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The Best Time and Concentration for Yeast Probiotic Enrichment of *Artemia urmiana* Nauplii

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Abstract: *Artemia* is a practical and suitable larvae food for both marine and fresh water crustaceans and fishes. Yeast probiotic enrichment of *Artemia urmiana* nauplii, found in Uromieh Lake of Iran, has not been documented in literature. The nauplii were enriched with Thepax, a commercial probiotic material containing yeast cells of *Saccharomyces cerevisiae* and the best time and concentration of the enrichment have been evaluated. To identify the optimal concentration of yeast cells, each group of nauplii (6-, 12- and 18-h after hatching) have been enriched with three treatments containing 10^4, 10^7 and 10^{10} colony forming units (CFU mL^{-1}). In result, the maximum survival rate of nauplii was in concentration of 10^7 CFU mL^{-1} ($p \leq 0.05$). As the most yeast cells have been seen in the 6-h group ($p \leq 0.05$); the best time of enrichment was at 6 hours after hatching. In contrast to probiotic enrichment of other *Artemia* species, this study showed that the earlier enrichment of *Artemia urmiana* nauplii has better results.

Key words: *Artemia* urmiana, *Saccharomyces cerevisiae*, probiotic, enrichment

INTRODUCTION

Artemia is a practical and suitable larvae food for both marine and freshwater crustaceans and fish (Bengston *et al*., 1991). In addition, live nauplii of brine shrimp have been used as a vector for delivering compounds of diverse nutritional (Lim *et al*., 2005) and therapeutic value as well as probiotic products to different developmental stages of aquatic animals (Fuller, 1992). As new findings emerged, several definitions of probiotics have been proposed e.g., a live microbial feeding supplement which beneficially affects the host animal by improving its intestinal balance (Fuller, 1992; Marqué *et al*., 2004; Patra and Mohamed, 2003).

The positive effect of probiotics may be attributed to its ability to compete with other opportunistic bacteria or produce of micronutrients which are important for larval development in addition to possession of antibacterial/bacterial properties (Lavens and Sorgeloos, 1984). Gomez-Gill *et al*. (1998) have also shown uptake of bacterial probiotic by the *Artemia franciscana* cause to increase survival rate. Verschuere *et al*. (2000) selected nine bacterial strains that positively influenced the growth and/or survival of juvenile brine shrimp cultured as a live food for other species.

Saccharomyces cerevisiae, the budding yeast, is the common yeast used in baking. Also, it is hosted by many animals and insects and found anywhere in nature. Because of easy culture, rapid growth and appropriate cell diameter (typically 5-10 μm), the yeast is well suited as a probiotic agent.

In this study, Thepax (Doxal Co., Italy) is used as enriching factor for *Artemia urmiana* found in Uromieh Lake of Iran (Azari-Takami, 1992). Thepax contains *S. cerevisiae* with controlled reproduction. The minerals, amino acids and vitamin B are the ingredients to increase useful intestinal microflora.

There was no document in literature about yeast probiotic enrichment of *Artemia* urmiana. In addition, the enrichment of *Artemia* species with the Thepax has not been documented before. The present study was aimed to determine the best concentration and time for enrichment of *Artemia urmiana* nauplii with Thepax. The concentration was identified by maximum survival rate of the nauplii and the time was concluded from highest number of yeast found within the nauplii body. In addition, the survival rate was compared between enriched and control nauplii.

MATERIALS AND METHODS

Probiotic medium: Pure cultures of *S. cerevisiae* were re-isolated on YGC Agar (Yeast Extract Glucose Chloramphenicol Agar FIL-IDE). YGC Agar medium contains yeast extract (Merk 5.0 g L^{-1}), glucose (20.0 g L^{-1}), Chloramphenicol (0.1 g L^{-1}) and Agar (14.9 g L^{-1}).

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***Artemia* cyst hatching and disinfection:** By a process known as decapsulation, the corions of the cysts were chemically removed according to Gomez-Gill *et al.* (1998). Hatching of the decapsulated cysts was performed in a sealed flask with 1000 mL of autoclaved saline water (3.0 g L^{-1} salinity). The cysts were stocked at a density of 2 g L^{-1} and incubated at 28±1 °C with pH = 7. To prevent bacterial contamination, the hatching environment was illuminated constantly and oxygen current was prepared through mechanical agitation.

After 24 h, the recently hatched nauplii were collected aseptically in a 120 μm-pore sieve by exploiting the positive phototactic behavior of the nauplii and washed thoroughly with sterile distilled water.

Enrichment treatments: To determine the optimal treating concentration, three groups of nauplii were considered (6, 12 and 18 h after hatching) and each group was assigned to feed with three yeast concentrations. (10^4, 10^7 and 10^{10} (CFU) mL^{-1}). Consequently, nine different subgroups were developed. In each subgroup, nauplii densities of 2 g L^{-1} were stocked in three separate flasks.

To determine the best time of enrichment, 6-, 12-, 18-h groups were enriched with the best concentration found in earlier section, three yeast-only and three nauplii-only flasks were prepared.

Sampling: After 6 h of enrichment, 1 mL from each flask of enriched and control nauplii were sampled and mean survival were estimated. In addition, for counting of the yeast within enriched nauplii, 200 mg nauplii from each enriched flask were collected concurrently in sterile conditions. The collected nauplii were disinfected completely with Benzachloniom 1% (Rengpipat *et al.*, 1998), washed and homogenized. Serial dilutions of the supernatant fluid (or from the flask water in the yeast-only control) were prepared. The dilutions were spread plated on YGC Agar media. The plates were incubated at 37 °C for 24-36 h and CFUs/nauplii were counted after incubation.

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/PC Software (Version 13.0). The significance was set at $p \leq 0.05$.

RESULTS

Enrichment experiment: Mean survival rates of the nauplii in different groups were shown in Table 1. As shown in this table, the survival of *Artemia* nauplii in concentration of 10^{10} CFU mL^{-1} was very low. The multiple range tests indicated that all groups of nauplii had highest survival in concentration of 10^7 CFU mL^{-1} in comparison to the other concentrations ($p \leq 0.05$). Hence, this concentration was selected for the enrichment later in the study. The survival rates of control groups were less than the survival of groups enriched by the best concentration (10^7 CFU mL^{-1}) ($p<0.05$). The 6-h nauplii showed the highest survival among the groups enriched by 10^7 CFU mL^{-1} but no significance was found between the survivals of 6- and 12- h groups in this concentration. ($p>0.05$)

Table 1: Mean survival rates of the nauplii groups enriched with different yeast concentrations

	Enriched with 10^4 CFU mL^{-1}	Enriched with 10^7 CFU mL^{-1}	Enriched with 10^{10} CFU mL^{-1}	Yeast-free control group
6-h nauplii	61.29±1.57 [1]	98.50±0.57	2.40±1.24	71.47±0.91
12-h nauplii	51.53±1.26	96.50±1.42	0.00	62.24±0.92
18-h nauplii	24.81±3.53	59.40±4.05	0.00	34.70±4.50

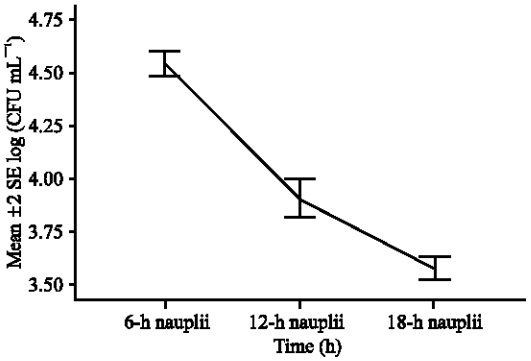

Fig. 1: The *S. cerevisiae* counts (CFU mL^{-1}) within nauplii body of the groups (6-, 12- and 18-h) enriched by the best concentration (10^7 CFU mL^{-1})

Bacterial counts: The *S. cerevisiae* counts within nauplii groups (6-, 12-, 18-h) enriched by the best concentration (10^7 CFU mL^{-1}) were shown in Fig. 1. The highest *S. cerevisiae* counts found within the bodies of 6-h nauplii ($3.4\times10^4\pm2.4\times10^2$ CFUs/nauplii) ($p \leq 0.05$). Therefore, the 6 h after hatching was selected as the best time for the enrichment. No changes were found in yeast count of yeast-only group ($p>0.05$).

DISCUSSION

There was no document in literature about yeast probiotic enrichment of *Artemia urmiana*. Use of *S. cerevisiae* as a probiotic has been reported in fish (Intriago *et al.*, 1998; Lim *et al.*, 2005; Marqué *et al.*, 2004) but Thepax has not been used. The yeast grows rapidly and can be cultured easily. Its diameter is 5-10 μm that is sufficient for enrichment of *Artemia* nauplii.

The survival rates of the nauplii were highest in 10^7 CFU mL^{-1}. The low survival in 10^{10} CFU mL^{-1} can be attributed to the presence of high numbers of the yeasts within the flasks. The profuse yeasts could impair the illumination as well as the biochemical features which are necessary for culture of the nauplii. The concentration of 10^4 CFU mL^{-1} was accompanied with a moderate survival in all groups. It may signify that enrichment with this concentration could not meet the optimal nutritional needs of nauplii.

In this study, the 6 h after hatching was found as the best time for enrichment of *Artemia urmiana*. The yeast probiotics tend highly to attach to intestinal mucus. In addition, the gastrointestinal floras of various organisms are developing during the neonatal period as shown by Ringo and Birkbeek (1999). Hence, the replacement of the flora with yeast probiotics is more successful during the primitive periods of life rather than other periods.

In contrast to our study, Bossier (2005) and other studies showed that the best time for enrichment of *Artemia sanfrancisco* nauplii was at the beginning of Instar II and about 12 or 18 h after Hatching of nauplii (Gomez-Gill *et al*., 1998; Patra and Mohamed, 2003).

The difference between our suggested optimal time and the times mentioned in these studies may be due to differences in environmental temperature, the hatching conditions and species of *Artemia*.

The optimal probiotic enrichment of *Artemia urmiana* nauplii was at the early stages of their life. This characteristic can help to preserve the nutritional values of enriched nauplii for feeding of crustaceans and fish. *Artemia urmiana* nauplii and Thepax showed acceptable features in enrichment process. Hence, it proposes to develop Thepax enrichment of *Artemia urmiana* nauplii in hatchery centers with access to the nauplii.

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