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Original Article

The effect of oral administration of lactic acid bacteria isolated from kefir on intestinal microbiota, growth performance and survival in juvenile rainbow trout, *Oncorhynchus mykiss*

Mohsen Ali[1], Siyavash Soltanian[*1], Ali Taheri Mirghaed[*2], Mostafa Akhlaghi[1], Seyed Hossein Hoseinifar[3], Atefeh Esmailnejad[4]

[1]Division of Aquatic Animal Health, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
[2]Department of Aquatic Animal Health and Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
[3]Department of Fisheries, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.
[4]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

Abstract: Lactic acid bacteria (LAB) are used in the aquaculture industry to improve growth indices and nutrition efficiency of farmed fish. Kefir is a natural probiotic that largely consists of these microorganisms. This study aimed to isolate LAB strains from kefir grains and investigate their effects on the intestinal microbiota and growth indices of juvenile rainbow trout. Based on the results, one isolate was identified as *Lactobacillus faraginis* (LF) and other one as *Enterococcus durans* (ED), both were gram positive, non-hemolytic, catalase and oxidase negative. Both strains showed resistance to acidic environments and gastric juice and were able to grow against bile salts i.e. the hydrophobicity potential of both strains was evaluated above 50%. The results showed that both strains had appropriate and acceptable probiotic properties. A total of 480 juvenile fish with were randomly divided into eight groups viz. Control (basal diet), LF1 and LF2 receiving diets supplemented with *L. faraginis* at 10^7 and 10^8 cfu/g, respectively, ED1 and ED2 diets supplemented with *E. durans* at 10^7 and 10^8 cfu/g, respectively, Bactocell group receiving a diet supplemented with commercial probiotic Bactocell PA-10 (10^{10} cfu/g), combined treatments LF1+ED1 (5×10^6 cfu/g) and LF2+ED2 (5×10^7 cfu/g). The strains were prepared in the form of lyophilized powder and added to the basal diet as supplements. Sampling was performed after 0, 30, 15, and 45 days feeding with the diets. In the *in vivo* investigation, the highest LAB colony counts were obtained for the ED2, Bactocell, LF2+ED2 and LF2 groups, respectively. The highest BWI, SGR, DWG, PER, and CF, and the lowest FCR were observed in the LF2+ED2, Bactocell, ED2, and LF2 groups, respectively. On the other hand, the LF2+ED2, Bactocell and LF2 treatments also had the highest survival rate. Apparently, the use of the tested LAB strains as probiotic in high doses (LF2, ED2) and in combination (LF2+ED2) could improve growth indices, the intestinal LAB population and survival rate in juvenile rainbow trout.

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Introduction

Lactic acid bacteria (LAB) and bifidobacteria are among the microorganisms most commonly used as probiotics, although other bacteria and some yeast strains are also used for this purpose (Didari et al., 2014). An important branch of the family of LAB is the genus *Lactobacillus*, which is an anaerobic or microaerophilic gram-positive bacterium. Other notable genera of this family include *Lactococcus*, *Enterococcus*, *Pediococcus*, *Streptococcus*, and *Leuconostoc* (Makarova et al., 2006). Some strains of the genus *Enterococcus* with probiotic properties are also used in the pig and poultry feed industry as well as pharmaceutical products (Liu et al., 2014). So far, many *Lactobacillus* bacteria have been categorized as GRAS (Generally Recognized as Safe) (Soccol et al., 2010). Although some strains of the *Enterococci* are known to have probiotic properties and are used as animal feed supplements for improving growth and preventing gastrointestinal disorders (Franz et al., 2011), they are yet to be cauterized as GRAS (Huys et al., 2013).

LAB are resistant to bile salts, gastric acid, and pancreatic enzymes and adhere to the mucosal

*Correspondence: Siyavash Soltanian and Ali Taheri Mirghaed
E-mail: siyavashsoltanian@yahoo.com, mirghaed@ut.ac.ir
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membrane of the gastrointestinal tract (Fioramonti et al., 2003). Research in the field of aquaculture has shown that the use of some probiotics in the form of live and dead microorganisms may have positive effects on the host and its environment (Lazado and Caipang, 2014; Hoseinifar et al., 2017, 2019; Gobi et al., 2018). The notable applications of LAB in the aquaculture industry include improving the nutrition efficiency and growth performance, increasing the survival rate, preventing digestive and intestinal disorders by neutralizing anti-nutritional and toxic substances in the diet, controlling and monitoring the growth of microorganisms in intestines, improving immune responses and preventing the growth of pathogenic organisms in fishes (Panigrahi et al., 2005; Suzer et al., 2008; Mohapatra et al., 2012a, b). Also, LAB have been reported to have beneficial effects on growth parameters of juvenile fish by improving the digestion of nutritional compounds of the diet and enhancing digestive enzymes and intestinal microbiota (Nayak, 2010). Moreover, the multiple strains of LAB can increase the growth and protein efficiency, reduce the feed conversion ratio, and improve digestion and food absorption of fish, which they have attributed to their role in reducing stressors (Gomez et al., 2000; Lara-flores et al., 2003). Under stressful situations in artificial and intensive fish culture systems, which disrupt the balance of the bacterial flora in the water and in the digestive system of the fish, the use of probiotic strains can be beneficial way of restoring microbial balance both in water and in the fish and increase the chance of proper growth (Ringo, 1998; Lara-flores et al., 2013). According to previous studies, the use of LAB probiotics in aquaculture can increase survival, enhance disease resistance, and improve growth parameters in various species of fish (Nayak, 2010; Estebanet al., 2014; Muñoz-Atienza et al., 2014; Hai, 2015).

Kefir is a traditional fermented milk product originating from the Caucasus Mountains, which is widely used in eastern Europe, southwest Asia, and many other parts of the world (Rodrigues et al., 2005). Since the onset of industrial-scale kefir production in eastern and central Europe, the microbiology of kefir grains has attracted the attention of many researchers (Atalan et al., 2003). Kefir grains contain complex probiotic microorganism produced following milk fermentation (Atalan et al., 2003; Rodrigues et al., 2005). The fermentation agents of kefir grains include the species of *Lactobacillus*, *Streptococcus*, *Saccharomyces*, and some other bacterial genera, which coexist in the form of gelatinous colonies (Mukai et al.,1988,1991). It has been shown that the microorganisms that exist in kefir grains include LAB and yeasts that are used as leaven in its preparation (Rodrigues et al., 2005). LAB species of kefir can be both homofermentative and heterofermentative (Gao and Li, 2016). In kefir grains, LAB and yeast are present in a matrix of proteins, fats, and sugars, which also contains important species of beneficial bacteria such as *Bifidobacterium* spp. and *L. acidophilus* (Guzel-Seydim et al., 2011; Uluköy et al., 2015). There is evidence suggesting that some of the microorganisms found in kefir can potentially exhibit probiotic properties (Zheng et al., 2013; Bolla et al., 2013). Studies carried out on the probiotic properties of *Lactobacillus* strains isolated from kefir grains has shown their high resistance to gastric acid and bile salts, their ability to attach and adhere to the gut epithelial cells in in-vitro conditions, and lack of their adverse enzymatic or hemolytic activity (Zheng et al., 2013; Leite et al., 2015). Also, some studies have reported the beneficial effects of the addition of kefir grains to the fish diet on the growth performance (Uluköy et al., 2016; Van Doan et al., 2017).

Rainbow trout is an important aquaculture species in Iran, with total annual production of more than 160000 tons (Hoseini et al., 2014; Hoseini et al., 2019a). It is cultured under semi-intensive and intensive conditions in tanks, concrete ponds and earthen ponds. Although previous studies have explored the impacts of direct use of kefir grains in the fish diet, they have not investigated the probiotic potential of specific LAB strains isolated from kefir when used as a fish diet supplement. In the present work, after isolating and identifying two strains of LAB from kefir grains, they were added to the diet of

juvenile trout to investigate the effects on growth parameters, the intestinal lactic acid bacteria population and survival rate.

Materials and Methods

Preparation of kefir: Imported kefir grains originating from the Russian Caucasus Mountains were purchased from an official sales representative in Iran (Oxan company). According to Unal and Arslanoglu (2013), 20 g of kefir in 200 ml of fresh cow milk was incubated for 24 hours at 25-28°C (Wszolek et al., 2006). Then, kefir grains were filtered from the fermented milk and stored at 4°C (Uluköy et al., 2016).

Isolation and purification of kefir bacteria: To isolate the bacteria, 1 g of kefir grains was homogenized with T18 basic ULTRA-TURRAX® homogenizer (IKA, Germany) and subsequently, serial dilution was made with sterile saline solution (0.9%). Strains were isolated and purified using direct and indirect methods. In the indirect method, primary enrichment of kefir with MRS broth and the steps were continued as in the direct method. In both cases (Krieg and Padgett, 2011), the serially diluted sample was cultured on MRS agar with pH=6.2 at 37°C under 5% CO_2 atmospheres for 48 hours in the NuAire DHD AutoFlow (NU-5510) incubator. Then, purification of LAB colonies was performed by streaking bacteria on MRS agar in three replications (Dworkin et al., 2006).

Strain identification: Gram staining method was used to identify the purified strains and investigate their morphological characteristics. Staining results were confirmed with the test of solubility in 3% KOH solution based on Suslow et al. (1982). Catalase test was performed with 3% hydrogen peroxide (H_2O_2) solution (Macfaddin, 2000) and oxidase test was performed using 1% oxidase (N, N, N', N'-Tetramethyl-p-phenylenediamine) reagent (Harrigan and McCance, 1990). Of the four types of colony purified by these tests, the two that were positive in gram-positive staining and negative in KOH, catalase, and oxidase tests were chosen for molecular identification. This identification was carried out by 16srRNA gene sequencing. Whole DNA extraction from the strains was performed with Cinnagen kits (Cinnagen, Iran) based on Marmur (1961) with some modifications. The polymerase chain reaction (PCR) was then performed using the universal primers (27F: 5'-AGAGTTTGATCMTGGCTCAG-3') and (1492R: 5'-GGTTACCTTGTTACGACTT-3') and confirmed on 1% agarose gel by electrophoresis (PowerPac™) and observation with a gel doc (Syngene Gbox EF). The product was sequenced with a Macrogen sequencer (Macrogen, Korea) and the results were analyzed using the Chromas software (v.2.01, Technelysium Pty Ltd). Finally, the software BLASTn was used to compare the results with the sequences of the NCBI gene database and the isolate with the highest similarity in terms of SrNA16 gene sequence was identified.

In-vitro evaluation of the probiotic potential of the strains isolated from kefir: Hemolytic activity of the isolated LAB strains was studied in Columbia Agar (Merck, 110455) (Leite et al., 2015). pH tolerance test was carried out using the method of Klayraung et al. (2008). Resistance of strains to gastric juice was evaluated based on Yanyan et al. (2010). Also, resistance to bile salts was measured according to Walker and Gilliland (1993), briefly the suspension of the strains was added to the MRS broth with different levels of bile salts (0%, 0.15%, and 0.3%), and the optical density (OD) of the media was measured during 0, 2, 4, 8 hours of incubation with a Novaspec II Visible spectrophotometer at 620 nm, then the coefficient of inhibition of the strains for bile oxalate was calculated by the equation of Gopal et al. (1996). Hydrophobicity was evaluated by the method of Goldberg et al. (1990) based on adhesion to hexadecane. To measure the antagonistic activity of the strains, as instructed by Balcazar et al. (2008), the strains were cultured in MRS broth, then a suspension with a concentration of 0.5 McFarland of *Lactobacillus garvieae* Ir-LGT-MS-1 (KF918779), *Streptococcus iniae* (GQ850377), *Yersinia ruckeri* (KC291153), and *Aeromonas hydrophila* (AH04) freshly grown on TSA medium was added to Mueller-Hinton agar plates and cultured. Multiple wells were created on each culture medium and filled with the

Table 1. specifications and chemical analysis of extruded rainbow trout feed (FF2-Extruded).

Proximate composition	Content (%)
Crude protein	40
Crude lipid	12
Crude fiber	2
Ash	7
Moisture	5
Phosphorus	1
Feed form	Floating
Feed size (mm)	4±0.3

bacterial filtrates, and finally, the inhibition zones around the wells were measured in mm (Balouiri et al., 2016).

Preparation of the lyophilized form of the strains for diet supplementation: Lyophilized powder of the strains was prepared according to John et al. (2007). In summary, a cold-trap was used to prepare a suspension of the strains from skim milk (protective lyophilization medium) and after freeze-drying, the lyophilized form of the strains was stored at 4°C until use. To determine the exact amount of powder that should be added to the diet, colonies in one gram of lyophilized powder were counted according to Li et al. (2012).

Design of experiment and treatments (*in vivo*): A total of 480 healthy rainbow trout with mean weight and length of 41.9±2.4 gr and 14.5±0.5 cm were obtained from a private fish farm, Haraz, Tehran Province. The fish were stocked in 24 tanks (20 fish per tank) and allowed to acclimatize for 10 days, during which, the fish were monitored for health problems. The water physicochemical conditions during the experiment were as follows: temperature = 18±1.5°C, pH = 7.5±0.1, DO = 8.5±0.5 mg/L, hardness = 180±15 $CaCO_3$ mg/L, total ammonia ≤ 0.01 mg/L and nitrite ≤ 10 mg/L. During the treatment period, water was changed on a daily basis and aeration was carried continuously so that the physicochemical conditions of water remained optimal for growth. A daily ration of 3% of biomass was divided into four meals. After acclimation, the tanks were divided into eight treatments as follow:

(1) Control: basal diet without any supplement
(2) LF1: basal diet + 10^7 cfu/g *L. ferraginis*
(3) LF2: base diet + 10^8 cfu/g *L. ferraginis*
(4) Bactocell: basal diet + commercial probiotic Bactocell PA 10 (Lallemand Animal Nutrition S.A., Blagnac, France) containing 10^{10} cfu/g *Pediococcus acidilactici* (according to manufacturer)
(5) ED1: basal diet + 10^7 cfu /g *E. durans*
(6) ED2: basal diet + 10^8 cfu/g *E. durans*
(7) LF1 + ED1: basal diet + 5×10^6cfu/g *L. farraginis* + 5×10^6 cfu/g *E. durans*
(8) LF2 + ED2: basal diet + 5×10^7 cfu/g *L. farraginis* + 5×10^7 cfu/g *E. durans*.

Basal diet specifications: Commercial extruded feed for the juvenile rainbow trout (FF2-Extruded) was purchased from Faradane Co. (Iran). The type, shape, size, and composition of the basal diet were decided according to the average weight and length of the fish (Table 1).

Addition of bacterial strains to the basal diet and preparation of daily feed: First, the daily feed requirements of the treatments were determined according to water temperature and weight of the fish. Then, the lyophilized strains (as defined for the treatments) were added to the diet based on Panigrahi et al. (2004) with minor modifications. For this purpose, the exact amount of lyophilized powder needed for each diet was calculated according to the number of colonies counted in one gram of that powder (*L. farraginis*: 10^{14}cfu/g; *E. durans*: 10^{12}cfu/g). These amounts of the lyophilized powder were dissolved in edible oil and sprayed uniformly on the commercial feed pellets and mixed with a drum mixer. For the Bactocell treatment, Bectocell powder was added to the diet in the same way. For the control diet, all of the above steps were performed without adding the strains. For control one, number of the bacteria added to the diet, the colonies in one gram of

Table 2. The average number of colonies of the strains per gram of diet.

Treatments	Lactic acid bacteria (cfu/g)
Control	-
LF1	1.1×10^{7}
LF2	1.9×10^{8}
Bactocell	1.01×10^{10}
ED1	1.2×10^{7}
ED2	1.7×10^{8}
LF1+ED1	2.1×10^{7}
LF2+ED2	2.65×10^{8}

diet were counted basedon Swanson et al. (1992). For this purpose, a suspension of the diet in buffered peptone water was prepared and then serially diluted with physiological serum (0.85%). Solutions of different dilutions were cultured in MRS agar and counting was performed after 48 hours of incubation at 37°C (Table 2).

Growth parameters and survival: The measurement of growth parameters was taken at days 0, 15, 30, 45 after the start of the experiment. From each treatment, a total of 15 fish (5 from each replicate) were randomly collected. Growth indices were calculated by following equations (Yousefi et al., 2016; Hoseini and Mirghaed et al., 2018):

Specific growth rate = Log (Wf-Wi)/ n * 100

Feed conversion ratio = FG(g) / WG (g)

Condition factor = Wf (g) / TL^3 (cm) *100

Protein efficiency ratio = WG (g) / TP (g)

Daily growth rate = Wf-Wi / n

Percentage weight gain = (Wf-Wi) / Wi * 100

Survival rate = (Nf / Ni) * 100

Where Wf is mean final weight, Wi = mean initial weight, n = number of treatment days, TL = final total length, WG = weight gain, FG = feed intake, TP = total protein received, Ni = initial number of fish and Nf = number of fish survive.

Evaluation of intestinal LAB: After sampling, the external surfaces of fish were first disinfected with benzalkonium chloride and then a portion of the intestinal tract was sampled 0, 15, 30, and 45 days after start feeding trial. The samples were homogenized with normal saline, serially diluted, and immediately cultured in MRS agar. Finally, the media were incubated at 25°C for 5 days and then numbers of the colonies were counted (Mahious et al., 2006). The number of colonies of each sample was calculated by the following equation:

cfu/g intestine = Number of colonies × Inverse of the dilution factor

Statistical methods and data analysis: To analyze the data, first, all data were compiled in Excel 2007. Normality of data was checked by Kolmogorov-Smirnov test and homogeneity of variance by Levene test. Analyses of the collected data were performed with one-way ANOVA. In the cases where there was a significant difference between treatments, the Tukey test was performed at 95% confidence level and $P\leq0.05$ significance level. All tests were performed using the statistical analysis software SPSS 19 (Chicago, IL, USA).

Results

Isolation and purification of LAB strains from kefir: Four bacterial strains were isolated and purified from kefir grains. One strain, LF, was bacillus-shaped and observed often in single units and occasionally in pairs. Another strain, ED, was cocci-shaped with slight elongation and observed often in clusters and sometimes in pairs and short chains. Both of these strains were gram positive (confirmed by the KOH test). The catalase and oxidase tests of these strains were negative. In terms of characteristics, these strains were very similar to the family of lactic acid bacteria. The other two strains were gram negative (confirmed by the KOH test) and did not belong to the LAB family.

Molecular identification of the isolated strains: LE strain had 99.7% phylogenetic affinity with

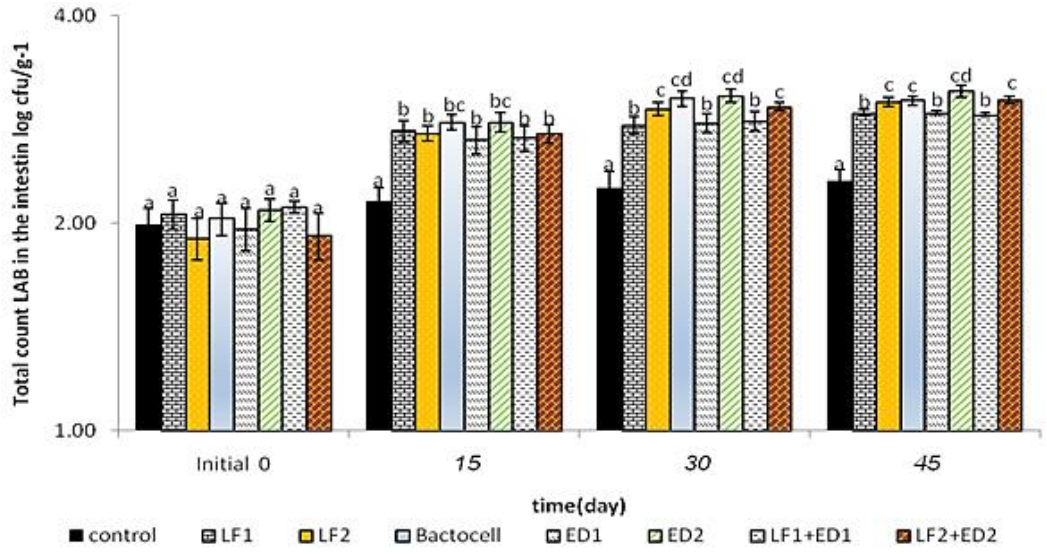

Figure 1. Total count of LAB colonies in the intestine of rainbow trout fed with different levels of the LAB strains isolated from kefir at days 0, 15, 30, 45 (Mismatching letters (a, b, c) signify a significant difference ($P \leq 0.05$) between means).

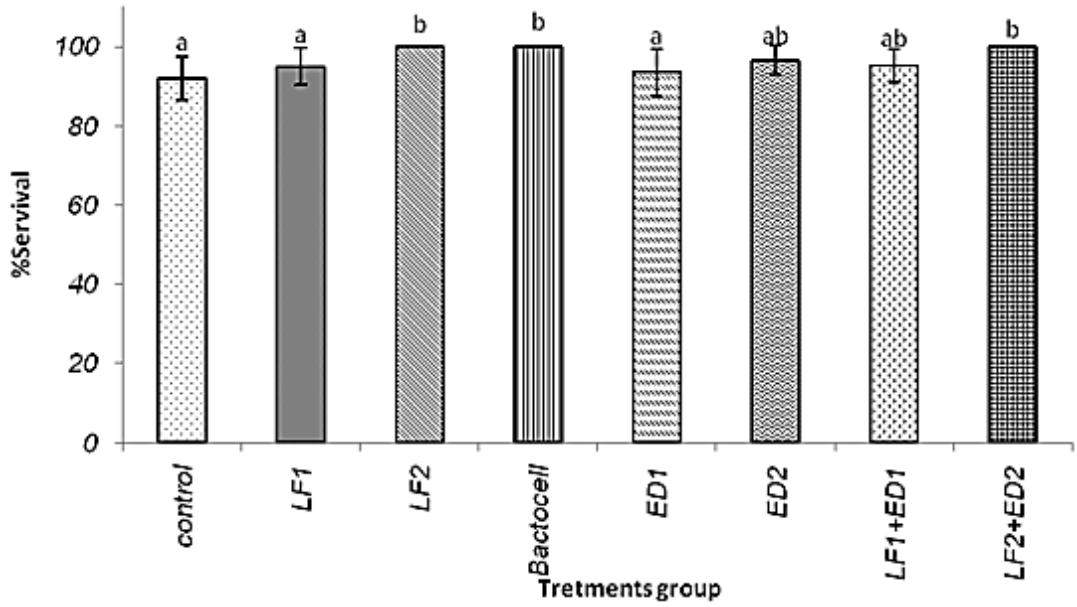

Figure 2. Comparison of percentage of survival rate of rainbow trout fed with different levels of the LAB strains isolated from kefir at the end of the treatment period (day 45) (Mismatching letters (a, b, c) signify a significant difference ($P \leq 0.05$) between means).

L. farraginis JCM 14108 (T) and the ED showed 99.9% affinity with *E. durans* NBRC 100479 (T).

Probiotic characteristics of the strains: Investigation of hemolytic activity of the strains LF and ED in blood agar did not show any type of hemolytic activity (α, β, or γ) around the colonies of these strains. Both strains showed resistance to acidic environments and gastric juice and were able to grow against bile salts. The hydrophobicity of the LF and ED strains was measured to 72.47% and 51.42%, respectively. The strains exhibited good *in vitro* antagonistic activity against *Lactobacillus garvieae* and *Aeromonas hydrophilia*, but their activity against *Streptococcus iniae* was extremely weak.

Results of analysis of intestinal LABs: According to the results (Fig. 1), comparison of the number of LAB in the midgut of fish at the first sampling time and the beginning of the treatment period (Initial: 0) showed no significant difference ($P>0.05$) between the treatments and control groups.

Analysis of the samples collected at three time points (days 15, 30 and 45) showed a significant

Table 3. Comparison of growth parameters and survival rate of rainbow trout fed with different levels of the LAB strains isolated from kefir at the end of the treatment period (day 45).

Parameters	Control	LF1	LF2	Bactocell	ED1	ED2	LF1+ED1	LF2+ED2
Initial weight (g)	41.42±1.21^{a}	41.82±1.55^{a}	42.16±0.82^{a}	42.48±1.08^{a}	42.40±1.26^{a}	41.98±1.51^{a}	41.78±0.91^{a}	42.16±0.83^{a}
Final weight(g)	84±3.60^{a}	86.56±1.72^{a}	92.15±2.71^{b}	99.79±2.69^{d}	94.67±2.46^{bc}	95.75±2.72^{c}	92.01±1.32^{b}	99.99±2.50^{d}
Weight gain(g)	42.2±2.60^{a}	44.76±1.71^{a}	50.35±2.71^{b}	57.99±2.68^{d}	52.87±2.46^{bc}	53.95±2.72^{c}	50.21±1.32^{b}	58.19±2.49^{d}
Initial length (cm)	14.60±0.51^{a}	14.66±0.50^{a}	14.70±0.31^{a}	14.62±0.46^{a}	14.54±0.53^{a}	14.64±0.50^{a}	14.78±0.52^{a}	14.56±0.32^{a}
Total length (cm)	20.9±0.37^{a}	20.95±0.4^{ab}	21.3±0.38^{bc}	21.5±0.27^{c}	21.25±0.36^{abc}	21.28±0.35^{abc}	21.31±0.18^{bc}	21.5±0.4^{c}
Length gain (cm)	4.17±0.36^{a}	4.22±0.40^{ab}	4.57±0.37^{bc}	4.77±0.26^{c}	4.52±0.36^{abc}	4.55±0.35^{abc}	4.58±0.18^{bc}	4.77±0.40^{c}
BWI (%)	100.95±8.6^{a}	107.08±4.11^{a}	120.45±6.50^{b}	138.73±6.43^{d}	126.49±5.89^{bc}	129.08±6.51^{c}	120.1±3.17^{b}	139.21±5.98^{d}
CF	0.92±0.06^{a}	0.94±0.06^{ab}	0.95±0.05^{ab}	1.01±0.05^{b}	0.98±0.06^{b}	0.99±0.05^{b}	0.95±0.03^{ab}	1.01±0.06^{b}
SGR (% day^{-1})	1.55±0.10^{a}	1.62±0.04^{a}	1.76±0.07^{b}	1.93±0.06^{d}	1.82±0.05^{bc}	1.84±0.06^{c}	1.75±0.03^{b}	1.94±0.06^{d}
PER	1.89±0.16^{a}	2.00±0.08^{a}	2.25±0.12^{b}	2.59±0.12^{d}	2.37±0.11^{bc}	2.41±0.12^{c}	2.25±0.06^{b}	2.60±0.11^{d}
DWG (gr)	0.93±0.08^{a}	0.99±0.04^{a}	1.11±0.06^{b}	1.28±0.06^{d}	1.17±0.05^{bc}	1.19±0.06^{c}	1.11±0.03^{b}	1.29±0.06^{d}
(FCR)	1.46±0.11^{a}	1.06±0.05^{b}	0.96±0.12^{cd}	0.91±0.04^{d}	1±0.05^{bc}	0.98±0.05^{bcd}	1.05±0.03^{b}	0.91±0.04^{d}

* Mismatching letters (a, b, c) in the rows signify a significant difference (P≤0.05) between means.

increase (P<0.05) in the total number of intestinal LABs in ED2, Bactocell, LF2+ED2 and LF2 treatments compared to others. Although no significant difference was observed between the intestinal LAB colony counts of LF1, ED1, and LF1+ED1 in any of the sampling times, the number of colonies in all of these treatments was significantly higher (P<0.05) than in the control. In addition, in all treatment groups except the control, the number of LAB colonies increased as the experiment progressed (Fig. 1).

Growth parameters and survival rate: According to growth indices (Table 3), the parameters BWI, SGR, DWG, PER, and CF were highest in the treatments LF2+ED2, Bectocell, ED2, and LF2, respectively (P<0.05). In these treatments, the least significant change compared to the control group was observed in FCR. The parameters length gain and total length in the treatments LF2+ED2, Bectocell, and LF2 showed the greatest increase in comparison with other groups (Table 3). Inaddition, the survival rate was found to be significantly (P<0.05) higher in LF2+ED2, Bactocell and LF2 than in other treatments (Fig. 2).

Discussions

Probiotics can contribute to aquaculture as beneficial and eco-friendly microorganisms with multiple mechanisms of positive effect on the host and environment (Lazado and Caipang, 2014). Indeed, some of the probiotic microorganisms are able to provide the essential nutrients for fish to grow more favorably (Balcazar et al., 2006; Panigrahi et al., 2007). Kefir grains, as a probiotic product, consist of many members of the family of lactic acid bacteria (Rodrigues et al., 2005) and can serve as a useful source for the extraction of new probiotic strains (Kumura et al., 2004). However, after molecular identification of LAB strains, they should be subjected to vigorous *in vitro* examinations to study their probiotic and health characteristics (Saarela et al., 2000; Hammes and Hertel, 2002; Mercenier et al., 2008). In the current study, two strains of the LAB family were isolated and purified in the anaerobic and microaerophilic conditions from the fermentation of kefir grains in the milk. Colonies of the strain *L. ferraginis* (LF) were showed 99.7% phylogenetic similarity with the *L. farraginis* strain JCM 14108 (T) in the NCBI database. Another study that isolated *L. farraginis* and *L. para ferraginis* strains from a traditional beverage called Shochu produced by fermentation of rice extract (Endo and Okada, 2007b). The incubation of kefir can take place not only in cow milk but also in soy milk, rice milk, and coconut milk (Semih and Cagindi, 2003). According to the taxonomic classification, LF strain belongs to the phylum Firmicutes, the class Bacilli, the order

Lactobacillales, the family Lactobacillaceae, and the genus *Lactobacillus*, and has a phylogenetic affinity with *L. hilgardii, L. buchneri, L. diolivoran, L. parakefi, L. kefiri* and *L. parabuchneri* species (Felis and Dellaglio, 2007; Endo and Okada, 2007a). The second strain isolated in the present study from kefir grains was *E. duran* (ED), which has 99.9% phylogenetic similarity with the strain *E. durans* NBRC 100479 (T). The strain *E. durans* was first isolated from kefir grains by Rosi (1978), who reported it as *Streptobacterium durans* and later reported by Yuksekdag et al. (2004) as *Streptococcus durans*, which is one of the strains of the LAB family (Rosi and rossi, 1978; Yuksekdag et al., 2004). In a study similar to the present work, three strains of *E. durans* were isolated from Turkish kefir (Yuksekdag et al., 2004), and recently, *E. durans* was isolated from the kefir incubated in goat milk and reported using API-50 biochemical methods (Aloklah et al., 2017). According to previous studies, *E. durans* is homologous to *E. faecium*, *E. hirae*, and *E. lactis* in terms of molecular structure (Farrow, 1983; Yu et al., 2011; Techo et al., 2018).

In the present study, the two isolates of kefir lacked any hemolytic activity in blood agar and they also presented no catalase activity. In a similar study, the hemolytic activity of *L. kefiri* isolated from kefir grains, which has a genetic affinity with *L. farraginis*, was also negative (Carasi et al., 2014). On the contrary, some *E. durans* strains have been reported to have α and β hemolytic activity in blood agar (Collins et al., 1984), and meanwhile, there are other studies indicating that *E. durans* strains have no hemolytic activity (Foulquie et al., 2006; Pieniz et al., 2014). These contradictory results are probably due to the large variety of strains of this species and the difference in the sources from which they have been isolated, because *E. durans* is one of the most prevalent of Enterococcus species (Morandi et al., 2006). Many studies have reported negative catalase activity for *L. farraginis* and *E. duran* (Devriese et al., 2002; Endo and Okada, 2007b). In general, it can be concluded that the two strains isolated from kefir belong to the family of LABs, because the members of this family are all gram-positive, negative catalase, and anaerobic or microaerophilic (Orla-Jensen, 1919), and also because these strains were isolated from kefir grains in milk and, according to 16SrRNA gene sequencing, they both belong to the phylum Firmicutes, which are all characteristics of the LAB family (Kandler and Weiss, 1986).

Another finding of this study was the resistance of the isolated strains to acidic conditions (pH = 2.5-4) of the gastrointestinal tract and pepsin and trypsin enzymes of gastric juice (pH = 2) for 1-3 hours. Also, the ED strain showed generally higher resistance levels than the LF strain. The strains were able to grow in the presence of bile salts with a concentration of 0.15%, but are not able to grow when bile salt concentration increases to 0.3%. However, there was no significant difference between the growth rates of the strains in these conditions. In a similar study, the strains isolated from kefir grains, including *L. acidophilus*, *L. plantarum*, and *L. kefiri* exhibited high resistance to gastric acid and bile salts (Zheng et al., 2013). A study on the strain *L. kefiranofaciens* isolated from kefir found it to be resistant to acidic conditions but unable to grow in the presence of bile salts at 0.20% level (Xing et al., 2017). In addition, *L. paracasei* isolated from kefir showed high resistance to acidic conditions, pepsin enzyme, and 0.15% bile salts (Bengoa et al., 2017). However, *E. durans* isolated from dairy products (fermented milk) can survive and grow under acidic conditions (pH = 2-3), gastric juice, and 1.5 and 0.3% bile salts (Pieniz et al., 2014; Guo et al., 2016). In general, one of the interesting characteristics of LABs is their resistance to acid, gastric juice, and bile salts (Fioramonti et al., 2003).

The findings of this study showed the in-vitro antagonistic activity of the LF and ED strains against *L. garvieae* and *A. hydrophila*, which are two important pathogens of fish farms, but they had very weak activity against *S. iniae*. The LF strain showed stronger antagonistic activity against *Y. ruckeri* than the ED strain. In a study of *Lactobacillus* strains isolated from kefir, they were able to inhibit the growth of *Salmonella enterica*, *Escherichia coli*,

Listeria monocytogenes, and *Staphylococcus aureus* (Zanirati et al., 2014). A study reported that *E. durans* species isolated from kefir can inhibit the growth of gram-positive and negative bacterial pathogens (Carasi et al., 2014). The strains isolated from cheese showed broad antimicrobial activity against *A. hydrophila, E. coli, L. monocytogens,* and *P. aeruginosa* (Pieniz et al., 2014). The antagonistic activity of ED and LF is probably caused by the production of antibacterial compounds by these strains, because the metabolites resulting from the function of LAB strains of kefir including acetic acid, lactic acid, ethanol, diacetyl, peptides, and bacteriocins, have sufficient antibacterial effect to act against pathogenic bacteria (Jamuna and Jeevaratnam, 2004).

Another important characteristic of probiotic strains that is highly associated with their stability and survival in the gastrointestinal tract is hydrophobicity (Nikoskelainen et al., 2001). The in-vitro investigation of hydrophobicity of the isolated strains showed that they both have a hydrophobicity level of more than 50%, with the LF strain (72.47%) being significantly more hydrophobic than the ED (51.42%). These results are consistent with the findings of a study conducted on *L. faraignis*, *L. plantarum kefiri*, and *L. acidophilus*, which showed a high level of adhesion to digestive tract epithelium in the culture medium (Zheng et al., 2013; Thamacharoensuk et al., 2017). The strain of *L. kefiranofaciens* XL10 isolated from Tibetan kefir was also reported to have 79.9% hydrophobicity (Xing et al., 2017). In contrast, a study on *E. durans* isolated from milk products showed low levels of hydrophobicity in the medium (Guo et al., 2016). In general, it can be argued that the hydrophobicity of the ED and LF strains signifies their ability to adhere to the intestinal mucus and avoid excretion with feces.

The analysis of LAB colony counts in the intestines of the fish fed with different doses of ED and LF showed that all treatment groups had significantly more LAB colonies in the intestine than the control group. Among the treatment groups, ED2, Bactocell, and LF2+ED2 had the highest colony counts. This also revealed that the number of LAB colonies in the intestines increased with increasing the dose of the strains in the diet. Also, the combined use of ED + LF in the diet increased the LAB colony count in the intestines. Similar to the results of the present study, the addition of *L. sakei* (CLFP 202) isolated from the intestines of Salmonids to the diet of *Salmo trutta* significantly increased the number of LAB colonies in the intestine, which was attributed to their high potential for adhesion and survival in the intestinal tract of the fish (Balcazar et al., 2007). Other studies have also reported that the addition of probiotics *E. faecium* and *E. casseliflavus* to the diet of common carp and rainbow trout, respectively, increased the LAB population in their intestine (Dehaghani et al., 2015; Safari et al., 2016). A study of oral administration of *L. kefiranofaciens* strain isolated from kefir to mice reported an increase in the LAB colony count in the intestine and a decrease in the number of pathogenic bacteria (Xing et al., 2017). Since the presence of LABs in the intestines of most fish species has been proved (Ringo and Gatesoupe, 1998), the increased number of colonies in the sampled intestines may be attributed to the introduction of probiotic strains into the diet, which significantly increases the share of LABs in the intestinal flora and limit the growth of other bacteria, including the harmful ones (Ziaei-Nejad et al., 2006). Both of the isolated strains seem to have a good survival in the gastrointestinal environments, as the conditions for adherence and colonization in the intestinal wall is favorable and this increases the population of LAB.

A significant increase in BWI, SGR, PER, CF, DWG, and TL indices of the fish fed with LF2 + ED2, Bectocell, ED2, and LF2 diets indicate that using higher doses of the strains (LF + ED) (10^8) and a combination of the two strains (LF + ED) improves growth parameters more successfully. In contrast, FCR ratio of these treatments was the lowest in comparison with the control group. The addition of *L. acidophilus* to diet of *Clarias gariepinus* improved the growth and FCR, SGR, and BWI (Al-Dohail et al., 2009). Adding a mixture of *B. subtilis*, *E. faecium* and

P. acidilactici to the diet of rainbow trout has shown significant effects on growth performance and nutrition efficiency (Giannenas et al., 2015). Supplementing of 2.5×10^6 CFU/g of *E. faecium* and *E. durans* isolated from the gastrointestinal tract of *Oreochromis niloticus* has been shown to improve the nutrition efficiency and growth parameters fish (Lara-flores et al., 2013). Probiotics can increase fish growth by positively affecting digestive functions such as digestion and absorption of nutrients, intestinal microbiota, appetite, and FCR (Ringo and Cratesoup, 1998; Panigrahi et al., 2005; Nayak, 2010). The combined use of probiotic strains in fish diets has also shown to increase SGR and PER (Mohapatra et al., 2012a, b).

In the present study, the treatments LF2 + ED2, bactocell and LF2 had the highest rate of survival. Adding probiotic bacteria has been shown to increase the survival of fry and larvae of the spotted seatrout (*Cynoscion nebulosus)*, turbot (*Scophthalmus maximus*), goldfish (*Carassius auratus*), swordtail (*Xiphophorus helleri*) (Gatesoup, 1994; Kennedy et al., 1998; Abraham et al., 2007) and *Catla catla* (Mohanty et al., 1996). Several studies suggest that LAB species increase the survival rate by preventing digestive system impairment, neutralizing anti-nutritional substances and toxins, regulating the growth of beneficial intestinal microorganisms, improving immune responses, and limiting the activity of pathogenic microorganisms (Ringo and Gatesoupe, 1998; Panigrahi et al., 2005; Suzer et al., 2008). In contrast, some researchers have not observed any significant difference in the survival rate after using probiotics (Farzanfar et al., 2006). This discrepancy is probably due to differences in the type of probiotic strains, growth conditions, and fish species. In general, LF and ED strains are likely to increase the resistance and survival rate of fish by improving their health condition and growth performance and stimulating the immune system.

In conclusion, the two LAB strains isolated from kefir showed suitable probiotic properties, including resistance to acidic conditions, gastric juice enzymes, and low concentrations of bile salts, as well as high hydrophobicity. They showed in-vitro antagonistic activity against some pathogens that are common in fish farms. The addition of LF and ED strains to the trout diet in higher doses (LF2, ED2) and in combined form (LF2 + ED2) increased the number of LAB colonies in the intestine, enhanced and improving the biometric indicator and growth performance and survival of the fish. Although both LF and ED appear to be good candidates for addition to the diet of juvenile rainbow trout, further studies with more in-vitro and in-vivo investigations seem necessary.

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