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Modulating gut microbiota and digestive enzyme activities of *Artemia urmiana* by administration of different levels of *Bacillus subtilis* and *Bacillus licheniformis*

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Abstract The aim of this study was to evaluate the effects of *Bacillus subtilis* and *Bacillus licheniformis* on growth, gut microbiota, and digestive enzyme activities of *Artemia urmiana*. Three diets containing 10^2 (T_1), 10^4 (T_2), 10^6 (T_3) CFU of probiotics/g feed, and a control diet (C) without probiotic were used through a completely randomized design (treatments with triplicates). Twelve plastic tanks with the capacity of 60- l and density of 20 nauplii/ml were used and the trial continued for 15 days. Results showed that probiotics significantly increased the total length of *A. urmiana* ($P < 0.05$). Although the total aerobic gastrointestinal bacteria count showed no significant differences among the treatments, the total *Bacillus* count significantly increased in experiments ($P < 0.05$). The ratio of TCBS to total aerobic bacteria count was significantly lower in T_1 (0.31 ± 0.05), T2 (0.27 ± 0.15), and T_3 (0.25 ± 0.05) compared to the control (0.76 ± 0.34) ($P < 0.05$). The probiotics were able to increase the protease and amylase activities ($P < 0.05$). No significant effect on lipase activity. The study determined T_2 and T_3 as the most effective treatments for improving growth, bacterial flora, and digestive enzyme activities. As less probiotic needed in T_2, using 10^4 bacteria per g diet is recommended for rearing Artemia up to the maturity stage.

Keywords Probiotics · *Artemia urmiana* · Growth · Microbiota · Digestive enzyme activity

Introduction

One of the main ways of transmission pathogenic bacteria to aquaculture systems is the use of live food including artemia. Controlling microbial communities in modern farming systems is necessary for increasing the productivity and preventing the spread of disease.

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Generally, water chlorination (Sorgeloos et al. 1986) or several antibiotics are used in this regard (Marques et al. 2005). These have resulted in bacteria resistant to antibiotics in the aquatic environment (Verschuere 1997; Marques et al. 2005). Controlling microbial populations in aquatic hatcheries is of paramount importance using alternative techniques such as probiotics. Probiotics are live microbial food supplements that can cause beneficial effects on the host through the modification of the intestinal microbial balance (Fuller 1989). The microbial ecology of the gastrointestinal tract (GI) of a variety of freshwater and marine fish has been investigated over the last decade (Denev et al. 2009). It has been proven that bacterial populations residing in the intestine affect the establishment of pathogenic microorganisms in the digestive tract (Huber et al. 2004). However, the role of each microbe in the GI is still not well understood. Probiotics including Yeasts (*S. cerevisiae*) or bacteria (*Lactobacillus*, *Bacillus* etc.) have been used through water or the feed (Ringø and Birkbeck 1999). The genus of *Bacillus* spp is among the most popular probiotics that have been employed in aquaculture. This Gram-positive bacteria are the natural flora of Artemia rearing environments and are able to produce and secrete a number of extracellular enzymes (Moriarty 1998) including proteases (bacitracin and subtilin) (Maget-Dana and Peypoux 1994; Sanders et al. 2003). These bacteria can participate in the process of digestion, through enhancing the digestive systems efficiency and ultimately improve the host growth. In particular, *Bacillus subtilis* and *Bacillus licheniformis* (as BioPlus 2B® product) have been successfully applied as probiotics for rainbow trout (Raida et al. 2003; Bagheri et al. 2008, Merrifield et al. 2010), pig (Alexopoulos et al. 2004; Link and Kovac 2006), chiken (Rahimi and Khaksefifi 2006). It is the first time that this product is used in Artemia.

Avella et al. (2010) tested a mixture of *Bacillus* probiotic bacteria in the gilthead sea bream (*Sparus aurata*) larviculture focusing on their effects on survival, growth, and general welfare. The mixture was composed of three *Bacillus* strains, *B. subtilis, B. licheniformis,* and *B. pumilus*. The *Bacillus* mixture significantly increased growth in terms of standard length and body weight. Using *Bacillus* spp as probiotic led to an increased digestibility of protein, fat, and starch of the diets in common carp (Wang and Xu, 2006). It has been shown that *Bacillus* bacteria could increase protease, amylase, and lipase activity in *Penaeus vannamei* (Wang 2007).

Due to the importance of Artemia in aquaculture and its significant biological indices, it has been introduced as an appropriate model for laboratory studies (Soltanian et al. 2007). Urmia Lake the unique habitat of *Artemia urmiana* has been threatened over the last decade due to the successive droughts and pollutants entrance. As a result, the Artemia population in the Lake has been severely threatened over the last decade. One possible approach to protect *A. urmiana* is to expand its large-scale artificial production. The aim of this study was to evaluate the effects of different dietary inclusion of *Bacillus subtilis* and *Bacillus licheniformis* on gut microbiota (total aerobic count, *Bacillus* count and TCBS count (as a sign of *Vibrio* total count)), and digestive enzymes (protease, lipase, and amylase) activity of *Artemia urmiana*.

Materials and methods

The probiotic

One of the probiotics that have been approved by the European Commission is Bioplus 2B, (German products, Biochem Company). This product contains genetically superior strains

of bacteria *Bacillus subtilis* (CH201) and *B. licheniformis* (CH200). The commercial probiotic containing equal proportion (1:1) of active bacteria spores (3.2×10^9 CFU per g) (SCAN 2000) was used in this experiment.

Artemia experimental design

Three levels of probiotics including 10^2 (T1), 10^4 (T_2), 10^6 (T_3) CFU/g feed and a control (C) diet (without probiotics) were used through applying a completely randomized design (four treatments with triplicates). Twelve plastic tanks with the capacity of 60 l and density of 20 nauplii per ml were used and the trial lasted for 15 days.

Hatching and rearing of Artemia

For hatching the cyst of Artemia, 5 grams of cyst was used in each experimental unit. Physicochemical properties of hatching and rearing water, including temperature, dissolved oxygen, salinity, and pH were daily monitored according to standard methods (Table 1) (Agh et al. 2007).

Artemia feeding

Over the first 5 days of the experiment, nauplii were fed with backers yeast (Lavens and Sorgeloos 1996). From the second day, after hatching 1.25 mg of baker yeast per 1,000 nauplii in 400 ml saline water (35 g/l) with the temperature of 28°C, the solutions were passed through a 150 micron mesh and then distributed in rearing water. From the sixth day onwards, a diet containing chickpea flour (44.38%), soybean meal (44.38%), and white wheat flour (11.24%), which was provided by Behparvar Co. (Iran), was used to feed the Artemia. Feeding was performed three times a day with a four-hour interval. Chemical composition of the diet was investigated according to the standard method (Peterson et al. 1999). Dry matter content was (97.5 ± 0.77) also ash (5 ± 0.35), crude protein (55 ± 1.2), and crude fat (12 ± 0.93). Artemia feeding schedule has been shown in Table 2.

Probiotics in determined quantities were completely mixed with Artemia food. Artemia was fed with the probiotic containing diets from the first exogenous feeding day, up to the fifteenth day. From the fifteenth day until the twentieth, the probiotics were not added to the diets, and all treatments were fed with the control diet.

Growth monitoring

To evaluate the growth, the biometry of Artemia was undertaken on the first, fifth, tenth, and fifteenth day of the experiment using a 10-ml pipette. Sampling was conducted in a manner that five 10 ml samples of water from each tank were removed, and the total length of Artemia was measured using a micrometer.

Table 1 Physico-chemical parameters of Artemia hatching and rearing water (mean ± SD)

Agent	Temperature (°C)	Dissolved oxygen (mg/l)	Salinity (g/l)	pH
Hatching	29 ± 1	4 ± 1	35 ± 2	8.3 ± 0.5
Rearing	29 ± 1	6 ± 1	60 ± 2	8.3 ± 0.5

Table 2 Artemia feeding schedule over the experiment

Experimental days	1	2	3	4	5	6	7	8	9	10	11–14
Feed amount (g/l)	–	0.02	0.03	0.05	–	0.05	0.06	0.062	0.07	–	0.07
Yeast	–	0.02	0.03	0.05	–	0.037	0.03	0.0155	–	–	–
Dry food	–	–	–	–	–	0.0125	0.03	0.0465	0.07	–	0.07

Microbial analysis

Samplings were performed on days 1, 5, 10, 15, and 20 (five days after cessation of the probiotics). After the Artemia was rinsed with sterilized distilled water, washed with 70% alcohol, and rinsed again with sterilized distilled water to eliminate the bacteria sticking to body surface (Gatesoupe 1999). Whole Artemia was homogenized in order to enumerate the total aerobic bacterial count, total *Bacillus* count, and total TCBS count in the GI of Artemia (CFU/g Artemia) (Ziaei-nejad et al. 2006). The homogenized samples were then prepared through gradually adding 5 ml sterile saline water (35 g NaCl/l). Then, 10 times of serial dilution were prepared, and total aerobic bacteria, *Bacillus,* and TCBS counts were performed through using mediums Bacillus Cereus Agar, Marine agar 2216, and Thiosulfate citrate bile salt agar (TCBS), respectively (Rengpipat et al. 1998). Incubation performed 24 h at 30°C for Bacillus Cereus Agar, 24 and 48 h at 29°C for Marine agar and TCBS plates.

Enzymatic assays

Samplings were performed on days 1, 5, 10, and 15 to determine protease, lipase, and amylase activities. After washing, the samples with cold fresh water and rinsing, the samples stored within 15-ml falcon tubes and immediately transferred to freezer (−80°C) (Brito et al. 2001). For enzyme extraction, samples defrosted in laboratory conditions. Extracts prepared in physiological saline solution (0.9% NaCl) were homogenized through adding saline solution to achieve a total volume of 1.6 ml per sample. The homogenized solutions were centrifuged at 5,000×g for 5 min. Then, the supernatants were used for the enzymatic assays. Protease activity assay was conducted through using casein hydrolysis at pH 8 (Hidalgo et al. 1999). To determine the amylase activity, starch was used as the substrate. (Bernfeld 1951; Worthingtone 1991). The lipase activity was measured using olive oil emulsion substrate-Gum Arabic through titration the thawed samples at room temperature (Worthingtone 1991).

Statistical analysis

All percentage data were transformed using the arcsine method. After confirming the homogeneity of variance and normality of the data using Leaven and Kolmogorov–Smirnov tests, respectively (Zar 1999), one-way ANOVA was used to study the data. Duncan's test was applied in order to compare the significant differences among the treatments ($P < 0.05$). Student's t test was used to compare total aerobic bacteria and Bacillus counts and the ratio of TCBS to total aerobic bacteria count in the GI of Artemia between the fifteenth and twentieth days. Statistical analysis was performed using the SPSS software.

Results

Effect of using probiotics on growth of Artemia

The effects of using probiotics on Artemia length have been shown in Fig. 1. Results showed that in fifth, tenth and fifteenth days of the experiment, the total length of treated Artemia significantly increased compared to the control ($P < 0.05$). Survival rate (Artemia/ml) was not affected by probiotics, and no significant difference observed between T_1 (4.30 ± 0.67), T_2 (5.89 ± 1.61), T_3 (4.03 ± 0.53), and control (4.73 ± 0.58) ($P>0.05$).

Effect of using probiotics on the gut microbiota of Artemia

Effects on total aerobic bacteria count

Total number of aerobic bacteria in the digestive tract of Artemia nauplii at stage I and before mouth opening showed that the GI was sterile and free of bacteria. As it has been shown in Table 3, in the fifth, tenth and fifteenth days of the experiment, there were no significant differences in terms of total aerobic bacteria count among the treatments and the control. Also in day 20, 5 days after stopping the use of probiotics in the diet, no significant differences were observed among the treatments and the control.

Effects on Bacillus count

The effects of different dietary levels of probiotic on the *Bacillus* count in the GI of Artemia are presented in Fig. 2. Results indicated a significant increase in the number of *Bacillus* bacteria in treatments compared to the control in the first five days of the experiment ($P < 0.05$). In the tenth day, the number of *Bacillus* bacteria in T_1 and T_2 showed no significant differences compared to the control, but differences between T_3 and control was significant ($P < 0.05$). On the fifteenth day, the number of *Bacillus* bacteria in different treatments was significantly increased compared to the control and the highest value was related to T_3 ($P < 0.05$). However, the difference between T_1 and T_2 was not significant. In day 20, the number of probiotic bacteria in the treatments was not significantly differed.

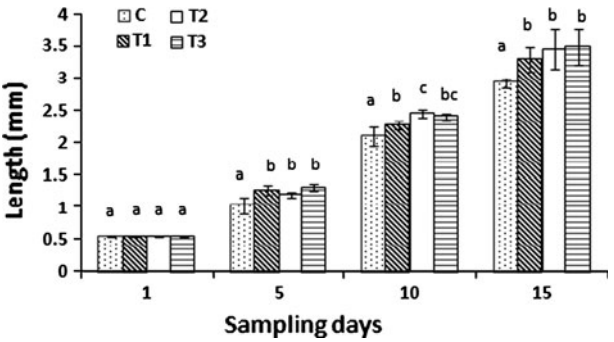

Fig. 1 Mean (±SD) of total length of *Artemia* in different treatments over the experiment ($r = 3$)

Table 3 Mean (±SD) of total number of aerobic bacteria ($\times 10^6$) in the digestive tract of Artemia over the experiment ($r = 3$)

	1	5	10	15	20
C	–	0.45 ± 0.12	3.20 ± 1.96	5.60 ± 2.01	6.08 ± 1.66
T_1	–	0.44 ± 0.24	5.06 ± 2.11	9.46 ± 2.20	7.99 ± 1.85
T_2	–	0.53 ± 0.30	7.80 ± 3.80	11.26 ± 3.23	9.38 ± 2.57
T_3	–	0.90 ± 0.97	7.00 ± 2.60	11.83 ± 1.50	9.35 ± 2.11

Effects on TCBS count

The effects of different dietary levels of probiotics on total TCBS count and the ratio of TCBS to total aerobic bacteria count in the fifteenth day are presented in Table 4. Results showed that different dietary levels of probiotics had no effect on the TCBS count in the GI of Artemia. However, the proportion of TCBS to total aerobic bacteria count was significantly decreased in the GI of different treatments compared to the control ($P < 0.05$).

Effects of stopping using probiotics on the total aerobic bacteria count

The results of stopping the use of probiotics in the diet on total aerobic bacteria and *Bacillus* count of the digestive tract of Artemia are presented in Table 5. There were no significant differences in terms of total aerobic bacteria count in the twentieth day compared to the fifteenth day in the control. However, total aerobic bacteria count was significantly decreased in the twentieth day compared to the fifteenth day in all experimental treatments ($P < 0.05$).

Effects of stopping using probiotics on the Bacillus count

There were no significant differences in terms of the number of *Bacillus* bacteria in the GI of control between the fifteenth and twentieth days, whereas the *Bacillus* bacteria count in all experimental treatments were significantly decreased in the twentieth day ($P < 0.05$).

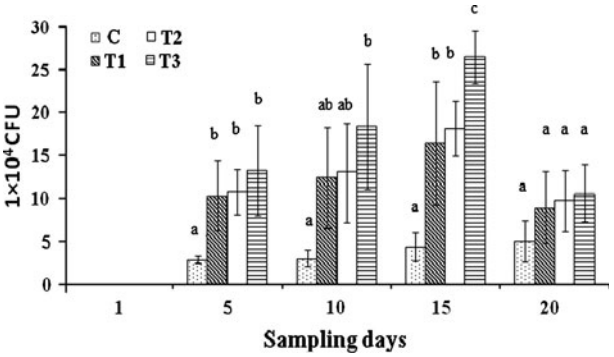

Fig. 2 Mean (±SD) of *Bacillus* bacteria count in different treatments over the experiment ($r = 3$)

Table 4 Mean (±SD) of total TCBS count and the ratio of TCBS count to total aerobic bacteria count in the fifteenth day ($r = 3$)

	Total TCBS count ($\times 10^4$)	Ratio (%) TCBS count to total aerobic bacteria count
C	4.00 ± 1.71^a	0.76 ± 0.34^b
T_1	2.93 ± 0.90^a	0.31 ± 0.05^a
T_2	2.77 ± 1.08^a	0.27 ± 0.15^a
T_3	2.93 ± 1.50^a	0.25 ± 0.05^a

Data presented in each column with non-common characters were significantly different ($P < 0.05$)

Table 5 Mean (±SD) of total aerobic bacteria count and *Bacillus* bacteria count in the fifteenth and twentieth days of the experiment ($r = 3$)

	Total aerobic bacteria count ($\times 10^6$)		*Bacillus* bacteria count ($\times 10^4$)	
	15	20	15	20
C	5.60 ± 2.01^a	6.08 ± 1.66^a	3.76 ± 1.74^a	4.34 ± 2.08^a
T_1	9.46 ± 2.20^a	7.99 ± 1.85^b	14.33 ± 4.93^a	7.77 ± 3.65^b
T_2	11.26 ± 3.23^a	9.38 ± 2.57^b	15.70 ± 2.82^a	8.44 ± 3.10^b
T_3	11.83 ± 1.50^a	9.35 ± 2.11^b	23.00 ± 2.60^a	9.21 ± 2.82^b

Data presented in each row with non-common characters were significantly different ($P < 0.05$)

Effect of using probiotics on the digestive enzyme activities of Artemia

Effects on protease activity

Results showed that there were no significant differences in terms of protease activity among the treatments and the control over the first five days of the experiment (Fig. 3). However, in the tenth day, protease activity was significantly higher in T_3 compared to the control ($P < 0.05$). Meanwhile, the protease activities were the same in all of the other treatments. On the fifteenth day, the protease activity in T_2 and T_3 was the same and was significantly higher than that of T_1 and the control ($P < 0.05$).

Effects on amylase activity

Results showed that there were no significant differences in terms of amylase activity among the treatments and the control over the first five days of the experiment (Fig. 4). However, in the tenth day, amylase activity was significantly higher in T_3 compared to the control ($P < 0.05$). Meanwhile, the amylase activities were the same in all other treatments. In the fifteenth day, the amylase activity in T_2 and T_3 was the same and was significantly higher than that of T_1 and the control ($P < 0.05$).

Effects on lipase activity

Although lipase activity were significantly increased ($P < 0.05$) in days 5, 10, and 15 as compared to day one; however, there were no significant differences in terms of lipase activity among the treatments in days 5, 10, and 15 (Fig. 5).

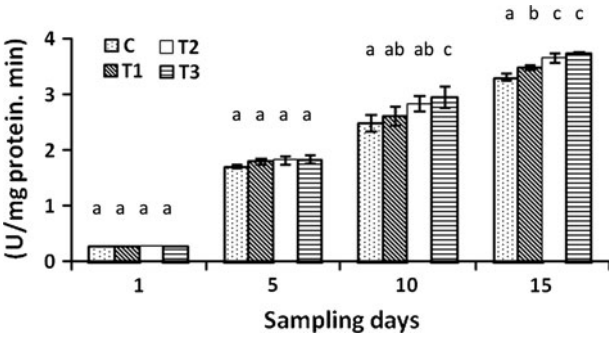

Fig. 3 Mean (±SD) of protease activity in different treatments over the experiment ($r = 3$)

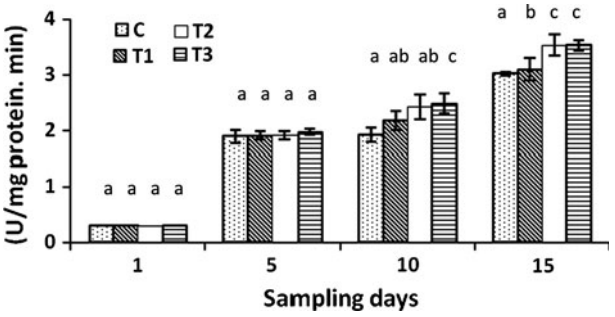

Fig. 4 Mean (±SD) of amylase activity in different treatments over the experiment ($r = 3$)

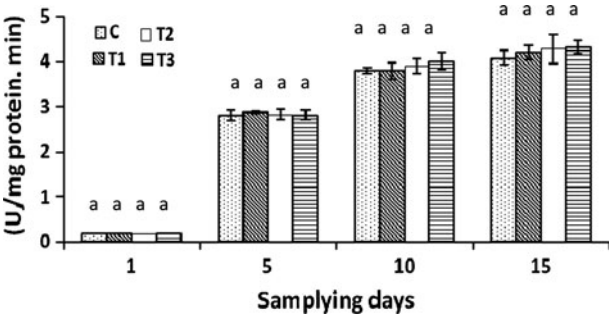

Fig. 5 Mean (±SD) of lipase activity in different treatments over the experiment ($r = 3$)

Discussion

Results of this study clearly showed that the employed probiotics (*B. subtilis* and *B. licheniformis)* could significantly improve the growth of Artemia.

The results of this study are in agreement with the results reported by other researchers that showed the positive effect of using probiotics on the growth of *Penaeus latisulcatus* (Van Hai et al. 2010) and *Gadus morhua* (Lauzon et al. 2010). In a study, nine different bacterial species were used to improve the nutritional value of dry food for Artemia. It was

found that the bacterial species, promoted the growth indices of *Artemia franciscana* (Verschuere 1997). Recent studies by Bagheri et al. (2008) and Merrifield et al. (2010) also demonstrated that the use of *B. subtilis* with *B. licheniformis* as a BioPlus 2B® product could improve the growth performance of rainbow trout fry. Probiotic strains in the GI can be used as a source of food supplements such as vitamins or essential amino acids (Balcázar et al. 2008; Skrodenytė-Arbaĉiauskienė 2007). Since Artemia is a non-selective filter feeder, the probiotic bacteria can be directly used as the main sources of protein and amino acids (Verschuere 1997). However, the number of studies that have separated the nutritional role or probiotic role of the bacteria is very limited (Ahmadnia motlagh et al. 2009; Verschuere 1997).

Effect of using probiotics on the gut microbiota of Artemia

This experiment was conducted to evaluate the effects of different levels of *B. subtilis* and *B. licheniformis* on gut microbiota and the digestive enzymes activities of Artemia over hatching to maturation. Results indicated that using probiotic bacteria could simply modify the gut microbiota in favor of the beneficial bacteria and suppress the potential opportunistic bacteria. In addition, probiotics had significantly improved the protease and amylase activities with no effects on lipase activity.

No bacteria were recorded in the gut at the first day of hatching (before starting exogenous feeding). This result is in agreement with other researcher's finding that showed GI of fish and crustaceans including Artemia are sterile and free of bacteria up to first feeding time (Ringø and Gatesoupe 1998). Bacteria are part of Artemia food, and gut microbiota could be a reflection of the bacterial population associated with the food items (Ringø and Birkbeck 1999).

The effect of different dietary levels of probiotic bacteria on the total number of aerobic bacteria in GI (Table 3) during the fifth, tenth, and fifteenth days of the experiment showed no significant increase in the experimental treatments compared to the control. The results of the current research are confirmed by other researcher's findings. Ziaei-nejad et al. (2006) showed that the administration of *Bacillus* bacteria had no effect on the total aerobic bacteria count in the digestive tract of *Fenneropenaeus indicus*. Similarly, the total aerobic bacteria count in the intestine of Persian sturgeon (*Acipenser persicus*) fry was not affected by *Bacillus* bacteria treatment (Jafarian et al. 2007). In contrast, the total number of aerobic bacteria in the digestive tract of *Sparus aurata* were significantly increased when lactic acid bacteria were applied (Suzer et al. 2008).

Due to the limitations of adhesion sites, there is a high competition between bacteria for adhering to the sites and establish a new microbiota in the digestive tract. Administration of probiotic bacteria before the first exogenous feeding facilitates the establishment of the new bacteria through adhering adhesion sites in the digestive tract and preventing the colony formation by other bacteria to some extent.

Using different levels of probiotics on *Bacillus* count (Fig. 1) showed that probiotic bacterial colonies were significantly higher in the treatments compared to the control. The results of the current research are in agreements with other researcher's findings (Ziaei-nejad et al. 2006; Suzer et al. 2008). This may be due to introducing the probiotics that significantly changed the ratio of *Bacillus* bacteria to total aerobic bacteria count in the intestine and the limitation of other bacteria (especially hazardous bacteria) by the probiotics (Ziaei-nejad et al. 2006). Bagheri et al. (2008) also showed that the number of intestinal *Bacillus* bacteria was significantly higher in the treatments compared to the

control when rainbow trout was treated by *B. subtilis* and *B. Licheniformis* (BioPlus 2B® product).

Intensive Artemia rearing, in most cases is associated with high mortality due to the development of opportunistic bacteria (Verschuere 1997). Results of the current study showed that although treatment of Artemia by probiotics had no effect on the number of TCBS bacteria; however, it significantly reduced the ratio of TCBS to total aerobic bacteria count in the experimental treatments compared to the control. This confirms the beneficial effects of used probiotics on the modulation of the bacterial communities in favor of establishing useful bacteria. The results of the current study are supported by other researcher's findings that showed the microbial community of the GI can be modulated by probiotics (O'Toole and Cooney, 2008). In another study, in which the commercial probiotic Biogen® (manufactured by ChinaWay Corporation) that consists of *B. licheniformis* and *B. subtilis* was used, it was found that probiotic could successfully prevent the establishment of opportunistic bacteria in the GI through nutrients and space competition, changing the microbial metabolism and antagonism with other bacteria (Haroun et al. 2006).

One of the main objectives in using probiotic bacteria is to temporarily or permanently modify the microbial community in digestive tract of the host (Marques et al. 2005). Adhesion to the gastrointestinal mucosa is a fundamental prerequisite for the establishment of a colony. Results of current study showed that, 5 days after excluding the use of probiotics in the diet, the total number of aerobic bacteria and *Bacillus* count in the GI of all treatments were significantly reduced. This is in contrast with other research findings, which implies the absence of significant difference in the total number of aerobic bacteria and *Bacillus* count in the digestive tract of *Fenneropenaeus indicus* after excluding the use of probiotics (Ziaei-nejad et al. 2006). Probably the physiochemical conditions in the current research especially the high salinity (60 g NaCl/l) largely affected the survival rate of the probiotics and reduced the *Bacillus* and total aerobic bacteria count within 5 days after excluding the use of probiotics. Meanwhile, it should be noticed that in the fifteenth day, the rearing water was completely exchanged to prevent transferring of probiotics into the environment. These may explain the substantial decreasing the *Bacillus* count in the GI between the twentieth and fifteenth days of the experiment. Similar results stated that *Lactobacillus rhamnosus* could not adhere strong enough to the intestinal mucosa and disappeared through the intestine while the administration of probiotics stopped (Panigrahi et al. 2005).

Effect of using probiotics on the digestive enzyme activities of Artemia

B. subtilis and *B. licheniformis* are capable of digesting proteins and carbohydrates. Results of the current research showed that probiotic bacteria could enhance the protease and amylase activities from the tenth day onward ($P < 0.05$); however, they had no effect on lipase activity. It seems that the used probiotic bacteria need at least 10 days for stimulating the digestive system for secreting the enzymes (protease and amylase). However, it have not been clarified whether the increase in the enzyme activity was due to the stimulation of digestive system or related to the bacteria activity in digestive tract. Ruminant animals could break down polysaccharides, oligosaccharides, and disaccharides by the bacteria exist in their digestive tract. However, monogastric animals could not fully digest these materials (Skrodenytė-Arbaĉiauskienė 2007). Possibly, probiotic bacteria could increase the utilization of carbohydrate exist in the diet by Artemia. The existence of the extracellular digestive enzymes produced by bacteria have been demonstrated in Chinese

shrimp (*P. chinensis*) (Wang and Xu 2006), *Rutilus rutilus* (Skrodenytė-Arbaĉiauskienė 2007), *Sparus aurata* (Suzer et al. 2008), Indian white shrimp (Ziaei-nejad et al. 2006), and *Penaeus vannamei* (Wang 2007). The digestive enzyme activities are affected by life stage, amount and the chemical composition of food, and the nutritional requirements of Artemia. The lack of significant differences in terms of lipase activity may be explained by the low fat content of the assimilated food items (two percent in yeast and 12 percent in the mixed diet).

Conclusions

T_2 and T_3 showed the highest effects on growth, GI microbiota, and digestive enzyme activities in *Artemia urmiana*. However, due to the lower using probiotics in T_2, it is suggested to use 10^4 bacteria per g of food for growing *A. urmiana* up to the maturity stage. As ceasing probiotics inclusion in the diet significantly reduced the *Bacillus* bacteria in the GI, it is recommended to keep the population of useful *Bacillus* bacteria in the digestive tract at an appropriate level to benefit from their positive effects. This issue should be further investigated in the future research.

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