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Potential of Brine Shrimp (*Artemia urmiana*) Enrichment with Two Species of Bacillus and Yeast (*Saccharomyces cerevisiae*)

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Abstract: Experimental features for brine shrimp (*Artemia urm*iana) enrichment with two bacillus species and yeast which isolated from Bluga sturgeon fish (*Huso huso*) gut have been prepared in laboratory of Gonbad Kavous University (Gonbad kavous- Iran). For present study three treatments with three replicates and three control treatments with three replicate was prepared. Brine shrimp cysts after incubation and isolation from other cysts and cysts sack was enriched with three suspension of *Bacillus subtilis* ($1/4997\times10^{8}\pm0/308\times10^{7}$ CFU/L), *Bacillus licheniformis* ($1/5017\times10^{8}\pm0/275\times10^{7}$ CFU/L) and *Saccharomyces cerevisiae* ($1/5057\times10^{7}\pm0/297\times10^{6}$ CFU/L). This two bacteria and bakery yeast are members of probiotics. Enrichment was carried out for 24 hours in four times period: from enrichment starting (t_0) respectively after 5, 10 and 24 hours sampling have been done and amount of probiotic consuming by nauplii was calculated. The significant enrichment was related to the treatment which fed with bakery yeast (541/95±44/79 CFU/nauplii) for 24 hours ($P<0.05$). The significant survival rate (93 %) was observed in treatment which enriched with *Bacillus licheniformis* for 24 hours ($P<0.05$). The present study has shown that *Artemia urmiana* nauplii have a good potential of enrichment and the brine shrimp (*Artemia urmiana*) has different response to different probiotic and density.

Key word: *Artemia urmiana* % Bacillus bacteria % Probiotic % Enrichment % *Saccharomyces cerevisiae* yeast

INTRODUCTION

The use of probiotic bacteria has been suggested as an important strategy to accomplish reproducible outputs through biocontrol in cultivation systems for marine fish larvae and crustaceans [1,2]. Live nauplii of the brine shrimp (*Artemia spp*.) have been used as vectors for delivering compounds of diverse nutritional [3, 4], and/or therapeutic [5-8], value to larval stages of aquatic animals, a process known as bioencapsulation. Probiotic bacteria are defined as a live microbial feed supplement, which beneficially affects the host animal by improving the intestinal microbial balance [9]. The bacterial flora in the larval gut originates from bacteria associated with the eggs, the water in the rearing tanks, and the live food [10-12]. Bacteria able to colonize the gut are adapted to the physical, chemical, and biotic conditions in the host, and can thus persist the activity of bile, digestive enzymes, the host's immune system, and variations in pH levels. Successful colonization involves competition with the established microflora for attachment sites and nutrients. The species-composition of the intestinal microflora of fish larvae can be influenced at an early stage of development, when few, if any, bacteria are present in the larval gut, by addition of specific bacterial strains to the live food or the water [13,14] *Artemia urmiana* nauplii are able to graze bacteria and the number of bacteria accumulated during a short-term incubation depends on the concentration of

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the bacterial suspension and the bacterial strain applied [15,16]. Inoculating the digestive tracts of target organisms with probiotic bacteria through bioencapsulation and feeding is another alternative use for *Artemia* nauplii . Many studies have been performed to improve the nauplii feature as a feed and or vector. Probiotic enrichment of *Artemia* nauplii by *Saccharomyces cerevisiae* has been used for both aims. As an example Patra and Mohamed [17], attempted to improved vector function of the nauplii by probiotic *Saccharomyces spp*. The present study was carried out to evaluate different concentration and time of enrichment and effect of it on probiotic concentration on vector (*Artemia urmiana*) body.

MATERIAL AND METHODS

Artemia Cyst Hatching: Artemia urmiana cysts were decapsulated using a chemical process (Sodium Hypochlorite) according to Sorgeloos *et al*. [18]. Decapsulated cysts were incubated with density of 5 gram per liter in incubation conical glass with temperature of 30°C degree, 2000 Lux light intensity, and sterile water with 30 ppt salinity with strong aeration (Gomes-Gill *et al*. 1998). To ensure for axenic incubation of nauplii three kinds of antibiotics (chlormphenicol 30µg/ml, Sulfamethoxazol 40 µg/ml and trimethoprim 8 µg/mL) were used in incubation environment [15].

Assessment of Antibiotic Fragment in Nauplii Body: After nauplii isolation and washing with sterile water according to Bauer *et al.* [19], Antibiotic susceptibility testing by a standardized single disk method have been done [15]. Polluted disks with *Artemia* nauplii extraction have been located into the agar plates with *Bacillus subtilis* and *Bacillus licheniformis*. In this stage *Escherichia coli* bacteria use as an indicator of sensitivity to antibiotics. We prepared another Muller Hinton agar plates and subjected to isolated *Bacillus spp* and after that polluted disk with cultivation water and nauplii extraction were added to this plates. After 24 hours of incubation in 37°C all distance of inhibitors were counted and diameter of them were measured.

Nauplii Enrichment with Probiotics: Sterile nauplii were added to a 250-ml conical glass with the bacterial suspension (sterile seawater and the desired bacterium) at a density of 200 nauplii per mL (0/5 g of hatched nauplii to 250 mL sea water) (Makridis *et al*. [16]. Enrichment process have been done for each suspension of *Bacillus subtilis* ($1/4997\times10^{8}\pm0/308\times10^{7}$ CFU/L), *Bacillus licheniformis* ($1/5017\times10^{8}\pm0/275\times10^{7}$ CFU/L) and *Saccharomyces cerevisiae* ($1/5057\times10^{7}\pm0/297\times10^{6}$ CFU/L) separately with three replicates. For each treatment we obtained three control treatments that contained: 1st control treatment; sterile nauplii with unsterile water that included natural microflora of environment. 2nd control treatment; sterile nauplii without enrichment with *Bacillus spp* in sterile water. 3rd control treatment; unsterile nauplii in sterile water.

Bacterial Experiments and Bacterial Present, Calculation in Nauplii Body (CFU): For this aim in period of enrichment from first of experiment (t_0) sampling has been done on 5, 10 and 24 hour of enrichment, and salty homogeny suspension (0/85 % w/v NaCl) has made. This suspension has been used for serial direction process from 10^{1} up to 10^{8}. From serial direction suspension, samples in amount of 100µl were prepared that subjected to agar cultivation plate that divided in to two kind of cultivation agar: Blood agar and TSA agar. For bakery yeast treatments PDA (Potato Dextrose agar) has been used. After incubation of them colonization of bacteria have been counted .

Statistical Analysis: Treatments were compared by One-way Analysis of Variance (ANOVA). In case of in homogeneity, comparisons of means were made using Duncan's multiple range test at 5 % level of significance using SPSS (Version 13.0). The significant level was set at $P<0.05$.

Table 1: Control treatment explanation

Control treatment	Sterile water	Unsterile water	Sterile nauplii	Unsterile nauplii
1st		*	*	
2nd	*		*	
3rd	*			*

RESULTS

Probiotic *Bacillus* (*Bacillus subtilis* and *Bacillus licheniformis*) and bakery yeast (*Saccharomyces cerevisiae*) were incorporated to the *Artemia* nauplii body successfully. As defined in figure 1 after 10 hours *Bacillus sp* and yeast have been located into the nauplii body and probiotic concentration was increased time to time, and after 24 hours we obtained the highest concentration of *Bacillus sp* and bakery yeast in *Artemia urmiana* nauplii body. Figure 1 contained all aspects of bacteria on nauplii body contained bacteria in to the body or bacteria which attached to the surface of nauplii body. This figure shows the nauplii which has been washed with Benzalkonium chloride. Bacteria and yeast density in nauplii body were improved with over of enrichment time (24 h). There was positive relation between time of probiotic adding and density of them (CFU/nauplii) in nauplii body. Between treatments T3 which included *Saccharomyces cerevisiae* yeast either, has had the highest density of *Bacillus* (541/95±44/79 CFU/nauplii) and after 24 hours it has significant difference with other treatments ($P<0.05$). The lowest density of *Bacillus* was observed in T1 (19/80±7.20 CFU/nauplii) treatment. In all treatments, there were different bacterial density and this is because of different enrichment time.

The highest survival percentage after 24 hours enrichment with *Bacillus* probiotic and bakery yeast was obtained from T1 (93%) that did not have significant difference with the other treatments ($P>0.05$). The results of performed study donated that *Artemia urmiana* has a high potential of enrichment with *Bacillus* and bakery yeast probiotic.

DISCUSSION

The use of bacteria and yeast can due to attach them to the surface of *Artemia* nauplii body or located into the body by *Artemia* nauplii grazing [15]. In performed study Bacillus (*Bacillus subtilis* and *Bacillus licheniformis)* probiotic and (*Saccharomyces cerevisiae*) yeast was located to the *Artemia urmiana* nauplii body successfully. Gomez-Gil *et al.* [15] had reported same method about (*Brachionus plicatilis*) rotifer. Negative relation between bacterial allocation and density of them in environment was observed. This observation have shown that locating of bacteria in to the nauplii body does not related to the bacteria concentration in the liquid environment. Thus it is related to the bacteria stability on surface of nauplii body and density of bacteria that entered to the digestive canal of nauplii [15].

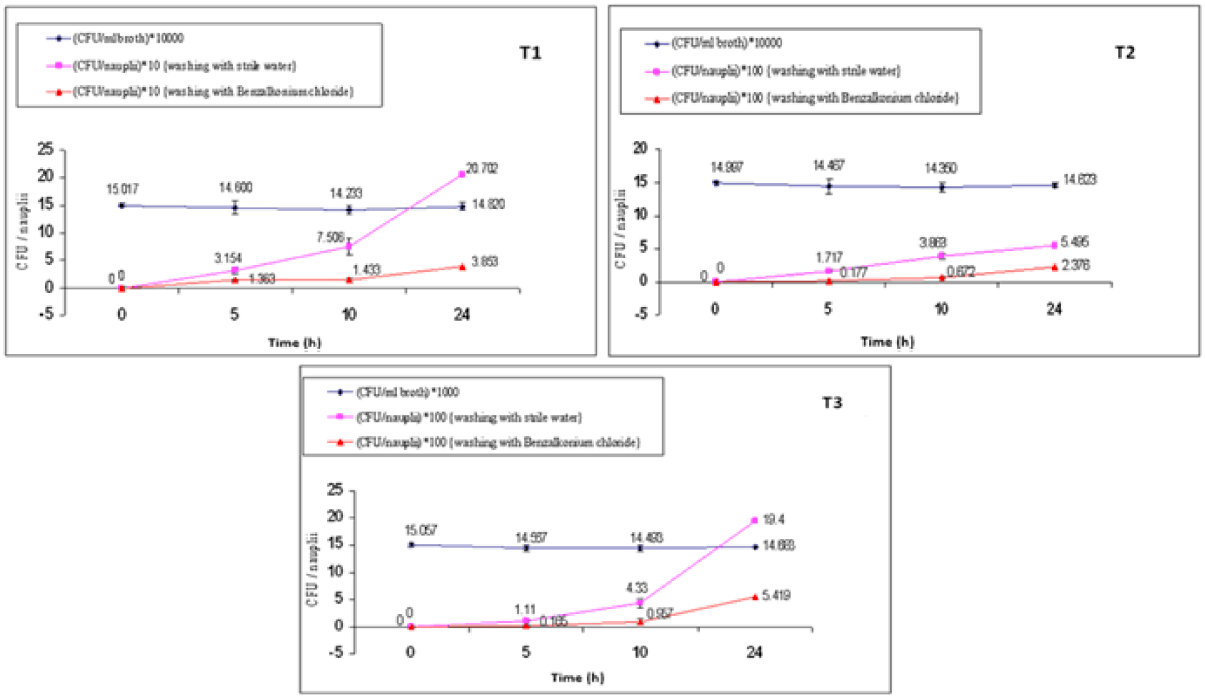

Fig. 1: The concentration of *Bacillus subtilis*, *Bacillus licheniformis* and *Saccharomyces cerevisiae* allocation to the *Artemia urmiana* nauplii in different enrichment time in experimental treatments

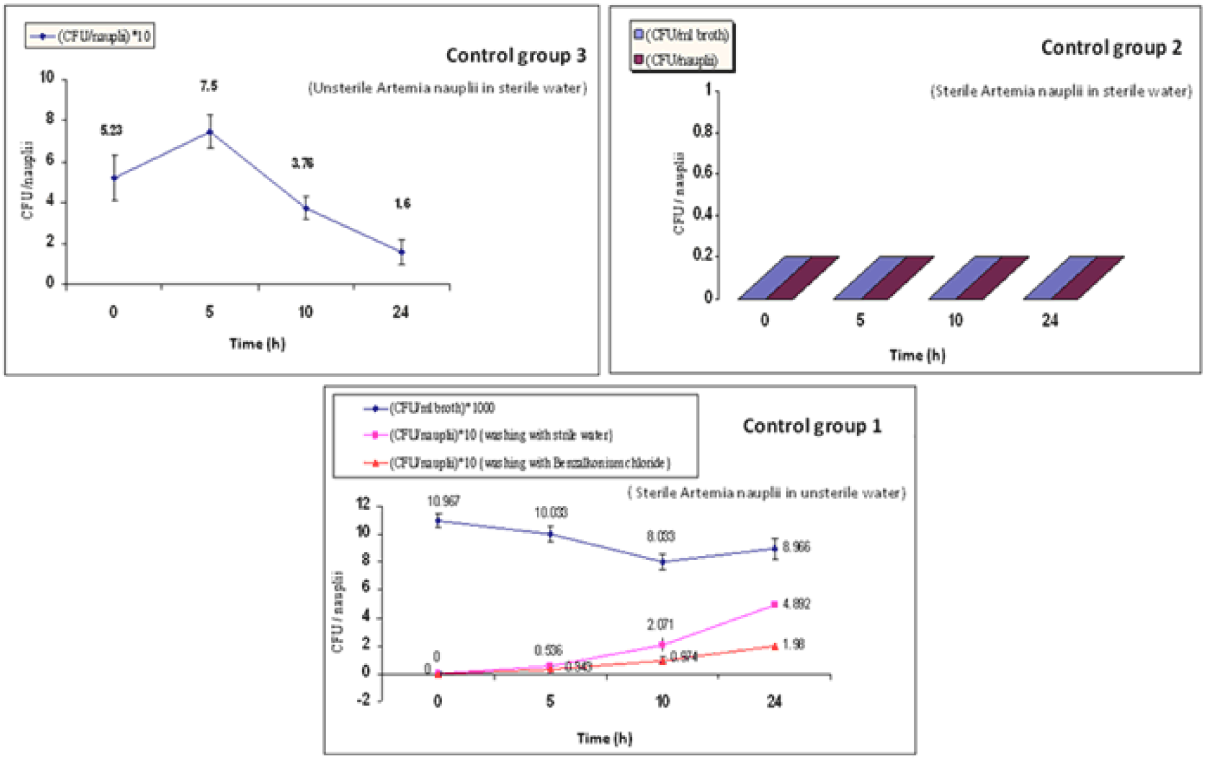

Fig. 2: The concentration of *Bacillus subtilis*, *Bacillus licheniformis* and *Saccharomyces cerevisiae* allocated to the *Artemia urmiana* nauplii in different enrichment time in control treatment.

If bacteria were passed away, concentration of them is the important factor in transferring to the nauplii body. T3 treatment that enriched with bakery yeast after 24 hours nauplii contains the lowest density of yeast in nauplii body. This result was observed in T2 which was enriched with low density of bacteria than T1 but contain the highest density of bacteria (CFU/nauplii). For enrichment process bacteria species and strain is important. In contrast of this study Jafariyan *et al*. [9], reported that *Artemia urmiana* has a good potential to use as vector. The present study has shown that *Artemia urmiana* has a high potential of enrichment with *Bacillus subtilis*, *Bacillus licheniformis* and *Saccharomyces cerevisiae*. This species of *Artemia* has different reaction with different probiotic. In control treatment we observed that *Artemia urmiana* has enough ability to keep its natural microflora in sterile water. Fazeli *et al.* [20], reported that enrichment of *Artemia urmiana* with yeast (Thepax) due to decrease in crude protein, crude lipid and energy contents. It means that *Artemia urmiana* enrichment with yeast has effect on body composition. Tovar *et al.* [21], showed decreases in secretion of amylase and tripsin following treatment of sea bass fish by *Saccharomyces cerevisiae*. In the case of *Artemia* nauplii, this finding can suggest that the cells of bakery yeast might be indigestible within the nauplii body. In contrast, Lim *et al*. [22], found significant increase in crude lipids by enriching *Artemia fransiscana* nauplii with wild *Saccharomyces cerevisiae* compared with the newly hatched nauplii ($p<0.05$). Results observed by Campbell *et al*. [23], with formalin-killed bacteria showed that maximum uptake of *V. anguillarum* occurred at 60 min in a bacterial concentration of $1.5\ 3\ 10^7$ CFU ml21, while at a lower concentration of $1.5\ 3\ 10^6$, a peak was observed after 120 min. A similar pattern was observed with *Brachionus plicatilis* when challenged with a *V. anguillarum* vaccine [24]. Bacterial colonization of the nauplii could occur externally, via attachment to the body surfaces or internally by ingestion [25]. After the nauplii were removed from the bacterial suspension, the bacterial content decreased rapidly. This decrease might be due to the removal of the external bacteria after the nauplii were washed and placed in sterile seawater. The bacteria still detected could be the ones colonizing the interior or firmly attached to the external surfaces. Similar trends were observed with rotifers after they were removed from a bacterial suspension [25].

CONCLUSION

Artemia nauplii with this potential can be use as specific vector to carry any kind of probiont matters or antibiotics. It means that this organism in near future will be use for bio-vaccination for diseases treatment and controlling. Time of enrichment of course is the most important parameter that do effect of concentration of probiont matters in nauplii body.

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