E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 76.37
(GIF) Impact Factor: 0.549
IJFAS 2023; 11(6): 96-101

www.fisheriesjournal.com
Received: 02-07-2023
Accepted: 09-08-2023

Ishori S Mahato
Fishery Research Station, Trishuli, Nuwakot, Nepal

Prem Timalsina
National Fishery Research Station, Godawari, Lalitpur, Uttar Pradesh, India

Gun Bahadur Gurung
Fishery Research Station, Trishuli, Nuwakot, Nepal

Krishna Paudel
Fishery Research Station, Trishuli, Nuwakot, Nepal

Abhimanyu Shrestha
Sichuan Agricultural University, Chengdu, China

Churamani Bhusal
National Fishery Research Station, Godawari, Lalitpur, Uttar Pradesh, India

Prakash Kunwar
National Fishery Research Station, Godawari, Lalitpur, Uttar Pradesh, India

Corresponding Author:
Ishori S Mahato
Fishery Research Station, Trishuli, Nuwakot, Nepal

Effects of dietary supplementation of a multi-strain commercial probiotic on growth performance, feed conversion ratio, and survival rate in rainbow trout *Oncorhynchus mykiss*

Ishori S Mahato, Prem Timalsina, Gun Bahadur Gurung, Krishna Paudel, Abhimanyu Shrestha, Churamani Bhusal and Prakash Kunwar

DOI: https://doi.org/10.22271/fish.2023.v11.i6b.2881

Abstract
In aquaculture, inefficient feed utilization and disease infections are main causes of delayed growth and mortality which results in economic losses. Research has demonstrated that by modifying the fish's gut microbiota, probiotic treatment can improve nutritional performance and reduce mortality. This study was organized to examine the effects of commercial multi-strain probiotic PRO-360 (*Bacillus subtilis, Bacillus licheniformes,* and *Bacillus magaterium*) on growth performance, feed conversion ratio, and survival rate of rainbow trout (*Ohcorhynchus mykiss*). For this, a total of 945 fish each weighing 29.65 ± 2.57 g were divided equally into 15 flow-through raceway tanks and fed experimental diets at rate of 5% body weight for 90 days. The first group (T1) was given a basal diet; the remaining four groups (T2, T3, T4, and T5) received a basal diet supplemented with PRO-360 (4% or 6% w/v) at two different concentrations (1.5 g/kg and 3 g/kg). While assessing the growth performance, feed utilization, and survival, after 90 days feeding on experimental diets, no significant improvement of weight gain, specific growth rate, feed conversion ratio, or survival rate were observed in the probiotic supplemented group. However, fish given 3 g/kg 6% PRO-360 had the lowest FCR, the highest weight gain and the specific growth rates, and all groups had 100% survival. These findings imply that rainbow trout growth, feed utilization, or survival are not impacted by PRO-360 supplementation in feed under typical rearing conditions.

Keywords: Probiotic, rainbow trout, gut microbiota, immunity, PRO-360

1. Introduction

Aquaculture is an industry dealing with the culture of aquatic creatures by involving human intrusion and ownership of the organism being produced. With the increasing demand for high-quality animal protein, the growth of the aquaculture industry has augmented over the past decade, making aquaculture the fastest-growing sector to produce animal protein [1, 2]. Due to this, the goal of the aquaculture industry has been to maximize the efficiency and production of aquatic food. Subsequently, this industry has inclined towards intensification day by day. Because rainbow trout is packed with high-quality protein and a good source of healthy fatty acids [3], it has become one of the most highly cultivated commercial cold-water species intensively throughout the world [4]. However, conditions in intensive aquaculture have subjected this high-value species to high-stress conditions, making them prone to pathogenic infections and decreasing their productivity [5]. This condition has posed a major hitch for the sustainable growth of the trout farming in Nepal, as evident by the increased incidence of fin and tail rot in rainbow trout at higher stocking density because of deteriorated water quality [6]. In intensive production systems, antibiotics are often used as feed additives by farmers in large quantities to prevent fish diseases. However, this practice has negative consequences, such as environmental and food contamination. In addition, the emergence of drug resistant pathogens, the disruption of the useful gut microbiota, and the accumulation of drug residues in the fish muscle that can affect the quality and safety of fish products for human consumption are some of the effects of antibiotics use in aquaculture [7–9].

In this context, adopting more environment friendly and acceptable approach to minimize disease infestation has become a matter of paramount importance through the nutritional and management approaches [10].

The manipulation and control of the gut-microbiota of fish using dietary probiotics have been identified as ways to lower the occurrence of invading pathogens, promote digestion and absorptive processes [11], and improve the growth performance [1]. Probiotics are the live microbes or microbial adjuncts added to a feed as a supplement to modify the host-associated microbial community to improve the intestinal balance while ensuring improved feed use [12]. This improvement in the performance and general health status of an organism can be the result of boosting the immunological response, increasing the competition for nutrition and binding sites, and the generation of anti-microbial compounds [12, 13]. Additionally, probiotics may supplement vitamins, essential fatty acids, and several digestive enzymes, which may be beneficial in providing nutrients to the host [14, 15].

From single-species to multiple-species products, a large number of bacteria are used commercially as probiotics for aquaculture [16]. *Lactobacillus* (Lactic acid bacteria) separated from the digestive tract of fish is widely used in aquaculture as a probiotic, as it is generally recognized as safe [12]. In trout, probiotics have been demonstrated to be useful in successful control of the disease, stimulating the immune system, and improving growth [17–19]. Specifically, probiotics of *Pediococcus acidilactici, Bacillus subtilis,* and *Enterococcus faecium* have successfully improved the growth performance, disease resistance, intestinal microbiota, and survival ability in rainbow trout [11, 20]. Moreover, probiotics such as *B. subtilis* and *B. licheniformis* have enhanced the growth, promoted better feed consumption and utilization of rainbow trout fry [21]. Despite these facts and widespread information regarding the efficacy of probiotics as growth, feed utilization and health promoter of rainbow trout, there is a dearth of statistics concerning these effects in a Nepalese context. In addition, the higher feed cost, disease outbreak, and slower growth of the rainbow trout have become obstacles to the growth of this business in Nepal [22]. For this reason, it is urgently needed to conduct research to promote the nutritional content of the trout feed, for which adding probiotics is one way. Probiotics would control and manipulate the microbial community in the digestive tract, improve feed efficiency, and ultimately reduce production costs by lowering the feed conversion ratio and improving fish welfare, as evident by the several studies conducted in other countries. Hence, the aim of the present study was to observe the effects of probiotic Pro-360, a consortium of probiotic bacteria, *Bacillus subtilis*, *Bacillus licheniformes*, and *Bacillus magaterium*, on growth performance, survival ability and feed utilization capacity of rainbow trout.

2. Materials and methods

2.1 Fish and rearing conditions

The experiment was executed at the Fishery Research Station, Trishuli Nuwakot, Nepal. A total of 945 rainbow trout fingerlings having an average initial biomass of 29.65 ± 2.57 g were equally distributed into 15 flow-through raceway tanks with 63 individuals in each. Fish were housed in each tank at the initial density of 50 fish per square meter at 13.56 ± 0.09 °C. The dissolved oxygen and pH of the rearing tanks were maintained at 7.9 ± 0.49 mg/L and 7.38 ± 0.04 mg/L, respectively. Tanks were cleaned by siphoning every four days.

Raw material, probiotic, and experimental diets

A commercial probiotic product called PRO-360 was sourced from Evoenzyme Technologies Pvt. Ltd., Hyderabad, India. To culture the bacteria in PRO-360, a combined substrate or broth of sugarcane molasses and baking yeast was prepared first. For this, 10% sugarcane molasses (w/v) were taken and centrifuged three times at 4000 rpm for 20 minutes each. The muddy precipitate of sugarcane molasses was discarded, followed by the addition of 2% baking yeast (w/v). Finally, bacteria were cultured by adding 4% and 6% PRO-360 to a certain volume of molasses-yeast broth. After 6 days of incubation at 30 °C with continuous stirring, probiotic cells were harvested by centrifugation. The basal diet was prepared within the farm according to the nutritional requirement of rainbow trout using finely ground prawn (Jwala) and soyabean full fat as the main protein source and other ingredients (Table 1). The basal diet was mixed with harvested probiotics to prepare the other four experimental diets. Altogether, five experimental diets were prepared and assigned as treatment groups, which included a control group given with a basal diet (T1), a group given with a basal diet supplemented with 1.5 g/kg 4% PRO-360 (T2), a group fed with a basal diet containing 3 g/kg 4% PRO-360 (T3), 1.5 g/kg 6% PRO-360 (T4), and a group given with a basal diet containing 3 g/kg 6% PRO-360 (T5). Fish were given experimental diets at 5% of individual body weight daily, in equal amounts in the morning (09:00) and in the evening (17:00) for 90 days. The proximate composition analysis of the experimental diets was done following the method of the Association of Official Analytical Chemists (AOAC) [23] at National Animal Nutrition Research Centre located at Lalitpur, Nepal (Table 1).

Table 1: Feed composition and results of proximate analysis of experimental diets.

Feed ingredients	Treatments (Composition of experimental diets expressed in g/kg)				
	T1	T2	T3	T4	T5
Fish meal	500.00	500.00	500.00	500.00	500.00
Soybean full fat	180.00	180.00	180.00	180.00	180.00
Wheat flour	200.00	200.00	200.00	200.00	200.00
Mustard oil cake	80.00	80.00	80.00	80.00	80.00
Rice bran	50.00	48.50	47.00	48.50	47.00
Vitamins mix*	10.00	10.00	10.00	10.00	10.00
Mineral mix**	10.00	10.00	10.00	10.00	10.00
Probiotic (PRO-360)	0	1.5	3	1.5	3
Proximate composition of experimental diets					
Dry matter (%)	92.69	93.92	91.70	92.33	93.70
Ash (%)	8.84	8.85	8.07	8.80	7.87
Crude protein (%)	39.19	39.62	41.98	38.80	41.51

Lipid (%)	17.80	18.31	17.66	16.55	17.44
Crude fiber (%)	12.5	12.49	12.69	11.49	12.29
Energy (MJ/kg)	22.81	22.84	23.33	22.22	21.13

*Vitamin mixture/kg premix containing the following: 33000 IU vitamin A, 3300IU, vitamin D3, 410I U vitamin E, 2660 mg Vitamin BI, 133 mg vitamin B2, 580 mg vitamin B6, 41 mg vitamin B12, 50 mg biotin, 9330 mg choline chloride, 4000 mg vitamin C, 2660 mg Inositol, 330 mg para-amino benzoic acid, 9330mg niacin, 26.60 mg pantothenic acid **Mineral mixture/kg premix containing the following: 325mg Manganese, 200 mg Iron, 25 mg Copper, 5 mg Iodine, 5 mg Cobalt. T1: basal diet; T2: 1.5 g/kg 4% PRO-360; T3: 3 g/kg 4% PRO-360; T4: 1.5 g/kg 6% PRO-360; T5: 3 g/kg 6% PRO-360. PRO-360: *Bacillus subtilis* + *Bacillus licheniformes* + *Bacillus magaterium*

2.2. Sampling and growth performance

All fish were sampled biweekly, following 24 hours of starvation, for their body weight and length, and the ration required for the next 15 days was adjusted accordingly. Before each fish was weighed, the water level of all tanks was slowly reduced, and the fish were transferred to another covered container using a gentle scoop net. To analyze the growth performance parameters; biomass gain (BWG), relative growth rate (RG), specific growth rate (SGR), condition factor (K), and survival rate (SR) were determined according to Munir *et al*. [24]. And, the feed conversion ratio (FCR) was calculated using formulae given below [25].

BWG (g): Final weight (g) – initial weight (g)

SGR (%): [In (Final weight) – In (initial weight) / No. of days] × 100

RG (%): (Final weight − initial weight) / Initial weight) × 100

CF (%): [Final weight (g) / L^3 (cm)] × 100

SR (%): [Final number of fish / Initial number of fish] × 100

FCR: Total feed consumption / Weight gain of fish

2.3. Data analysis

The statistical analysis of the data was performed using the SPSS program (Version 25). All the obtained raw data were subjected to Shapiro-Wilk and Levene test for the normality and homogeneity of variance test, respectively. Then the results were subjected to a one-way ANOVA. Differences among the means were tested using the Tuckey post-hoc test when ANOVA showed significant differences between means. A 5% probability level was considered to test differences among the means.

2.4. Results

No fish mortality was observed in this experiment, resulting in a 100% survival rate across all treatments. The initial weight of the rainbow trout used for this experiment varied between 30.85g and 33.13g, and they were statistically comparable. The growth performance and feed consumption parameters obtained at the completion of the experiment, following a duration of 90 days, are summarized in Table 2. With the exception of T3, which was fed 3 g/kg 4% PRO-360 probiotic, all probiotic supplemented groups exhibited higher growth performance than the group fed on basal diet (T1). The fish biomass experienced a growth of more than 200% in all groups. The group that showed the highest weight gain (93.39±2.08g) was T5, which was fed with 3 g/kg 6% PRO-360. On the other hand, the lowest biomass gain was observed in T3 (84.57±1.71g). The maximum specific growth rate was reported in T5 (1.54), followed by T4 (1.50), T2 (1.49), T1 (1.46), and the lowest rate of 1.41 in T2 (1.5 g/kg 4% PRO-360). Nevertheless, after a period of 90 days, no significant difference was observed in the final weight, weight gain, or growth rates, irrespective of the inclusion of probiotic supplements in the feed. Similarly, the fish in T5 had the greatest condition factor of 1.35, followed by T4 (1.29) and T2 (1.24). The condition factor of fish in T3 and T1 exhibited no noteworthy variation, with both groups having an identical value of 1.22. In addition, the FCR was rather low in T5 (2.89) while the greatest ratio was reported in T1 (3.15). Although, the FCR value in T5 was better than that of the control and other treatments, there average did not differ statistically.

Table 2: Results of growth performance and feed utilization of rainbow trout after 90 days (mean ± SEM)

Parameters	T1	T2	T3	T4	T5	P-Value
Initial weight (g)	32.02±1.42	31.85±1.25	33.13±1.17	30.85±1.98	31.11±1.04	0.80
Final weight (g)	119.77±6.81	121.97±7.66	117.7±2.82	118.93±5.42	124.5±1.05	0.90
Weight gain (g)	87.75±6.35	90.12±6.82	84.57±1.71	88.08±6.94	93.39±2.08	0.82
SGR (%)	1.46±0.06	1.49±0.05	1.41±0.02	1.50±0.12	1.54±0.05	0.69
RG (%)	274.8±21.69	282.82±17.22	255.57±4.81	290.5±40.13	301.32±16.24	0.70
K	1.22±0.03	1.24±0.05	1.22±0.06	1.29±0.05	1.35±0.05	0.73
FCR	3.15±0.17	2.98±0.23	3.06±0.09	3.06±0.34	2.89±0.09	0.92
Survival rate (%)	100	100	100	100	100	

SGR: specific growth rate; RG: relative growth rate; K: condition factor; FCR: feed conversion ratio; T1: basal diet; T2: 1.5 g/kg 4% PRO-360; T3: 3 g/kg 4% PRO-360; T4: 1.5 g/kg 6% PRO-360; T5: 3 g/kg 6% PRO-360.

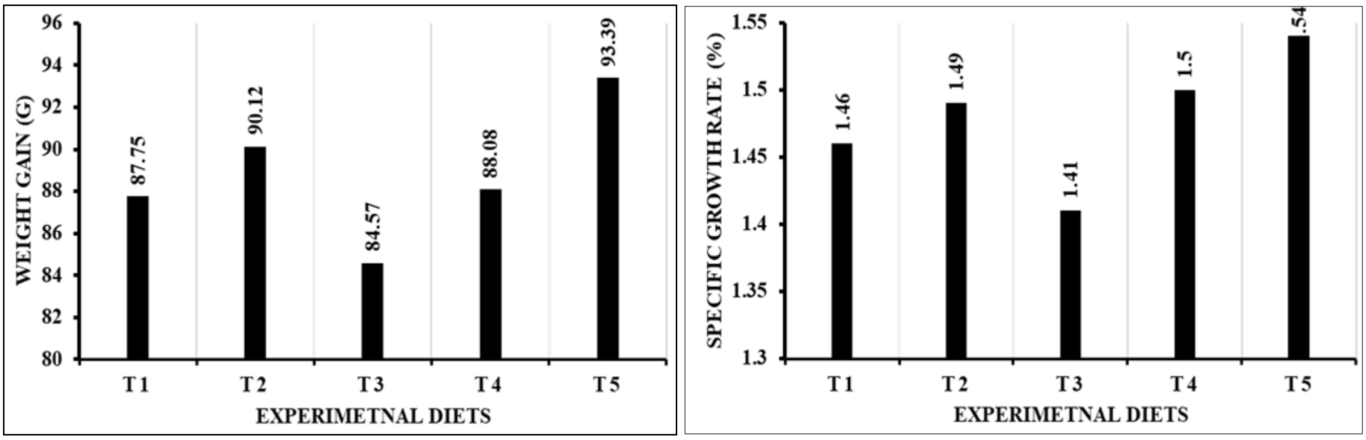

Fig 1: Weight gain (g) and SGR of rainbow trout fingerling fed with experimental diets for 90 days

3. Discussion

In this experiment, a commercial probiotic, PRO-360, a consortium of probiotic bacteria, *B. subtilis, B. licheniformes, and B. magaterium*, was supplemented into the rainbow trout diet at two concentrations (4% and 6%), each at two different levels (1.5 and 3 g/kg). The highest value of final weight, weight gain, SGR and RG in the T5 may indicate that a diet containing 3 g/kg PR0-360 probiotic at 6% concentration (w/v) apparently contributed to the growth performance of rainbow trout, but it should not be taken as a takeaway from this experiment because it did not differ significantly from the control and other treatment groups. To our best knowledge, this experiment is the first to use the PRO-360 in feed for rainbow trout in Nepal. In addition, there is a lack of studies to compare the effectiveness of PRO-360 on the growth and health status of rainbow trout or any aquatic species, which has posed a significant challenge to determining the effectiveness of these probiotics in aquaculture. Today, several commercial probiotics are available on the market which single or multiple microorganisms and have been introduced in aquaculture to boost the production of aquatic animals [1]. A study showed that, a commercial probiotic with multiple species (*Bacillus sp., Lactobacillus sp., and Enterococcus sp.*) significantly improved the growth and specific growth rate of juvenile rainbow trout (*Oncorhynchus mykiss*) compared to the control group, where a single-species probiotic (*Pediococcus acidilactici*) did not affect the weight gain and SGR of trout [13] when fed for 56 days. Other studies that used only *P. acidilactici* for two to five months to supplement the rainbow trout di*et al*so did not observe any significant improvement in weight gain [26,27]. In fact, according to Shelby *et al*. [28], when *P. acidilactic* was combined with *E. faecium*, it decreased the weight gain of young catfish (*Ictalurus punctatus*). Similarly, in agreement with our findings, when rainbow trout juveniles [29], catfish [28] and tilapia [30] were fed a diet containing *B. licheniformis* and *B. subtilis* (BioPlus2B®), there was no significant effect observed on weight gain. In contrast to our findings, a study conducted in rainbow trout fry demonstrated that combined *B. subtilis* and *B. licheniformis* improved the growth performance at a dietary level of log 9 cells/g. Similarly, the combination of *B. licheniformis* and *B. subtilis* notably enhanced the weight gain and SGR of trout larvae during the initial feeding phase in a dose-dependent manner [21,31]. As such, comparing the results of the previous studies that used *B. subtilis* and *B. licheniformis*, and the current findings, probiotics containing *B. licheniformis* and *B. subtilis* appeared to be more efficient when they were used in the early stages (fry) compared to the later stage (fingerlings) of the fish, including rainbow trout. However, the use of *B. magaterium* has been scarcely reported in aquaculture despite its ability to grow easily on many carbon sources and secrete several enzymes such as α-amylase, β-amylase, penicillin amidase, and many others [32]. In a recent study by Afrilasari *et al.* [33], probiotic *B. magaterium* (PTB 1.4) has been demonstrated to foster the activity of digestive juice and the growth of catfish (*Clarias* sp.). Similarly, *B. magaterium*, when administered in the feed and water for the post-larval stage of tiger shrimp (*Penaeus monodon*), significantly improved the growth rate and FCR compared to control group [34]. However, no study has reported the use of *B. magaterium* as a probiotic for rainbow trout culture. Another reason for its inclusion in PRO-360 could be because it is a major producer of vitamin B12 and falls within the group of *B. subtilis* [32].

Generally, probiotics in aquaculture are administered to improve fish resistance to bacterial infections via enhancing the immunological response and creating a competitive environment for harmful microorganisms [12,13]. In addition, probiotic incorporation in the diet of aquatic animals enhances growth as a result of improved nutrient assimilation, thereby increasing the availability of metabolic substrates like digestive enzymes [35] and vitamins [36] produced by the microbiota. In this regard, the results of the present study exposed that PRO-360 supplementation at 1.5–3 g/kg may have slightly improved the growth performance of rainbow trout fingerlings, although not significantly significant. Therefore, it is important to analyze the mechanism of action of this commercial probiotic to determine the effect on gut microbiota. It is because the different stains have different metabolic capacities and can also enhance the growth of cohabiting probiotic strains or useful strains in the intestine because of the secretion of metabolites [13]. It is also important to analyze the gut microbiota because probiotics can enhance the growth of indigenous microbiota and therefore enhance the digestibility of diet [37].

In conclusion, this study has given a slight insight that the incorporation of dietary PRO-360, consisting of *B. subtilis*, *B. licheniformes*, and *B. magaterium*, may enhance the survival rates, growth rate, and feed utilization of rainbow trout at the rate of 3 g/kg feed. However, the complete spectrum of its benefits may not have been fully elucidated within the confines of this investigation. Given that the efficacy of probiotics is often more pronounced in adverse rearing conditions, stressful environments, or in the face of disease challenges, it is advisable for future research endeavors to focus on such scenarios. Moreover, considering the recent introduction of PRO-360 to the Nepalese market and its novel application in rainbow trout feed, further studies are warranted to comprehensively explore its potential impacts and ascertain the optimal dosage for incorporation in aquafeed.

Acknowledgements
The authors heartedly acknowledge the Evoenzyme Technologies Pvt. Ltd, Hyderabad, India for providing the probiotic product with necessary details. The authors would also convey thanks to National Animal Nutrition Research Center, Lalitpur, Nepal for conducting the proximate analysis of the experimental diets.

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