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Evaluation of the Impacts of Long-Term Enriched *Artemia* with *Bacillus subtilis* on Growth Performance, Reproduction, Intestinal Microflora, and Resistance to *Aeromonas hydrophila* of Ornamental Fish *Poecilia latipinna*

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Abstract

The present study investigated the effect of enriched *Artemia* with *Bacillus subtilis* on growth performance, reproductive factors, proximate composition, intestinal microflora, and resistance to *Aeromonas hydrophila* of ornamental fish, *Poecilia latipinna*. Using a completely randomized design, the experiment included three groups. The first group was fed with commercial food without any probiotic. The second group was fed with unenriched *Artemia*, and the last group consumed long-time enriched *Artemia* with *Bacillus subtilis*. The bacteria *B. subtilis* with a density of 1×10^5 CFU mL^{-1} was added daily to *Artemia* culture medium. The total microflora and *Bacillus subtilis* counts were significantly increased in enriched *Artemia* compared to the unenriched group ($P < 0.05$). In fish fed groups, growth factors did not show any significant difference ($P > 0.05$). The maximum relative fecundity (28.65 ± 2.52 egg number g^{-1}), fry production (62.93 ± 4.6 individual per female), and fry survival ($70.97 \pm 1.56\%$) obtained in the third group were found to be significantly more than those in the first and the second groups. Moreover, intestinal bacterial count for *Bacillus* revealed that the higher concentration of bacteria was significantly related to the third group (6.24 ± 0.11 log CFU g^{-1}) ($P < 0.05$). Maximum protein and fat contents were observed in fish fed with *Bacillus*-enriched *Artemia*; however, no significant difference was found between control and unenriched *Artemia* groups ($P > 0.05$). The highest amount of ash was observed in fish fed with commercial food without any probiotic ($P < 0.05$). At the end of the feeding period, each of the three groups along with positive group (oxytetracycline 100 mg kg^{-1} of commercial food) was exposed to *A. hydrophila* (BCCM5/LMG3770) bacteria intraperitoneally. Based on the results, the lowest cumulative mortality was significantly found in group three ($68.75 \pm 3.6\%$) and positive group ($62.5 \pm 7.0\%$) compared to control and unenriched *Artemia* groups ($P < 0.05$). Hence, *B. subtilis* with a concentration of 1×10^5 CFU mL^{-1} during the period of *Artemia* culturing can improve the reproductive parameters, intestinal microflora, and resistance to pathogenic bacteria of *Poecilia latipinna*.

Keywords *Aeromonas hydrophila* · *Artemia* · *Bacillus subtilis* · *Poecilia latipinna* · Probiotics · Reproduction

Introduction

Ornamental fish is important for the development of aquaculture in developing countries. Reproduction and cultivation of the ornamental fish species have increased dramatically and have received attention in a number of studies. Considering that the economic value of the ornamental fish is high, to support their sustainability, it is important to study various aspects of ex situ cultivation of broodstock in order to avoid relying on animals in the wild. With over 2500 species, freshwater aquarium fish trade is the biggest section of fish industry. New methods for the cultivation and reproduction of ornamental fish have been investigated worldwide in the recent decades [1].

Health and nutrition of the ornamental fish are of paramount importance in ornamental fish trade. Probiotics are microorganisms enhancing fish health via microbial balance of the host gut [2]. Probiotics have no negative effects [3], using several mechanisms such as production of essential digestive

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enzymes and vitamins and increase access to minerals, and trace elements [4] play a key role in preventing and controlling pathogens, thereby promoting growth performance, increasing larval survival, enhancing immunity, and improving tolerance as confirmed by findings of many studies [3, 5–10]. The *Bacillus* group is one of the most common probiotics used in aquaculture, which have beneficial impacts on fish [11–13] and shrimp [14–16]. The role of probiotics in increasing growth in ornamental fish, including *Poecilia reticulata*, *Xiphophorus helleri* [11, 17], and *Cyprinus carpio* koi [18, 19], was investigated by adding *B. subtilis* to their diets. The use of appropriate live food in the culture of ornamental fish is nutritionally significant. In this regard, *Artemia* is important due to its size, high nutritional value, non-selective feeding, and its role as carriers in the transfer of various nutrients and bacteria to fish [20]. A number of studies [5, 21] reported that *Artemia* had the ability to bio-encapsulate (short-time enrichment) bacteria, but this kind of enrichment causes disturbance [22] in the overall nutrition or energy balance in live food. There is also a force-feeding in this type of enrichment in the form of "gorging" in aquaculture. Nonetheless, a long-term enrichment during *Artemia* culture can be more effective. The selection of probiotic bacteria in the long-term enrichment is important because if the probiotic bacteria show antagonistic relationship with algae, it will not deem to be suitable for nutrition. In order to confirm the hypothesis that the use of the long-term enrichment in growing *Artemia* can have a positive effect on target animals, the present study examined the influence of long-time enriched *Artemia* with *Bacillus subtilis* on the growth performance, reproduction, proximate composition, gastrointestinal microflora, and resistance to *Aeromonas hydrophila* of ornamental fish *Poecilia latipinna*.

Materials and Methods

Preparation of Probiotic and Algae

Bacillus subtilis (strain number: IBRC-M 10742) was obtained from the National Center for Genetic and Biological Diseases of Iran and was grown in tryptic soy broth (TSB) medium (Merck, Germany) at 37 °C and aerobic condition in a shaking incubator (160 rpm) [23]. Bacteria number in each bacterial suspension was determined based on colony unit (CFU) per milliliter [24]. To feed *Artemia franciscana*, the cyst of this *Artemia* was obtained from Iran *Artemia* Co., Kerman, Iran, and algae, *Dunaliella tertiolecta*, was obtained from *Artemia* and Aquaculture Institute, Urmia, Iran. According to the method proposed by Sorgeloos [25] Valene, culture medium was used to culture algae. The number of algae was counted using a homocytometric lam, and its number per milliliter was determined.

Artemia franciscana Supplementation with Probiotic

Artemia franciscana cysts were hatched through the method suggested by Sorgeloos [25]. Filtered and autoclaved sea water (FASW) with a salinity of 30 ppt, temperature of 28 °C, pH of 8–8.5, light intensity of 2000 lx, and DO of 6 mg L^{-1} was selected as standard conditions for *Artemia* culture. According to Coutteau et al. [26] technique, *Artemia* was fed with 30% algae and 70% wheat bran in growing period (15 days). Based on Niu et al. [27] method, the minimum concentration of *B. subtilis* having a positive effect on disease response was 1×10^5 CFU mL^{-1}, so during the growth period, *B. subtilis* with a density of 1×10^5 CFU mL^{-1} was daily added to *Artemia* culture tank. After 15 days of feeding *Artemia* with the mixture of algae at a density of 3×10^6 cell mL^{-1} and wheat bran, the grown *Artemia* were harvested for fish feeding and were stored at − 20 °C.

Experimental Design

Using a completely randomized design, three groups were included in this study. The first group was fed with commercial food without any probiotic (T1). The second group was fed with unenriched *Artemia* (T2), and the last group consumed enriched *Artemia* with *Bacillus subtilis* (T3). A total of 400 molly fish, *Poecilia latipinna*, with an average weight of 1.2 ± 0.2 g were obtained from Urmia Ornamental Fish Reproduction Center. The fish were fed at 4% body weight with the commercial food (Bio-Mar, France, with 46% crude protein and 6% crude lipid) or frozen *Artemia* (wet weight) for 60 days twice daily. On the 30th and 60th day, nine fish were randomly selected from each treatment and anesthetized with a solution of cloves powder (200 ppm) [28], and their total length and weight were measured by a ruler with a precision of 1 mm and by a digital scale with a precision of 0.001 g, respectively.

Bacterial Counts of *Artemia* and Fish Intestine

In order to evaluate colonization of bacteria in enriched *Artemia* and the intestinal tract of fish, the samples were harvested from each treatment. To eliminate the surface bacterial populations, first, the samples were placed in 0.1% benzalkonium chloride solution for 60 s. After homogenization of the *Artemia* and fish intestine, serial dilutions were prepared at a range of 10^{-1} to 10^{-3} with sterile saline solution (NaCl, 0.87 *w/v*, 1 mol). Under sterile conditions, 0.1 mL from each dilution was transferred to a plate containing tryptic soy agar (TSA) medium [29, 30]. They were incubated at 37 °C for 5 days, and finally, the number of bacteria in each sample was counted according to the log CFU g^{-1} of intestine or *Artemia* [31]. After counting colonies, *Bacillus subtilis* was identified with gram-positive staining and different

biochemical tests (e.g., positive catalase, positive citrate, negative indole, positive movement, positive nitrite reduction, positive spores, and variable oxidase) by Holt method [32].

Reproductive Performance

Breeders were identified based on specifications such as abdominal bulge, anal appearance, black and white spots on the abdomen, and specific behavior of the fish. They were transmitted to the maternity ward that had previously been filled with fresh water. During spawning period, born larvae pass from the maternity ward and go down to avoid being eaten by the breeders. After larvae being born, the mother was removed, and the length and weight of the fish and born larvae were recorded.

Several parameters including relative fecundity (total number of born larvae in the experimental period/weight of brood stocks), reproductive efficiency (after 3 days, the number of healthy larvae/all born larvae ×100), larval survival (after a 3-day period, total live larvae/total number of born larvae), and the number of healthy, dead, and deformed larvae were used to determine reproductive performance [33].

Aeromonas hydrophila Challenging Test

After 60 days of feeding with enriched *Artemia*, experimental infection with strain of *A. hydrophila* (BCCM5/LMG3770) was induced [28]. Bacteria *A. hydrophila* were obtained from Belgian Coordinated Collections of Microorganism, Belgium, and cultured for 24 h at 27 °C in BHI medium (Merck, Darmstadt, Germany). A total of 16 fish were selected from each group, and then, 0.1 mL of *A. hydrophila* with 1.1×10^7 CFU mL^{-1} (10 times of LD50 concentration) [34] was injected intraperitoneally to the samples in all three groups and the positive group (oxytetracycline 100 mg kg^{-1} of commercial food). After injection, fish were examined twice daily in aquariums of 30 L for 8 days in terms of mortality and symptoms. The typical signs of infection including irregular hemorrhages, ulcers, and sero-hemorrhagic fluids in abdominal were observed. Water quality parameters were similar to those recorded during the nutritional trial. Daily death was recorded, and bacteriological examination was carried out to confirm infection in dead fish.

Proximate Composition Analysis

According to AOAC [35], body composition of fish fed with different treatments was investigated. The moisture and dry matter content of carcasses were determined using weight difference. By the Kjeldahl method and nitrogen coefficient of 6.25, crude protein was calculated, and the fat content was determined by dissolving fat in ether. The amount of ash by burning fish samples in 600 °C for 2 h was determined.

Statistical Analysis

First, data normalization and homogeneity of variances were investigated using Shapiro-Wilk and Levene's tests. Then, the data were analyzed through one-way ANOVA, and the difference between means was assessed by Duncan's test. The minimum significance level for all tests was set to be $P < 0.05$. SPSS software (version 21) was employed for statistical analysis. Charts were also plotted in the Excel software (version 2013).

Results

Artemia Bacterial Count

The bacterial counts of enriched *Artemia* and unenriched *Artemia* are shown in Table 1. The *Bacillus subtilis* count in enriched *Artemia* was significantly higher than that of unenriched *Artemia* ($P < 0.05$). In addition, the enriched *Artemia* had significantly higher total bacterial count than unenriched *Artemia* ($P < 0.05$).

Growth Factors

The results of survival and length and weight indices of male and female fish are presented in Table 2. Based on statistical analysis, no significant differences were found between groups ($P > 0.05$). In both males and females, the survival rate in the control group was less than that of the other two groups, and the length gain of male samples was more than that of female ones.

Reproductive Factors

The results of reproductive factor in fish fed with different treatments are illustrated in Table 3. Maximal fry production (62.93 ± 4.6 individual per female), relative fecundity (28.65 ± 2.52 number per weight of female), and fry survival ($70.97 \pm 1.56\%$) were found to be significantly higher in fish fed with enriched *Artemia* with *B. subtilis* than other groups. About larval indices, no abnormal larvae were observed in group 3, and the dead larvae in this treatment were less than other treatments. However, there was no significant difference among the treatments ($P > 0.05$).

Intestinal Bacterial Count

The bacterial count of fish intestinal is shown in Table 4. In group 3, the *Bacillus* count (6.24 ± 0.11 log CFU g^{-1}) significantly was higher than that of others ($P < 0.05$). Additionally, the highest total bacterial count (7.00 ± 0.02 log CFU g^{-1}) was significantly observed in group 3 ($P < 0.05$).

Table 1 The log CFU/g *Artemia* of total microflora and *Bacillus* group in unenriched and enriched *Artemia* with *B. subtilis* in growing days (after 15 days)

Treatments	Colony type	
	Bacillus group	Total microflora
Unenriched *Artemia*	5.56 ± 0.050[b]	5.76 ± 0.042[b]
Bacillus-enriched *Artemia*	5.95 ± 0.061[a]	6.41 ± 0.098[a]

Mean ± SD values represent three replicates of each treatment. Different letters in each column have a significant difference ($P < 0.05$)

Proximate Compositions

The results of body composition of the fish fed different treatments are presented in Fig. 1. The maximum protein and fat contents were observed in group 3, but no significant difference was found between other treatments ($P > 0.05$). The highest amount of ash (14%) was significantly observed in the control group ($P < 0.05$). Fish fed with enriched *Artemia* with *B. subtilis* significantly showed the lowest moisture content compared to other groups ($P < 0.05$).

Fish Mortality in Challenge of *A. hydrophila*

After injection of *A. hydrophila*, the symptoms of disease and mortality were recorded for 8 days (Fig. 2). The lowest cumulative mortality (68.75 ± 3.6%) was recorded in group 3 compared to the control group with 81.25 ± 3.6% mortality ($P < 0.05$). No significant difference was observed in cumulative mortality between group 3 and the positive group (tetracycline) ($P > 0.05$).

Discussion

Special care must be taken to ensure health and nutrition of fishes including ornamental fish, as this type of fish, in suboptimal conditions or during intensification of aquaculture, tends to overcome opportunistic bacteria. Probiotics are biocompounds optimizing the colonization of intestine microbiota in fish and enhancing immunity, survival, and growth performance [3, 5–10]. The positive effects of probiotics on growth performance, reproductive factors, and immune response of fish and shellfish species are reported [2, 8, 36–43]. Probiotic bacteria produce components such as fatty acids, vitamins, and enzymes (protease, lipase, and amylase) through stimulating appetite and enhancing microbial metabolism, thereby improving fish feeding [8]. By increasing digestibility, enhancing absorption of ingested food, and having antibacterial properties, probiotics increase feeding efficiency in fish [17].

In this study, 60 days after feeding, no significant differences were observed in fish growth indices among groups. In line with our results, *Bacillus pumilus and Bacillus clausii* with 10^8 CFU g^{-1} of diet had not significant effect on growth indices of *Epinephelus coioides* after 60 days [44]. Contrariwise, the use of *Bacillus subtilis* efficiently increased the growth performance of live-bearing ornamental fishes [17]. Furthermore, employing enriched rotifer or *Artemia* with a combination of *Bacillus pumilus*, *B. subtilis*, and *B. licheniformis* efficiently improved body weight and growth rate in sea bass *Sparus auruta* [41].

The insignificant effect of probiotics on length and weight of the fish examined in this study can be attributed to the use of fish with a high weight. Nonetheless, employing *B. subtilis* had no negative influence on the fish growth indices. The impact of *B. subtilis* probiotic on guppies (*Poecilia reticulata* and *P. sphenops*) and swordtail species (*Xiphophorus helleri* and *X. maculatus*) had been investigated [17]. An increase in length and weight in treated fish was ascribed to an increased activity of amylase and protease enzymes. In clownfish, *Amphiprion ocellaris*, larval development was doubled by adding probiotic bacteria to water and live feed compared to control treatment [45].

Due to the non-selective nutritional behavior of *Artemia*, it has the capability of transmitting probiotics to fish [46–48]. In this study, the total microflora and *B. subtilis* counts in on-growing *Artemia* with probiotic were increased, indicating the ability to colonize probiotics by *Artemia*. Hence, by changing

Table 2 Average growth factors of fish, *Poecilia latipinna*, after 60 days feeding with different treatments in both male and female genus

Genus*	Treatments	Initial weight (g)	Weight gain (g)	Initial length (mm)	Length gain (mm)	Survival (%)
Female	T1	1.17 ± 0.03	0.98 ± 0.04	44.11 ± 0.7	3.47 ± 0.10	83.33 ± 5.55
	T2	1.16 ± 0.03	1.01 ± 0.05	44.72 ± 0.27	3.50 ± 0.32	96.29 ± 3.71
	T3	1.12 ± 0.02	1.06 ± 0.06	45.66 ± 1.06	3.07 ± 0.11	94.44 ± 3.21
Male	T1	1.36 ± 0.07	0.52 ± 0.04	44.40 ± 0.94	6.36 ± 0.83	83.33 ± 3.21
	T2	1.26 ± 0.05	0.48 ± 0.06	44.73 ± 1.28	6.33 ± 0.57	98.15 ± 1.85
	T3	1.15 ± 0.07	0.51 ± 0.02	43.94 ± 1.56	5.62 ± 0.64	90.73 ± 1.85

T1 indicates fish fed commercial feed; T2, fish fed unenriched *Artemia*; T3, fish fed enriched *Artemia* with *Bacillus subtilis*. Mean ± SE values represent three replicates of each treatment

*Data are analyzed in each gender separately

Table 3 Reproductive factors in fish, *Poecilia latipinna*, fed different treatments in 60 growing days

Treatment[1]	Reproductive factors						
	TFP (number of fry per fish)[2]	RF (number of fry per gram of fish)[3]	FS (%)[4]	AF (%)[5]	DF (%)[6]	FW (mg)	FL (mm)
T1	31.33 ± 2.19[b]	14.63 ± 1.09[b]	33.03 ± 2.01[b]	1.01 ± 1.01[a]	2.21 ± 1.13[a]	12.37 ± 0.57[a]	11.73 ± 0.10[a]
T2	37.00 ± 1.53[b]	17.06 ± 0.55[b]	27.87 ± 0.73[c]	0.85 ± 0.85[a]	1.73 ± 0.87[a]	12.62 ± 0.56[a]	11.75 ± 0.09[a]
T3	62.33 ± 4.10[a]	28.65 ± 2.52[a]	70.94 ± 1.56[a]	0.00 ± 0.00[a]	1.02 ± 0.51[a]	12.57 ± 0.59[a]	11.78 ± 0.12[a]

1) T1 indicates fish fed commercial feed; T2, fish fed unenriched *Artemia*; T3, fish fed enriched *Artemia* with *Bacillus subtilis*; TFP, total fry production; RF, relative fecundity; FS, fry survival; AF, abnormal fry; DF, dead fry; FW, fry weight; FL, fry length. Mean ± SE values represent three replicates of each treatment. Different letters in each column have a significant difference ($P < 0.05$)

2) Average per female

3) Total fry production throughout experimental period/mean weight of female in gram

4) Total live fry after time/total fry production × 100

5) Abnormal fry/total fry production × 100

6) Dead fry/total fry production × 100

the microbial flora of the intestinal tract, probiotic-enriched *Artemia* increases the growth and survival of target organisms [49]. The intestinal microflora of fish fed with *Bacillus*-enriched *Artemia* indicated that *Bacillus* was effectively colonized in the gastrointestinal tract after 60 days. The number of colonies of *Bacillus* was highest in the third group. It seems that there was competition between useful bacteria (*Bacillus*) and harmful ones, and *Bacillus* succeeded in replacing itself with other microorganisms by killing harmful bacteria by producing bacteriocins components [50]. Additionally, it was reported that using *Lactobacillus delbrueckii* (probiotic bacteria) led to bacterial colonization in the intestine of sea bass, *Dicentrarchus labrax*, and T cell production and intestine immune system were improved [51].

The nauplius of *Artemia urmiana* was found to have a high potential for enrichment with *B. subtilis* [52]. It was reported that if microorganisms were used for a long time, they could be colonized in the digestive system. For example, by using *Bacillus* sp. during 20 days, the microflora of the intestine was increased 500 times than normal condition [53].

Table 4 The log CFU/g intestine of total microflora and *Bacillus subtilis* in fish, *Poecilia latipinna*, fed enriched *Artemia* with *B. subtilis* compared to other groups after 60 days

Treatments	Colony type	
	Bacillus subtilis	Total microflora
T1	5.78 ± 0.06[b]	6.69 ± 0.10[b]
T2	5.33 ± 0.14[c]	6.22 ± 0.12[c]
T3	6.24 ± 0.11[a]	7.00 ± 0.02[a]

T1 indicates fish fed commercial feed; T2, fish fed unenriched *Artemia*; T3, fish fed enriched *Artemia* with *Bacillus subtilis*. Mean ± SE values represent three replicates of each treatment. Different letters in each column have a significant difference ($P < 0.05$)

Based on Table 2, reproductive factors in terms of high number of fry per fish, high number of fry per gram of fish, high fry survival, reduction of abnormal fry, and dead fry in fish fed with *Bacillus*-enriched *Artemia* were higher than those in other groups. B vitamins promoting maturation of intestinal secretory cells and synthesizing essential components in the host gut, probiotic bacteria enhance the efficiency of protein and fat conversions in diet of broodstock [11]. Likewise, Ghosh et al. [11] reported that including *B. subtilis* in ornamental fishes' diet led to the production of B vitamins, thereby increasing larval production, fecundity, and larval survival and declining the number of dead larvae and abnormal larvae. Furthermore, the gonadosomatic index, fingerling production, and relative fecundity were increased in the ornamental fish, *X. helleri*, fed with PrimaLac (commercial probiotic containing four species of lactic acid bacteria) [54]. Essential fatty acids, B vitamins, and proteins support oocyte development, high rate of vitellogenesis energy [55], and spawning activities [56] in fish fed with probiotic. Avella et al. [57] indicated that continuous administration of external probiotics affects host development; sexual maturity in *Danio rerio* was observed by 2 months feeding with *Lactobacillus rhamnosus*. Munro et al. [58] showed that bacterial flora had an essential role in larval survival, although there may be no relationship between the number of bacteria in the intestine and the survival rate of larvae. Gram-positive bacteria lead to an increased survival, uniformity of size, and high growth of larvae in fish [59]. The synthesis of vitamin B_{12} and B_1 by *B. subtilis* could be a reason for the reduced abnormal and dead larvae number in the group fed with *Bacillus*-enriched *Artemia*. Previous studies had reported the positive effect of thiamine in dropping the number of abnormal fry in *Poecilia sphenops*, *P. reticulata*, *Xiphophorus helleri*, *X. maculatus* [11], Atlantic salmon [60], and the Pacific salmon [61].

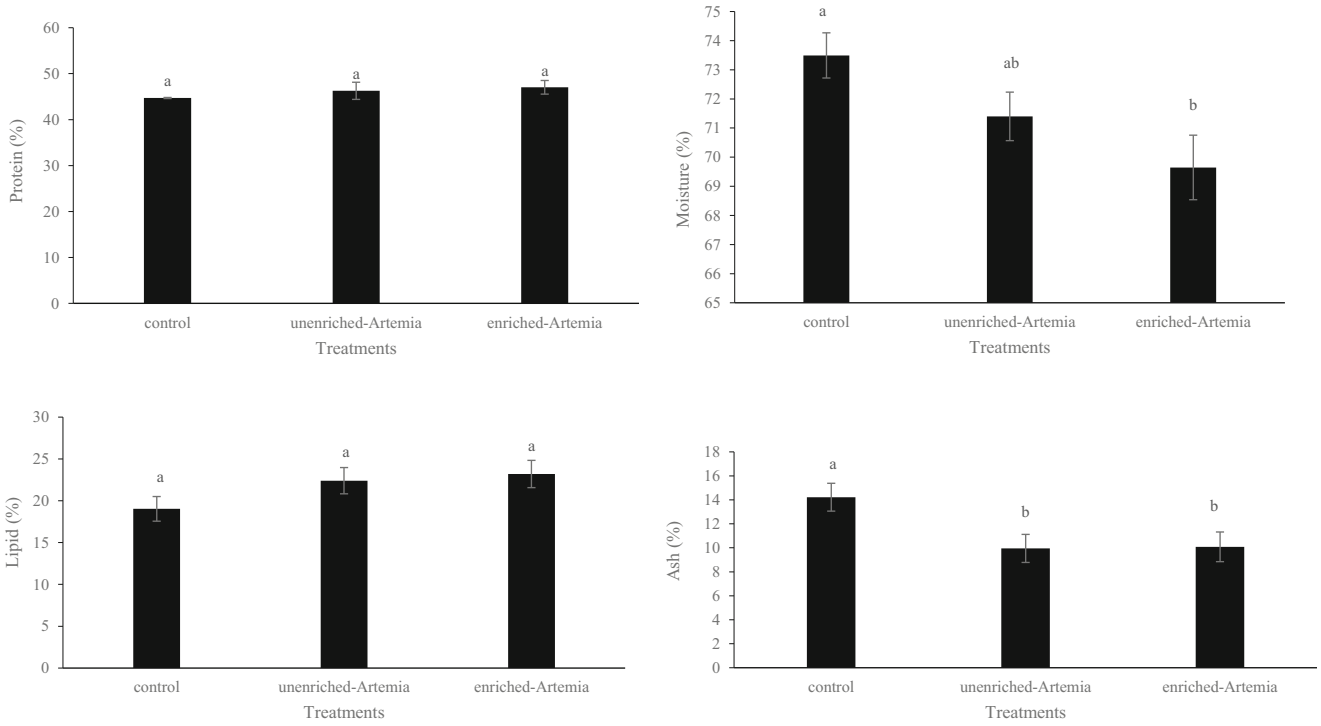

Fig. 1 Proximate composition of fish fed enriched *Artemia* with *Bacillus subtilis* after 60 days in comparison to other groups. Different letters have a significant difference ($P < 0.05$). Mean ± SE values represent three replicates of each treatment

The enhancement of the immune system's efficiency by probiotics had been reported in various aquatic species. Probiotic bacteria with positive effect on immunological components such as white blood cells (monocytes, lymphocytes, neutrophils, and macrophages) increase immune response [7, 43]. In this study, *Bacillus*-enriched *Artemia* reduced about 13% of cumulative mortality after 8 days of exposure to *A. hydrophila* compared to the control group (Fig. 2). By adding *B. subtilis* to *Artemia* culture media, the density of *Bacillus* group bacteria was increased in fish intestine (Table 3), confirming the presence of this bacterium in the intestinal mucosa. *Bacillus* group bacteria prevented the establishment of *A. hydrophila* in the fish body and in turn, reduced mortality when exposed to pathogens. It was stated that successful probiotics were those that have the ability to colonize in the intestinal mucus, to prevent the placement of pathogens, and to clean infected digestive tract [62]. It is indicated that probiotic bacteria secrete compounds that have the potential to

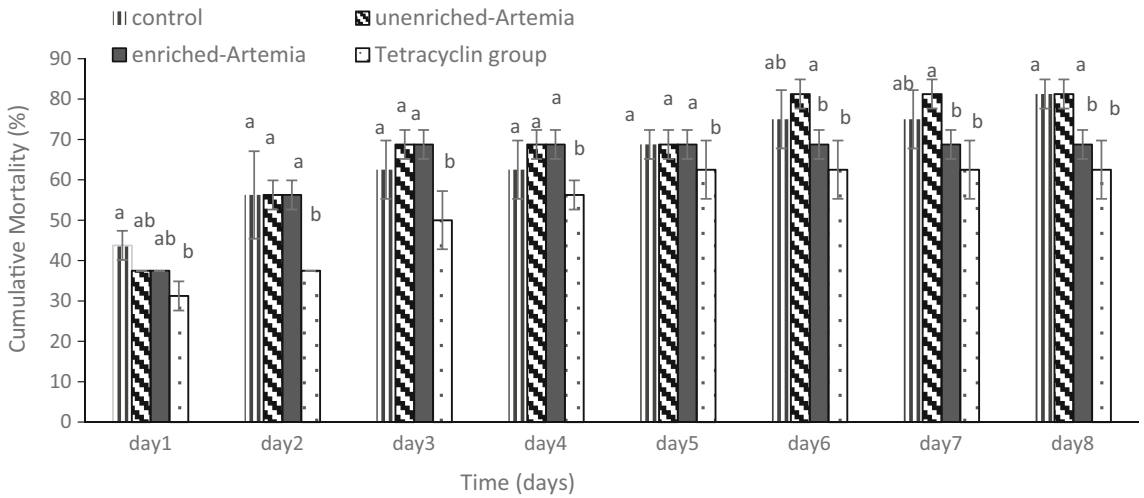

Fig. 2 Cumulative mortality (%) in fish fed enriched *Artemia* with *Bacillus subtilis* in growing days in comparison to other groups. Different letters in each day have a significant difference ($P < 0.05$). Mean ± SE values represent three replicates of each treatment

inhibit pathogenic bacteria [63]. Gerard et al. [64] recorded the production of a peptide antibiotic by *Bacillus* sp. and stated that this antibiotic prevents pathogens' growth.

According to Fig. 1, the highest moisture content was related to the control treatment. In line with findings of this study, using *Bacillus* in *Huso huso* diet, Jafaryan et al. [65] showed a significant difference in moisture content among different groups. In contrast, using *Saccharomyces cerevisiae* in Nile Tilapia, *Oreochromis niloticus*, Allam [66] did not find a significant difference in moisture content of fish fed with yeast and control treatments. Based on Fig. 1, the percentage of crude protein in *Bacillus*-enriched *Artemia* group increased, but the increase was not significant. Tukmechi and Bandboni [9] showed that *S. cerevisiae* had an effect on increasing the protein content of rainbow trout larvae. Jafaryan et al. [65] found a significant difference in crude protein content of the larvae of fish fed with *Bacillus* sp. Similarly, Allam [66] reported a significant increase in the amount of crude protein in Nile Tilapia, *Oreochromis niloticus*, fed with 10% yeast compared to the control group. He attributed protein increase in these fish to a rise in the total serum protein. It also was found that crude protein significantly increased in common carp when fed on yeast [67]. In the present study, the percentage of fat in all groups did not show any significant difference. In line with the results of our study, using 10% and 20% yeast in the Nile Tilapia diet, Allam [66] showed that there was no significant difference in the crude fat content compared to that of the control group.

In conclusion, the use of *Bacillus*-enriched *Artemia* in feeding *Poecilia latipinna* had a positive impact on gastrointestinal tract microflora, reproductive factors, and resistance to *A. hydrophila*. According to the results of this study, the concentration of 1×10^5 CFU mL^{-1} of *B. subtilis* can be used indirectly through *Artemia* culture to increase productivity and resistance to pathogens in fish.

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