haematological and biochemical parameters to near normal values. The present work shows that the chosen probiotic bacteria might have an impact on the specific and innate immunity of fish.

They may also trigger the immune system of Tilapia *Oreochromis mossambicus* infected with *Aeromonas hydrophila* (Venkatalakshmi & Michael 2001, Nikoskelainen et al. 2003). It can be concluded that the probiotic-enriched diet is more effective than the triherbal extract-enriched diet, and that *Lactobacillus* was superior to Sporolac in affording protection against pathogens and promoting survival. Further extensive testing, including field and commercial cost benefit analysis, is necessary before recommending its widespread application in aquaculture. The optimal dose of the probiotic bacteria to be administered may depend on the size and species of cultivated fish.

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