The comparative effects of dietary supplementation with *Pediococcus acidilactici* and *Enterococcus faecium* on feed utilization, various health-related characteristics and yersiniosis in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792)

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Abstract

This study examined the effects of feeding of two dietary probiotics on growth performance and feed utilization, intestinal colonization and related health criteria, biochemical parameters, hematological indices, and immune parameters and protection against *Yersinia ruckeri* in *Oncorhynchus mykiss* during a period of 56 days. The study included 3 groups:1(a control group of fish, 2) a group of fish fed with a basal commercial diet supplemented with 2×10^6 CFU g^{-1} *Pediococcus acidilactici* (PA-group and 3) a group of fish fed with a basal commercial diet supplemented with 2.5×10^8 CFU g^{-1} *Enterococcus faecium* strain IR5 (EF-group). Each group was run in triplicate. The PA-group and EF-group showed significant improvement with respect to WG, SGR, FCR, PER, lactic acid bacteria (LAB) (%) in intestinal, intestinal colonization (log CFU g^{-1}), survival (%), RBCs, WBCs, hematocrit percentage (Hct %), and respiratory burst activity (RBA) levels, each of which was significantly higher than that in the control group ($p<0.05$). The WG and PER in the EF-group were significantly higher than that in the PA and control groups ($p<0.05$). Levels of immune-system response across a variety of measures, some of the measured hematological indices, LAB (%) in intestines, and survival rate (%) were higher in the PA group than that in the EF group on 56th days of feeding. Mortality in the PA group (20%) and EF group (33.3%) were significantly lower compared with that in the positive control (73.3%) during the 10 days following exposure to *Y. ruckeri* ($p<0.05$).

Keywords: Rainbow trout, Probiotic, Growth performance, Hemato-immunological parameters, *Yersinia ruckeri*

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Introduction

Cold-water fish are commercially valuable in all parts of the world (Nafisi Bahabad and Azhar, 2015). Iran is the world's largest producer of rainbow trout in fresh water (Kalbassi *et al.*, 2013). In 2013, Iran's farmed rainbow trout production was 143,917 tons, up from 23,138 tons in 2003. However, production declined in 2014 to 126,515 tons (FAO, 2016). This decline was precipitated by management and disease problems in the cold-water aquaculture industry (Baba Alian *et al*., 2014; Nafisi Bahabad and Azhar, 2015). Probiotics may activate immune function and improve health status, growth performance, and feed utilization of teleosts (Austin, 2015). Further research efforts are needed to draw comprehensive conclusions on the effects and benefits of adding probiotics to fish diets in order to provide the aquaculture industry, the scientific community, regulatory bodies, and the general public with the necessary information (Ringø *et al*., 2010). In fact, it is important to distinguish the differences between probiotics (Merrifield *et al*., 2010a).

This study determined the effects of two probiotics drawn from a group of lactic acid bacteria (LAB) in relatively identical conditions, with the aim to increase production in the rainbow trout culture industry. The current study compared the effects of dietary supplementation with *P. acidilactici* (CNCM MA 18 / 5 M) live cells (as a common commercial probiotic) to those of *E. faecium* strain IR5 (JX154575) live cells (as a native probiotic that is the most common species from the enterococcus family being developed for use as a probiotic in fish) on growth performance, feed utilization, intestinal colonization, related health parameters, immune response, hematological indices, and incidence of yersiniosis (as a major bacterial disease) in farmed rainbow trout.

Materials and methods

Experimental animals

Two hundred and seventy rainbow trout with an average weight of 35.6 ± 3.8 g were obtained from a local commercial farm (Amol, Iran), and the *in vivo* study was carried out in a wet-laboratory at the farm. Water was supplied from a private well (Table 1).

Table 1: **The average physical and chemical indices of the water supply during the study period (mean±SD).**

Location	Temperature (°C)	pH	DO (mg L^{-1})	EC (µs cm^{-1})	TDS (mg L^{-1})
Source	18.8±0.1	7.4±0.0	7.2±0.1	820±0.0	458±0.0
Wet-Lab	19±0.7	7.6±0.3	7.6±0.2	832.1±2.3	464.2±2.1

DO: dissolved oxygen, EC: electrical conductivity, TDS: total dissolved solids.
Source: water well, Wet Lab: water for experimental tanks at the wet-laboratory.

Fish were acclimatized for 20 days in a rectangular tank (5,000 L) with a spray nozzle aerator.

The water supply was initially pumped into the reservoir tank; this was later transferred to the wet-laboratory by a pressure pump. During the adaptation period (20 days), fish were fed with the commercial trout diet (38% crude protein, 16% crude fat, 3% crude fiber and 10% moisture; EXG1, Kimiyagaran-e-Taghziyeh Co., Shahrekord, Iran) 3 times per day at 2.2% of body weight. These fish had no history of elevated mortality or abnormalities on the first and last days of the adaptation period. On day 21, the fish (average weight, ~52 g) were transferred to the wet-laboratory, where they were subsequently randomly distributed into 3 groups, with each group containing 90 fish (30 fish per tank). Nine cube-shaped concrete tanks (250 L) were used with a flowing water-well system, in which each tank was equipped with an inlet, outlet, and continuous aeration with a spray nozzle aerator. The tanks were maintained under natural light/dark conditions.

During the experimental period, water quality parameters (mean ± SD) were monitored with a multi-parameter water analyzer (HQ40d, Hach®, USA). No significant changes in water quality parameters were observed during the treatments. Fish were fed with the experimental diets, and the daily mortality rate was recorded for each tank for 8 weeks.

On days 28 and 56 of the trial, 45 fish (5 fish per tank) were transported live within 1 hr of capture in 9 plastic bags (1 plastic bag per tank) containing oxygen-enriched water in an icebox to the laboratory of the Caspian Sea Ecology Research Center (CSERC), Sari, Iran, to obtain blood and serum samples (Harikrishnan *et al*., 2010; Gopalakannan, 2011). Comprehensive hematologic, immunologic, and microbiologic analyses were performed at CSERC laboratories.

Probiotic bacteria

E. faecium strain IR5 (GenBank accession no: JX154575) (EF) was prepared from CSERC, Sari, Iran. This bacterium was isolated from the intestinal tract of the cultured rainbow trout (*Oncorhynchus mykiss*) in Mazandaran province, Iran, and had been stored in glycerol (20%) at −20°C. The preparation of inoculants proceeded as follows: EF was cultured in MRS broth (De Man, Rogosa, and Sharpe) (Difco; Detroit, Michigan, USA) and incubated at 37°C for 24 hr under aerobic conditions in a shaker incubator at 150 rpm. EF cells grown in the MRS broth were harvested by centrifugation at 5010 rpm for 15 min at 4°C, and the cell pellets were washed 3 times with distilled water and stored at 4°C until use (Daum *et al*., 1982). The final cell concentration was adjusted to 10^{10} cells mg^{-1}. *P. acidilactici* (PA) was sourced from a commercial probiotic product (Bactocell®; Lallemand, France). The preparation method for PA was similar to that of EF.

Experimental diets and design

Three experimental diets were formulated to commercial trout feed (EXG1, Kimiyagaran-e-Taghziyeh Co., Shahrekord, Iran) (control group), *P. acidilactici* (2×10^6 CFU g^{-1} of feed) (PA-group) (Anderson, 2013), and *E. faecium* (2.5×10^8 CFU g^{-1} of feed) (EF-group) (Neissi *et al*., 2013) (Table 2). After live cultures were incorporated into basal diets (Lazado *et al*., 2012), the experimental feed was dried at room temperature and stored in sealed plastic bags at –4°C until use (Balcázar *et al*., 2007a,b). New batches of the experimental feed were produced every 3 days. Fish were fed 2.2% of their biomass per day in equal rations at 8:00, 12:30, and 16:00 hours for 8 weeks. Lactic acid bacteria (LAB) counts and survival of the probiotics were confirmed after incorporation in the feed. Five-gram quantities of food were homogenized in 45 mL of sterile saline, and serial dilutions prepared to log 6 CFU mL^{-1}, after 0.1 mL^{-1} were spread on triplicate plates of MRS media. Colony counts were determined after incubation for 48 hr. at 30°C.

Growth performance, health status, and feed utilization

All fish were deprived of food for 24 hr before weighing and sampling. The fish weight was measured at the beginning (0 days), the middle (28 days) and at the end of the trial (56 days). The results for growth performance, feed utilization, and health status (measured via wet weight gain [WG], specific growth rate [SGR], feed conversion ratio [FCR], protein efficiency ratio [PER], and survival rate [%]) were calculated using the following equations:

WG = final weight (g) – initial weight (g)

Specific growth rate (SGR) =100 × (ln final weight (g) - ln initial weight (g))/56d

Feed conversion ratio (FCR) = feed intake (g)/weight gain (g)

Protein efficiency ratio (PER) = weight gain per fish (g)/protein intake per fish (g)

Survival (%) = 100 × [(total population at beginning of study – number of deaths during 56d)/total population at beginning of study]

Table 2: Proximate composition and probiotics levels (log CFU g^{-1}) of experimental diets.

Group	Commercial trout feed	Probiotics inclusion (log CFU g^{-1} of feed)
Control	EXG1	_
EF	EXG1	84
PA	EXG1	6.3

Dietary groups: EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*. EXG1, the basal diet.

Intestinal bacterial counts

All fish were deprived of food for 24 hr before the day of the middle of the trial (28 days) and also at the end (8 weeks). Two fish per tank (6 fish per diet) were euthanized by administration of a lethal dose of clove oil (Barijessance pharmaceutical co, Kashan, Iran) followed by destruction of the brain. The entire body of each fish was immersed in ethanol (ethyl alcohol, C_2H_5OH) 70% for 10 min and placed on a sterile laboratory dissection tray. After aseptic dissection, approximately 1.0 g of the intestinal sections was mechanically homogenized for 5 min in 9.0 mL of sterile saline. Samples were serially diluted to 10^{-6} with physiological saline, after 10 µL was spread on the TSA (Merck, Germany) plate to obtain the total bacteria count (TBC) (Merrifield *et al*, 2010b), and 100 µL was spread on DeMan, Rogosa, and Sharpe (MRS) plates to determine the number of viable LAB (Safari *et al*, 2016). Colony counts were enumerated after incubation for 48 hr at 36°C (Capkin and Altinok, 2009). These study procedures were performed in triplicate.

Hematological parameters

After 28 and 56 days of the trial, blood samples were randomly taken from 5 fish per tank (15 fish per group). Individual fish were euthanized using clove oil (Barijesans, 1 mg L^{-1} for 4 min). Blood was sampled from the caudal vein using a 25-gauge needle and 5-mL syringe. The blood samples were pooled according to the experimental diet received and immediately divided into two halves. One half was transferred to a micro-tube containing heparin anticoagulant for the respiratory burst assay and hematological examination, while the other half was transferred to a non-heparinized micro-tube. Sera samples were obtained by blood centrifugation (7906 rmp for 10 min at 4°C) and then stored at –20°C until use for the biochemical and immunological studies enumerated earlier (Blaxhall and Daisley, 1973; Binaii *et al*., 2014). The total red blood cells (RBCs: 10^6 mm^3) and white blood cells (WBCs: 10^3 mm^3) were enumerated using a hemocytometer. Hematocrit was determined using the standard micro-hematocrit method and expressed as a percentage (Hct %). The hemoglobin level (Hb, g dL^1) was estimated using the cyan–metahemoglobin method using a wavelength of 540 nm (Binaii *et al*., 2014). Furthermore, differential leukocyte types were measured by preparing Wright–Giemsa stained smears. Blood smears were studied using light microcopy to obtain blood cell counts (Blaxhall and Daisley, 1973). The hematological indices, such as mean cell hemoglobin (MCH, pg), mean cell hemoglobin concentration (MCHC, g dL^{-1}), and mean cell volume (MCV, fL) were calculated using the total RBC, Ht, and Hb content (Binaii *et al*., 2014).

Immunological parameters

Serum lysozyme activity was determined by turbidimetric assay, as

described by Ellis (1990), with some modifications. Sera (25 mL) was added to 1-mL *Micrococcus lysodeikticus* (Sigma Aldrich; St Louis, Missouri, USA) suspension (0.2 mg mL^{-1}, 0.05 M PBS, pH 6.2), and absorbance was measured at a wavelength of 670 nm after 30 s and 180 s by spectrophotometer (Biophotometer Eppendorf). Respiratory burst activity (RBA) was measured using a chemiluminescent assay (CL) method described by Khoshbavar-Rostami *et al*. (2006) using an automated system for CL analysis (LUMI scan Ascent T392, Finland). Finally, levels of IgM and complement components C3 and C4 in serum were measured using a biochemical auto analyzer (Eurolyser, Belgium) and commercial kits (Pars Azmoon, Tehran, Iran) (Shahsavani *et al*., 2010).

Biochemical assay in serum

Total protein (TP) and albumin (ALB) levels in the serum samples were measured using commercial kits (Pars Azmoon, Tehran, Iran) using the auto analyzer method (Shahsavani *et al*., 2010). The globulin level was calculated by subtracting albumin values from total serum protein; the albumin/globulin (A/G) ratio, by dividing the albumin values by the globulin values.

Y. ruckeri challenge

A virulent strain of *Y. ruckeri* biotype 1 (GenBank accession no: KC291153, Department of Biotechnology, CSERC, Sari, Iran) was used for the experimental challenge. A lyophilized ampule of the second passage of the bacterium was first grown in tryptic soya broth (Merck, Germany) and incubated at 25ºC for 48 hr. The bacterial cells were then collected in sterile PBS after centrifugation of the culture at 4°C for 15 min. The pellet cells were washed 3 times in distilled PBS (Soltani *et al*., 2014). The sample was serially diluted to 10^8 with PBS and stored at 4ºC until being used. After the end of the trial (56 days), 15 fish per diet (5 fish per tank) were randomly selected. These fish were intraperitoneally (IP) injected with 0.1 mL of a virulent strain of *Y. ruckeri* before being placed in separate tanks (5 injected fish per tank).

The challenge study was performed using injection (IP) of *Y. ruckeri* (KC291153) containing 1.2×10^8 viable bacteria mL^{-1} in 0.1 mL of PBS per fish. Fish fed with non-supplemented diet (the control group) were divided into two halves. In one half of the total group of fish, 15 fish (5 fish per tank) from the control group were given *Y. ruckeri* in the same way as the 2 groups described above (the positive control group); in the other half, 15 fish (5 fish per tank) were given an intraperitoneally injection (IP) with 0.1 mL of PBS (the negative control group). Fish were kept for 10 days in their tanks (which had aeration systems); water temperature was maintained at 19.1 ± 0.3°C with a DO of 8.27 ± 0.27 mg L^{-1}.

During the challenge study, fish were fed with the commercial trout

feed (EXG1, Kimiyagaran-e-Taghziyeh Co., Shahrekord, Iran) once daily (Welch and La Patra, 2015). Any signs of abnormalities or adverse health effects, including all deaths, in the fish were recorded during the experimental period (10 days). *Y. ruckeri* infection was re-isolated in the fish that died in each tank by plating kidney tissue onto blood agar (5% Sheep Blood in TSA, Merck, Germany). This procedure was performed in all fish that died and for all tanks. The mortality (%) was calculated as:

Mortality (%) = (no. of deaths in a specified period/initial number of fish) × 100

The protective index was calculated according to Amend (1981) as the relative percentage survival (RPS):

RPS =1- [mortality (%) in the treatment with probiotic group/mortality (%) in the control group] ×100

Statistical analysis

This experiment used a completely randomized design (CRD), and all tests were performed in three exact replications. Statistical analyses were carried out using SPSS version 18 (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used for comparison between groups, with a P-value of <0.05 considered statistically significant. Data were described as mean ± SD.

Results

Intestinal microbial analysis

After 28 and 56 days of feeding, the total intestinal-tract bacteria count of the fish was different in all groups. The populations of the LAB colonies in the intestinal tracts of the fish treated with an EF diet or a PA diet were significantly higher than those for the control diet (p<0.05; Table 3). After 56 days, these values accounted 5.76% and 6.43% of the bacterial population for fish fed EF-supplemented and PA-supplemented diet, respectively, versus 0.21% of the bacterial population in the control group (p<0.05; Fig. 1).

Growth performance parameters

The WG, PER, and survival (%) for the fish receiving the EF diet or the PA diet significantly increased when compared to the control group (p<0.05). Additionally, the FCR with the EF diet and PA diet were significantly lower than that for the control group (p<0.05). The WG and PER in the EF-group was significantly higher than in the PA-group after 8 weeks of feeding (p<0.05). The findings are presented in Table 4.

Hematology

Table 5 demonstrates the total hematological indices. The RBC and WBC, along with the neutrophils in fish fed the EF-diet versus the PA-diet were greater than that in the control group after 8 weeks.

Table 3: Average total count of bacteria (log CFU g^{-1}) in intestine tract of rainbow trout for 8 weeks.

Group	Time	TBC (log CFU g^{-1})	LAB (log CFU g^{-1})
Control	Week 4	7.45±0.07^{a}	4.28±0.36^{b}
EN		7.81±0.51^{a}	6.37±0.63^{a}
PA		7.41±0.16^{a}	6.03±0.57^{a}
Control		6.99±1.32^{a}	4.31±0.97^{b}
EN	Week 8	7.78±0.08^{a}	6.54±0.14^{a}
PA		7.74±0.11^{a}	6.55±0.09^{a}

Dietary groups: EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*.
TBC, Total bacterial count; LAB, lactic acid bacteria
Values in the same column with different superscripts show significantly different (p< 0.05).

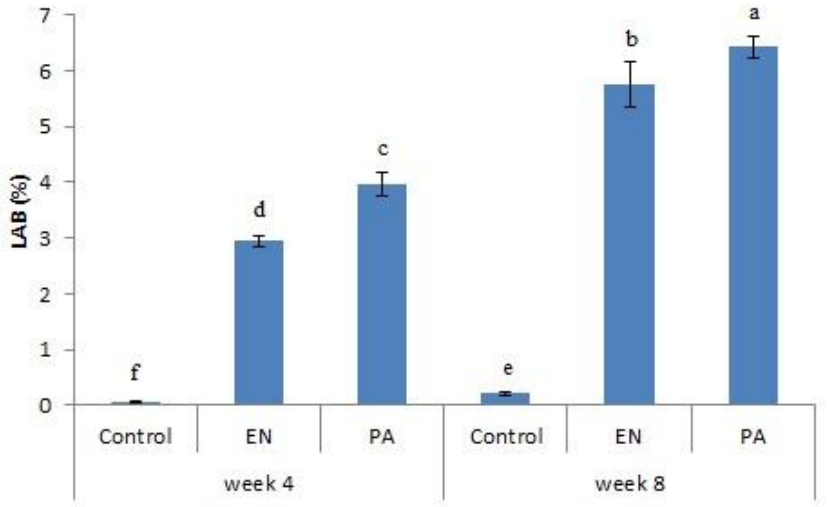

Figure 1: Proportion (%) of Lactic Acid Bactria from the cultured rainbow trout in intestinal microbiotic for 8 weeks feeding on the experimental diets.
LAB, Lactic Acid Bactria; EF, diet enriched with *Enterococcus faecium*; PA, diet enriched with *Pediococcus acidilactici*.
Data presented as mean±SD of 6 individual fish (n=6). Significant difference (p<0.05) with control is indicated by different letter notation.

Table 4: Growth performance of rainbow trout after 8 weeks feeding on experimental diets, n = 15 per group (mean ± SD).

Index	Control	EF	PA
WG (g)	52.83±7.73^{c}	71.08±2.32^{a}	66.67±1.95^{b}
SGR (% day^{-1})	1.09±0.17^{b}	1.53±0.13^{a}	1.48±0.12^{a}
FCR	1.38±0.2^{a}	1.18±0.9^{b}	1.22±0.04^{b}
PER (g)	1.9±0.28^{c}	2.23±0.19^{a}	2.15±0.09^{b}
Survival (%)	95.5±9.7^{b}	100.0±0.0^{a}	100.0±0.0^{a}

Dietary groups: EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*.
WG, weight gain; SGR, Specific growth rate; FCR, Feed conversion ratio; PER, protein efficiency ratio.
Values in the same row with different superscripts show significantly different (p<0.05).

Table 5: Hematological indices of rainbow trout for 8 weeks of feeding on experimental diets. n=15 per group (mean ± SD).

Group	Time	RBC (10^6 mm^3)	WBC (10^3 mm^3)	Hct (%)	Hb (g dL^{-1})	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})	Lymph. (%)	Neut. (%)	Mon. (%)
Control	Week 4	1.35± 0.25^{c}	4.98± 0.91^{c}	33.78± 4.87^{c}	7.11± 0.73^{a}	250.22± 8.23^{a}	55.6± 5.6^{a}	20.52± 3.68^{a}	98.56± 1.94^{a}	1.44± 0.94^{a}	0.0± 0.0^{a}
EF		1.57± 0.11^{b}	8.77± 3.05^{b}	37.78± 3.8^{b}	7.68± 0.88^{a}	243.64± 6.8^{a}	50.92± 2.43^{a}	19.2± 1.42^{a}	97.89± 2.42^{a}	2.11± 1.42^{a}	0.0± 0.0^{a}
PA		1.57± 0.25^{b}	9.7± 2.32^{b}	38.78± 2.94^{b}	7.83± 0.57^{a}	261.21± 6.6^{a}	51.87± 1.7^{a}	18.34± 1.03^{a}	97.44± 3.17^{a}	2.33± 1.77^{a}	0.0± 0.0^{a}
Control	Week 8	1.53± 0.15^{b}	9.32± 2.97^{b}	37.24± 1.0^{b}	7.90± 0.32^{a}	253.22± 16.2^{a}	53.1± 9.59^{a}	21.37± 0.44^{a}	99.33± 1.0^{a}	0.67± 0.7^{a}	0.0± 0.0^{a}
EF		1.75± 0.27^{a}	10.87± 4.45^{b}	42.11± 3.55^{a}	8.36± 0.88^{a}	240.62± 6.4^{a}	50.28± 3.09^{a}	19.85± 0.66^{a}	98.78± 1.09^{a}	1.22± 0.85^{a}	0.0± 0.0^{a}
PA		1.75± 0.55^{a}	14.1± 3.15^{a}	44.0± 3.64^{a}	8.7± 0.27^{a}	251.43± 7.17^{a}	51.2± 1.1^{a}	19.77± 1.13^{a}	98.7± 2.2^{a}	1.22± 0.9^{a}	0.11± 0.04^{a}

Dietary groups: EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*.
RBC, red blood cells; WBC, white blood cells; Hct, hematocrit; Hb, hemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Lymph, lymphocytes; Neut, neutrophils; Mon, monocyte.
Values in the same column with different superscripts indicate significant differences (p<0.05).

The RBC and hematocrit (%) in both the PA-diet and EF-diet groups were significantly higher than in the control group (p<0.05). The WBC in the PA-diet group was significantly higher than in the EF-diet and control groups (p<0.05).

Immunological parameters

Respiratory burst activity level of serum in fish that were fed the PA and EF diets were significantly higher than in the control group (p<0.05). After 56 days, lysozyme activity and levels of the complement components C3 and C4 were not significantly different between the groups, whereas the results showed an increase in the immune parameters for the PA and EF diets when compared with those in the control group (Table 6).

Biochemical parameters (blood)

The results of total protein, albumin, globulin, and A/G levels in serum are displayed in Fig. 2.

Challenge test

10 days after IP injection of *Y. ruckeri* (biotype 1) in fish, the mortality rate (%) in the positive control group was the highest (73.3%) and significantly higher than that in fish fed the PA diet (20%) and EF diet (33.33%) (p<0.05). The lowest mortality rate (%) was observed in the negative control group (0.0%), which in turn was significantly lower than that in the fish fed the EF diet (p<0.05). Table 7 displays the details of these results.

Table 6: Immune parameters levels on sera of rainbow trout after 8 weeks of feeding on experimental diets. n=15 per group (mean±SD).

Group	Time	Lysozyme activity (µg mL^{-1})	IgM (mg dL^{-1})	RBA (RLU S^{-1})	C3 (mg dL^{-1})	C4 (mg dL^{-1})
Control	Week 4	1.48±1.14^{b}	72.65±16.65^{b}	965.63±272.61^{b}	22.28±11.85^{a}	11.9±2.74^{a}
EN		3.6±0.86^{a}	86.71±18.32^{b}	1481.7±399.24^{a}	22.74±5.58^{a}	11.96±2.87^{a}
PA		3.75±1.04^{a}	96.46±13.2^{b}	1717.57±548.22^{a}	23.76±4.00^{a}	13.99±1.75^{a}
Control	Week 8	3.75±2.63^{a}	75.94±13.19^{b}	1214±319.24^{b}	22.15±4.39^{a}	8.54±1.11^{a}
EN		4.33±2.13^{a}	97.39±14.43^{b}	1485.11±324.02^{a}	22.59±8.15^{a}	11.44±5.59^{a}
PA		5.23±1.28^{a}	135.13±32.46^{a}	1955.56±683.61^{a}	28.03±7.67^{a}	23.58±16.7^{a}

Dietary groups: EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*; IgM, total immunoglobulin; RBA, Respiratory burst activity; C3, complement component C3; C4, complement component C4.
Values in the same column with different superscripts indicate significant differences (p<0.05).

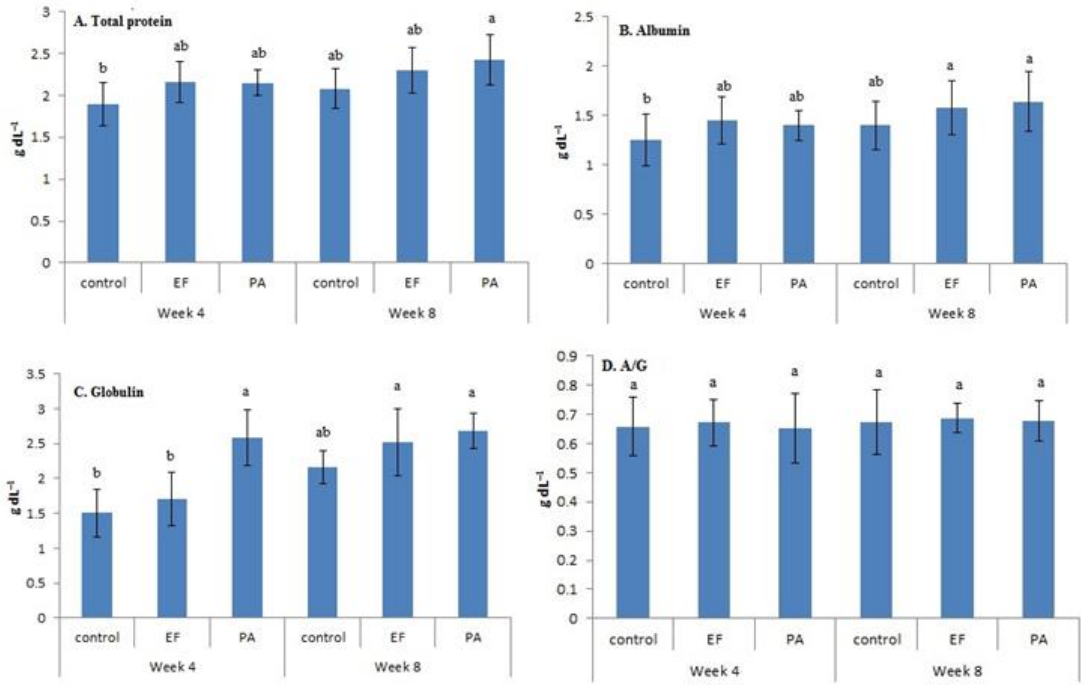

Figure 2: Biochemical indices present on sera of rainbow trout for 8 weeks of feeding on experimental diets. n = 15 per group (mean ± SD).
Dietary groups: EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*; A, total portion; B, albumin; C, globulin; D, albumin/globulin ratio.
Significant difference (p<0.05) with control is indicated by different letter notations.

Swimming near the surface, exophthalmia and darkening of the skin, petechial hemorrhages on the surfaces of the liver and pyloric caeca, hemorrhagic ulcers in the lateral muscles, and bladder distention were observed in the moribund fish. The spleen was often enlarged and almost black in color, with the lower intestine becoming reddened and filled with an opaque, yellowish fluid.

Table 7: Rainbow trout mortality (%) and relative survival (%) after 8 weeks feeding with probiotics and IP injection of *Yersinia ruckeri* KC291153 for 10 days. n = 15 per group.

Group	*Y. ruckeri* (Cells mL^{-1})	Dose (mL)	No. of mortality (10 days), n=15	Mortality(%)	Relative percentage survival
Positive control	1.2×10^8	0.1	11	73.3^a	-
EF	1.2×10^8	0.1	5	33.3^b	54.6
PA	1.2×10^8	0.1	3	20^b	72.8
Negative control	PBS	0.1	0	0.0^c	-

Dietary groups: EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*. PBS, phosphate-buffered saline.
Values in the same column with different superscripts indicate significant differences (p<0.05).

Discussion

Kosaza (1986) published the details related to the first empirical application of probiotics in aquaculture. However, large gaps of knowledge on this subject remain (Merrifield *et al*., 2010a).

In the present study, total viable counts (CFU g^{-1}) of bacteria in the rainbow trout distal intestinal lumen had a range that roughly indicated an average of 10^7 CFU g^{-1}, which is about the same as the range previously found in studies in rainbow trout (Gram *et al*., 1999; Merrifield *et al*., 2010a, c). The LAB population of the intestinal tract of fish fed a PA diet or an EF diet was significantly higher than that of the control group (Table 3) (p<0.05). Several previous studies demonstrated that *E. faecium* (Panigrahi *et al*., 2007; Merrifield *et al*., 2010a) and *P. acidilactici* (Merrifield *et al*., 2011) increased the LAB population in the intestinal tract of farmed rainbow trout when compared with the control group. Studies have reported that *E. faecium* may be better adapted than other probiotics to the environmental conditions of reared rainbow trout (Panigrahi *et al*., 2007; Merrifield *et al*., 2010b). The present study showed that a *P. acidilactici* (with log 6.3 CFU g^{-1}) supplemented diet led to development of a similar LAB population to that of an *E. faecium* (log 8.4 CFUg^{-1}) supplemented diet in the intestine of juvenile rainbow trout after 8 weeks (Table 3). It seems that *P. acidilactici* may lead to better colonization of the intestinal tract of reared rainbow trout than the IR5 strain of *E. faecium*.

The general effects of probiotics on increased production and increased resistance to stress in fish and other aquatic animals in aquaculture have been confirmed to aid in the digestion of food and the absorption of vitamins, help stimulate the immune system, and break down cellulose and other polysaccharides (Gatesoupe, 1999; Verschuere, 2000; Sun *et al*., 2011; Mahmoudzadeh *et al*., 2016). The results of the present study demonstrated that either an EF-diet or a PA-diet significantly improved the growth performance and feed utilization (along with related health criteria) in farmed juvenile rainbow trout when compared to the control

group (p<0.05). Overall, it appears that *E. faecium* (log 8.4 CFU g^{-1} feed) may be more effective in enhancing growth and improving feed utilization in juvenile rainbow trout in comparison to *P. acidilactici* (log 6.3 CFU g^{-1} feed) in 8 weeks (Table 4). Previous research findings showed that sources of probiotics in diet can affect mode and function of the bacteria in fish the intestinal track (Merrifield *et al*., 2010c; Ramos *et al*., 2013).

A hematological assay may provide an index of the physiology status of fish (Adel *et al*., 2015). Many authors have agreed that the number of erythrocytes in the typical rainbow trout is between 0.8 and 1.5 $\times 10^6$ (Saglane-Unal *et al*., 2003; Farahi *et al*., 2010; Docan *et al*., 2011). Significant elevations in RBC were also recorded with probiotic treatment in rainbow trout by Irianto and Austin (2002) (p<0.05).

Hemoglobin (Hb) is the protein contained in red blood cells that is responsible for delivery of oxygen to the tissues. To ensure adequate tissue oxygenation, a sufficient hemoglobin level must be maintained (Srivastava and Sahai, 1987; Docan *et al*., 2011). In the current study, the results in hemoglobin and RBC levels were above normal in fish fed either a PA-diet or an EF-diet after 4 and 8 weeks of feeding. This could be a reaction to increasing the metabolism of cells in the PA and EF groups (Table 4) (Fadl *et al*., 2013; Gado *et al*., 2014) and with the increase in respiratory burst activity (Table 6) in probiotic groups (Walker *et al*., 1990) in comparison to the control.

The Hct (%) is the volume percentage (vol %) of RBCs in blood (Walker *et al*., 1990). A significant increase of the Hct (%) in the EF-group and PA-group was observed when the number of red blood cells in the probiotic groups was significantly higher than that in the control group (p<0.05). This finding was supported by the study of Safari *et al*. (2016).

Fish fed a PA or EF diet showed no significant difference versus the control group in MCV, MCH, and MCHC levels. From the physiological point of view, the MCV, MCH, MCHC levels registered for all groups in this study are normal values for rainbow trout (Ghittino, 1983; Saglane-Unal *et al*., 2003; Farahi *et al*., 2010; Zorriehzahra *et al*., 2010).

Leukocytes (or WBCs) are actively engaged in the destruction or neutralization of invading microorganisms and are quickly transported to the vicinity of infection or inflammation (Magnadóttir, 2006). Several previous studies showed a high WBC count in rainbow trout when fed specific types of LAB-supplement diets (Fadl *et al*., 2013; Khoshghalb *et al*., 2013; Safari *et al*., 2016), in agreement with the results of this study. The probiotic groups showed an improvement in innate immunity versus the control group with increases in WBC counts.

The neutrophils and monocytes are phagocytic (Magnadóttir, 2006). In the current study, the monocytes were only

observed in the PA-group after 8 weeks. The neutrophils (%) in the EF-group and PA-group were higher than that in the control group after 4 and 8 weeks of feeding. The results showed that neutrophils (%) in the PA-group were higher than in the EF-group after 4 and 8 weeks feeding. After 56 days, neutrophils (%) declined and lymphocytes (%) increased in all the groups when compared with 4 weeks after feeding (p>0.05). Lymphocytes have been shown to have a positive effect on the humoral and cellular immunity of fish (Parra *et al*., 2015). These findings are similar to those of Safari *et al*. (2016) and Khoshghalb *et al*. (2013).

Lysozyme activity is another defensive factor against invasion by microorganisms. In addition to having a direct antibacterial effect, lysozymes promote phagocytosis as an opsonin, or by directly activating polymorphonuclear leukocytes and macrophages (Klockars and Roberts, 1976; Jollès and Jollès, 1984). The serum lysozyme activity level may be increased when rainbow trout are fed LAB-supplemented diets (Panigrahi *et al*., 2004, 2005; Kim and Austin, 2006; Balcázar *et al*., 2007a, b). In this study, serum lysozyme activity levels in the PA-group and EF-group were higher than in the control group after 28 and 56 days of feeding; this increase was significant at 28 days (p<0.05). Serum lysozyme activity level in the PA-group was higher than EF-group after 28 and 56 feeding (Table 6). Concentrations of complement components C3 and C4 in serum are used as indicators of complement consumption in immune complex diseases (Koelle and Bartholomew, 1982). Complement is the major humoral component of the innate immune system, which plays a key role in alerting the host immune system of the presence of potential pathogens as well as their clearance. Complement is initiated by one or a combination of three pathways; namely, the classical, alternative, and lectin pathways. All three pathways merge at a common amplification step involving C3, and proceed through a terminal pathway that leads to the formation of a membrane attack complex, which can directly lyse pathogenic cells (Boshra and Sunyer, 2006). Mayer (1961) showed that some gram positive bacteria can activate the alternative pathway, leading to C3 deficiency and susceptibility to bacterial infections. The most important action of complement components is to facilitate the uptake and destruction of pathogens by phagocytic cells. C4b also acts as an opsonin but has a relatively minor role, largely because so much more C3b than C4b is generated (Janeway *et al*., 2001). Panigrahi *et al*. (2007) and Safari *et al*. (2016) reports confirmed the C3 and C4 levels in this study.

The previous studies have shown a significant increase in the respiratory burst activity level in fish fed diets including various probiotics specifically from certain types of LAB (p<0.05) (Nikoskelainen *et al*., 2003; Salinas *et al*., 2005; Safari *et al*., 2016).

Respiratory burst activity (RBA) is an important innate defense mechanism of fish (Nayak *et al*., 2010). Respiratory burst is the rapid release of reactive oxygen species (superoxide radical and hydrogen peroxide) from different types of cells, and usually denotes the release of these chemicals from immune cells, e.g., neutrophils and monocytes, as they come into contact with different bacteria (Sharp and Secombes, 1992; Sharp and Secombes, 1993; Inoguchi *et al*., 2003). In the present study, an increase in neutrophil counts in the PA- and EF-group, along with an increase in monocytes in the PA-group when compared to the control group. RBA levels in the PA- and EF-groups were significantly higher than in the control group in this study ($p<0.05$). The PA-group showed higher RBA levels when compared to the EF-group ($p>0.05$; Table 6).

IgM may be increased in rainbow trout fed LAB-supplement diets (Sun *et al*., 2011; Khoshghalb *et al*., 2013; Safari *et al*., 2016). IgM is an immunoglobulin that includes the humoral-specific immune system. It increases neutralization, agglutination, opsonization, and concentrations of complement component in fish (Soltani, 2008). In addition, total immunoglobulin is an important component of the humoral defense against pathogens in teleost fish (Ingram, 1980). By these findings, it would appear that PA (log 6.3 CFU g^{-1}) and EF (log 8.4 CFU g^{-1}) supplemented diets enhance cellular and humeral immunity when compared with the control group. PA-supplemented diet can also cause a greater increase in cellular and humeral immunity in juvenile rainbow trout after 4 and 8 weeks of feeding when compared to the EF-supplement diet.

Globulin is the main resource of immunoglobulin production, and increased levels in serum have an immunostimulatory potential (Anderson, 2013). In this study, the albumin/globulin ratio does not indicate a significant difference compared to the control group. The results for total plasma protein and serum albumin levels in the present study are in accordance with previous studies on *E. casseliflavus* or *P. acidilactici* supplemented diets in rainbow trout (Merrifield *et al*., 2011; Safari *et al*., 2016).

Amend (1981) reported that the positive effect of probiotics is indicated by relative percentage survival (RPS) values over 50%. *P. acidilactici* (log 6.3 CFU g^{-1}) and *E. faecium* strain IR5 (log 8.4 CFU g^{-1}), with relative survival rates of 72.8% and 54.5% respectively, can be used to prevent and control yersiniosis in rainbow trout.

These findings suggest that *P. acidilactici* (commercial probiotic) at a dosage of 2×10^6 CFU g^{-1} or *E. faecium* strain IR5 at a dosage (native probiotic) of 2.5×10^8 CFU g^{-1}, as supplements to the rainbow trout diet for 8 weeks, can be useful to up-culture rainbow trout production by stimulating the immune response (which improves resistance against yersiniosis), reduce the cost of feed used, and improve

growth and health status. It appears that a diet supplemented with *E. faecium* strain IR5 (log 8.4 CFU g^{-1}) is more acceptable for growth performance and feed utilization improvement, while *P. acidilactici* (log 6.3 CFU g^{-1}) supplemented diet is more acceptable for stimulating the immune response and resistance to yersiniosis in cultured rainbow trout.

It was reported by Araújo *et al*., (2015) that 17 (26.6%) from 64 enterococcus isolates from trout did not harbor any antibiotic resistance or virulence factor thus considered safe for application as probiotics. The *E. faecium* IR5 can be considered relatively safe for *in vivo* application as it was isolated from a healthy fish and the experimental groups exposed to these microorganisms did not present any clinical signs of infection or any other abnormalities.

This study, to our knowledge, is the first investigation on the effects of an *E. faecium* strain IR5–supplemented diet in fish and the first report from resistance improvement against *Y. ruckeri* infection in rainbow trout (*Oncorhynchus mykiss*) by *E. faecium*. We would recommend investigation on the effects of a diet containing *P. acidilactici* and *E. faecium* supplements (with a focus on the bacteria dosage) in other farmed fish in the future.

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