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Original Article

The study of enrichment capability of adult *Artemia franciscana* with singular or combined administration of *Pediococcus acidilactici* and fructooligosaccharide

Mahmood Azimirad*[1], Saeid Meshkini[2], Nasrollah Ahmadifard[1], Seyed hossein Hoseinifar[3]

[1]Fisheries Department, Faculty of Natural Resources, Urmia University, Iran.
[2]Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Iran.
[3]Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

Abstract: The present study investigates the possibility of enriching adult *Artemia franciscana* with singular or combined administration of *Pediococcus acidilactici* and fructooligosaccharide (FOS). The experiment was conducted in a completely randomized design with four treatments, including synbiotic, *P. acidilactici* +FOS (T1), probiotic, *P. acidilactici* (T2), prebiotic, FOS (T3) and control (T4). To evaluate the enrichment of adult *Artemia* with each treatment, sampling was performed at 2, 4 and 6 hrs post enrichment. The bacterial counts was measured using the microbial culture and expressed as log CFU per g *Artemia*. A pre-experiment has been designed and probiotic was used in three levels (10^7, 1^{08} and 10^9 CFU per litter of suspension) and prebiotic was used in three levels of 1, 2 and 5 g per litter of suspension. Based on pre experiment results, 10^8 CFU per litter of probiotic and 5 g per litter of prebiotic was selected. The results of this experiment showed that over time, consumed bacteria increased by adult *Artemia* and there was a significant difference between sampling in terms of ingested bacteria. The highest bacterial count (6.78±0.03 log CFU g^{-1}) was observed 6 hrs after the start of enrichment. Based on microbial culture, the number of bacteria *P. acidilactici* in T1 and T2 was significantly higher than those in T4 (control) and T3 (prebiotic). There was no significance difference between T2 (probiotic) and T1 (synbiotic). In conclusion, the results of this study showed that adult *Artemia* in a short time (about 4 hrs) can retain a large amount of probiotic bacteria.

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Introduction

During the past decade, the use of probiotics in aquaculture is become prevalent and can overcome many of the problems associated with bacterial diseases. The use of probiotics as a food supplement for farmed animals goes back to the 1970s (Fuller, 1989). Various types of microalgae (*Tetraselmis*), yeasts (*Phaffia* and *Saccharomyces*), Gram-positive bacteria (*Bacillus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Streptococcus* and *Weissella*) and Gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorhodobacterium*, *Pseudomonas* and *Vibrio*) have been studied as probiotics (Gatesoupe et al., 2010). The doubts in the use of probiotics such as the non-guaranteed viability of the probiotics in the gastrointestinal tract, necessity of competition autochthonous microbiota, the colonization ability and the long-term sustainability of the colony, caused the researchers to suggest the idea of prebiotic (Gibson, 2004; Mahious and Ollevier, 2005).

The prebiotics increase numbers and dominance of beneficial bacteria due to selectively fermentation (Roberfroid, 2007). Researches in this field have shown that non-digestible oligosaccharides such as inulin and oligofructose are the most important materials that have prebiotic function (Flickinger et al., 2003). Because of the inability of probiotic species to form stable masses and maintain dominance in the aquatic microbiota, simultaneous

* Corresponding author: Mahmood Azimirad
E-mail address: mahmoodazimirad@gmail.com

use of probiotics species with appropriate prebiotics (synbiotic) as a substrate to increase dominance and sustainable growth of probiotics bacteria has been suggested (Hoseinifar et al., 2015).

Regarding the use of synbiotics in aquaculture, few studies have been performed and their positive effects on physiology and immunity have been reported (Rodriguez-Estrada et al., 2009; Merrifield et al., 2010; Montajami et al., 2012; Abid et al., 2013; Hosseinifar et al., 2015). However, the use of synbiotics during the early life stages of fish through the enrichment of live food and the effects on growth, physiology and immunity has not been considered. The use of synbiotic in *Artemia* could be considered as a food for *Artemia*, and also could affect the intestinal microbiota, immune system and increase resistance to pathogenic bacteria, enhance health and reduce the risk of disease outbreaks.

Artemia is among the live foods that widely used in the culture of ornamental fishes due to the high nutritional value, the proper size and the enrichment capability (Sorgeloos et al., 2001). *Artemia* can be used as the carrier of particles used in aquaculture such as nutrients (fatty acids, vitamins, etc.), antimicrobial substances, vaccines and probiotics (Ziaei-Nejad et al., 2006)

Application of live, useful and non-pathogenic bacteria to culture medium or *Artemia* culture can positively affects cultured fish species by improving the intestinal microbial microbiota, eliminating harmful bacteria and improving the nutritional value of *Artemia* (Havennar et al., 1992; Ringo et al., 1992). The number of bacteria in the *Artemia* exponentially increases at the time of *Artemia* hatching and enrichment processes by nutrients (Ritar et al., 2004). It also has been observed that during the early stages of fish development, the increase in the number of bacteria in the intestinal microbiota of fish, is mainly associated with the bacteria in live food (Makridis et al., 2000). It can be concluded that mortality increases in the intensive culture of early life stages of fish along with elevation of the number of opportunistic bacteria in the fish intestine. Therefore, control of bacterial population in the live feed may lead to higher survival rates of fish larvae and profitability in hatcheries (Olafsen, 2001). Therefore, this study was conducted to study enrichment capability of adult *Artemia franciscana* with singular or combined administration of *Pediococcus acidilactici* and fructooligosaccharide (FOS) as probiotic and prebiotic, respectively.

Materials and Methods

Artemia culture conditions and Bacterial strain: *Artemia* cysts (*A. franciscana*) was obtained from Great Salt Company, USA. Chorionic layer of cysts were separated using sodium hypochlorite during decapsulation. Hatching of the decapsulated cysts was performed by a cone-shaped container with a volume of 120 litters and sea water (with salinity of 30 g per litter). Cysts were incubated with a density of 5 g per litter at 30°C with 2000 lux lighting conditions and vigorous aeration (Sorgeloos et al., 1986).

Artemia naupliis were transferred to culture environment after hatching. The culture environment was a 150L cone-shaped plastic containers that were aerated by aeration pipes connected to the central pump. Nauplii were fed during the first few days by spirulina algae (*Spirulina platensis*), and then by a mixture of rice bran, baker's yeast and spirulina. Feeding was performed three times a day with an interval of 4 hrs. Stocking density was three nauplii per ml and culture period was 20 days to reach sexual maturity (Teresita et al., 2005). During culture period, all physical and chemical parameters were measured and recorded daily. Physical and chemical factors, including water temperature, salinity, dissolved oxygen, light and pH were 28.69°C, 32 g L^{-1}, 7.75 mg $L^{-}1$, 1500 lux and 7.88, respectively. The used commercial probiotic used in this experiment was obtained from Tak Gene Company with Pedi-guard brand name contains bacteria *P. acidilactici* to amount of 1×10^{10} CFU g^{-1}. Prebiotic, Oligofructose (Raftilose P95) was supplied from Orafti Company, Belgium.

Enrichment of synbiotic to adult Artemia: For

Table 1. The enrichment condition adult *Artemia* in different treatments.

Treatments	Rapeseed oil suspension (ml L^{-1})	Probiotic, *P. acidilactici* (mg L^{-1})	Prebiotic, FOS (mg L^{-1})
Synbiotic (T_1)	150	700	100
Probiotic (T_2)	150	700	0
prebiotic (T_3)	150	0	100
Control (T_4)	150	0	0

enrichment of the adult *Artemia* by synbiotic, combinations of the probiotics and prebiotics were used along with singular administration of the probiotic and prebiotic as described in Table 1. For preparation of the synbiotic suspension, first a ratio of 0.1:10 lecithin and water at 40°C were poured into a clean and dry beaker and mixed using an electric mixer. Then, the rapeseed oil was added to the solution and mixed very well by mixer. The ratio of lecithin, oil and water in suspension was 0.1, 1 and 10, respectively. To evaluate the diameter of oil particle, some samples were poured on slide and observed under light microscope. 150 ml was separated from the prepared suspension, 700 mg probiotic, *P. acidilactici* and 100 mg of prebiotic, FOS were transferred to the beaker and were uniformed with an electric mixer, then mixed in 2 litters of seawater. The adult *Artemia* with the number of 4000 was placed inside the culture container (Agh and Sorgeloos, 2005; Daniels et al., 2013) (Table 1).

To determine the best level of the probiotic, *P. acidilactici* and prebiotic FOS in enrichment suspension of *Artemia*, a pre-experiment has been designed and probiotic was used in two levels with an amount of 10^7 CFU and 10^8 CFU per litter of suspension, and prebiotic was used in two levels of 2 and 5 g per litter of suspension. The results of this pre-experiment has been used as level of probiotic, prebiotic and synbiotic in this experiment.

To examine the process of enrichment, sampling was performed from the all treatment at 2, 4 and 6 hrs (Dhont and Lavens, 1996). In each sampling time, 100 ml (containing 0.5 g of adult *Artemia*) were collected using a sterile pipette and were transferred to a filter with a mesh size of 300 micrometer, then to elimination of the bacteria in the external surface of *Artemia* body, were washed for 60 seconds in a salt solution, Benzalkolium chloride (0.1%) and again were washed with sterile water and after that, water of samples was taken after a while (Makridis et al., 2000). The sterile samples were weighted and transferred to sterile porcelain mortar. After the homogenization of samples using a sterile saline solution (0.87% w/v), dilutions of 10^{-1} to 10^{-7} were prepared. From prepared dilutions, under sterile conditions, 0.1 mm was removed and spread on surface of the MRS agar plates (for determine the number of lactic acid bacteria). The incubation of plates was conducted for 3-5 days in an incubator at a temperature of 30°C and under aerobic conditions. After the incubation period, the bacteria were counted, and recorded according to the logarithm of the colony unit (the number of bacterial colonies grown on culture medium × dilution coefficient $^{-1}$) per g of *Artemia* (Rengpipat et al., 1998). *Pediococcus acidilactici* was identified based on apparent characteristics, gram staining and also standard biochemical tests such as phenol red, citrate, indole, motion and methyl red (Peter and Sneath, 1986).

Statistical analysis: Statistical analysis was performed using the SPSS software package (version 18). One-way ANOVA was used for comparison between treatments and Duncan's multiple range test was used for the comparison of means at confidence level of 0.05% (P<0.05).

Results

The effects of different treatments and sampling time on the amount of bacteria in the *Artemia* is shown in Table 2. The results indicated that probiotic bacteria in each sampling time, were successfully enriched in *Artemia*. The enrichment trend of *A. franciscana* was

Table 2. The cultivable lactic acid bacteria levels (log CFU g^{-1} *Artemia*) in *A. fransiscana* enriched in pre-, pro- and symbiotic.

Hours	Treatments			
	Synbiotic (T_1)	Probiotic (T_2)	Prebiotic (T_3)	Control (T_4)
2	5.50±0.07^b	5.58±0.04^b	1.15±0.02^a	1.09±0.05^a
4	6.61±0.07^b	6.67±0.03^b	1.04±0.02^a	1.23±0.04^a
6	6.71±0.04^b	6.78±0.03^b	0.83±0.07^a	1.15±0.04^a

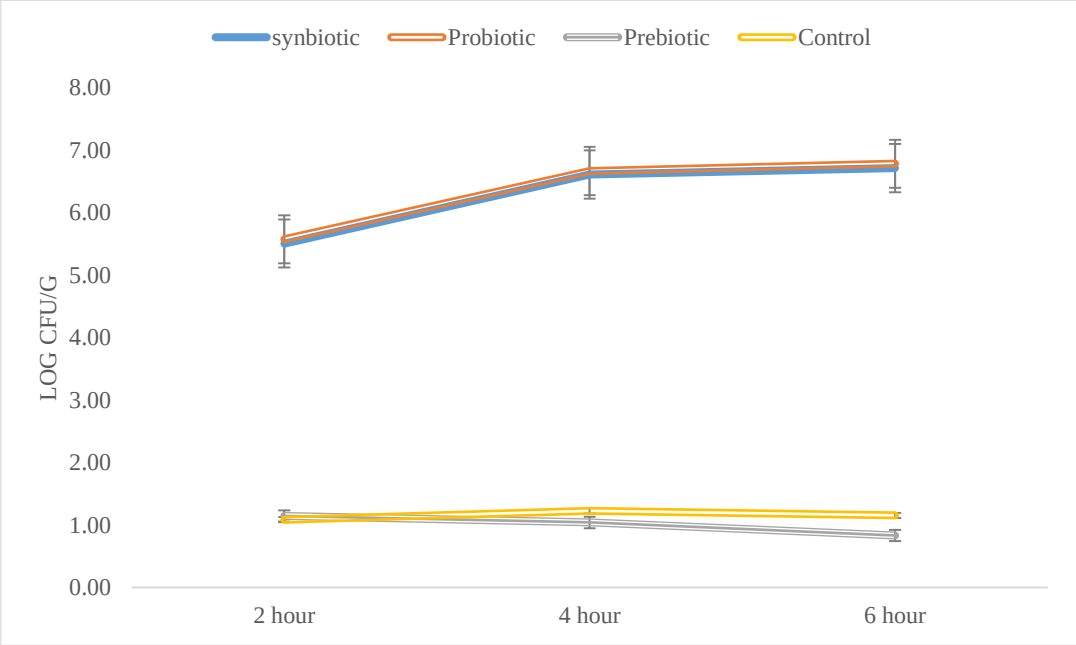

Figure 1: The process of enrichment adult *Artemia* enrichment at different times in different treatments.

different at different sampling times. In terms of the enrichment time, the results showed significant difference in the capability of *Artemia* enrichment (P<0.05). Regarding the synbiotic and probiotic treatments at 4 and 6 hrs enrichment, there was no significant difference in the number bacteria per g of *Artemia* (P>0.05). The results of bacterial count in prebiotic and control treatments showed that the concentration of lactic acid bacteria in these treatments were lower than 20 CFU g^{-1} and no significant difference were observed between sampling times (P>0.05).

The bacterial counts in treatments enriched by probiotic and synbiotic were almost at the same level, but increasing trend was observed along with enrichment period (Fig. 1). However, no statistically significant differences was observed between bacterial level in adult *Artemia* at 4 and 6 hrs of enrichment (P>0.05).

Discussion

In this experiment, bacterial levels used in the enrichment solutions at all sampling times were at a level of 10^{10} CFU g^{-1}. Gomez-Gil et al. (1998) were applied the concentrations of 10^7 CFU g^{-1} and 10^8 CFU g^{-1} of *Vibrio parahaemolyticus* and *V. alginolyticus*, respectively, during enrichment experiment of *A. franciscana* and reported the same pattern in their changes at different sampling times. Similar study were not observed regarding to enrichment of adult *Artemia* with probiotic and synbiotic. Based on the results, the concentration of bacteria in adult *Artemia* showed a positive correlation with the duration of enrichment, similar to the results of Parta et al. (2003) during the enrichment of *A. franciscana* nauplii with yeast (*Saccharamyces baulardii*) which revealed accumulation of yeast in nauplii at a level of 3.5×10^3 CFU g^{-1}. However, enrichment of *A. franciscana*

nauplii with two strains of *Vibrio* sp. showed different patterns, so that, attached bacteria to *Artemia* nauplii began to increase at first 30 minutes of enrichment, then suddenly declined at 8 hrs after enrichment and again a sharp rise occurred at 24 hrs at the levels of bacteria in nauplii which all naupliis died at the end of this time (Gomez-Gil et al., 1998). The *A. urmiana* showed a gradual trend in enrichment with mentioned probiotic increased over time. Furthermore, Campbell et al. (1993) enriched *A. franciscana* with the formalin-killed *V. angualiurum* and showed that when the concentration of bacteria in enrichment solution is 1.5×10^7 CFU g^{-1}, the maximum accumulation of *Vibrio* sp. in the *Artemia* nauplii is happened at 60 min. Moreover, in concentrations lower than 5×10^6 CFU g^{-1}, the maximum accumulation is occurred at 120 min after the start of enrichment. Changes in the number of bacteria in the *A. franciscana* is not limited by the number of bacteria in enrichment suspension and the same results reported by Makridis et al. (2000) in the enrichment of *A. franciscana* nauplii with the probiotic bacteria.

In conclusion, the results of this experiment indicated that adult *Artemia* has high ability to be enriched with the probiotic bacteria, *P. acidilactici* and bacterial levels in *Artemia* that is increased along with enrichment time.

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چکیده فارسی

مطالعه امکان غنی‌سازی آرتمیا بالغ، *Artemia franciscana* با کاربرد منفرد یا همزمان *Pediococcus acidilactici* و فروکتوالیگوساکارید

محمود عظیمی راد*[1]، سعید مشکینی[2]، نصرالله احمدی فرد[1]، سید حسین حسینی فر[3]

[1]گروه شیلات، دانشکده منابع طبیعی دانشگاه ارومیه، ارومیه، ایران.
[2]گروه بهداشت مواد غذایی، دانشکده دامپزشکی، دانشکده منابع طبیعی دانشگاه ارومیه، ارومیه، ایران.
[3]گروه تکثیر و پرورش آبزیان، دانشکده شیلات و محیط زیست، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران.

چکیده:

در این مطالعه امکان غنی سازی آرتمیا فرانسیسکانا (*Artemia franciscana*) بالغ با سین‌بیوتیک ترکیبی از *Pediococcus acidilactici* و فروکتوالیگوساکارید مورد بررسی قرار گرفت. این آزمایش به صورت طرح کاملاً تصادفی در قالب چهار تیمار شامل سین بیوتیک *P. acidilactici* و فروکتوالیگوساکارید (T1)، پروبیوتیک (T2) *P. acidilactici*، پری بیوتیک فروکتوالیگو ساکارید (T3) و تیمار شاهد (T4) اجرا گردید. جهت ارزیابی غنی‌سازی آرتمیای بالغ با هر یک از تیمارها، در زمان‌های ۲، ۴ و ۶ ساعت پس از شروع غنی‌سازی، نمونه‌برداری انجام و تعداد باکتری‌های موجود در داخل بدن آرتمیا پس از کشت میکروبی، تعداد باکتری‌ها بر حسب لگاریتم CFU در هر گرم آرتمیا شمارش گردید. نتایج نشان داد که با گذشت زمان، باکتری‌های مصرف شده توسط آرتمیای بالغ بیشتر بوده و بین زمان غنی‌سازی و تعداد باکتری الحاق شده به آرتمیای بالغ نسبت معنی‌داری وجود دارد ($P>0/05$). بعد از ۶ ساعت غنی‌سازی، بیشترین تعداد باکتری ($5/2 \times 10^{6} \pm 0/06$) به آرتمیای بالغ الحاق گردید که با زمان ۲ ساعت اختلاف معنی‌دار نشان داد ولی با مدت زمان ۴ ساعت غنی‌سازی اختلاف معنی داری نشان نداد. همچنین نتایج نشان داد که تعداد باکتری *P. acidilactici* در تیمارهای ۱ و ۲ به میزان معنی‌داری بیشتر از تیمار شاهد و تیمار واجد فقط پری بیوتیک می‌باشد ($P>0/05$). اما اختلاف معنی‌داری بین تیمار پروبیوتیک و سین‌بیوتیک مشاهده نگردید. نتایج همچنین نشان داد که آرتمیای بالغ در مدت زمان کوتاهی (حدود ۴ ساعت) می‌تواند میزان بالایی از باکتری پروبیوتیک را در خود ذخیره نماید.

کلمات کلیدی: سین‌بیوتیک، *Pediococcus acidilactici*، فروکتوالیگوساکارید، غنی سازی، آرتمیا فرانسیسکانا.