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Original Article

Effect of probiotic *Pediococcus acidilactici* on growth, reproductive and bacterial count of marine rotifer *Brachionus plicatilis*

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Abstract: This study investigated the effects of different concentrations of probiotic *Pediococcus acidilactici* on the total number, specific growth rate, attached egg number and microbial count (lactic acid and total aerobic bacteria) in culture water and body of marine rotifer *Brachionus plicatilis*. The experiment was conducted as a completely randomized design in four groups: first group fed probiotic at 0.5×10^6 CFU ml^{-1} (P1), the second group fed probiotic at 1×10^6 CFU ml^{-1} (P2), the third group fed probiotic at 1.5×10^6 CFU ml^{-1} (P3), and the last group fed without probiotic (NP, control). Rotifers were cultured in standard conditions at an initial density of 30 ind.ml^{-1} using a 2.5×10^6 cell mL^{-1} of *Nanochloropsis oculta*. Based on the results, the maximum number (215±4.91 ind.ml^{-1}) of rotifers were significantly obtained after ten days in the third group in comparison to other treatments (P<0.05). In addition, the highest growth rate (0.46±0.023 ind.ml^{-1}) and shortest doubling time (1.5±0.14 days) were obtained in the treatment P3 but no significant difference was found between NP and other groups (P>0.05). Moreover, it was revealed that the total aerobic bacteria was significantly related to the 3rd group that found be 1.80×10^3 CFU $rotifer^{-1}$ and 34.0×10^4 CFU ml^{-1} in rotifer body and culture water, respectively (P<0.05). Nonetheless, lactic acid bacteria of rotifer body and culture water was not concentration-dependent and the highest number of lactic acid bacteria in rotifer body (50.58±6.08 CFU $rotifer^{-1}$) and rotifer culture water ($4.43\pm3.28\times10^3$ CFU ml^{-1}) was obtained in the treatments of P1 and P2, respectively. In conclusion, the present study showed that the bacteria *P. acidilactici* with *N. oculta* algae provide a higher growth rate and total aerobic bacteria in rotifer *B. plicatilis*.

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Introduction

In the early stages of aquatic larvae, the initial establishment of microflora is associated with their foods that introduced into the system as live food and water of the culture system (Makridis et al., 2000; Planas et al., 2004). Live foods, especially rotifer, are the main cause of transmission of the bacteria to the digestive tract of the marine larvae (Bergh et al., 1994; Makridis et al., 2000; Ringø and Birkbeck, 1999) and the transmission of pathogenic bacteria may result in high mortality rates (Grisez et al., 1997). Keys et al. (1935) suggested that bacteria may be a significant source of food for zooplankton. Besides, it is expressed that in aquaculture system with high stocking densities, bacteria act as a direct food source for rotifers and may also contribute to the nutrition of the target organisms (Nevejan et al., 2018).

In recent years, many efforts have been made to develop microbial control methods to reduce the use of chemicals and antibiotics and to promote the use of natural resources for sustainable aquaculture development (Cabello, 2006). Probiotics, defined as living organisms such as bacteria, yeast, and microalgae, when used at appropriate rates, have beneficial effects on host organisms and play a role in reducing mortality in aquatic organisms (Kesarcodi-Watson et al., 2008; Verschuere et al., 2000). In aquaculture, probiotic bacteria may be introduced directly into the culture water or through live foods, including rotifers to aquatic animals. Rotifers play an important role as live food for larvae, and their microbial community is important (Støttrup and

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McEvoy, 2008). Different kinds of food such as *Nanochloropsis, Isochrysis* and *Chlorella*; and *Saccharomyces* have been used in rotifers feeding. Moreover in rotifer cultures, *Vibrio*, *Pseudomonas* and *Acinetobacter* are common opportunistic bacteria which may be important additional food sources for rotifers (Verdonck et al., 1994). However, Gatesoupe et al. (1989) and Gatesoupe (1989) indicated that inoculation of probiotic bacteria (*Bacillus toyoi*) into disinfected rotifers increases the growth rate of Japanese flounder*, Paralichthys olivaceus* and turbot larvae, *Scophthalmus maximus*, respectively. Also, it is determined that adding *Lactobacillus plantarum* to rotifer culture water inhibit the growth of *Aeromonas salmonicida* and increases the population growth rate and the nutritional value of rotifers (Gatesoupe, 1991).

Most applicable probiotics in aquaculture belong to the genera *Lactobacillus*, *Carnobacterium*, *Vibrio alginolyticus*, *Bacillus*, *Pseudomonas* and *Pediococcus* (Verschuere et al., 2000). *Pediococcus acidilactici* is a gram-positive lactic acid bacteria that can grow in a wide range of pH, temperature, and osmotic pressure and can colonize the digestive tract of aquatic animals (Azimirad et al., 2016; Bhunia et al., 1988; Klaenhammer, 1993). This probiotic is a facultative anaerobic bacteria that has a lower sensitivity to oxygen and in the intestines has inhibitory effects on the growth of pathogens in fish due to the ability to produce bacterial and organic acids (acetic and lactic acid) (Vázquez et al., 2005). Moreover, *P. acidilactici* has the ability to overcome the normal microbiota of the digestive tract of endothermic animals and had shown promising results in animal and human experiments (Barros et al., 2001). This group of bacteria is not usually found in fish, but an improvement of growth, reproduction, and immunity in angelfish, *Pterophyllum scalare* were obtained by using of *P. acidilactici*-enriched *Artemia* (Azimirad et al., 2016). The growth and resistance to pathogenic bacteria in turbot (*Psetta maxima*), by using *P. acidilactici* in culture water and through enriched rotifer were studied, and the results showed the increase of transmission of this bacterium to fish by enriched-rotifer (Villamil et al., 2010). Also, the positive effect on the weight of pollack, *Pollachius pollachius,* was found using of *P. acidilactici* enriched-*Artemia* nauplii (Gatesoupe, 2002).

Based on resource overview, probiotic *P. acidilactici* may have the potential to convey a wide range of benefits but is less studied in rotifer culture, an important live food in the larval stages of fish. Therefore, in this study, the effect of this probiotic on growth factors of rotifer *B. plicatilis* was investigated. In addition, the number of bacterial colonies in the rotifer body and in culturing medium was investigated.

Materials and Methods

Preparation the organisms: The primary stock of algae *Nanochloropsis oculta,* as a food source, and rotifer *B. plicatilis* were obtained from shrimp research institute of Bushehr, Iran. The algae were cultured in standard methods as suggested by Nematzadeh et al. (2018) with Valne culture medium, harvested 4-7 days after the culture period, counted with Hemocytometer Lam and stored in the refrigerator for the feeding of rotifer. Commercial probiotic (Pedi-guard®) was prepared from Tak Gene Company (Tehran, Iran) contains 1×10^{13} CFU kg^{-1} of *P. acidilactici*.

Experimental design: The initial stock of rotifer *B. plicatilis* (210 μm average length) was up-scaled using algae *N. oculta* at a density of 1.5×10^{6} cell ml^{-1} in a 4 L glass vessels. For rotifer cultivation, saltwater obtained from Urmia Lake, filtered using 0.2 μm mesh, autoclaved at 121°C for 15 minutes and finally diluted with sterilized distilled water. Glass flasks (working volume: 200 ml) were filled with saline water of 30 ppt, and then rotifers were introduced with a density of 30 individual ml^{-1}. The flasks were maintained at temperature 24±1°C with submersible heaters and provided with gentle continuous aeration using a central pump and without air stone. Along with adding bacteria to the culture medium, rotifers were daily fed with 2.5×10^{6} cell mL^{-1} of algae *N. oculta*. Probiotic *P. acidilactici* with 24 h intervals was added to each flask with three replicates as follows: P1: 0.5×10^{6} CFU mL^{-1}, P2: 1×10^{6} CFU mL^{-1} and P3: 1.5×10^{6} CFU mL^{-1} and P4 (NP): (None-probiotic, NP,

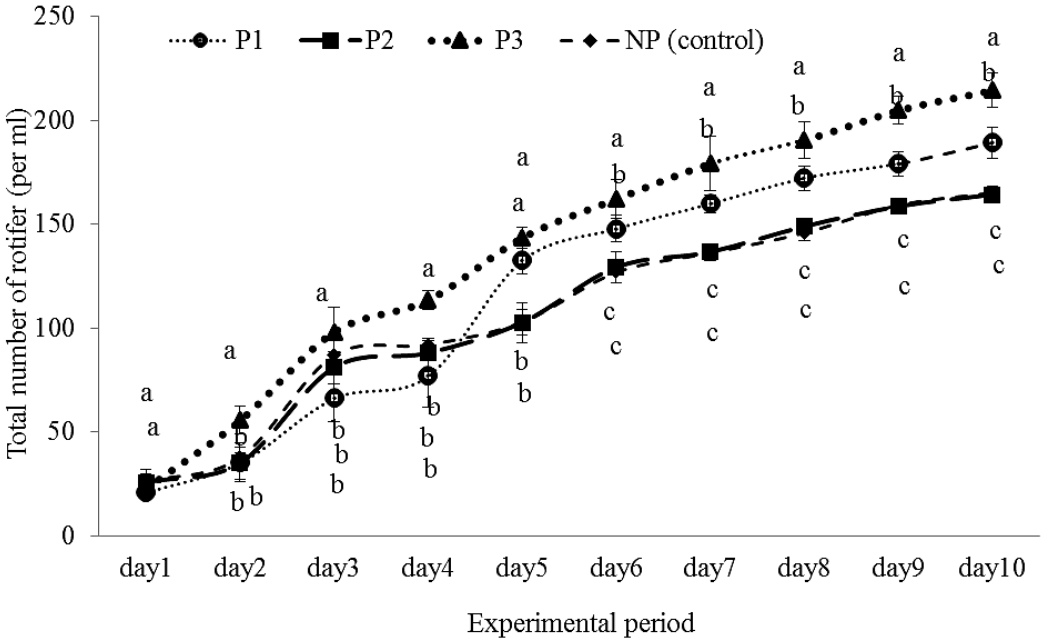

Figure 1. The total number (Mean±SE) of rotifer fed with 3 concentrations (P1: 0.5×10^6 CFU ml^{-1}, P2: 1×10^6 CFU ml^{-1} and P3: 1.5×10^6 CFU ml^{-1}) of *Pediococcus acidilactici* compared to the NP, control at 10 days.

control).

To evaluate the effect of probiotic on rotifer population, a total number of rotifers and attached eggs were daily counted via 3 replicates of each treatment for 10 days. Lugols' solution was used to fixation the rotifers (Nematzadeh et al., 2018). Finally, using the following equations, the specific growth rate (SGR) and the doubling time (DT) were calculated (Krebs, 1995; Planas and Estevez, 1989).

$$SGR = \frac{\text{Ln Nt} - \text{LnN0}}{t}$$

$$DT = \frac{Ln\ 2}{SGR} = \frac{0.693}{SGR}$$

Where N_0 = initial population density and N_t = density of population after time t (days).

Microbiological assays: 24 h after the feeding period, rotifer specimens were sieved and washed with autoclaved saltwater at a salinity of 30 ppt and then were homogenized under sterile conditions into falcon tube with sterile saline solution (%87 w/w, 1m). The homogenate at two serial dilutions of 10^{-1}-10^{-2} was spread onto MRS agar (Merck, Germany) and TSA (Merck, Germany) media via 3 replicates for lactic acid bacteria and total viable heterotrophic aerobic bacteria cultivation, respectively. Plates were placed at 28 °C for 24-48 h and colony forming units (CFU) were counted as CFU $rotifer^{-1}$ (Shiri Harzevili et al., 1998). In addition, those mentioned bacteria were investigated in culture water of rotifer to obtain the viability of those in water.

Statistical analysis: After survey of the normalization of data by Shapiro-Wilk test, the homogeneity of variances was investigated by Levene's test. One-way ANOVA was used to investigate the effects of different concentrations of probiotic and Duncan's test was used for multiple comparisons of means. The minimum significance level of the test was considered as $P<0.05$. SPSS software version 21 was used to check the data and Excel software was used to draw charts.

Results

Growth and reproductive factors: During the 10-day experiment, the addition of 3 concentrations of bacteria *P. acidilactici* along with algae had a significant effect on the population density, specific growth rate and doubling time of rotifers (Fig. 1 and Table 1; $P<0.05$). The highest population density (N_{max}) (215±4.91 ind.ml^{-1}) was observed in the treatment P3 that was significantly more than the other groups; however, there was no significant difference in maximum density between NP and P2 treatments. ($P>0.05$). Among the examined treatments, the

Table 1. Specific growth rate (SGR), N max and doubling time (DT) (Mean±SE) in rotifer fed with 3 concentrations of *Pediococcus acidilactici* compared to the NP (control) at 10 days.

Treatment	CFU mL^{-1} of probiotic	N_{max} (ind mL^{-1})	DT (days)	SGR (day^{-1})
NP	0	165±2.08^{c}	2.04±0.55^{b}	0.340 ± 0.044^{b}
P1	0.5×10^{6}	189±7.51^{b}	1.78 ±0.16^{ab}	0.389 ± 0.020^{ab}
P2	1×10^{6}	164±4.16^{c}	2.13 ± 0.37^{b}	0.326 ± 0.035^{b}
P3	1.5×10^{6}	215±4.91 ^{a}	1.50 ± 0.14^{a}	0.461 ± 0.023^{a}

Table 2. The total number (Mean±SE) of attached eggs in rotifer fed with 3 concentrations of *Pediococcus acidilactici* at 10 days.

Experimental groups	Experimental days									
	1	2	3	4	5	6	7	8	9	10
NP	19.0±1.00^{a}	11.3±0.33^{b}	19.0±3.46^{a}	7.3±0.33^{b}	13.0±0.58^{a}	13.0±1.53^{b}	9.3±1.20^{a}	15.3±3.93^{a}	11.3±2.33^{a}	12.0±2.08^{a}
P1	18.7±1.76^{a}	16.0±2.08^{a}	16.0±1.00^{a}	18.0±1.15^{a}	18.7±7.42^{a}	17.3±3.28^{ab}	9.3±1.20^{a}	12.7±2.40^{a}	12.0±1.15^{a}	10.3±0.88^{a}
P2	17.3±1.20^{a}	11.7±0.88^{b}	18.3±2.03^{a}	8.0±2.08^{b}	13.0±1.53^{a}	13.7±0.88^{b}	11.0±2.08^{a}	10.3±0.88^{a}	11.0±1.53^{a}	10.3±1.67^{a}
P3	18.7±1.20^{a}	17.0±1.00^{a}	19.3±2.85^{a}	9.3±0.88^{b}	16.0±2.31^{a}	21.7±2.19^{a}	14.3±1.76^{a}	11.0±4.58^{a}	12.3±3.58^{a}	15.0±2.65^{a}

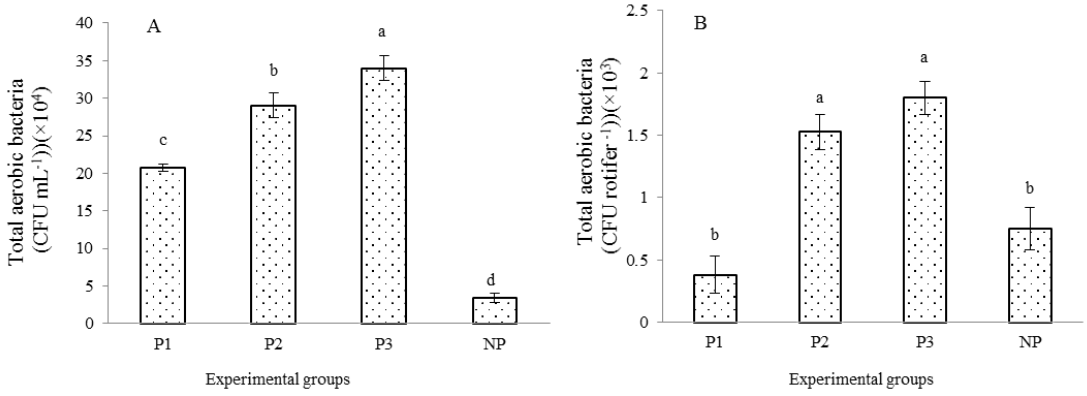

Figure 2. The CFU (mean±SE) of total microflora in culture water (A) and rotifer (B) fed with 3 concentrations of *Pediococcus acidilactici* at 10 days. NP, control, P1: 0.5×10^{6} CFU ml^{-1}, P2: 1×10^{6} CFU ml^{-1} and P3: 1.5×10^{6} CFU ml^{-1} of *P. acidilactici*.

highest growth rate (0.46±0.023 ind.ml^{-1}) and shortest doubling time (1.5±0.14 days) were related to rotifers fed with treatment P3. Nevertheless, there was no significant difference between the growth rates of NP and treatments P1 and P2 (P>0.05). Based on Table 2, the total number of attached eggs in rotifers fed with 3 concentrations of *P. acidilactici* fluctuated from initial day until 10th day; however on the second and fourth day the treatment P1 and on the 6th day of treatment P3 significantly showed the highest values of attached eggs (P<0.05).

Microbiological Analysis: The total number of aerobic bacteria in culture water and rotifers fed with three concentrations of the probiotic *P. acidilactici* are shown in Figure 2. As shown with increasing the concentrations of probiotic in treatments, the total aerobic bacteria of rotifer body increased and the maximum colony count of 1.80×10^{3} CFU $rotifer^{-1}$ was significantly observed in treatment P3 (P<0.05). Also, the total aerobic bacteria in culture water was significantly concentration-dependent and the highest number (34×10^{4} CFU ml^{-1} of culture water) of those was observed in treatment P3. In the group without probiotic was found the lowest total number of bacteria colony in rotifer culture water.

The total lactic acid bacteria in different treatment of culture water and rotifers are shown in Figure 3. Contrariwise, the total number of lactic acid bacteria in both of culture water and rotifers was not significantly concentration-dependent. In rotifers, the higher amount of lactic acid bacteria was significantly observed in treatment P1 (P<0.05), and with increasing the concentration of probiotic, the colony count of bacteria was similar to NP treatment. Also, the maximum number (44.33±3.28×10^{2} CFU mL^{-1} of culture water) of lactic acid bacteria in culture water

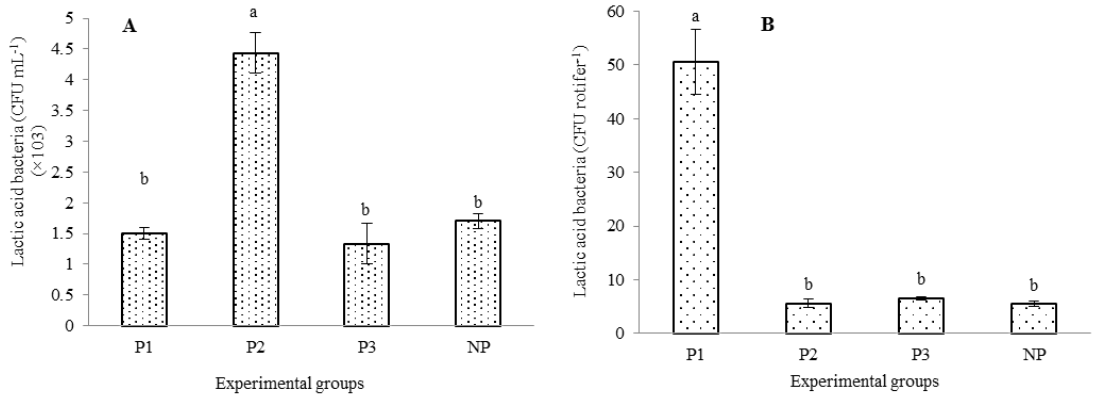

Figure 3. The CFU (mean±SE) of lactic acid bacteria in culture water (A) and rotifer (B) fed with 3 concentrations of *Pediococcus acidilactici* at 10 days. NP, control, P1: 0.5×10^6 CFU ml^{-1}, P2: 1×10^6 CFU ml^{-1} and P3: 1.5×10^6 CFU ml^{-1} of *P. acidilactici*.

was observed in treatment P2. There were no significant differences in the number of lactic acid bacteria between treatment NP and treatments of P1 and P3 ($P>0.05$).

Discussions

In the present study, lactic acid bacteria, *Pedicoccus acidilactici* had a beneficial effect in term of population growth and microbial composition of rotifer *B. plicatilis*. The total population of rotifers as well as the specific growth rate at 1.5×10^6 CFU ml^{-1} of probiotic showed the maximum amount during 10 days of the culture period compared to other treatments. In finfish and shellfish larvae culture, adding the benefit bacteria not only improve the health of live foods but also can be transmitted to fish larvae (Zambonino and Cahu, 2010). These positive effects of probiotics on live foods (Gatesoupe, 2002) and target organisms (Gatesoupe, 2002; Gatesoupe and Lesel, 1998) have been indicated. Shiri Harzevili et al. (1998) has been reported that lactic acid bacteria had no effect on growth rate of rotifer *B. plicatilis,* but modified the micro-flora of them. Also, survival of Turbot larvae, *Scophthalmus maximus* fed with lactic acid bacteria increased when exposed to pathogenic *Vibrio sp.* (Gatesoupe, 1994). Nonetheless in the study of Planas et al. (2004), by increasing the concentration of *P. acidilactici* in rotifer culture resulted in an increase in growth factors in comparison to the non-probiotic treatment, and the optimum concentration of probiotic was reported at a rate of 1×10^9 CFU ml^{-1}. Similarly, in the current study, the SGR, doubling time, and attached egg of rotifer population increased but this was not concentration-dependent. First, at low concentration, positive effects of bacteria on population growth and SGR were observed, but with increasing the probiotic concentration, those factors were first reduced and then increased at 1.5×10^6 CFU ml^{-1} of *P. acidilactici*. These trends may be due to two major factors, including (1) bacteria as a food source and (2) as a microbial component of these animals is providing essential nutrients in the aquatic environment (Nevejan et al., 2018). With using the high levels of bacteria, they can be used not only as a microbial component but also it acts as food sources. Since rotifers can ingest bacteria forming an aggregate (Abell and Bowman, 2005), the increase of population growth rate and SGR in a high concentration of probiotic can be related to providing of carbon, protein, B-complex vitamins and polyunsaturated fatty acids from direct consumption of bacteria. Planas and Estevez (1989) indicated the correlation between reproductive factors and quality of food ingested, so with that, the former is shorter when the food quality is better.

The selection of probiotic bacteria in long-term enrichment of live food is very important because probiotic bacteria can be unsuitable for aquatic nutrition with an antagonistic activity with algae (Planas et al., 2004). In this study, lactic acid bacteria only increased at low concentration of *P. acidilactici* and the higher concentration of probiotic had no increased effect on lactic acid bacteria colony in comparison to NP treatment. This no incremental

effect could be due to the antagonistic of algae with probiotic when fed to rotifer (Shiri Harzevili et al., 1998). However, based on the results, total number of aerobic bacteria colonies in the body of rotifers showed an increasing trend with increasing probiotic concentration, and the maximum number of total microflora (1.8×10^3 CFU per rotifer) was about 2.4 times higher than total microflora of NP treatment. The methods and foods used for cultivation can effect on the microflora of rotifers (Skjermo and Vadstein, 1993). In this study, the positive trend of the probiotic concentration on the composition of the rotifers and the culture water, indicate a non-selective uptake of bacteria from the culture water by rotifer (Skjermo and Vadstein, 1999). On the other hand, low density of lactic acid bacteria in treatment with high amount of probiotic indicates that this bacteria in the environment by the production of compounds such as water-soluble vitamins (Phillips, 1984) have been responsible for the growth of other bacteria in rotifer culture water, which was confirmed by high density of total microflora. The microbiological assay only measures the living cells and does not measure dead and digested cells. Planas et al. (2004) used a combination of three probiotic *Pediococcus acidilactici*, *Lactococcus casei* and *Lactobacillus lactis* in the culture water of rotifer *B. plicatilis*, and found that at the end of the trial the total aerobic bacteria of rotifers was approximately 5×10^3 CFU rotifer^{-1}. The number of probiotic cells that incorporate to live food are depending on the probiotic type, exposure time, and the state (dead or live) of the living organism (Gomes et al., 1998). If the level of bacterial load in live foods is too high, it can reach a level that negatively affects the health of the host. In the current study, the loading of the probiotic *P. acidilactici* at higher concentration without positive effect on colony count of lactic acid bacteria increased the population growth rate of rotifer as well as the total aerobic bacteria. In otherwise, Olsen et al. (1999) found that overloading bacteria through *Artemia* is caused poor growth of halibut larvae *Hippoglossus hippoglossus* during the feeding period with live prey. On the other hand, Gatesoupe (2008) stated that a microorganism, with long-term exposure in the environment, can multiply in the alimentary canal. For example, when *Bacillus* sp. was added to water for 20 days, it increased the intestinal bacterial flora of treated microorganism in comparison to the non-probiotic group. Also, Ahmadifard et al. (2018) indicated that long-term enrichment of *Artemia* with 1×10^5 CFU ml^{-1} of *B. subtillis* increased the total microflora from 5.76 to 6.41 log CFU in *Bacillus*-enriched *Artemia*. In the present study, in culture water of rotifer, the maximum number of lactic acid bacteria was obtained in 1×10^6 CFU ml^{-1} of *P. acidilactici*. However, the total aerobic bacteria increased with positive correlation with increasing the probiotic concentration. Bacteria *P. acidilactici* is a terrestrial bacterium, therefore its survival rate is important in saline water. This study showed that by increasing the concentration of this bacterium, the total aerobic bacteria concentration in the water environment increased, which indicates the positive of the survival rate of this bacterium within 10 days of the experiment. In conclusion, the present study showed that the bacteria *P. acidilactici* along with algae *N. oculta* through direct consumption or providing essential material could result in higher population growth rate and higher total aerobic bacteria in rotifer *B. plicatilis.*

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چکیده فارسی

تاثیر پروبیوتیک *Pediococcus acidilactici* بر رشد، تولیدمثل و تعداد باکتری‌های روتیفر آب شور *Brachionus plicatilis*

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چکیده:

در تحقیق حاضر تاثیر غلظت‌های مختلف باکتری پروبیوتیکی *Pediococcus acidilactici* بر تعداد کل، نرخ رشد ویژه، تخم‌های متصل و تعداد کلنی باکتریای در محیط کشت و بدن روتیفر *Brachionus plicatilis* مورد بررسی قرار گرفت. آزمایش حاضر در چهار گروه شامل (۱) گروه تغذیه شده با غلظت $0/5\times10^{6}$ CFU ml^{-1} از باکتری، (۲) گروه تغذیه شده با 1×10^{6} CFU ml^{-1} از باکتری، (۳) گروه تغذیه شده با غلظت CFU ml^{-1} $1/5\times10^{6}$ از باکتری و (۴) گروه شاهد (گروه بدون پروبیوتیک) انجام شد. روتیفرها در شرایط استاندارد و در ظروف پلاستیکی ۵۰۰ میلی‌لیتری با جلبک *Nanochloropsis oculta* با تراکم $2/5\times10^{6}$ cell ml^{-1} کشت شدند. تراکم اولیه روتیفرها در تیمارهای مختلف ۳۰ عدد در میلی‌لیتر بود. براساس نتایج حداکثر تراکم روتیفر (۴/۵±۲۱۵ عدد در میلی‌لیتر) بعد از ۱۰ روز در تیمار سوم به‌دست آمد که به‌طور معنی‌داری بیشتر از سایر تیمارها بود ($P<0/05$). همچنین بالاترین نرخ رشد ویژه و کمترین زمان دو برابر شدن جمعیت در تیمار سوم به‌دست آمد؛ ولی بین تیمار شاهد با سایر تیمارها تفاوت معنی‌داری یافت نشد. علاوه بر آن تعداد کل باکتری‌های هوازی در روتیفر ($1/81\times10^{3}$ CFU rotifer^{-1}) و محیط کشت آن ($34/0\times10^{4}$ CFU ml^{-1}) در تیمار سوم باکتری یافت شد. با این حال تعداد باکتری‌های اسید لاکتیکی در محیط کشت و بدن روتیفر وابسته به غلظت باکتری پروبیوتیکی مورد استفاده در تیمارها نبود و بالاترین میزان باکتری‌های اسید لاکتیکی در روتیفر (۶/۰۸±۵۰/۵۸ CFU rotifer^{-1}) و محیط کشت ($3/28\times10^{3}$±۴/۴۳ CFU ml^{-1}) به‌ترتیب در تیمار اول و دوم به‌دست آمد. مطالعه حاضر نشان داد که باکتری *P. acidilactici* به‌همراه جلبک *N. oculta* رشد و تعداد باکتری‌های هوازی بیشتری را در روتیفر *B. plicatilis* ایجاد می‌نماید.

کلمات کلیدی: کلونی باکتری، پروبیوتیک، روتیفر، نرخ رشد ویژه.