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RESEARCH PAPER

Dietary Effects of Two *Bifidobacterium* Strains on Growth, Length-weight Relationships and Biological Indices on *Oncorhynchus mykiss* (Walbaum, 1792)

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Abstract

This study aimed to evaluate the effects of *Bifidobacterium animalis* and *Bifidobacterium lactis* on growth, length-weight relationship and some biological indices of rainbow trout, *Oncorhynchus mykiss* fry. An iso-nitrogenous (50% crude protein) diet was supplemented with three different concentrations of a mixture of equal quantities of the two strains of *Bifidobacterium*, namely T1 (1×10^7CFU/g), T2 (2×10^7CFU/g), T3 (3×10^7CFU/g) and a control (without bacteria). A total of 480 trout fries with an initial mean body weight of 0.53±0.19 g were randomly divided into four groups, each with 4 replicates. The fries were distributed into 16 fiberglass tanks (20 L total capacity) each filled with 15 L of water (water exchange rate of 2 L min^{-1} and permanent aeration) at a density of 2 fries per liter. During the 8-week feeding trial, the mean water temperature was 17.66±1.33°C and that of pH was 7.63±0.08. All fish groups demonstrated positive allometric growth. Trout fries fed with diet T1 recorded significantly higher ($P<0.05$) gonadosomatic index, specific growth rate and mean weight gain. The highest final mean length (14.12±4.57 cm) was recorded in fry fed with diet T2. The lowest concentration of *Bifidobacterium*, T1 induced the best growth and it enhanced the biological indices of *O. mykiss*.

Introduction

Probiotics possess several critical properties such as efficient adherence to intestinal epithelial cells to reduce or even suppress the colonization of pathogens (Kirjavainen *et al.,* 1998; Raheem *et al.,* 2021), balance the bacterial flora in the digestive tract (Verschuere *et al.,* 2000; Raheem *et al.,* 2021; Wuertz *et al.,* 2021), and secretion of extracellular enzymes (Jafaryan *et al.,* 2014; Sahandi *et al.,* 2022). The use of probiotics for larviculture is gaining much attention in the scientific community considering their potential benefits such as controlling diseases, enhancing immune response, providing nutritional and enzymatic contributions to the digestion of the host, and improving water quality (Sahandi *et al.,* 2022). Probiotics act as sources of nutrients and electrolytes, sources of extracellular enzymes for better digestion and promoters of immunity in the host organisms. The probiotics with secreting enzymes and organic acids could enhance growth performance of the host organisms (Akhter *et al.,* 2015; Lara-Flores *et al.,* 2003; Sahandi *et al.,* 2022).

Among the probiotics, the use of lactic acid bacteria is the most popular. Several studies have shown

the beneficial effects of lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* on different aquatic organisms' growth (Lara-Flores *et al.,* 2003; Balcazar *et al.,* 2008; Beck *et al.,* 2015; Shimada *et al.,* 2007; Huang *et al.,* 2022). The uses of *Bifidobacterium* as probiotics have been recognized and accepted (Charteris *et al.,* 1998; Gill *et al.,* 2001; Huang *et al.,* 2022), but still, many unanswered questions such as appropriate concentrations and form or application exist. Microbial additives in-feed have particular relevance in the development of rearing technologies for larviculture purposes (Sahandi *et al.,* 2022).

Bifidobacterium is a gram-positive bacteria that ferment various carbohydrates mainly lactate and acetate to produce lactic acid. These bacteria can produce biogenic compounds such as bioactive peptides and fatty acids. These biogenic compounds are essential for enhancing health properties (Gobbetti *et al.,* 2010). Appropriate feed supply is essential for fish growth (Elliott, 1976; Jones, 2002; Akhter *et al.,* 2015). However, several components such as electrolytes and bioactive peptides are critical compounds that must be presented in-feed for appropriate growth. Modifying the microbial community of the larval digestive tract using appropriate strains of probiotics in-feed could enhance the feed's nutritional value, and increase the growth performance. The most apparent method seems to be incorporating probiotics in-feed and administering them regularly to fish, as proven in a study on juveniles of common carp (*Cyprinus carpio*), (Jafaryan *et al.,* 2014). In general, the larval digestive system is poor in microbes and enzymes. After hatching, the digestive tract develops, which could be the critical time for inoculation of beneficial microbes (Jafaryan *et al.,* 2014).

The rainbow trout, *Oncorhynchus mykiss* has always been known as a potential fish species for aquaculture. The typical diet for raising this species must contain appropriate levels of protein, fat and energy. However, in the last few years, there has been international interest in the nutritional requirements of this species for better performance. Several studies have suggested using probiotics in the larviculture of rainbow trout (Irianto and Austin, 2003; Aubin *et al*., 2005; Merrifield *et al*., 2009). However, no study has been carried out on the length-weight relationship of this species after treatment with *Bifidobacterium* probiotics. This study aimed to investigate the length-weight relationships and growth characteristics of *O. mykiss* fry in an in-vitro study under different concentrations of *B. animalis* and *B. lactis* in-feed.

Materials and Methods

Experimental Animal and Design

Healthy fries of rainbow trout (*Oncorhynchus mykiss*) were obtained from a private trout farm (Mazandaran, Iran). Before the start of the experiment, the fish were fed on a control diet four times a day and acclimatized to the experimental environment for two weeks. The fries with an initial mean weight of 0.53±0.19 g and length of 3.06±1.24 cm were randomly distributed in 16 fiberglass tanks. For each group, four replicates were maintained and each fiberglass tank (15 L) was stocked at a density of 2 larvae L^{-1}. The fish were hand-fed to apparent satiation four times daily at 8:00, 12:00 am, 4:00, and 8:00 pm for 8 weeks. The 12: 12 h light-dark cycle was applied.

The water temperature in the tanks was measured twice a day at 6:00 and 18:00. At the end of the study, the total number of fish in each tank was counted, the mean body weight (using a digital scale with an accuracy of 0.001 g) and the length (using a caliper with an accuracy of 0.01 mm) were measured and recorded to determine the length-weight relationship and growth. Physico-chemical parameters including pH, total dissolved solids (TDS), and electrical conductivity (EC) were measured using a portable instrument (Hannah H198311, Washington, US), alkalinity and total hardness were measured using commercial kits.

Bacterial Strain and Culture Condition

Bifidobacterium animalis PTCC-1631 isolated from rat faeces and *Bifidobacterium animalis subsp lactis* PTCC-1736 isolated from yogurt (fermented milk) were obtained from the Persian type culture collection (PTCC) (Tehran-Iran). The freeze-dried samples were cultivated on de Man, Rogosa and Sharp (MRS) (Merk, Germany) broth at 37°C for 24–48 h with 160 rpm under aerobic conditions. The cell-free supernatants (CFS) were obtained separately for each bacterium according to an optical density (OD) after Gomez-Gil *et al.* (1998). About 10mL of fresh *Bifidobacterium* culture was centrifuged at 5000rpm for 10 min. The liquid supernatant was discarded, and the pellet was suspended in a sterile saline solution. Cell density after preparation was calculated by spectrophotometer (model Libera S22: Biochrom, Cambridge, UK) of 1.00 at 610 nm, and values were correlated with the colony-forming unit (CFU) counts using serial dilution. Then two strains with an equal concentration were mixed together and the final solution was used for adjustment of concentrations. Three concentrations of the used strains were 1×10^7 (T1), 2×10^7 (T2), and 3×10^7 (T3) CFU per gram of dry feed respectively.

Preparation of Diets

A fish feed was obtained from a commercial producer (BEYZA, Fars—Iran) containing 50% crude protein, 14% crude fat, 3% crude fiber, and 4300 Kcal kg^{-1} digestible energy. The final bacterial concentrations were mixed with an equal volume of the autoclaved saline solution (0.9% w/v) and the mixture was slowly sprayed onto 100 g of dry feed under sterilized conditions. The resultant feed was dried at 30°C for 2-4 h in an oven (Shimaz-55L, Tehran, Iran) and it was stored

in sealed plastic bags in a refrigerator (−20°C). The supplemented feed with *Bifidobacterium* for each treatment was prepared every week. The fish were fed manually four times a day for 8 weeks. The feeding rates were approximately 4–8% of the fry body weight during rearing time. The daily feed fed was recorded.

Fish Sampling and Estimation of Growth Indices

Sampling was performed from each treatment after the experimental period. Rainbow trout larvae were collected at random with hand-held fishing nets with 0.1 mm mesh size from each tank. In the sampling location, fish specimens were immediately anesthetized with the extraction of *Eugina caryophillata*. The fish specimens were weighed (W, ±0.01) on a digital scale (Kern model, Germany) and the total length (TL, ±0.1) was measured with calipers. Length-weight relationships were calculated for the entire population of all treatments. The exponential equation: $W=aTL^b$, where W is the weight (g), L is the length (cm), a and b are regression parameters.

At the end of the feeding trial, the growth indicators of trout larvae were assessed in terms of weight gain, specific growth rate, condition factor, gasterosomatic index, gonadosomatic index, hepatosomatic index and kephaleosomatic index. The growth parameters were estimated based on standard equations as follows:

Mean weight gain (WG)=final mean weight (g) – initial mean weight (g) (Tacon, 1983)

Specific growth rate (SGR %)=(ln final mean weight of fish) – (ln (initial mean weight of fish)) ×100/days of feeding trial (Helland *et al.*, 1996).

Condition factor (CF)=final weight/ (final length)^3 × 100 (Douillet & Langdon, 1994)

Gasterosomatic index (GSI)=(alimentary canal weight/whole body weight) × 100 (Desai, 1970)

Gonadosomatic index (GSI)=(Gonad weight/whole body weight) × 100 (Wootton, 1991)

Hepatosomatic index (HSI)=(Liver weight/whole body weight) × 100 (Marzouk *et al.*, 2008)

Kephaleosomatic index (KOI)=(Head weight/whole body weight) × 100

Data Analysis

Data were analyzed using the SPSS software version 21. One-way analysis of variance (ANOVA) was performed. Duncan's multiple range tests were used to identify the significant differences among different treatments when P<0.05. The slope of length-weight regressions was compared for differences using ANOVA.

Results

The water quality parameters were found to be in the range of temperature (17.66±1.33°C), pH (7.63±0.08), EC (3012.62±450.03 µmhos cm), TDS (2.01±0.13 mg L^{-1}), alkalinity (240 mmol L^{-1}), and total hardness (391.6 mg L^{-1}) throughout the experiment period. The results of growth parameters are presented in Table 1. The results showed that the trout fries of group T1 had the highest growth performance (P<0.05) compared to the other treatments. The weight gains (WG) of T1 and T2 were significantly higher (P<0.05) than the control. Frequent sizes of 12 to 13.5 cm were observed in T2 and T3 groups (Figure 3).

Length-weight regression is presented in Table 2. The weight gain of fish over 8 weeks of feeding with *Bifidobacterium* strains is presented in Figure 1. The T1 fed with the lowest microbial concentration had significantly higher weight gain (P<0.05) than the other groups and the control.

Significant differences were observed in length-weight relationships (Figure 2), while the *b*-value implied that the body shape displayed positive allometric growth in all experimental groups.

Table 1. Growth response of rainbow trout, *Oncorhynchus mykiss* fries fed with diets containing graded levels of two *Bifidobacterium* strains for 8 weeks

	Treatments			
Parameters	Control	T1 (1×10^7) CFU/g	T2 (2×10^7) CFU/g	T3 (3×10^7) CFU/g
Initial mean weight (g)	0.57±0.02	0.50±0.01	0.52±0.01 01	0.54±0.01
Final mean weight (g)	20.98±0.42^c	24.98±0.62^a	23.06±0.47^b	20.70±0.47^c
Final mean length (cm)	12.26±0.88^c	12.98±1.14^b	14.12±4.57^a	12.25±0.88^c
Mean weight gain (g)	20.41±0.42^c	24.48±0.62^a	22.55±0.47^b	20.15±0.40^c
Specific growth rate (%)	5.95±0.03^c	6.46±0.04^a	6.29±0.03^b	6.01±0.03^c
Condition factor	1.12^a	1.12±0.01^a	1.11^a	1.10^a
Gonadosomatic index	2.01±0.8^b	2.73±0.96^a	2.32±0.93^{ab}	2.10±0.84^{ab}
Gasterosomatic index	6.14±0.4^a	6.83±0.39^a	6.23±0.27^a	6.32±0.2^a
Hepatosomatic index	1.32±0.07^a	1.57±0.11^a	1.47±0.06^a	1.51±0.06^a
Kephaleosomatic index	15.92±0.48^a	16.9±0.48^a	17.19±0.39^a	16.06±0.32^a

Different superscripts on the same row showed significant different (P<0.05) determined by Duncan's test. Data are expressed as mean ± S.E (n=480)

Discussion

The physico-chemical parameters among the different groups were the same and showed no significant difference (P>0.05). The fish fed with the supplemented diet with the lowest concentration (1×10^7CFU/g) of two *Bifidobacterium* strains exhibited a significant difference in weight gain and specific growth rate when compared to other experimental groups and the control (P<0.05). Hence, the increase in the concentration of probiotics does not necessarily result in better growth (Bagheri *et al.,* 2008; Goda *et al.,* 2012). Reduced growth performance at higher levels of *Bifidobacterium* strains could be an indicator of depressed nutrient utilization. The same finding was reported previously by Opiyo *et al.,* (2019). Another possible reason for depressed nutrient utilization might be unwanted changes in the digestive tract microbiota of trout fries. The successful inoculation of probiotics could increase nutrient utilization and absorption of essential compounds, finally ending better growth (Jafaryan *et al.,* 2014). The colonization procedure of probiotics has been well explained by several researchers (Bagheri *et al.* 2008; Jafaryan *et al.,* 2014;

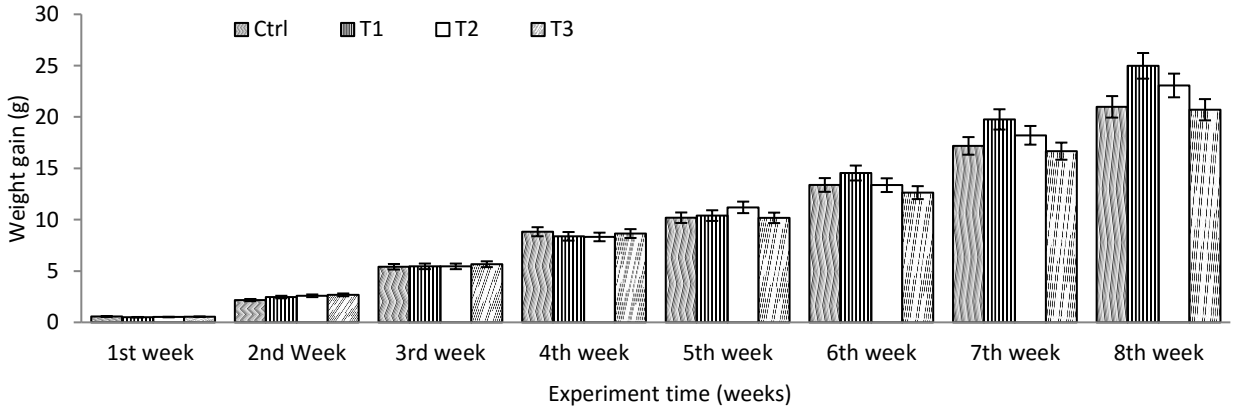

Figure 1. Weight gain change of rainbow trout, *Oncorhynchus mykiss* fries fed with diets containing graded levels of two *Bifidobacterium* strains for 8 weeks. Data are expressed as mean ± S.D (n=120)

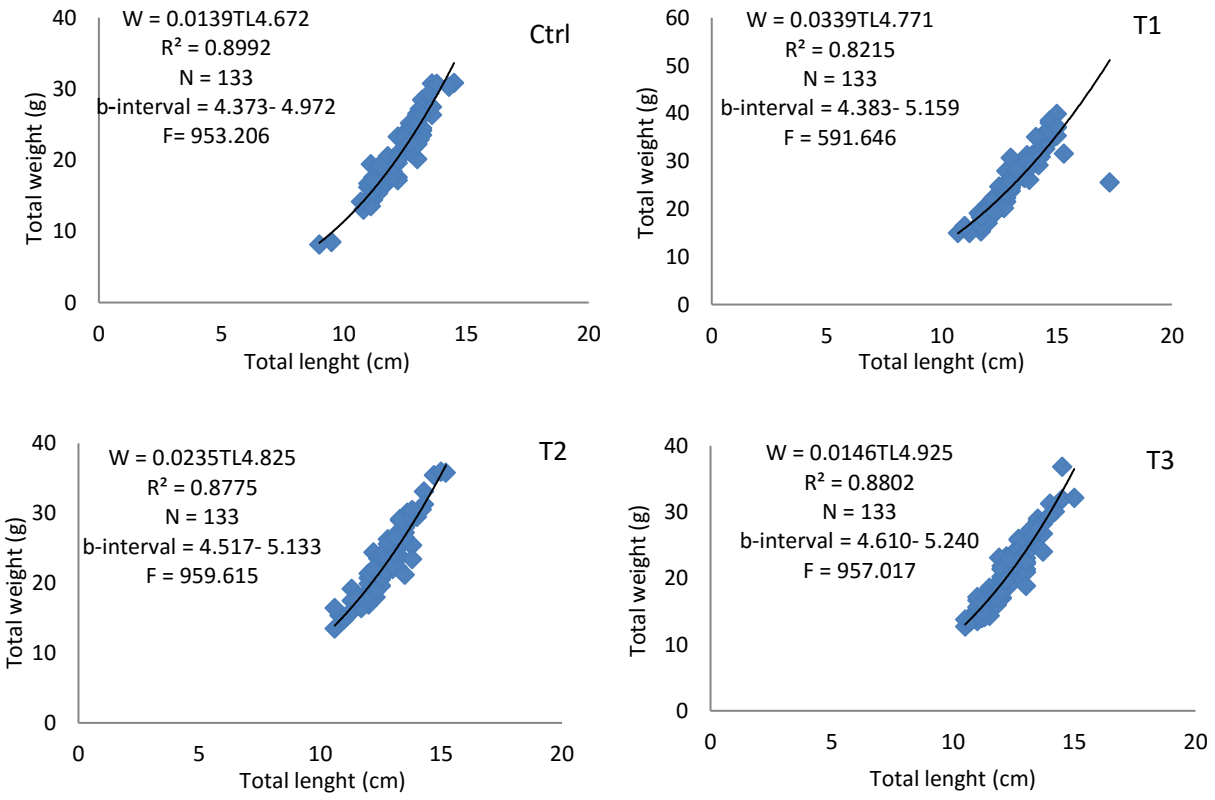

Figure 2. Relative growth curves (total length – total weight) of rainbow trout fries fed with two *Bifidobacterium* strains for 8 weeks

Jafaryan *et al.,* 2008). According to De-Vrese and Marteau (2007), the mechanism and function of the probiotic effects depend mainly on the interactions between probiotic strains and microbiota of the host or immune-competent cells of the intestinal mucous. The results showed a significant increase in weekly weight gain and the condition factor of T1 compared to other groups and the control ($P<0.05$). However, T2 showed the highest final length. Besides, both T1 and T2 had positive allometric growth, thus there is no significant difference in length-weight relative growth between T1 and T2 ($P>0.05$; Table 2; Figure 2 and Figure 1). Group T2 had the most frequent size classes of 12-13.5 cm and T1 had the most frequent size classes of 13.5-15 cm (Figure 3). The growth in length might be caused by the application of appropriate concentrations of probiotics which could increase mineral absorption (Sanders *et al*., 2014). Sahandi *et al.,* (2012) also reported similar results that demonstrated among different concentrations of *Bacillus* spp*.* on the length of silver carp (*Hypophthalmichthys molitrix*), the highest length was observed in a treatment received 2×10^6 CFU L of *Bacillus* however; this was 2.85±0.37 mm in control. The gonadosomatic index demonstrated that the use of *B. animalis* and *B. lactis* significantly increased the size of the gonad in T1 (2.73±0.96%) and T3 (2.10±0.84%). Peters, (1983) reported that fish fecundity is positively correlated with the length and the amount of energy available for egg production. This suggests the use of *B. animalis* and *B. lactis* with lower concentrations might increase the fecundity in *O. mykiss*. A similar finding was reported by Lawson (2011). Improving fish fecundity in aquaculture will create a sustainable expanse in this field. The organic acid, secrets by *Bifidobacterium* strains could be a possible reason for the increase in the gonadosomatic index. The organic acid increases the microbiota by controlling the pH (Grattepanche and Lacroix, 2013). Also, acidifying the digestive tract and building up an optimum pH for enzyme operation might be another reason for the high gonadosomatic index. Enzymes in optimum pH would have a high reaction rate and result in high utilization of ingested feed. Ghosh *et al.* (2007) reported that using probiotics could increase the fish gonadosomatic index; however, overdosing or under dosing could be useless.

No significant differences were observed in gasterosomatic, hepatosomatic and kephaleosomatic indices among the experimental groups and the control; however, T1 had the highest rate. The kephaleosomatic index which is suggested in this study refers to the size of the fish head compared to the whole-body size.

The exponents of the rainbow trout's length-weight relationship (Table 2) showed positive allometric growth in all the experimental groups (T1: *b*=4.77; T2: *b*=4.82; T4: *b*=4.92 and the control: *b*=4.67). Similar to our finding Jafaryan *et al.* (2008) reported that the probiotic *Bacillus* caused positive allometric growth of *Acipenser nudiventris* when compared to the control (without *Bacillus*). Ismen (2005) reported that the functional regression of the *b* value is directly related to weight and is affected by ingested food. The growth coefficient values showed that trout fries fed with diets containing the two strains of *Bifidobacterium* approached the asymptotic length faster than that of

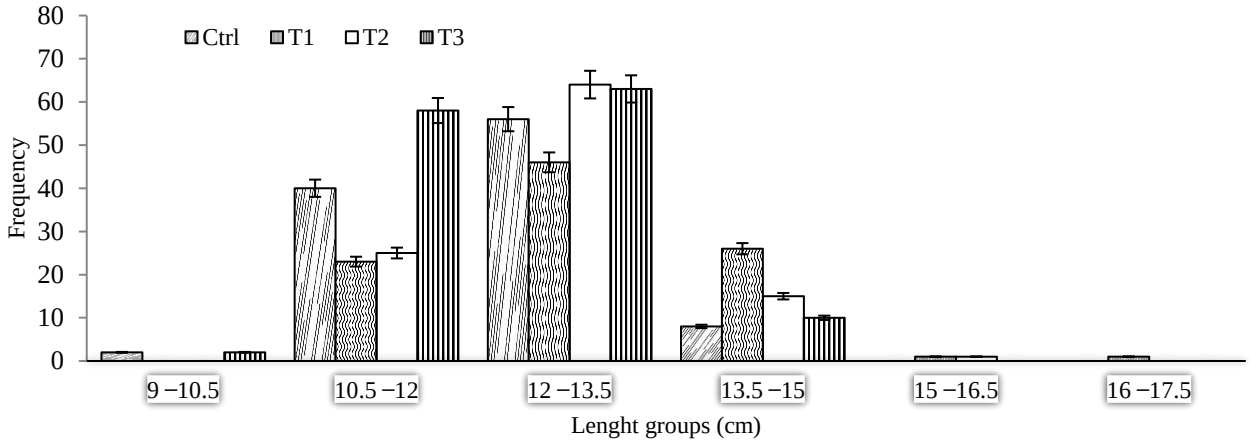

Figure 3. The total length (cm) frequency of different treatments of rainbow trout larvae. Data are expressed in mean ± S.D (n=480)

Table 2. Parameters of the regression ($W=aTL^b$) between total length (TL, cm) and total weight (W, g) of rainbow trout, *Oncorhynchus mykiss* fries fed with diets containing graded levels of two *Bifidobacterium* strains for 8 weeks

Treatment	a	b	b-interval	n	R^2	F
Control	0.013	4.67	4.37-4.97	133	0.90	953.2
T1	0.033	4.77	4.38-5.15	133	0.82	591.6
T2	0.023	4.82	4.51-5.13	133	0.88	959.6
T3	0.014	4.92	4.61-5.24	133	0.88	957.0

the control. The fish length is the best indicator of production efficiency which could be used for grading fish (Ghorbani *et al.,* 2012). The increase in the length of trout of T2 under the *Bifidobacterium* strains consumption is not completely understood but, according to Lilley and Stillwell (1965), probiotics improved the gastrointestinal microbial populations and this could increase enzyme secretion (Jafaryan *et al.* 2008). The increase in enzyme activity would increase the utilization of nutrients, protein anabolism and absorption of minerals such as calcium which is essential for growth (Figure 1).

Conclusion

Our findings showed that the applied strains with the lowest concentration (1×10^7CFU/g) significantly enhanced *Oncorhynchus mykiss* fries growth (weight gain and SGR). The secretion of organic acids and electrolytes by applied *Bifidobacterium* strains at an appropriate concentration might be a possible reason for growth performance. Among biological indexes in the present study, only the result related to the gonadosomatic index was significantly higher. The use of the lowest concentration (1×10^7CFU/g) of two *Bifidobacterium* strains showed the highest gonadosomatic index. The lowest applied concentration (T1) showed better performance, meaning the use of a high probiotic concentration (3×10^7CFU/g) does not necessarily result in a better performance. Our study also demonstrated that the applied *Bifidobacterium animalis* and *B. lactis* at a concentration of 1×10^7CFU/g dry feed could be a reliable additive in the culture of rainbow trout larvae (*O. mykiss*).

Ethical Statement

In this study the rainbow trout larvae were treated under standard condition and no hazardous activity against animal welfare was carried out. All operational sampling was performed after anesthesia the fish with high concentration of clove powder to minimize the suffering

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Author Contribution

JS; Project administration, Conceptualization, Formal analysis, Data curation, Writing - original draft, HJ; Supervision, Methodology Funding acquisition, Writing - review and editing

Conflict of Interest

Authors have no conflict of interest.

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