Aquaculture International **11:** 505–514, 2003.

Enrichment of *Artemia* nauplii with the probiotic yeast *Saccharomyces boulardii* and its resistance against a pathogenic *Vibrio*

S.K. PATRA and K.S. MOHAMED*

*Central Marine Fisheries Research Institute, PO Box 1603, Tatapuram P.O., Cochin – 682014, Kerala, India; *Author for correspondence (e-mail: mfdcmfri@vsnl.com; phone: +91 484 2394867; fax: +91 484 2394909)*

Received 20 February 2002; accepted in revised form 17 April 2003

Key words: *Artemia* nauplii, Challenge, Enrichment, Pathogen, Probiotic, *Saccharomyces boulardii*, *Vibrio*

Abstract. Enrichment of *Artemia* nauplii with a known probiotic yeast *Saccharomyces boulardii* (SB) and its role in enhancing resistance against the pathogen *Vibrio harveyi* was investigated. SB was cultured, then fed to instar II *Artemia* nauplii in three dierent treatments; 10^2 (T1), 10^3 (T2) and 10^4 (T3) colony forming units (CFU) per ml in triplicate. The algae *Nanochloropsis* sp. was used as control diet. Survival and total count of CFU nauplii^{-1} was observed on dierent media (Sabouraud, for enumerating yeasts, thiosulphate citrate bile salts sucrose, for enumerating *Vibrio* and seawater agar, for enumerating total aerobic flora) for each replication. Enhanced survival of nauplii was observed in treatments as compared to control. Results indicated that enrichment of SB in *Artemia* nauplii proceeded in a linear fashion, and up to 3500 CFU of SB could be detected in one nauplii at 10^4 CFU ml^{-1} treatment. No conclusive trend could be observed in the count of *Vibrio* and total aerobic flora due to treatment. Enriched nauplii were then challenged with the pathogen *V. harveyi* for 24 and 48 h at a concentration of 6.1×10^6 CFU ml^{-1}. The survival counts at 48 h showed that the resistance of the nauplii was significantly ($P < 0.01$) improved in those fed with 10^4 CFU ml^{-1} SB (90% survival rate after 48 h of challenge versus less than 40% for the infected control group without SB and treatments T1 and T2). This study shows that SB, which has been used for the first time in an aquatic live feed organism, has a profound beneficial eect on the nauplii by increasing its resistance to a pathogenic *Vibrio* infection.

Introduction

Artemia nauplii are widely recognized as the best natural storable live feed available, and are widely used in marine finfish and crustacean hatcheries around the world because of their nutritional and operational advantages (Lavens and Sorgeloos 1986). They have been used as a vector for the delivery of dierent materials, such as nutrients (Watanabe et al. 1983), antimicrobial agents (Dixon et al. 1995), vaccines (Campbell et al. 1993), and probiotics (Gatesoupe 1994) and all these may have a positive eect on host organism by improving properties of the native microflora. This positive eect of probiotics may be attributed to its ability to compete with other opportunistic bacteria or produce micronutrients important for development of larvae (Sugita et al. 1991; Ringo et al. 1992) or possession of antibacterial/bactericidal properties.

Studies (Gatesoupe 1991, 1993) have been made on the ecacy of *Artemia* nauplii in bioencapsulating bacteria and this indicates that it strongly depends on the type of bacteria used, time of exposure, and status (live or dead) of the bacteria.

Gatesoupe (1993) has also shown that uptake of spores of the bacteria (*Bacillus* IP 5832) by the rotifer usually takes place within an hour and is then digested during the following hours. This probiotic treatment modified the rotifer flora and increased the resistance of turbot larvae challenged with a pathogenic *Vibrio*. Makridis et al. (2000) reported the change in composition of bacterial microflora of the live food organisms (rotifer and *Artemia*), with the bioencapsulated strains comprising up to 100% of the total colony forming units (CFU).

Saccharomyces boulardii (SB) is well known as a human probiotic yeast, isolated from lychee fruits, which is active against *Clostridium dicile* (Buts et al. 1993) and *Vibrio cholera* toxin (Vidon et al. 1986; Czerucka et al. 1994). This yeast is well suited as a probiotic agent because it is able to achieve high concentration in the colon quickly, maintain constant levels, does not permanently colonize the colon, and does not translocate easily out of the intestinal tract compared to other colonic flora (McFarland and Bernasconi 1993). It is also eective and safe for oral ingestion in case of adults as well as children infected with acute diarrhoea. Although it is used as both a preventive and as a therapeutic agent in human beings and farm animals, its eects has not been studied in aquatic organisms so far. *Vibrio* induced mortality are common in crustacean and finfish hatcheries. The fact that SB is eective against *V. cholera* toxin prompted us to examine whether SB would be equally eective against marine pathogenic vibrios. Besides, the availability of suitable probiotic species for use in shrimp and fish hatcheries is limited.

The aims of the present study were: (i) to decide the maximum level of enrichment of *Artemia* nauplii by the known probiotic yeast SB, (ii) to examine changes in other micro flora resulting from such treatment, and (iii) to investigate its role in enhancement of resistance against a pathogenic *Vibrio*, by means of a challenge test.

Materials and methods

Probiotic

Pure cultures of the yeast SB were reisolated on Sabouraud broth (HiMedia Laboratory, India) from dried powder in gelatine capsules available from Laboratories Biocodex, France, and stored at 4 °C. Characterization of SB was carried out by means of the Api 20C Aux (Bio Merieux, France) test. The yeast was cultured at room temperature (25–28 °C) with 10% innoculum, in Sabouraud broth under continuous agitation. The concentration of the yeast was determined by counting total CFU on Sabouraud Agar (Pothoulakis et al. 1993) after incubation at 37 °C for 48 h.

Artemia nauplii

Artemia franciscana (San Francisco Bay brand) cysts were decapsulated, incubated for 28 h, at 28 °C in 33 ppt salinity seawater under strong illumination (2000 lux) and aeration (Lavens and Sorgeloos 1986). After 28 h post-hatch, instar II *Artemia*

nauplii (600 µm average length) were harvested by exploiting the positive phototactic behaviour of the nauplii.

Enrichment treatments

To determine experimental treatment concentrations, instar II nauplii of *Artemia* were fed with SB concentration ranging from 10^2 to 10^5 CFU ml^{-1} and their survival was assessed after 24 h. Above 10^4 CFU ml^{-1} concentration, the survival of *Artemia* nauplii was very low due to the polluting nature of the media and hence concentrations ranging from 10^2 to 10^4 were selected for the experiments. Instar II *Artemia* nauplii were stocked at rate of 20 nauplii ml^{-1} in total volume of 2 l water in 3 l capacity glass containers and aerated continuously. Nauplii were fed with *Nanochloropsis* sp. at concentration of 10^6 cells ml^{-1} in control. The three dierent treatments (T1, T2 and T3) were 10^2, 10^3 and 10^4 CFU ml^{-1} of SB supplemented with 10^4, 10^3 and 10^2 cells ml^{-1} of *Nanochloropsis* sp. respectively, enriched for 24 h in triplicate. Enrichment feed was delivered in two doses at 12 h intervals. Two trials of enrichment were conducted.

Sampling

Mean survival in control and the three dierent treatments were estimated separately after 24 h enrichment. Later 1 ml well mixed samples (enriched nauplii, with counts varying from 13 to 30 per ml) were taken in sterilized vials of 5 ml capacity from each replication for bacteriological sampling.

During bacteriological sampling, nauplii were rinsed with sterile saline water (18 ppt) and homogenized in 5 ml sterile saline water. Serial dilutions of the homogenate in sterilized saline water were spread plated on dierent media, namely, Sabouraud plates for enumerating yeasts, seawater agar (SWA, HiMedia Laboratories Pvt. Ltd., Mumbai, India; composition in $g\,l^{-1}$: Part A contained yeast extract, 5; peptone, 5; beef extract, 3; agar, 15; pH 7.2 ± 0.2 and Part B contained sodium chloride, 24; potassium chloride, 0.7; magnesium chloride, 5.3; magnesium sulphate $7H_2O$, 7; pH 7.2 ± 0.2) plates for enumerating total aerobic flora and thiosulphate citrate bile salts sucrose (TCBS, HiMedia Laboratories Pvt. Ltd., Mumbai, India; composition in $g\,l^{-1}$: yeast extract, 5; protease peptone, 10; sodium thiosulphate, 10; sodium citrate, 10; Ox bile, 8; sucrose, 20; sodium chloride, 10; ferric citrate, 1; bromothymol blue, 0.04; thymol blue, 0.04; agar, 15; pH 8.6 ± 0.2) plates for enumerating vibrios. Sabouraud plates were incubated at 37 °C for 24–48 h, whereas SWA and TCBS plates were incubated at room temperature (25–28 °C) for 16–20 h respectively. CFUs and CFUs/nauplii were counted after incubation for each replication.

Challenge test

After enrichment, control and treatment nauplii of trial 2 were challenged with *Vibrio harveyi*, a luminous strain identified as pathogenic in shrimp hatcheries

(Lavilla-Pitogo et al. 1990). The identification was made based on keys provided by West and Colwell (1984). The challenge was made at a rate of 5 nauplii ml^{-1} in a total of 200 ml water for 48 h in triplicate and without aeration. *V. harveyi* was grown in nutrient broth with NaCl enrichment (3%) and up to 10^8 CFU ml^{-1} was obtained within 24 h. The concentration of *V. harveyi* dispensed was 6.1×10^6 CFU ml^{-1} in each challenge bottle. The survival was counted by sampling the nauplii after 24 and 48 h of challenge.

Statistical analysis

Treatment means were compared by one-way analysis of variance (ANOVA) and in the case of enrichment experiments, two way interactions between treatment and colony counts were made using two-way 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 8.0).

Results

Standardization of SB culture for enrichment

With 10% innoculum in Sabouraud broth under constant agitation, SB grew to 10^6 CFU ml^{-1} within 48–72 h. Spread plates revealed uniform o-white round colonies. The colour of the broth at this time was pale white. The Api 20C Aux coding obtained was 6000072 corresponding to SB.

Enrichment experiment – survival of nauplii

After 24 h of enrichment, the survival of *Artemia* II instar nauplii was uniformly above 85% in all experimental treatments in trial 1 (Figure 1). In the same trial, the control jars showed relatively less survival and the dierence was statistically significant ($P < 0.01$). The treatment T2 showed marginally higher survival than trial 1. Uniformly high survival was noticed in all treatments in trial 2 (Figure 1) and the dierences in survival means were not significant ($P > 0.05$).

Bacterial counts

Trial 1. SB counts per nauplii in treatments T1, T2 and T3 showed an increasing trend (Figure 2). At an enrichment level of 10^4 CFU ml^{-1} it was possible to detect up to 35×10^2 CFU in each nauplii in T3 treatment. Dierences in SB counts were statistically significant ($P < 0.01$). CFU on TCBS agar (Figure 2) were not significantly dierent ($P > 0.05$). Both yellow and green colonies were detected on

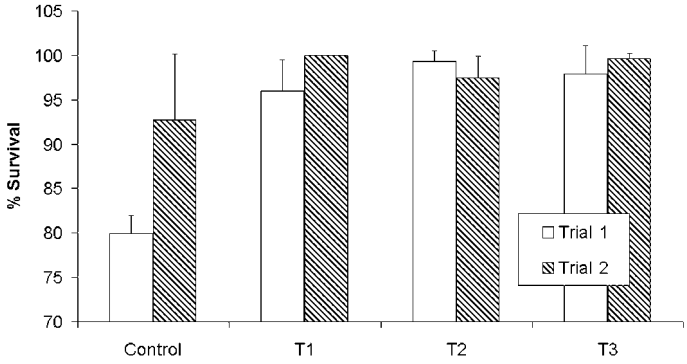

Figure 1. Survival of instar II *Artemia* nauplii after 24 h enrichment with SB in dierent treatments and control of trial 1 and 2. Error bars indicate standard error (SE).

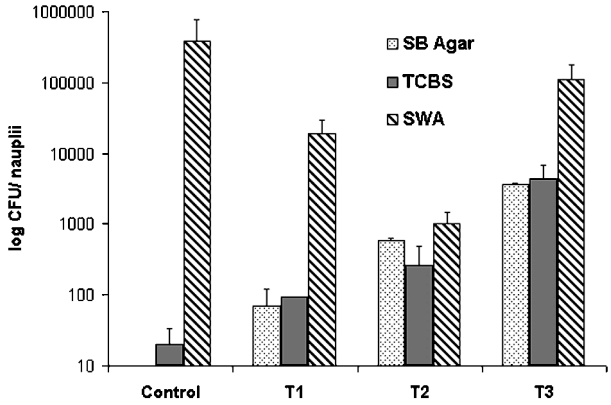

Figure 2. Mean bacterial counts of yeasts (SB agar), vibrios (CFU on TCBS) and aerobic flora (SW agar) after 24 h enrichment in dierent treatments and control in trial 1. Error bars indicate SE.

TCBS agar. The total aerobic flora showed large variation between treatment and replicates (Figure 2) and the mean colony counts were not significantly ($P > 0.05$) dierent.

Trial 2. In this trial also the treatments T1, T2 and T3 showed increasing level of SB counts (Figure 3). In T3 the level of SB was 22×10^2 CFU nauplii^{-1}. The SB counts among treatment groups were significantly ($P < 0.01$) dierent. Similar to trial 1, the CFU on TCBS agar were relatively low in control, T1 and T2, while it was high in T3 treatment. Both green and yellow colonies were present, and generally, yellow colonies were predominant in both control and treatments. Unlike trial 1, the total aerobic flora counted on SWA plates were significantly ($P < 0.01$) less in treatments T1 and T2 as compared to control and treatment T3. Many dierent types of strains were observed on SWA plates.

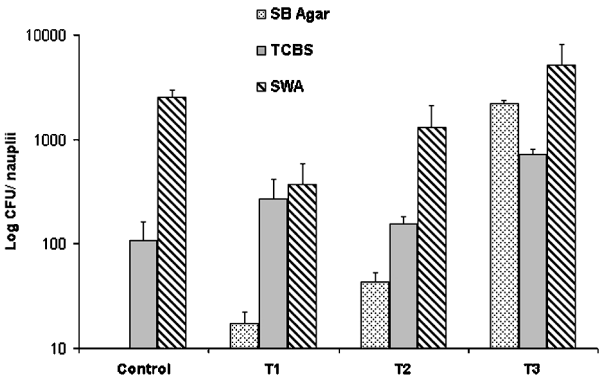

Figure 3. Mean bacterial counts of yeasts (SB agar), vibrios (CFU on TCBS) and aerobic flora (SW agar) after 24 h enrichment in dierent treatments and control in trial 2. Error bars indicate SE.

Table 1. Mean survival and SE of SB enriched II instar *Artemia* nauplii after challenge with the pathogen *V. harveyi* in 24 and 48 h. Non-identical superscripts within rows indicate significant dierences ($P < 0.05$).

Period	Control	T1	T2	T3
24 h				
Mean	80.0[a]	90.7[a]	88.0[a]	96.0[a]
SE	16.0	9.4	6.1	4.0
48 h				
Mean	33.4[a]	32.0[a]	26.7[a]	90.7[b]
SE	9.4	10.6	7.4	4.8

Challenge experiment

After challenge with the pathogen *V. harveyi* for 24 h, the dierences (Table 1) in mean survival among treatments were not significant ($P > 0.05$). After 48 h challenge, there was considerable mortality in control and treatments T1 and T2 (less than 40% survival), while in T3 treatment mortality due to challenge was minimal (90% survival). The dierence in survival means was statistically significant ($P < 0.01$).

Discussion

Use of SB as probiotic has not been reported previously in aquatic animals. However, the beneficial eect of SB in humans and farm animals has been reported by several workers (Buts et al. 1993; McFarland and Bernasconi 1993; Czerucka et al. 1994). In the present study the use of Sabouraud agar and broth for culture of SB proved to be adequate for mass culture. Similar results were observed by

Pothoulakis et al. (1993). A yeast extract–peptone–dextran medium with moderate shaking was used to obtain SB culture in good quantity by Czerucka et al. (1994). The present study shows that under tropical conditions and at 37 °C luxuriant (up to 10^6 CFU ml^{-1}) growth of SB was obtained within 48 to 72 h in broth culture with constant shaking. Besides, it was possible to standardize the colour of broth culture to the concentrations of CFUs. One of the primary reasons for selecting SB for use as an aquatic probiotic was that earlier reports indicated that SB has been used extensively in patients infected with *V. cholera* toxin (Czerucka et al. 1994). Since many of the larvae of aquatic organisms are prone to *Vibrio* induced mortality it was presumed that the SB colonized in the gut of the organisms could prevent action of other *Vibrio* toxins too. SB has also been shown to inhibit secretion induced either by cholera toxin in jejunum or enterotoxinogenous *Escherichia coli* in new born mice (Vidon et al. 1986). Furthermore, SB has been reported to help in the prevention of antibiotic associated diarrhoea (McFarland and Bernasconi 1993). This fact assumes significance in aquatic larval rearing where the use of antibiotics in larval rearing medium is reportedly very high (Mohamed 1995).

Artemia enrichment or bioencapsulation is widely applied in marine fish and crustacean hatcheries all over the world for enhancing the nutritional value of *Artemia* with essential fatty acids (Dhert et al. 1993) and for drug delivery (Aguila et al. 1994). Because of its primitive feeding characteristics, it allows a very convenient way to manipulate its biochemical composition by exploiting the non-selective feeding behaviour in instar II *Artemia* prior to oering them to the predator larvae. Similarly, *Artemia* nauplii and rotifers are also being used for the delivery of probiotics to fish larvae (Gatesoupe 1994), and shrimp larvae (Rengpipat et al. 1998). In the present study, two trials were conducted to enrich instar II *Artemia* nauplii wherein both control and treatments received 10^6 cells ml^{-1} of either algae or algae and yeast combined. Survival of *Artemia* nauplii up to 85% in all treatments was observed where as, control showed relatively less survival than treatments in both trials. Earlier, Intriago and Jones (1993) have reported that *Artemia* grown on a diet of bacteria (*Flexibacter* sp.) alone showed a survival of 88% from nauplii to pre-adults within 8 days.

Both control and experimental treatments in the present experiments were tested for the presence of SB colonies and the result showed that SB counts per nauplii showed an increasing trend as concentration increased, in both trials. At an enrichment level of 10^4 CFU ml^{-1} it was possible to detect up to 35×10^2 CFU $nauplii^{-1}$ (range – 22×10^2 to 37×10^2 CFU $nauplii^{-1}$). In an experiment where turbot larvae were fed with LAB (lactic acid bacteria) enriched rotifers, counts of 10^4 to 10^5 CFU per turbot larvae were retrieved in the groups fed with the increasing levels of LAB (Gatesoupe 1994). But no LAB colonies were detected for control group without LAB. Higher amounts of LAB could be enriched into *Artemia* nauplii than SB. This could be due to the larger size of the yeast cells. Further, the duration of enrichment also determines the level of enrichment. CFU on TCBS, especially in trial-1, showed a SB dose dependant increase. A comparable increase in *Vibrio* counts on TCBS plates was observed in turbot larvae after 24 h enrichment as concentration of LAB increases (Gatesoupe 1994). In contrast, total aerobic flora counts in T1 and T2 in the

present study, were significantly less compared to control and T3 in trial-2 but there was no significant dierence in trial-1. High degree of bacterial variability has been reported earlier in crustacean digestive tracts (Dempsey et al. 1989), making it dicult to draw definite conclusions.

Makridis et al. (2000) reported that it is possible after a short term incubation, to replace opportunistic bacteria present in the live food cultures with other bacteria, which persist as a dominant part of the bacterial flora of the live food (*Brachionus* and *Artemia*) for a relatively long period of time (4–24 h). In contrast, no decrease in total floral counts of cod-larvae was noticed among treatments after enrichment of LAB directly into the rearing medium (Strom and Ringo 1993). The results obtained in the present study also indicate that SB does not significantly change the composition of both vibrios and other aerobic flora in *Artemia* nauplii. In a study on healthy human volunteers given 1 g SB/per day no significant changes in selected population of normal colonic flora after 4–5 day exposure to the yeast was reported (Klein et al. 1993). When there is an overgrowth of pathogenic organisms, SB has been shown to reduce concentrations of several aetiological agents of diarrhoea or their associated toxins (McFarland and Bernasconi 1993). More trials have to be carried out to confirm this advantage in aquatic organisms.

When 24 h enriched nauplii of dierent treatments and control were challenged with the pathogen *V. harveyi* for 48 h, the results showed less than 40% survival in control, T1 and T2, compared to 91% survival in T3. However, the same challenge after 24 h did not show any significant dierence in survival. Similarly, the survival rate of turbot larvae fed with rotifers enriched with LAB and then challenged with a *Vibrio* pathogen for 72 h was higher than treatment without LAB (Gatesoupe 1994). According to his study, 53% survival rate was observed after 72 h of challenge in treatments of concentration between 10^7 and 2×10^7 CFU ml^{-1} LAB versus 8% for the infected control group, without LAB. In the present study, the *Vibrio* concentration used for the challenge was 6.1×10^6 CFU ml^{-1}, which is comparable to the *Vibrio* concentrations between 4×10^5 and 2×10^6 CFU ml^{-1} used by Gatesoupe (1994). The challenge results in the present study indicate that enrichment of *Artemia* II instar nauplii with SB at the level of 10^4 CFU ml^{-1} (T3) helped the nauplii to surmount an artificial *Vibrio* infection proving the probiotic value of SB in invertebrate larval rearing. The levels of enrichment in treatments T1 and T2 were not sucient to withstand the infection in 48 h. Therefore, it can be concluded that at least 2000 CFU nauplii $^{-1}$ of SB colonies are necessary to overcome a *Vibrio* infection in *Artemia* nauplii.

Research in probiotics for aquaculture is at an early stage of development and much work is still needed, as the available information is inconclusive (Gomez-Gil et al. 2000). The multitude of organisms and techniques used in larviculture, in addition to the increasing number of potential probionts necessitates experimental trials in all possible host–target combinations before critical assessments can be made. *Artemia* metanauplii are a widely used live feed organism in larviculture and have the potential to be used as a means for probiotic feeding of active prey-capturing larvae.

In conclusion, this study shows for the first time that the yeast SB a universally used probiotic in higher animals, is equally eective in an aquatic live feed organism

like *Artemia* nauplii to overcome *Vibrio* infections. Besides at an enrichment level of 10^4 CFU ml^{-1} these *Artemia* nauplii can be used as a vehicle to deliver probiotic to other aquatic larvae feeding on them.

Acknowledgements

The authors are grateful to the International Foundation for Science, Sweden for a research grant (A-2713/1) with which this work was carried out. They are thankful to Dr. F.J. Gatesoupe, IFREMER, Brest, France for providing gelatine capsules of the probiotic yeast. Dr. I.S.B. Singh, CUSAT, Cochin kindly provided the pathogenic strain and helped to identify it. We also thank Dr. K.K. Appukuttan, CMFRI, Cochin and two anonymous reviewers for critical comments on the manuscript.

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