

USE OF *PSEUDOMONAS STUTZERI* AND *CANDIDA UTILIS* IN THE IMPROVEMENT OF THE CONDITIONS OF *ARTEMIA* CULTURE AND PROTECTION AGAINST PATHOGENS

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ABSTRACT

To evaluate the effect of two bacterial strains isolated from *Artemia* cysts and yeast (*Candida utilis*) on the survival, growth and total biomass production of its larvae, challenge tests were performed with *Candida utilis*, *Pseudomonas stutzeri* and *Pasteurella haemolytica*. In addition, a pathogenic strain of *Vibrio alginolyticus* was tested for comparative purposes.

Pseudomonas stutzeri and *Candida utilis* have no impact on survival, but enhance growth and total biomass production of the larvae. However, we noted that *Pasteurella haemolytica* affect negatively *Artemia* larvae. The adhesion and antagonism assay demonstrates that *Candida utilis* and *Pseudomonas stutzeri* are fairly adherent and play an important role in the enhancement of the protection of *Artemia* culture against pathogens. On the basis of these results, it's suggested that it's possible to use *Candida utilis* and *Pseudomonas stutzeri*, potential candidates, as probiotic for the culture of *Artemia* larvae.

Key words: *Artemia*, Probiotics, Adhesion, Yeast, Bacteria.

INTRODUCTION

One of the most significant advances in aquaculture farms and industrial hatcheries has been the use of *Artemia* as a food for marine finfish and crustacean around the world because of their nutritional profiles (15). It is presumed that *Artemia* nauplii have been used as a vector for the delivery of different nutrients (24), antibiotics (35) and probiotics (9, 24). However, these feeds can be a source of pathogenic bacteria in aquaculture farms (34) like the genus of *Vibrio* and *Pasteurella*, such as *Pasteurella haemolytica* which is one of

the most common bacterial pathogen involved in veterinary diseases such as pneumonic pasteurellosis in sheep all over the world (30). It is responsible of much mortality in hatchery environments, in animal farming industry and it considered as an aetiological agent of fish pasteurellosis (13, 28, 25). For these reasons, it is necessary to study the bacterial interaction with crustaceans in order to distinguish between pathogens and potential probiotic candidate, because it is found that bacteria had a beneficial effect such as providing nutritional elements like vitamins, essential amino acid, fatty acids and enzymes (10, 36). Other microbiological studies have demonstrated that

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Bacillus sp. and *Aeromonas hydrophila* can protect *Artemia* against pathogens such as *Vibrio proteolyticus* and *Vibrio campbelli* (19).

Some yeast strains were used in the hatchery farms. Indeed, a high percentage of survival (100 % after 96 hour and more than 80 % after 144 hour) was obtained when autoclaved baker's yeast was used as food (23). *Saccharomyces baulardii* has been also utilized and it's found that this yeast can enhance protection of enrichment *Artemia* nauplii against *Vibrio sp.* (24). Recently, Marques *et al.* (19) used *Saccharomyces cerevisiae* and demonstrated that this yeast can provide protection against different pathogens. This promising source of protein can also supply the feed with vitamins, mineral and other components, which could stimulate the resistance of aquatics animals (26). This study aimed to investigate the effects of *Candida utilis* and *Pseudomonas stutzeri* on the *Artemia* culture and to evaluate their role in the enhancement of *Artemia* nauplii resistance to *Pasteurella haemolytica* and *Vibrio alginolyticus*.

MATERIALS AND METHODS

Isolation and identification of bacterial strains

Bacterial strains associated with *Artemia* cysts were isolated according the following procedure: 0.01 g of *Artemia* cysts recovered from Sebkhath El Adhibet in the South East of Tunisia (33°44 N – 10°46 E) were incubated in 60 ml sterile falcons containing 30 ml of filtered and autoclaved sea water (FASW) (salinity 34 g l⁻¹ and pH 7.99) at Shaker incubator (28°C, 120 rpm) and exposed to constant incandescent light of Shaker (Lab-Line Orbit Environ-Shaker). Under these conditions, hatched larvae appeared after 18 to 20 hours of incubation. Water samples (1 ml) were enriched 24 h at 30°C in nutrient broth sea water (NBSW) (salinity 34 g l⁻¹ and pH 7.99) and spread on nutrient agar plate and incubated at 30°C. The most appeared colonies were reisolated on Petri plates with nutrient agar. Gram negative rods were identified using Rapid NF Plus strips (Remel, USA) according the manufacture's recommendation.

Isolation and identification of yeast strain (*Candida utilis*)

Pure culture of *Candida utilis* used in this study was isolated on Sabuoraud agar plate from the American biomass known as "Kambautcha" in our Laboratory. Biochemical characterization of *Candida utilis* was carried out by Api 20 C Aux (Bio Merieux, France) test.

Well diffusion agar assay (WDAA)

Potential probiotic strains were tested for their antagonistic activity using the well diffusion agar assay (WDAA) (34) against three target strains: *Vibrio parahaemolyticus* ATCC17802, *Vibrio alginolyticus* ATCC17749, and *Vibrio alginolyticus* (S1) isolated from infected fish previously described by Ben Kahla-Nakbi *et al.*(2). The pathogenic bacteria were grown in 10 ml of nutrient broth and cultured for 24 hours on nutrient agar at 30°C. The common colonies from pure culture were suspended in 10 ml of physiological medium and well mixed during 5 min. One ml was spread over the agar plates. Potential probiotic strains were cultured in 10 ml nutrient broth for 24 hours, 100 µl of the supernatant were introduced into the wells of the MH agar medium and incubated for a period of 24 h at 30°C. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the well.

Adhesion assay

Adhesion ability of *Pseudomonas stutzeri* and *Candida utilis* grown in Trypticase Soy broth (TSB, Bio-Rad, France) was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark), as described previously (6, 18) with some modifications. Following overnight incubation at 37°C, the optical density at 620 nm (OD₆₂₀) of these strains was measured. An overnight culture grown in TSB at 37°C was diluted to 1:100 in TSB with 2% (w/v) glucose. A total of 200 µl of these cell suspensions was transferred in a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). Each strain was tested in triplicate. The plates were incubated aerobically at 37°C for 24 h. The cultures were removed and the microtiter wells were washed twice with phosphate-buffered saline (7 mM Na₂HPO₄,

3 mM NaH₂PO₄ and 130 mM NaCl at pH 7.4) to remove non-adherent cells and were dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µl of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 620 nm (OD₆₂₀) using an automated Multiskan reader (GIO. DE VITA E C, Rome, Italy). Adhesion ability was interpreted as highly (OD₆₂₀ ≥ 1), fairly (0.1 ≤ OD₆₂₀ < 1), or slightly (OD₆₂₀ < 0.1).

Inoculation of yeast and bacterial suspensions

Sterile 60 ml falcons were inoculated with bacterial strains and yeast (10⁶ CFU ml⁻¹) from pure culture in the exponential growth phase. The concentration of each bacterial strain and yeast was estimated through a regression analysis of the optical density of the pure culture. The number of CFU ml⁻¹ was determined using Petri plates with marine agar.

Artemia gnotobiotic culture

Experiments were performed with *Artemia* cysts, collected from Sebkhath El Adhibet in the South East of Tunisia (33°44 N – 10°46 E). Bacteria-free cysts and nauplii were obtained via decapsulation as described by Sorgeloos *et al.* (32). Decapsulated cysts were washed with filtered and autoclaved sea water (FASW) over a 50µm sterile filter net. This procedure was repeated nine times, using new filtered and autoclaved sea water (FASW). After this step, washed decapsulated cysts were transferred to a sterile falcon containing 30 ml of FASW. The Falcon was capped and placed on Shaker incubator (28°C, 120 rpm) and exposed to constant incandescent light of Shaker. After 18–20 h, ten axenic nauplii were picked and transferred to sterile falcons containing 30 ml of FASW, together with the amount of feed scheduled for day one. All manipulations were carried out under sterile conditions.

Experimental design

To evaluate the effect of the bacterial strains on *Artemia* culture, twelve challenge tests were performed. The first test was *Artemia* alone (Axe), the second was *Artemia* with *Candida utilis* (Art+Y). Tests 3, 4 and 5 were made with three bacterial strain: PS : *Pseudomonas stutzeri* (Art+y+PS); PH : *Pasteurella haemolytica* (Art+Y+ PH); VA : *Vibrio alginolyticus* (Art+y+ VA). Test 6, 7 and 8 were carried out using *Candida utilis* and the same three bacterial strains: (Art+Y+PS); (Art+Y+ PH) and (Art+Y+ VA). Tests 9 and 10 were made using a mixture of these bacterial strains: (Art+PS+PH); (Art+PS+VA), during tests 11 and 12 we added to the mixture of the bacteria used, the yeast *Candida utilis*: (Art+Y+PS+PH); (Art+Y+PS+VA). All challenge tests were made in two experiments: Experiment 1, Y and PS were added only at first day and PH and VA were provided at day three. Experiment 2, Y and PS were added during the first three day and pathogenic bacteria strains were provided only at day 3. *Vibrio alginolyticus* strain (S1) used for comparatives purposes was isolated from infected fish (2). Treatment of *Artemia* with *Candida utilis* (Art +Y) was chosen as a control treatment in the two experiments because larvae survival showed a higher percentage after 6 days of culture.

Methods used to verify axenity

At the beginning and end of each run of the procedure, absence of bacteria was monitored by transferring larvae and 100 µl of water to Petri plates in triplicate with marine agar.

Survival, growth and total biomass production of *Artemia*

During the challenge tests, the number of swimming larvae (survival percentage) was daily determined and the percentage of survival was calculated. At the end of each experiment, alive *Artemia* were fixed with lugol solution to measure their individual length (IL) under a binocular magnifying glass containing a graduated micrometer. As a criterion that combines both the effects of survival and IL, the total biomass production (TBP) was determined according to the following formula (19): TBP (millimeters per Falcon — mm / F) = number of survivor's × means IL.

STATISTICAL ANALYSIS

Values of survival larvae (percentage) were arcsine transformed, while values of IL and TBP were square root transformed to satisfy normal distribution and homoscedasticity requirements and differences on survival, individual length and total length of *Artemia* under different treatments were investigated with analysis of variances (ANOVA) and with Duncan's test using the Statistica 5.5 software.

RESULTS

Identification of bacterial strains

Bacterial strains isolated from *Artemia* cysts were identified as *Pseudomonas stutzeri* (PS) and *Pasteurella haemolytica* (PH).

Adherence and antagonisms assay

Pseudomonas stutzeri and *Candida utilis* are fairly adherent and showed inhibitory effect against *Vibrio parahemolyticus* ATCC 17802, *Vibrio alginolyticus* ATCC 17749, and *Vibrio alginolyticus* (S1). The diameters of the inhibitory zones around the growth of pathogenic strains tested were about 12-34 mm (Table 1 and 2).

Table 1. Adhesion ability

Strains	Adhesion value
<i>Pseudomonas stutzeri</i>	0.5 ± 0.09
<i>Candida utilis</i>	0.104 ± 0.007

OD₆₂₀ ≥ 1: highly adherent, 0.1 ≤ OD₆₂₀ < 1: fairly adherent, OD₆₂₀ < 0.1: slightly adherent.
For each average, the respective standard deviation is added (mean ± S.D.).

Table 2. Antagonism assay

Inhibitory zone of a potential candidates probiotic (mm)

Pathogens <i>vibrio</i>	<i>Pseudomonas stutzeri</i>	<i>Candida utilis</i>
<i>Vibrio parahemolyticus</i> (ATCC 17802)	26.6 ± 0.3 mm	22.3 ± 0.11mm
<i>Vibrio alginolyticus</i> (ATCC 17749)	25.6 ± 0.4 mm	23.6 ± 0.05 mm
<i>Vibrio alginolyticus</i> (S1)	12.3 ± 0.05 mm	13.6 ± 0.05 mm

For each average, the respective standard deviation is added (mean ± S.D.).

Larval survival

During the first experiment, larval survival rates resulted in 0% on culture day 5 in treatment *Artemia* axenic (Art axe) (Fig. 1A). The addition of yeast (Y) on *Artemia* culture resulted in higher survival rate (76% at the end of culture day 6). Treatment “*Artemia* with PS” resulted in low survival rate (8% on culture day 5) (Fig. 1A) and showed difference with control treatment (Art + Y) ($P < 0.05$). When yeast (Y) and bacteria (PS) were used, *Artemia* culture presented a high survival rate and no significant difference was found in comparison to the

control treatment ($P > 0.05$) (Fig. 1A). In presence or absence of yeast, *Artemia* nauplii challenged with PH and VA resulted in a low larval survival (0 % on culture day 5). Compared with control treatment, a significant difference was improved ($P < 0.05$) (Fig. 1B). Treatments *Artemia* with bacteria (Art+PS+PH and Art+PS+VA) and treatment with yeast and bacteria (Art+Y+PS+PH and Art+Y+PS+VA) resulted in low survival rate (0% on culture day 5) and no additional protection against PH and VA was noted in comparison to the control treatment ($P < 0,05$) (Fig.1C).

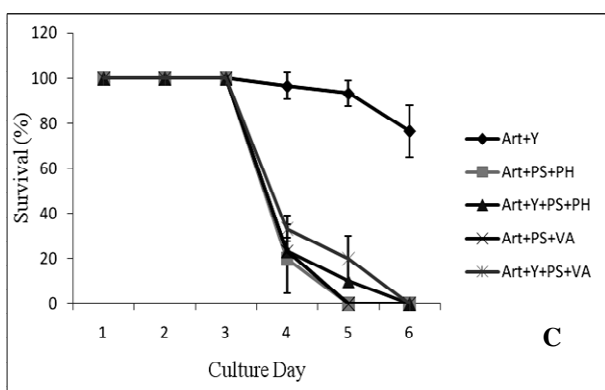
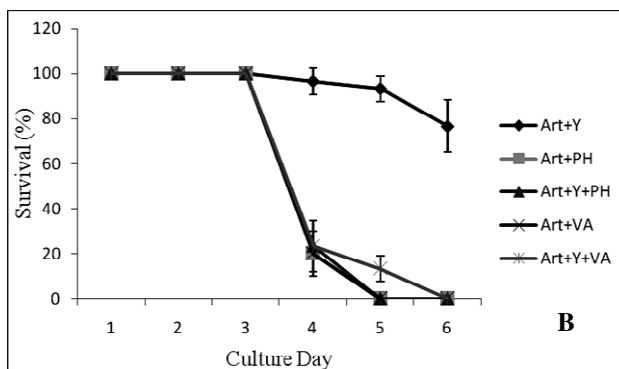
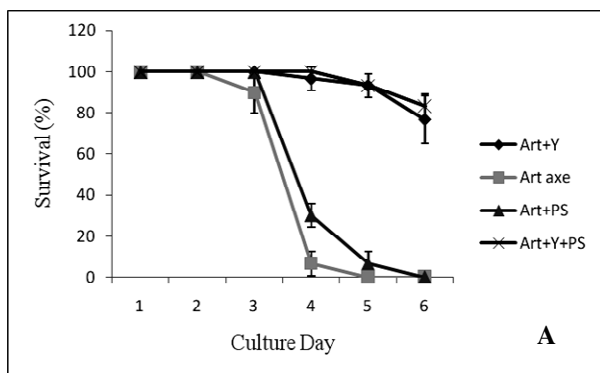


Figure 1. (Experiment 1) Survival of *Artemia* larvae after various treatments: (A) *Artemia* with yeast (Art + Y); *Artemia* axenic (Art. axe); *Artemia* with bacteria (Art + PS); *Artemia* with yeast and bacteria (Art + Y + PS), (B) *Artemia* with yeast (Art + Y); *Artemia* with bacteria (Art + VA); *Artemia* with yeast and bacteria (Art + Y + VA), (C) *Artemia* with yeast (Art + Y); *Artemia* with bacteria (Art + PS+PH; Art+PS+VA); *Artemia* with yeast and bacteria (Art + Y + PS+PH; Art +Y+PS+VA).

In the second experiment, results showed an improvement of survival rate in treatment *Artemia* with yeast (Y), with bacteria (PS) and *Artemia* with yeast (Y) and bacteria (PS), but no significant difference was found ($P > 0.05$) (Fig. 4 A and B). It was demonstrated that when *Artemia* nauplii were fed during

the first three days and challenged with PH and VA, the survival rate was not affected under treatments when yeast (Y) was used only or associated with bacteria (PS). No significant difference in comparison with control treatment was noted ($P > 0.05$) (Fig. 4 A, B and C).

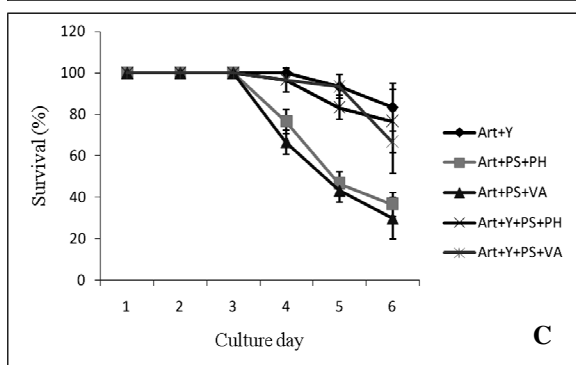
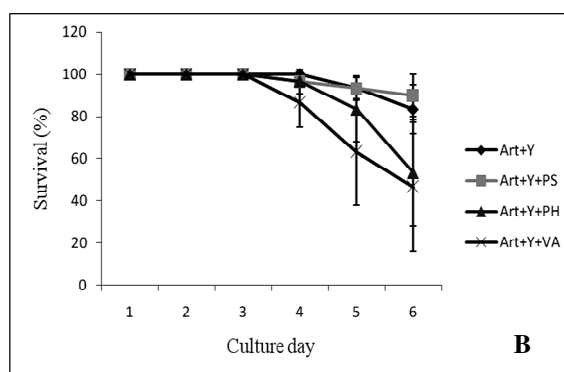


Figure 4. (Experiment 2) (A) *Artemia* with yeast (Art + Y); *Artemia* axenic (Art. axe); *Artemia* with bacteria (Art + PS; Art+PH). (B) *Artemia* with yeast (Art + Y); *Artemia* with yeast and bacteria (Art + Y + PS; Art +Y +PH; Art +Y +VA). (C) *Artemia* with yeast (Art + Y); *Artemia* with bacteria (Art + PS+PH; Art+PS+VA); *Artemia* with yeast and bacteria (Art + Y + PS+PH; Art +Y+ PS+VA).

Larval development

In experiment 1, under axenic conditions, *Artemia* nauplii have an individual length ranged between 0.45 and 0.55 mm. In treatment “*Artemia* with yeast (Art + Y)”, larvae reached a length varied between 0.9 – 1.2 mm. When *Artemia* was treated with *Pasteurella haemolytica*, in presence or in absence of yeast, the length of dead larvae was between 0.6 – 0.8 mm and a significant difference with control treatment was improved ($P < 0.05$). The same results were obtained when *Artemia* nauplii was challenged with VA (Fig. 2 A). The

introduction of PS alone in *Artemia* culture increases the development (from 0.48 to 0.8 mm). When it is used in the presence of yeast, the larvae have a length of 1.4 mm and significant differences were found in comparison with the control treatment (Art + Y) ($P > 0.05$) (Fig. 2 A). Results of interaction showed that the two strains, yeast (Y) and bacteria (PS), used separately or associated, did not confer protection for the larvae against pathogens. However, there was a significant difference in comparison with control treatment ($P < 0.05$) (Fig. 2 B).

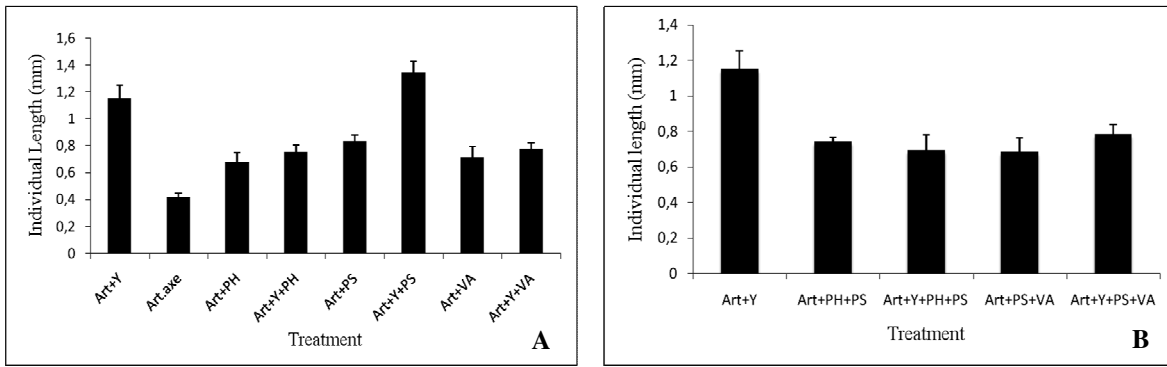


Figure 2. (A) *Artemia* with yeast (Art + Y); *Artemia* axenic (Art .axe); *Artemia* with bacteria (Art + PH; Art + PS; Art+VA); *Artemia* with yeast and bacteria (Art + Y + PS; Art + Y + PH; Art+Y+VA).(B) *Artemia* with yeast (Art + Y); *Artemia* with bacteria (Art + PH+PS; Art+VA+PS); *Artemia* with yeast and bacteria (Art + Y +PH+ PS); (Art + Y +VA+ PS).

During experiment 2, when bacteria (PS) was first used alone and then associated with yeast (Y), an improvement of the growth was demonstrated compared with treatment when PS was added just during the first day ($P < 0.05$). A significant difference was found compared to the control treatment ($P < 0.05$) when *Artemia* was supplied with PS in the presence of yeast. (Fig. 5). The addition of pathogens strains in culture medium enriched with Yeast (Y) didn't affect the growth of larvae which have a length ranging between 1.2 and 1.4 mm when pathogenic strains PH and VA were added. Also, treatment when bacteria PS was supplied with yeast, pathogens didn't affect the growth of *Artemia* larvae which have an individual length ranged between 1.4 and 1.5 mm. A significant difference was found compared with control treatment ($P < 0.05$) (Fig. 5). Compared with the first experiment, results demonstrate an improvement of growth in

all challenged tests and a significant difference were found ($P < 0.05$) (Fig. 2 A and B; Fig. 5).

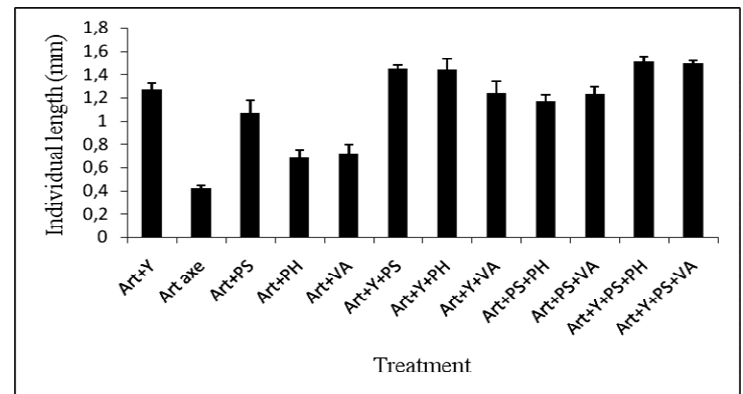


Figure 5. Total length of *Artemia* larvae: *Artemia* with yeast (Art + Y); *Artemia* Axenic(Art .axe); *Artemia* with bacteria (Art + PH; Art + PS; Art+VA; Art + PS + PH; Art + PS + VA); *Artemia* with yeast and bacteria (Art + Y + PS; Art + Y + VA; Art +Y + PH; Art + Y + PS + PH; Art + Y + PS + VA).

Total biomass production

During the first experiment, in treatment *Artemia* with yeast (Art +Y) and bacteria PS (Art + Y+ PS), a light increase in the production of biomass, which is about 10.78 mm/F, was noted and a no significant difference was shown in comparison with the control treatment (Art + Y) ($P > 0.05$) (Fig. 3).

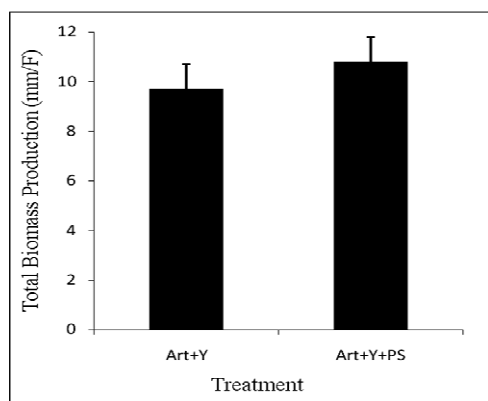


Figure 3. Total Biomass Production of *Artemia* larvae: *Artemia* with yeast (Art + Y); *Artemia* with yeast and bacteria (Art + Y+ PS).

During the second experiment, in all challenged tests when PS was used only, *Artemia* nauplii demonstrate a low TBP, significant difference were found ($P < 0.05$), but the addition of yeast during these challenged tests resulted in an improvement of TPB were found. No significant difference was noted ($P > 0.05$) (Fig. 6).

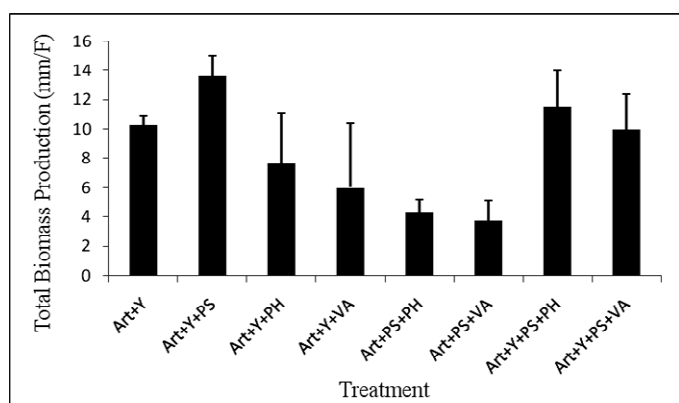


Figure 6. Total Biomass Production of *Artemia* larvae after treatments: *Artemia* with yeast (Art + Y); *Artemia* with bacteria (Art + PS + PH; Art + PS + VA); *Artemia* with yeast and bacteria (Art + Y + PS; Art + Y + PS; Art +Y + PH, Art +Y + VA; Art + Y + PS + PH; Art +Y+ PS + VA).

DISCUSSION

The two identified strains have been isolated from *Artemia* culture and aquatic environments. Sahul Hameed and Balasubramanian, (29), have isolated *Pseudomonas* strains from *Artemia* nauplii. *Pasteurella haemolytica* was also isolated from egg, fry of Mekong giant catfish, water flea, brine shrimp *Artemia*, and from fish farms (*Dicentrarchus labrax*) with a percentage of 50% (25, 28).

The use of *Candida utilis*, for *Artemia* culture showed a positive effect. On the basis of these results, this yeast can be used as a source of nutrients for *Artemia* culture and in the enhancement of protection against pathogens *Pasteurella haemolytica* and *Vibrio alginolyticus*. The protection conferred by *Candida utilis* when it was used for three days can be related to its capacity of adhesion, its ability to inhibit the growth of pathogenic strains and to stimulate the innate immune system. In fact, some components presented in yeast cell wall are likely to boost the non specific immune response in *Artemia* against pathogenic bacteria such as β -glucans (5, 20) and chitin (1). Siwicki *et al.* (31) tested several immunostimulants such as lyophilised *Candida utilis* and *S. cerevisiae*, β -glucans, deacylated chitin on rainbow trout and the most significant stimulations were generally observed with the two yeasts. The improvement of *Artemia* performance can be justified by the better quality of *Candida utilis* which is used for Tilapia culture as a source of protein and classified among the most interesting microorganisms for their protein content, which can account for up to 50% of the dry weight (22, 16). The apparent higher survival rate and resistance of *Artemia* in early developmental stages against pathogenic bacteria could be related to a variety of factors such as fewer attaching sites for bacteria which are available in the naupliar gastrointestinal tract and the presence of certain compounds sustaining naupliar resistance just after hatching. Stabili *et al.* (33) detected lysozyme and proteases in *A. franciscana* cysts and envelope before hatching. Lysozyme is a well-known antibacterial substance (e.g. direct bacteriolytic action, phagocytosis stimulation) in organisms unable to produce immunoglobulins

(37) and proteases are involved in protein digestion and in various aspects of immunology (4).

The positive effect of PS and their potential to inhibit growth of pathogenic strains confirm that PS play an important role for *Artemia* culture. This role can be due to high doses which can induce its artificial and temporary dominance (8, 11), the ability of some microorganisms to adhere to intestinal tract and their effect in the digestive processes of aquatic animals (19, 27). However, in the interaction between *Artemia* and bacteria, a number of factors have been proposed such as the provision of additional digestive abilities and the use of bacterial biomass as food (7, 10, 14). Lone *et al.* (17) found that *Pseudomonas fluorescens* is able to inhibit *Vibrio anguillarum* and can be used as a possible probiotic treatment of fish.

Result obtained in the treatment “*Artemia* with bacterial strains PH and VA”, showed a lower survival and *Artemia* nauplii did not survive for more than 4 days (Fig. 1 A and C). These results can be explained by the negative effect of PH because of the components of extracellular matrix (ECM) such as fibronectin (Fn), collagen (Cn), laminin, and proteoglycans (3, 21). In addition, the attachment of some pathogenic bacteria like *V. alginolyticus* and *V. parahaemolyticus* to the body surface of *Artemia*, causing a deleterious effect, can explain the negative effect of *V. alginolyticus* (12).

In conclusion, this study shows that *Artemia* culture conditions can be controlled using *Candida utilis* and *Pseudomonas stutzeri*. Data suggest that protection against pathogenic bacteria depends on the nature of treatment and the quality and quantity of potentials probiotics used. It is hoped that more studies are required in order to clarify the exact contribution of potential candidates probiotics used in this study an improvement of *Artemia* performance and protection against pathogens using host gene – expression and overcome disease in aquaculture farms.

REFERENCES

1. Anderson, D.; Siwicki, A. (1994). Duration of protection against

- Aeromonas salmonicida* in brook trout immunostimulated with glucan and chitosan by injection or immersion. *Prog. Fish. Cult.* 56, 258-261.
2. Ben Kahla-Nakbi, A.; Chaieb, K.; Besbes, A.; Zmantar, T., Bakhrouf, A. (2006). Virulence and enterobacterial repetitive intergenic consensus PCR of *Vibrio alginolyticus* strains isolated from Tunisian cultured gilthead sea bream and sea bass outbreaks. *Vet. Microbiol.* 117, 321-327.
3. Bresser, P.; Virkola, R.; Jonsson-Vihanne, M.; Jansen, H.; Korhonen, T., Van Alphen, L. (2000). Interaction of clinical isolates of nonencapsulated *Haemophilus influenzae* with mammalian extracellular matrix proteins. *FEMS Immunol. Med. Microbiol.* 28, 129-132.
4. Canicatti, C.; D'Ancona, G. (1990). Biological protective substances in *Marthasterias glacialis* (Asteroidea) epidermal secretion. *J. Zool. Lond.* 222, 445-454.
5. Cerenius, L.; Liang, Z.; Duvic, B.; Keyser, P.; Hellman, U.; Palva, E.I.; Wanaga, S., Soderhall, K. (1994). Structure and biological activity of α 1,3-beta-D-glucan binding protein in crustacean blood. *J. Biol. Chem.* 269, 29462-29467.
6. Christensen, G.D.; Simpson, W.A.; Younger, J.J.; Baddour, L.M.; Barrett, F.F.; Melton, D.M., Beachey, E.H. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J. Clin. Microbiol.*, 22: 996-1006.
7. Douillet, P. (1987). Effect of the bacteria on the nutrition of the brine shrimp *Artemia* fed on the dried diets. In: Sorgeloos, P.; Bengtson, D.A.; Decler, W., Jaspers, E. (Eds.), *Artemia* Research and its Applications. Ecology, Culturing, Use in Aquaculture, vol. 3. Universa Press, Wetteren, Belgium, p. 295-308.
8. Fuller, R. (1992). History and development of probiotics. In: Fuller, R. (Ed.), *Probiotics: The Scientific Basis*. Chapman and Hall, London, p. 1-8.
9. Gatesoupe, F.J. (1994). Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus* against pathogenic *Vibrio*. *Aquat. Living Resour.* 7, 277-282.
10. Gorospe, J.; Nakamura, K.; Abe, M., Higashi, S. (1996). Nutritional contribution of *Pseudomonas sp* in *Artemia* culture. *Fish Sci.* 62, 914 - 918.
11. Gullian, M.; Rodríguez, J. (2002). Immunostimulant qualities of probiotic bacteria. *Global Aquacult. Advocate.* 5, 52-54.
12. Gunther, D.C.; Catena, A. (1980). The interaction of *Vibrio* with *Artemia* nauplii. In: Persoone, G.; Sorgeloos, P.; Roels, O., Jaspers, E. (Eds.) *The Brine Shrimp Artemia*. Morphology, Genetics, Radiobiology, Toxicology, vol. 1. Universa Press, Wetteren, Belgium, p. 213-221.
13. Highlander, S.K. (2001). Molecular genetic analysis of virulence in *Mannheimia (Pasteurella) haemolytica*. *Frontiers Biosci.* 6, 1128-1150.
14. Intriago, P.; Jones, D.A. (1993). Bacteria as food for *Artemia*. *Aquaculture.* 113, 115-127.
15. Lavens, P.; Sorgeloos, P. (1996). Manual on the production and use of live food for aquaculture. *FAO Fish. Tech. Pap.* 361, 295.

16. Lawford, G.R.; Kligeman, A., Williams, T. (1979). Production of high-quality edible protein from *Candida* yeast grown in continuous culture. *Biotechnol. Bioeng.* 21, 1163-1173.
17. Lone, G.; Jette, M.; Bettina, S.; Ingrid, H., Torben, F. N. (1999). Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a Possible Probiotic Treatment of Fish. *Appl. Environ. Microbiol.*, Mar. 65, 969-973.
18. Mack, D.; Bartscht, K.; Fischer, C.; Rohde, H.; de Grahl, C.; Dobinsky, S.; Horstkotte, M.A.; Kiel, K., Knobloch, J.K. (2001). Genetic and biochemical analysis of *Staphylococcus epidermidis* biofilm accumulation. *Method. Enzymol.* 336, 215-239.
19. Marques, A.; Huynh Thanh, T.; Verstraete, W.; Dhont, J.; Sorgeloos, P., Bossier, P. (2006). Use of selected bacteria and yeast to protect *Artemia* against different pathogens. *J.Exp.Mar.Bio.Ecol.* 334, 20-30.
20. Misra, C.; Das, B.; Pradhan, J.; Pattnaik, P.; Sethi, S., Mukherjee, S. (2004). Changes in lysosomal enzyme activity and protection against *Vibrio* infection in *Macrobrachium rosenbergii* (De Man) post larvae after bath immunostimulation with β -glucan. *Fish Shellfish Immun.* 17, 389-395.
21. Molinari, G.; Talay, S.R.; Valentin-Weigand, P.; Rohde, M., Chhatwal, G.S. (1997). The fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, is involved in the internalization of group A streptococci by epithelial cells. *Infect. Immun.* 65, 1357-1363.
22. Olvera-Novoa, M.A.; Martinez-Palacios, C.A., Castillo-Olivera, L. (2002). Utilisation of yeast (*Candida utilis*) as a protein source in diets for tilapia (*Oreochromis mossambicus* Peters) fry. *Aquaculture Nutrition.* 8, 257-264.
23. Orozco-Medina, C.; Alejandro Maeda-Martinez, M., Lopez-Cortés, A. (2002). Effect of aerobic Gram-positive heterotrophic bacteria associated with *Artemia franciscana* cysts on the survival and development of its larvae. *Aquaculture.* 213, 15-29.
24. Patra, S.K.; Mohamed, K.S. (2003). Enrichment of *Artemia* nauplii with the probiotics yeast *saccharomyces baulardi* and its resistance against a pathogenic *Vibrio*. *Aquacult.Int.* 11, 505-514.
25. Purivirojkul, W.; Areechon, N.; Lawhavinit, O., Purukkiate, C. (2005). Bacterial and Fungal Infection in Mekong giant catfish (*Pangasianodon gigas* Chevey) hatchery. *Kasetsart J. Nat. Sci.* 39, 57-62.
26. Raa, J. (1990). Biotechnology in aquaculture and the fish processing industry: A success story in Norway. In: Voigt, M.N.; Botta J.R. (Eds.), *Advances in Fisheries Technology and Biotechnology for Increased Profitability.* Technomic Publishing Co, Inc, Lancaster, PA, USA.
27. Ringø, E.; Strøm, E., Tabacheck, J. (1995). Intestinal microflora of salmonids: a review. *Aquacult. Res.* 26, 773-789.
28. Saavedra, M.J.; Brito, R.D.; Sousa, M.; Alves, A., Rema, P. (2004). Isolation of *Pasteurella* spp. and *Vibrio* spp. in European sea bass (*Dicentrarchus labrax*). Susceptibility to different antibiotic groups. *Arg. Bras. Med. Vet. Zootec.* 56, 277-279.
29. Sahul Hameed, A.S.; Balasubramanian, G. (2000). Antibiotic resistance in bacteria isolated from *Artemia* nauplii and efficiency of formaldehyde to control bacterial load. *Aquaculture.* 183, 195-205.
30. Shewen, P.E.; Wilkie, B.N. (1988). Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. *Can. J. Vet. Res.* 52, 30-36.
31. Siwicki, A.K.; Anderson, D.P., Rumsey, G.L. (1994). Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection
32. against furunculosis. *Vet. Immunol. Immunopathol.* 41, 125-139.
34. 32. Sorgeloos, P. (1986). Characterization of *Artemia* from different localities in Tunisia with regard to their use in local Aquaculture. *Artemia* research and its application. 199- 209.
35. 33. Stabili, L.; Miglietta, A., Belmonte, G. (1999). Lysozyme-like and trypsin-like activities in the cysts of *Artemia franciscana* Kellog, 1906: is there a passive immunity in a resting stage? *J. Exp. Mar. Biol. Ecol.* 237, 291-303.
36. 34. Vaseeharan, B.; Ramasamy, P. (2003). Abundance of potentially pathogenic micro-organisms in *Penaeus monodon* larvae rearing systems in India. *Microbiol. Res.* 158 (4), 299-308.
37. 35. Verpraet, R.; Chair, M.; Léger, P.; Nelis, H.; Sogeroos, P., De Leenheer, A. (1992). Live-food mediated drug delivery as a tool for disease treatment in larviculture. The enrichment of therapeutics in rotifers and *Artemia* nauplii. *Aquac. Eng.* 11, 113-139.
38. 36. Verschuere, L.; Rombaut, G.; Sorgeloos, P., Verstraete, W. (2000b). Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655-671.
39. 37. Zachary, D.; Hoffmann, D. (1984). Lysozyme is stored in the granules of certain haemocyte types in *Locusta* sp. *J. Insect. Physiol.* 30, 405-411.