Journal of
Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Journal of Fisheries and Aquatic Science

ISSN 1816-4927
DOI: 10.3923/jfas.2020.42.50

Research Article
Probiotic Supplements Modulates Growth Performances and Muscle Hypertrophy of Minnow, *Labeo ariza*

[1]A.K. Shakur Ahammad, [1]Ashraful Haque, [1]Nahid Sultan Lucky, [1]Borhan Uddin Ahmed, [1]Biraj Kumar Dutta, [1]Fazla Rabbi, [1]Kamal Hossain and [2]Erik Anderson

[1]Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
[2]Quest Diagnostics LLC, Department of Manufacturing and Development, Lenexa Kansas, USA

Abstract

Background and Objective: Growth performances of minnow, *Labeo ariza* using probiotic with commercial feeds is necessary to develop its muscle growth and culture potentiality. The objective of the present study was to determine the effect of probiotics on the growth rate of *L. ariza*. **Materials and Methods:** Four treatments namely T_1, T_2, T_3 and T_4 having three replications of each where Power PS probiotic with commercial feed, Safegut probiotic with commercial feed, VC-7 probiotic with water and only commercial feed, respectively were used. Muscle histochemistry was done to confirm muscle growth in each treatment. One way ANOVA of data were performed for statistical analysis. **Results:** Mean length, weight, specific growth rate and survival rate were found to be highest in T_2 followed by T_3, T_1 and T_4, respectively. Further, muscle cellularity analysis unveiled both hyperplastic and hypertrophic muscle fiber in all treatments of *L. ariza* whereas the highest number of hyperplastic (small diameter) fibers were recorded at the post-larval stage of *L. ariza* in T_2 followed by T_3, T_1 and T_4, respectively. Moreover, it was observed that the average number of hypertrophic (medium and large diameter) muscle fibers was found to be highest at late juvenile stage in T_2 followed by T_3, T_1 and T_4, respectively. **Conclusion:** Increased growth rate of *L. ariza* in T_2 was mostly governed by hypertrophic growth rather than hyperplastic growth which indicated that Safegut probiotic with commercial feed has a potential role in muscle progression of slow growth minnow in aquaculture.

Key words: growth, hyperplasia, hypertrophy, *L. ariza*, muscle fiber, probiotics

Citation: Ahammad, A.K.S., A. Haque, N.S. Lucky, B.U. Ahmed, B.K. Dutta, F. Rabbi, K. Hossain and E. Anderson, 2020. Probiotic supplements modulates growth performances and muscle hypertrophy of minnow, *Labeo ariza*. J. Fish. Aquat. Sci., 15: 42-50.

Corresponding Author: A.K. Shakur Ahammad, Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh Tell: 01719599249

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bangladesh is a land of rivers that are blessed with a wide range of aquatic diversity. About 293 freshwater fish species are found in rivers, ponds, streams, haors, baors, beels, floodplains, lakes and ditches of Bangladesh where 13 orders and 61 families were recorded[1-2]. *Labeo ariza* is one of them under the order Cypriniformes and family Cyprinidae. It is well known as *Cirrhinusariza*, *Cyprinusariza*, *Tylognathusariza*, or *Banganaariza*as well as locally and commonly called as Bhagna and minnow/Arizalabeo, respectively[3]. *L. ariza* inhabits freshwater, found in clear rivers and tanks, ponds, beels and inundated fields. It breeds in flooded shallows from April to September[4-5]. *L. ariza* is found from the Indus plains and adjoining hilly areas in Pakistan; Ganges-Brahmaputra basin, Barak basin in India, Nepal and Bangladesh; Mahanadi, Krishna, Cauvery and some smaller basins in southern India; Karnaphuli and adjacent smaller basins in Chittagong Hill Tracts in Bangladesh and Myanmar[6]. In Bangladesh, this species is reported from Chalan Beel, Natore[3] and Halda River, Chittagong[7]. According to the International Union for Conservation of Nature (IUCN), this fish is a vulnerable freshwater fish of Bangladesh[3]. This fish is decreasing due to several anthropogenic activities that include aquatic pollution, building embankment through the river, loss of natural habitat for spawning and growth of this species, dumping of industrial chemicals[5,8]. Although they are listed as threatened and vulnerable, a small number of *L. ariza* is still available in different rivers, beels, haors and baors[1].

Bangladesh Fisheries Research Institute (BFRI) had developed an induced breeding technique of *L. ariza* successfully, but its growth pattern showed very slow in culture condition[7]. So, this fish species need to be improved their growth performance and muscle growth. For the increasing growth rate and muscle development of *L. ariza*, different supplemented diets or probiotics with commercial feed can be administered in pond culture conditions. Regards, fish muscle development has occurred through the generation of newly recruited muscle fibers (stratified and mosaic hyperplasia) between the medium and large diameter muscle fiber (hypertrophy)[9-10]. Various researches have demonstrated that newly recruited muscle fibers in fish are relatively small in size (hyperplastic muscle fiber) and that these fibers increase in the cross-sectional area through hypertrophic growth[11]. The contributions of hyperplasia and hypertrophy appear dependent on both the species and the ultimate somatic size of the fish[12]. These findings provide strong indications that the overall contribution of hyperplastic and hypertrophic muscle growth is responsible for determining the ultimate attainable size of a fish[11]. Hence, the determination of the muscle growth of *L. ariza* is essential to identify its effectiveness in pond aquaculture using commercially available probiotics in Bangladesh.

Probiotics are beneficial microorganisms that increase the digestibility and maintain water quality and immunity[13]. There are a vast range of micro and macro-organisms, such as microalgae (*Tetraselmis*), yeast (*Debaryomyces*, *Phaffia* and *Saccharomyces*) and gram-positive (*Bacillus*, *Lactococcus*, *Micrococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Streptcoccus*, *Weisslla*) and gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorhodobacterium*, *Pseudomonas* and *Vibrio*) that have been evaluated as probiotics[13]. Different scientists have reported auspicious results of probiotics application in fish farming which assists the probiotics to replace the antibiotics as growth promoters[14-17]. In fish, they are usually administered orally mixed with feed in order to develop the growth of microbial flora of the intestine[18-19].

L. ariza is an important source of, proteins, fats, vitamins, minerals, iron and calcium[20]. The fish flesh is oily, tasteful and people would like to eat due to its high nutrition, attractive flavor and a smaller amount of spines[6,21]. On the contrary, farmers are less interested to culture this species due to slow growth rate[7]. So optimization of growth for farming of this species is very important and demanding among the fish farmer of Bangladesh. A little information is available on the induced breeding, growth and morpho-genetic analysis of minnow, *L. ariza* in Bangladesh[10,22]. No initiative seems to have been taken to improve the growth performances of this slow-growing fish species in aquaculture and to conserve this tasty and nutritious endangered fish. On account of this issue, the present research was aimed to evaluate the growth performance of *L. ariza* using different commercially available probiotics as well as to find out the best muscle growth of *L. ariza* using cellular analysis through histology.

MATERIALS AND METHODS

Site selection, experimental design and rearing of *L. ariza*: The experiment was carried out from November 2017 to April 2018 in the Fisheries Faculty Field Laboratory Pond Complex, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh. Twelve ponds (18×14 m $\times$ 1.3 m^3 each) equipped with the same physical facilities (inlet-outlet, water exchange, aeration) were selected for four treatments with three replications of each. The treatments were designed by using different probiotics with commercial feed (35% protein) (Mega Feed Limited, Bangladesh) shown in Table 1. In each pond, approximately 500 post larvae (15 day after hatching) having an average length and weight (3.74 cm and 0.57 g,

Table 1: Experimental design during 180 days of experimental period

Treatments	Probiotics	Amount/quantity and application methods	Stocking density (No/Dc)	Replications
T_1	Power-PS probiotic (Photosynthetic bacteria along with other useful bacteria) with commercial feed	Every day before application of feed, 10 mL Power-ps were mixed with one glass of water thereafter mixed with feed and subjected to sunlight for 10 minutes. This feed were applied at 5% of body weight of the biomass	500	3
T_2	Safegut probiotic (*Bacillus subtilis, B. licheniformis, Lactobacillus sporogrnrs, Saccharomyces boulardii, S. cerevisiae, Asdpergillusoryzae, A. niger*) with commercial feed	Every day before application of feed, 3 g Safegut were mixed with one glass of water then this mixture were mixed with feed and subjected to sunlight for 10 min. This feed were applied at 5% of body weight of the biomass		
T_3	VC-7 probiotic (*Bacillus subtilis* along with other bacteria) with water	20 g VC-7 was mixed with 20 g adhesive molasses and 5 L water. Mixture was kept overnight in the laboratory. This mixture was applied in the pond at an interval of 20 days		
T_4	No probiotics only commercial feed	Only commercial feed at 5% of body weight of the biomass were given		

respectively) of *L. ariza* were stocked. The post-larvae were reared for six months providing a probiotic supplement with commercial feed, twice a day at 5% of their body weight. Water quality parameters of the ponds such as dissolved oxygen (DO), pH and ammonia (NH_3) were checked fortnightly using DO and pH meter (AND, Japan).

Determination of growth parameters: During the experimental period samplings were done randomly at 15 days interval. Length and weight of 60 fishes from each treatment were measured using a meter scale and a digital balance (TANITA Corporation, China). Growth performance of *L. ariza* was observed in terms of mean final weight (g), weight gain (g), mean final length (cm), length gain (g) and specific growth rate (% day^{-1}). These parameters were estimated by using the following formulae given by Bagenal[23]:

$$\text{Weight gain (g)} = \text{Mean final weight} - \text{Mean initial weight} \quad (1)$$

$$\text{Length gain (cm)} = \text{Mean final length} - \text{Mean initial length} \quad (2)$$

$$\text{Specific growth rate (SGR) \% day}^{-1} = [\ln W_2 - \ln W_1/T_2 - T_1] \times 100 \quad (3)$$

Where:

ln : Natural log function
W_1 : Mean initial weight (g)
W_2 : Mean final weight (g)
T_1 : Time at the start of the experiment
T_2 : Time at the end of the experiment

Cellular analysis through histology: Identification of the earlier presence of new muscle fiber was done through myotomal cross-sections of the post larvae (21 days after hatching), early juvenile (90 days after hatching) and late juvenile stages (180 days after hatching) of *L. ariza* following the histochemistry techniques according to Johnston *et al.*[12]. In this regard, the preserved trunk muscle was taken out in a perforated plastic holder covering by perforated steel plates. Cleaning, infiltration and dehydration processes were carried out in an automatic tissue processor unit using a series of alcohol concentrations, two times changes of xylene and finally through three series of molten wax. Paraffin-embedded blocks were cut by microtome knife at 4.0 μm size and the sections were kept into a water bath at a temperature of 52°C. The sections were then placed on a glass slide and kept overnight on a slide drier hot plate at 20°C. Then the sections stained routinely with hematoxylin and eosin[24]. At the end, the sample containing slides were covered by coverslip and mounted by Canada balsam. After that, photographs were taken under a compound microscope (Olympus, CX41, Shinjuku, Tokyo, Japan) and different sized fibers were counted and categorized in three size group (small<20 μm, medium 21 to 30 μm and large >30 μm) according to Johnston *et al.*[12].

Statistical analysis: MS Excel 2016 and SPSS-V.21 software (IBM SPSS for windows, Armonk, New York, USA) were used to analyze and interpret experimental data obtained from growth parameters in terms of length and weight. For the statistical analysis of data, a one-way ANOVA using Duncan's multiple range test was done at 5% level of significance. Muscle fiber sizes were measured using image analysis software through Sigma Scan Pro Software version 5. Thirty photos from each treatment were considered for the result.

RESULTS

Effect of probiotics on the growth performances of *L. ariza*: The results of growth in terms of length, weight, SGR and survival rate of fishes in the four treatments are presented in Table 2. During the investigation, the initial length and weight of *L. ariza* were 3.74±0.17 cm and 0.57±0.0 g, respectively in

all treatments. At the end of the experiment, T_2showed higher value in terms of length and weight followed by T_3, T_1 and T_4, respectively. There was no significant ($p>0.05$) differences in mean final length among the probiotics treatments (T_1, T_2, T_3) whereas these three treatments significantly ($p<0.05$) differed from the control treatment (T_4). On the other hand, there were significant ($p<0.05$) differences among the four treatments in terms of mean final weight.

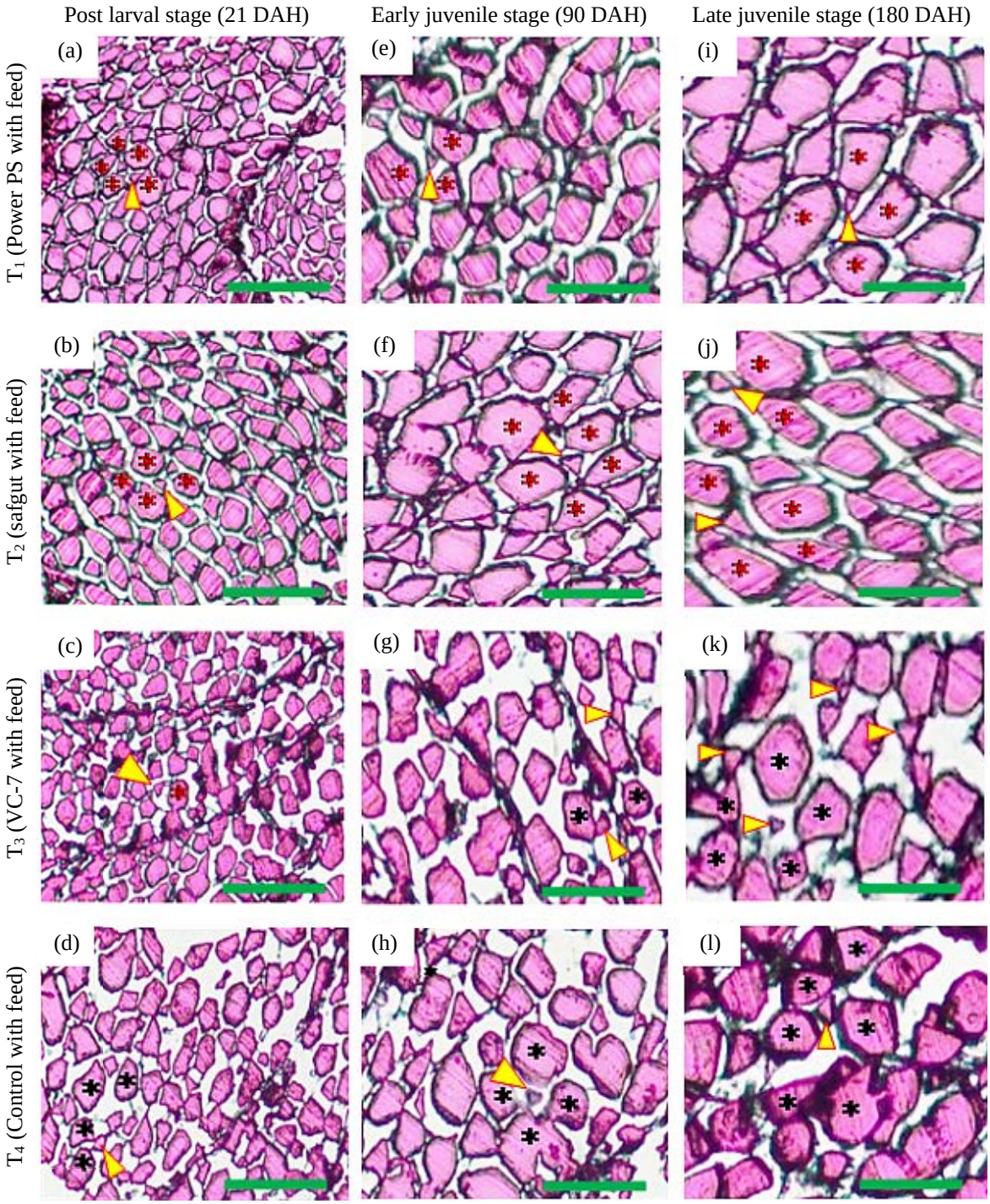

Fig. 1(a-l): Horizontal section of fast skeletal muscle in minnow, *L. ariza* at post larval (21 DAH), early juvenile (90 DAH) and late juvenile stages (180 DAH) in the T_1 (Power PS with feed), T_2 (Safegut with feed), T_3 (VC-7 with feed) and T_4 (Control with feed)

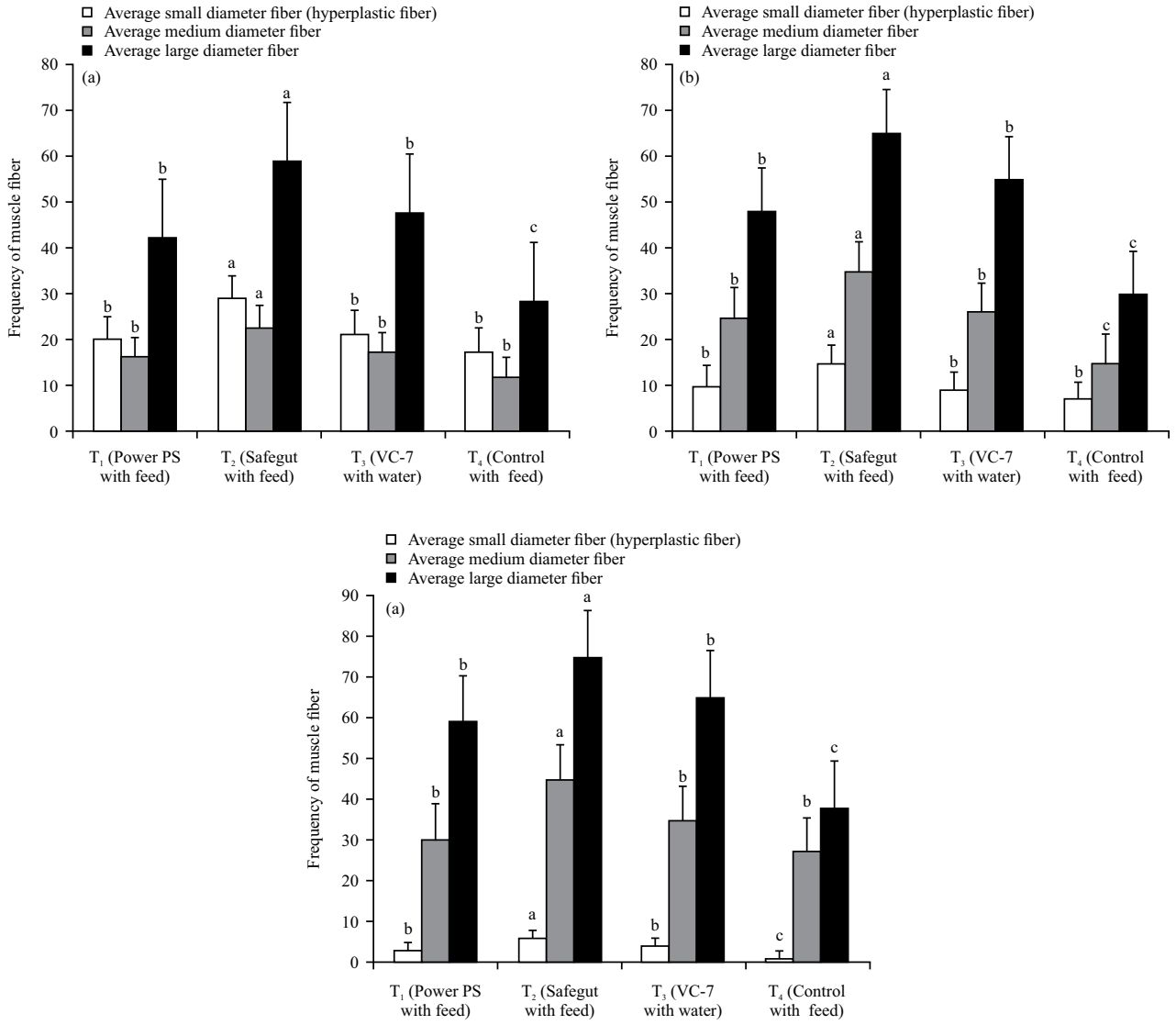

Fig. 2(a-c): Frequency distribution of skeletal muscle fibers of (a) post larvae stage (21 DAH), (b) early juvenile stage (90 DAH) and (c) late juvenile stage (180 DAH) in all treatments of *L. ariza*, The percentage values followed by different superscript letters in each diameter class indicate difference ($p<0.05$) among different treatments

Table 2: Growth performance of *L. ariza* using supplemented diet with probiotics after 180 days of experimental period

	Treatments			
Parameters	T_1 (Power PS with feed)	T_2 (Safegut with feed)	T_3 (VC-7 with water)	T_4 (Control with feed)
Initial length (cm)	3.74±0.17^a	3.74±0.17^a	3.74±0.17^a	3.74±0.17^a
Initial weight (g)	0.57±0.00^a	0.57±0.00^a	0.57±0.00^a	0.57±0.00^a
Final length (cm)	13.85±0.89^a	15.16±0.98^a	14.35±0.98^a	10.54±1.11^b
Final weight (g)	17.39±0.38^b	20.85±0.62^a	18.90±0.98^b	15.93±0.65^c
Length gain (cm)	10.21±0.97^a	11.42±0.90^a	10.61±0.88^a	6.80±1.31^b
Weight gain (g)	16.82±0.37^c	20.28±0.64^a	18.33±0.89^b	15.35±0.51^c
SGR (% day^{-1})	1.90± 0.06^a	2.01± 0.14^a	1.95±0.07^a	1.87± 0.18^a
Survival rate (%)	84.33±3.78^b	90.26±1.10^a	86.65±0.87^{ab}	78.47±0.94^c

Values in the same row having different superscripts are significantly different ($p<0.05$)

Effects of probiotics on muscle fibers morphometry of *L. ariza*

Muscle fibers morphometry at post-larval stage (21 days after hatching): Horizontal sections of fast skeletal muscle of *L. ariza* from the post-larvae of *L. ariza* at 21 days after hatching (DAH) in different treatments is shown in Fig. 1a-d. During post-larval stage (21 DAH) of *L. ariza*, muscle fibers were dispersed in a mosaic pattern which was categorized by fibers of different diameter sizes such as newly recruited small-diameter muscle fiber (i.e. hyperplastic muscle fiber) between medium and large diameter muscle fiber (hypertrophic muscle fiber) indicated by arrowheads and asterisks, respectively. Therefore, muscle morphometric analysis through histology showed that commercial feed with probiotics influenced both hyperplastic and hypertrophic muscle fiber in all treatments of *L. ariza* whereas the highest average number of hyperplastic and hypertrophic muscle fibers were recorded at post-larval stage (21 DAH) of *L. ariza* in T_2 followed by T_3, T_1 and T_4, respectively (Fig. 2a). Likely, all-fiber diameter classes in T_2 treatment were significantly ($p<0.05$) different from other treatments (Fig. 2a).

Muscle fibers morphometry at early juvenile stage (90 days after hatching): Figure 1e-h represents the cross-sections of fast skeletal muscle of *L. ariza* from the early juvenile stage at 90 days after hatching in different treatments. At this stage (90 DAH), the generation of newly recruited muscle fibers (hyperplastic muscle fiber) were reduced and enlarged in size in a mosaic pattern. It was also observed that the average number of small, medium and large diameter fibers was higher at early juvenile stage (90 DAH) in T_2 followed by T_3, T_1 and T_4, respectively (Fig. 2b). Similarly, all-fiber diameter classes in T_2 treatment were significantly ($p<0.05$) different from other treatments (Fig. 2b).

Muscle fibers morphometry at late juvenile stage (180 days after hatching): Withal, Figure 1i-l demonstrates the horizontal sections of the fast skeletal muscle of *L. ariza* from the late juvenile stage (180 DAH) of *L. ariza* in different treatments. Throughout this stage, the generation of small-diameter muscle fibers was greatly reduced and strongly enlarged in size. Here, the average number of medium and large diameter fibers (hypertrophic fiber) was higher at the late juvenile stage (180 DAH) in T_2 followed by T_3, T_1 and T_4, respectively (Fig. 2c). However, all-fiber diameter classes in T_2treatment were significantly ($p<0.05$) different from other treatments (Fig. 2c). So, cellular analysis through histology confirmed that the increased growth rate of *L. ariza* in T_2 was mostly governed by hypertrophic muscle growth rather than hyperplastic muscle growth.

DISCUSSION

Probiotics are living bacterial cells that improve fish growth, immunity and digestive processes. The findings of the current experiment confirm the findings from other studies that inclusion of probiotics in the commercial feed can improve growth performance in terms of length and weight gain, SGR and survival rate. In the current experiment, production was higher in all probiotics treated feeds which are in agreement with findings of other studies where better growth performance using probiotics containing bacteria with commercial feed were reported[25-28]. In the present study, higher growth performances were achieved by using safe gut probiotics which is consistent with the findings of Khatun and Saha[29] who conducted a comparative study on *Oreochromis niloticus* using different probiotics mixed feed where safe gut probiotic was the best. They also concluded as the formation of total heterotrophic bacteria by using safe gut was higher (1.65 ± 0.494 CFU mL^{-1}). So, this highest growth in T_2 (safe gut with commercial feed) might be due to the production of the highest total heterotrophic bacteria than other treatments. Since the first use of probiotics in aquaculture, a growing number of studies have demonstrated their ability to increase the growth rate and welfare of farmed fish[17,30-34]. Nevertheless, other investigators revealed that growth performances of fishes were not significantly influenced by dietary supplementation of yeast (*S. cerevisiae*) at different levels[35]. This reason might be due to the difference in aquaculture where they were cultured fish in cage system while the present experiment was conducted in earthen ponds as well as physiological conditions of fish and the type of basal ingredients in diets.

The highest survival rate and SGR were obtained using safe gut which is in agreement with the survival rate and SGR of Nile tilapia (*O. niloticus*) that were higher using probio aqua and safe gut probiotic[29]. Al-Faragi and Al-Saphar[36] demonstrated that the higher survival rate of *Cyprinuscarpio* was 94.4% by using bacteria *S. cerevisiae, B. subtilus* and Lactic acid bacteria that were present in safe gut probiotic in the present study. However, in the present study, a higher survival rate (90.26 ± 2.65) was close to the result designated by Muchlisin *et al.*[37]. The average survival rate in all probiotic ponds was higher than the control pond in the present study which is similar to the findings of Munirasu *et al.*[38].

Muchlisin *et al.*[37] also revealed that the average survival rate of *Tor tambra* was lower compared to the present study. The reason behind this was probably due to the application of a low dose of probiotics in case *T. tambra*.

Assay of muscle fiber morphometry in different treatments showed small-diameter fibers (hyperplastic muscle fiber) surrounded by medium and larger fibers (hypertrophic muscle fiber). This prototype is seen in maximum fish species during muscle development and is a characteristic feature of hyperplasia and hypertrophy[12,39,40]. In most fish species, this pattern of the mechanism are employed throughout their life cycle and have been well documented in a variety of species, particularly in species with a large potential for aquaculture[39,41,42]. Regard to present investigation, less than 20 µm fibers in size denoted as hyperplasia which is occurred as like mosaic groups of fibers and those exceeding this size characterized fibers which had successively grown by hypertrophy[43]. In the present study, it has been observed that post-larval stage (21 DAH) of *L. ariza*in safe gut with commercial feed treatment showed significantly the highest number of newly recruited small-diameter muscle fiber (hyperplastic fiber), demonstrating hyperplasia is the main muscle growth mechanism at this stage (Fig. 1b). Respectively in early and late juvenile stage of *L. ariza* in the diet supplemented with safe gut (T_2) showed that highest average number of medium and large diameter muscle, representing intense muscle hypertrophy (Fig. 1f,j) rather than hyperplasia possibly correlated with variation of mRNA expression of several muscle growth-related genes in different stages or physiological conditions of *L. ariza*. Findings of Carani *et al.*[44] support the present findings as he reported this mechanism was responsible for fish muscle growth. An almost similar observation has been made by Asaduzzaman *et al.*[9] in *Tor tambroides* fed different probiotic supplemented diets. Therefore, the present study reports the effects of different dietary probiotics on the muscle growth of *L. ariza* at the cellular level. There are many notable characteristics of teleost muscle growth, but little is known about the underlying probiotics enhanced hypertrophic muscle growth which will provide new insights not only into aquaculture but also in skeletal muscle biology.

In addition, this study has provided a technique for increase the growth of minnows using probiotics with their feed. This will insist farmers to culture this fish in Bangladesh. The description of muscle structure in this study will help the further researcher to work on these topics. However, further investigation is needed on the effects of these probiotics on the relative expression level of the muscle developmental related genes such as Myoblast determining factor (*MyoD*), *Myogenin*, Myosite enhancer factor (*MEF*), Paired box protein (*Pax3*) etc. Since the present study was conducted in the post-larval and juvenile stages of *L. ariza*. Next research needs to be carried out using larval, juvenile and adult fish for understanding the comprehensive functional role of these probiotics on the quantitative gene expression related to muscle development.

CONCLUSION

In conclusion, our findings designated that safe gut can be used as a probiotic with commercial feed for improving the growth performance of slow-growing minnow, *L. ariza*. Our study further confirmed that the improved growth performance of *L. ariza* was mostly governed by muscle fibers hypertrophy rather than hyperplasia. So farmers should use this probiotic to gain higher growth of this fish in culture condition.

SIGNIFICANCE STATEMENT

This study discovered the growth improvement of *L. ariza* that can be beneficial for fish culturists. Muscle growth has been improved in the present study which ultimately leads to higher production of the fish. This study will help the researchers to uncover the critical areas of the underlying causes of the slow growth rate of fishes and improve the growth rate of other slow-growing fish like *L. ariza* that many researchers were not able to explore in the past. Thus a new theory on growth improvement may be arrived by the present research.

ACKNOWLEDGMENT

This research was partially supported by the Ministry of Science and Technology from the People Republic of Bangladesh (BS-107). The authors thank Head, Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh, for providing the equipments and laboratory facilities used in this study.

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