Effect of aerobic Gram-positive heterotrophic bacteria associated with *Artemia franciscana* cysts on the survival and development of its larvae

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Abstract

The study of bacterial interaction with crustaceans used in aquaculture, such as the brine shrimp *Artemia*, is gaining importance. It is presumed that some bacteria provide nutritional elements, and/or have the capacity to function as probiotics. In this work, aerobic Gram-positive heterotrophic bacteria associated with commercial *Artemia* cysts were isolated. According to molecular analyses, these bacteria corresponded to the genera *Microbacterium* and *Exiguobacterium*. No previous record of these bacteria in association with *Artemia* cysts exists, nor are there studies of their effect on *Artemia* culture. In this study, nauplii of *Artemia franciscana* Kellogg, 1906 were challenged in 6-day-monoxenic cultures with three bacteria strains: *Microbacterium* sp. A, *Microbacterium* sp. B, and *Exiguobacterium* sp. Also, a putative pathogenic strain of *Vibrio parahaemolyticus* was tested for comparative purposes. Our objective was to evaluate the effect of these bacteria on the survival, growth, and development of *Artemia* larvae. *Microbacterium* sp. B and *V. parahaemolyticus* negatively affected *Artemia* larvae (survival < 17%). *Microbacterium* sp. A and *Exiguobacterium* sp. were harmless, having no impact on survival, growth, and development of the larvae when compared with the control treatment (survival > 80%). However, the mixture of the harmless bacteria in a dixenic culture had a significant positive effect on the growth and development of *Artemia* larvae. On the basis of these results, it is suggested that *Microbacterium* sp. A and *Exiguobacterium* sp. are potential candidates as probiotic bacteria for the culture of *Artemia* larvae. The results of the challenge tests demonstrated that the protocol to obtain and culture bacteria-free *Artemia* larvae, using autoclaved baker's yeast as food, was a useful standardized tool to evaluate the effect of bacteria strains on the survival and development of this crustacean. The method was

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an vivo, small-scale (monoxenic or dixenic) test, which was in line with the rationale for the search for probiotics in aquaculture.

1. Introduction

Artemia larvae are important for the production of commercial fish and crustaceans (Opstad et al., 1991; Léger and Sorgeloos, 1992; Pillay, 1993). The study of bacterial interaction with crustaceans, such as *Artemia* and shrimp (penaeids) is important, since it is presumed that the bacteria provide, directly or indirectly, nutritional elements like vitamins, essential amino acids, fatty acids, polyamines, and enzymes (Austin, 1988; Bergh, 1995; Griffith, 1995; Gorospe et al., 1996; Verschuere et al., 2000b). Also, it is claimed that some bacteria in *Artemia* and shrimp cultures have the capacity to control pathogenic or potentially pathogenic bacteria by means of competitive exclusion, or by producing inhibiting effects (Gómez-Gil, 1995; Verschuere et al., 2000a). Douillet (1987) found that dried foods (Cerophyl, lactoserum, defatted rice bran, soybean, *Spirulina*, and Fleisch-mann's yeast) resulted in low or no *Artemia* larvae survival under axenic conditions. However, the same foods resulted in more than 60% larval survival when axenic cultures were inoculated with "selected microflora". Intriago and Jones (1993) obtained successful *Artemia* cultures of nauplii to preadult by using a single strain of *Flexibacter* sp. as food. Rico-Mora and Voltolina (1995) challenged *Artemia franciscana* larvae with bacterial strains isolated from the cultures of the algae *Skeletonema costatum* and two species of *Vibrio*. They reported a negative impact on larval survival with *Vibrio parahaemolyticus* and *V. alginolyticus*. However, a positive impact on *Artemia* larval survival was obtained with two strains of *Flavobacterium* and *Aeromonas*. Gorospe et al. (1996) used rice bran for the culture of *Artemia*, and found that the highest values of survival and growth were obtained when the bacteria *Pseudomonas* sp. were present.

Microbiological studies have demonstrated that *Artemia* cysts carry bacteria in the shell (Austin and Allen, 1982; Prieto et al., 1987; García et al., 1988; López-Torres and Lizárraga-Partida, 2001). Austin and Allen (1982) studied the aerobic heterotrophic bacteria associated with commercial cysts of *Artemia* (San Francisco Bay Brand), and reported *Bacillus*, *Erwinia herbicola*, *Micrococcus*, *Staphylococcus*, *V. parahaemolyticus*, Gram-negative rods, and Gram-positive rods from dehydrated cysts, and *Aeromonas*, *Bacillus*, *Micrococcus*, *Staphylococcus*, Gram-negative rods, and Gram-positive rods from cyst-hatching water. From dehydrated commercial *Artemia* cysts (Nippai), Prieto et al. (1987) and García et al. (1988) isolated Gram-positive *Deinococcus radiourans*, *Micro-coccus halobius*, Gram-negative *Erwinia* sp., and *Serratia* sp. López-Torres and Lizárraga-Partida (2001) studied 26 samples of commercial *Artemia* cysts and found bacterial concentrations between 10^6 and 10^8 colony-forming units (CFU) per milliliter of *Artemia* homogenate. Also, these authors reported that from 617 bacterial isolates on TCBS media, 94% was Gram-positive (e.g. *Staphylococcus* spp., *Micrococcus* spp.) and only 6% was Gram-negative. In our laboratory, several aerobic Gram-positive heterotrophic bacteria

associated with commercial *Artemia* cysts have been isolated. According to molecular analyses using the 16S rRNA gene, these bacteria corresponded to the genera *Exiguobacterium* and *Microbacterium* (molecular data will be published elsewhere). To our knowledge, no previous record of these bacteria associated with *Artemia* cysts exists, and no study of their effect on *Artemia* culture has been carried out. In this paper, data about the interaction of specific strains of *Exiguobacterium* and *Microbacterium* with *Artemia* larvae are presented. For comparative purposes, a putative pathogenic strain of *V. parahaemolyticus* was additionally tested. The objective was to evaluate the effect of these bacteria on the survival and development of *Artemia* larvae.

2. Materials and methods

2.1. Isolation of bacterial strains

The bacterial strains associated with *Artemia* cysts were obtained by using the following procedure. Cysts (0.01 g) of *A. franciscana* Kellogg, 1906 (San Francisco Bay, Argentemia BP03051) were incubated in sterile screw-cap glass test tubes (20×150 mm) with 20 ml of sterile artificial sea water (ASW) (Instant Ocean, 35 g l^{-1}) at room temperature ($24–27$ °C). At the end of the incubation period ($18–20$ h), the water showed a pH range of $7.3–7.7$ and dissolved oxygen of $4.6–5.5$ mg l^{-1}. Under a laminar flow chamber, groups of 10 nauplii were transferred to each of the three sterile screw-cap glass test tubes (20×150 mm). These tubes contained 20 ml of sterile ASW, and 0.1 ml of a sterile yeast solution (see Challenge tests). After 96 h of culture, the three replicates showed 100% larval survival. Water samples of 1 ml were taken from each tube and spread on Petri plates with marine medium 2216 (Zobell, 1941) and incubated at 30 °C. From the bacterial community established on the plates, the most common colonies were sampled and incubated on new plates with marine medium 2216. A total of seven bacterial strains was isolated and coded as 8L, 8N, 8R, 8T, 9AN, 9T, and 9V. For the phylogenetic identification of these strains, sequence analyses of genes encoding the subunit 16S rRNA were carried out. DNA extraction was done according to Marmur (1961), and amplification was made by the PCR method (Saiki et al., 1988) using the GeneAmp PCR System 9700. The primers used were the 27F (Field et al., 1997) and RGEN-R (Genmoto et al., 1996). Sequences were obtained by using the genetic analyzer ABI Prism 310. A comparison of obtained sequences with sequences in the National Center for Biotechnology Information data base (NCBI) (Bethesda, MD, USA, http://www.ncbi.nlm.nih.gov/) was carried out by using the Basic Local Alignment Search Tool (BLAST). The phylogenetic identification of the strains was made upon phylogenetic trees using "neighbour-joining" (Saitou and Nei, 1987) and "bootstrap" (Felsenstein, 1985) methods. Strains 8L and 8R had 85% and 90% identity with *Microbacterium* sp. (accession number AJ391205), respectively; strain 9V had 87% identity with *Microbacterium esteraromaticum* (accession number Y17231); strains 8N and 8T had 97% identity with *Exiguobacterium aurantiacum* (accession number X70316); and strain 9AN had 97% identity with *Exiguobacterium* sp. (accession number X86064). These molecular analyses will be published elsewhere (manuscript in preparation.). In this study, the three most common strains (i.e. 8L (*Microbacterium* sp. A), 8R (*Microbacterium* sp. B), and 8N

(*Exiguobacterium* sp.)) found in the successful larval culture described above were used to evaluate their effect on the survival and development of *Artemia* larvae.

2.2. Challenge tests

To evaluate the effect of the bacterial strains on the survival and development of *Artemia* larvae, five challenge tests were performed. The first three challenge tests were made with the three bacterial strains as follows: Test 1, strain 8L (*Microbacterium* sp. A); Test 2, strain 8R (*Microbacterium* sp. B); and Test 3, strain 8N (*Exiguobacterium* sp.). Given that the single bacterial strains 8L and 8N did not affect the survival and development of *Artemia* larvae (see Results), Test 4 was carried out using a mixture of these strains (8L + 8N). Test 5 was made using a putative pathogenic strain of *V. parahaemolyticus* (catalog number 588, Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, Spain). Molecular analysis using the 16S rRNA gene confirmed the identity of this *Vibrio* strain (unpublished data). All challenge tests included the following treatments: (1) Control treatment: "*Artemia* with yeast solution" (AfSc). In preliminary tests, the bacteria-free larvae reared in axenic conditions with sterile yeast always showed a high survival (>80%) after 6 days of culture. For this reason this method was chosen as a control treatment. (2) Treatment "*Artemia* with yeast solution and bacteria" (AfScB). (3) Treatment "*Artemia* with bacteria" (AfB). The treatment "*Artemia* alone" (Af) was carried out only in Test 1. The treatments were run with four replicates, with 10 bacteria-free *Artemia* nauplii each. These nauplii were placed into the test vessels after the inoculation with the bacteria. A yeast solution was prepared with 0.2 g of commercial dehydrated Baker's yeast (Nevada SAF-MEX, México) in 10 ml of ASW. After manual agitation for 3 min, the solution was autoclaved (120 °C, 1.2 kg $(cm^2)^{-1}$) for 40 min. A volume of 0.1 ml of this solution was added to each replicate for those treatments, which included the yeast solution. The challenge tests were carried out at room temperature (26–29 °C). The duration of the tests was 6 days. Sterile screw-cap glass test tubes (20 × 150 mm) were used as test vessels containing 20 ml of sterile ASW. All the materials used in the cultures (e.g. glass tubes, volumetric and Pasteur pipettes, ASW, and distilled water) were previously autoclaved (120 °C, 1.2 kg $(cm^2)^{-1}$) for 40 min.

2.3. Inoculation and monitoring of bacteria

Test vessels were inoculated with the bacterial strains obtained from pure cultures in the exponential growth phase. The concentration of bacteria inoculated in the test vessels was estimated to be about 1×10^6 CFU ml^{-1}. In the case of Test 4, which included the mixture of strains 8L and 8N, the estimated bacterial concentration for inoculation was also about 1×10^6 CFU ml^{-1} for each strain. Pure cultures of each bacterial strain were performed in triplicate in 250-ml Erlenmeyer flasks containing 100 ml of liquid marine medium 2216 (Zobell, 1941) under an environ-shaker incubator (30 °C, 100 rpm, Lab-Line Orbit). The concentration of the inoculum was estimated through a regression analysis of the optical density of the pure culture and the number of CFU ml^{-1}. The number of CFU ml^{-1} was determined using Petri plates with marine agar 2216 in duplicate. During the challenge tests, monitoring of bacteria was made twice for each replicate. The first monitoring was carried out 4 h after inoculation with bacteria (culture day 0) and the second monitoring at the end of

the test (i.e. after 144 h) (culture day 6). The monitoring consisted of spreading samples from a dilutionseries in Petri plates with marine agar 2216 in duplicate. The first dilution was made by taking 0.1 ml of the culture water, diluted in 0.9 ml sterile ASW. Thus, the average number of CFU ml^{-1} for each treatment was determined from the numbers of CFU on eight plates. The absence or presence of bacteria in the treatments was also confirmed through observations with a light microscope (1000 × ; phase contrast Nikon Labophot).

2.4. Bacteria-free Artemia nauplii

Bacteria-free nauplii were obtained by the following method. Commercial *Artemia* cysts (0.01 g) (San Francisco Bay, Argentemia BP03051) were placed in a sterile screw-cap glass test tube (20 × 150 mm) with 20 ml of sterile ASW at 30 °C. The tube was agitated (100 rpm, Lab-Line Orbit Environ-Shaker) for 2 h. Hydrated cysts were decapsulated by transferring them to a sterile screw-cap glass test tube (16 × 125 mm) containing 10 ml of a solution 1:1 of sterile ASW and sodium hypochlorite (Cloralex, 60 g l^{-1} of active chlorine) and gently shaken by hand for 10 min. Decapsulated cysts were then washed by transferring them to a new sterile screw-cap glass test tube (16 × 125 mm) containing 15 ml of ASW, gently shaken by hand for 5 min. This procedure was repeated six times in total, using new ASW and new screw-top glass test tubes. Washed cysts were transferred to a new sterile screw-top glass test tube containing 20 ml of ASW and maintained at room temperature. The incubation tube was gently agitated manually every 8 h. Hatched larvae were collected after 18 to 20 h of incubation. At the beginning and end of each run of the procedure, absence of bacteria was monitored by transferring larvae and 0.1 ml of water to Petri plates
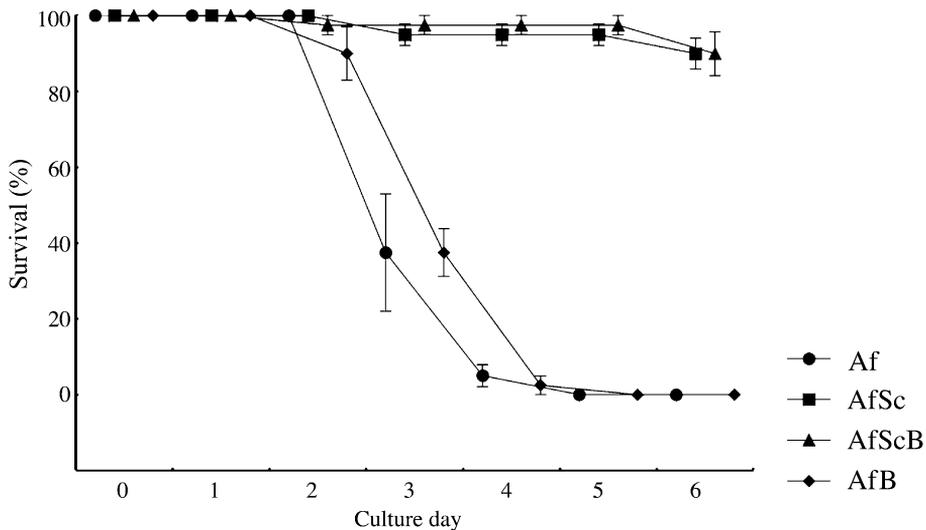

Fig. 1. Survival of *A. franciscana* larvae in Test 1 (strain 8L: *Microbacterium* sp. A). Treatments: *Artemia* alone (Af), *Artemia* with yeast solution (AfSc), *Artemia* with yeast solution and bacteria (AfScB), and *Artemia* with bacteria (AfB).

in triplicate with marine agar 2216. Absence of bacteria was also confirmed through observations with the light microscope (1000 ×).

2.5. Survival and development of Artemia

During the challenge tests, the number of swimming larvae (survival percentage) was determined daily. At the end of each test, the living larvae were fixed in 70% ethanol. Growth was determined by measuring their total length. The developmental stage of these larvae was determined according to Schrehardt (1987) with the aid of a phase contrast microscope (100−200 ×). Fixed larvae were deposited at the crustacean collection of the Centro de Investigaciones Biológicas del Noroeste, S.C., La Paz, Baja California Sur, México (CIB-507). Values of larval survival (percentage) were transformed to:

$$\text{ArcSen}\sqrt{\text{survival}} + 1,$$

and then analyzed by a one-way ANOVA and by the multiple comparison Tukey's test. Total length was analyzed by a one-way ANOVA, and the larval stages were analyzed by the Kruskal–Wallis distribution test (Statistica, 6.0).

3. Results

3.1. Larval survival

Larval survival rates are shown in Figs. 1−5. In Test 1, treatment "*Artemia* alone" (Af) resulted in 0% on culture day 5 (Fig. 1). Also, in all the tests, treatment "*Artemia* with
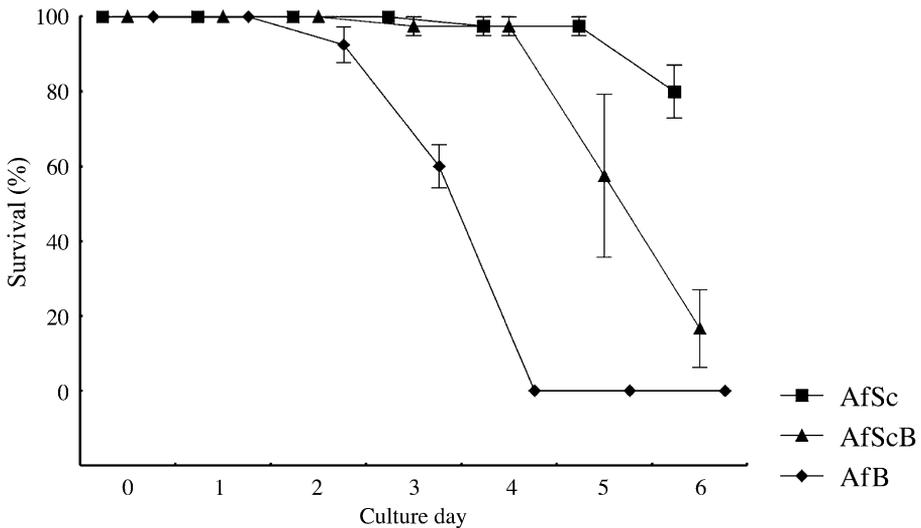

Fig. 2. Survival of *A. franciscana* larvae in Test 2 (strain 8R: *Microbacterium* sp. B). Treatments: *Artemia* with yeast solution (AfSc), *Artemia* with yeast solution and bacteria (AfScB), and *Artemia* with bacteria (AfB).
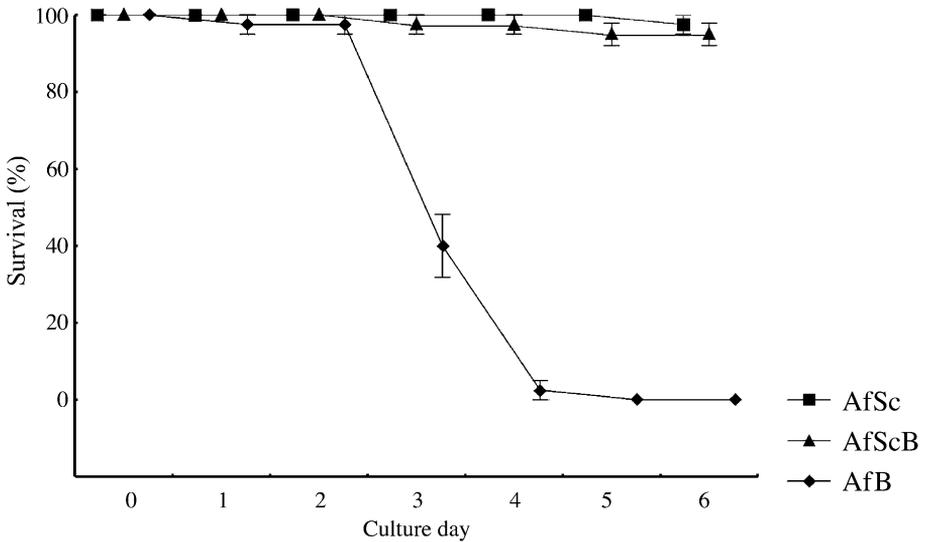
Fig. 3. Survival of *A. franciscana* larvae in Test 3 (strain 8N: *Exiguobacterium* sp.). Treatments: *Artemia* with yeast solution (AfSc), *Artemia* with yeast solution and bacteria (AfScB), and *Artemia* with bacteria (AfB).

bacteria'' (AfB) resulted in 0% on culture day 5 (Figs. 1–5). As expected, in all the tests, the control treatment ''*Artemia* with yeast solution'' (AfSc) resulted in a high survival (>80%) at the end of culture day 6 (Figs. 1–5). Treatment ''*Artemia* with yeast solution and
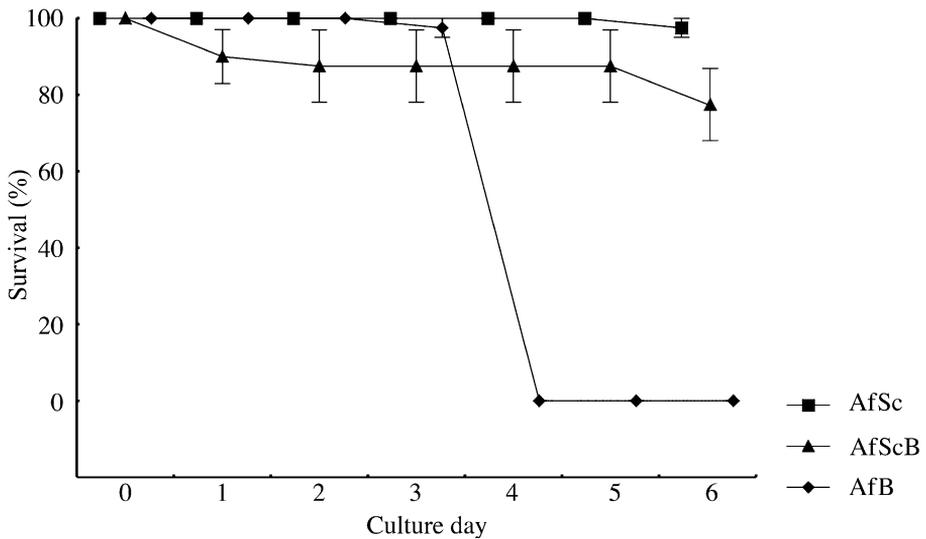

Fig. 4. Survival of *A. franciscana* larvae in Test 4 (strains 8L + 8N: *Microbacterium* sp. A + *Exiguobacterium* sp.). Treatments: *Artemia* with yeast solution (AfSc), *Artemia* with yeast solution and bacteria (AfScB), and *Artemia* with bacteria (AfB).
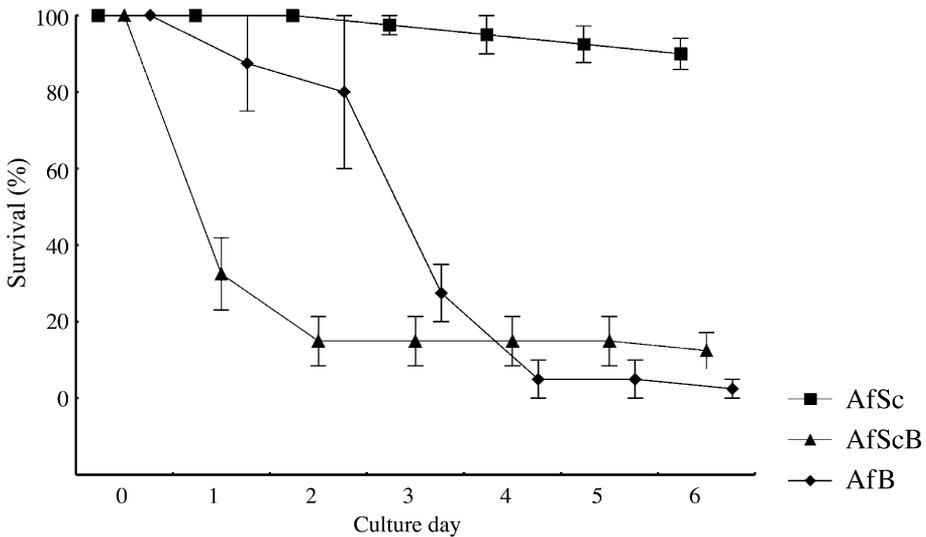
Fig. 5. Survival of *A. franciscana* larvae in Test 5 (strain *V. parahaemolyticus*). Treatments: *Artemia* with yeast solution (AfSc), *Artemia* with yeast solution and bacteria (AfScB), and *Artemia* with bacteria (AfB).

bacteria'' (AfScB) using the strains 8L (Test 1), 8N (Test 3), and the mixture of strains 8L and 8N (Test 4), resulted in a high survival (77.5% to 95%), and showed no significant differences with the control treatment (AfSc) ($P > 0.01$) (Figs. 1, 3 and 4). However, the same treatment (AfScB), but using strains 8R (Test 2) and *V. parahaemolyticus* (Test 5), resulted in a low larval survival (16.7% and 15.5%), and showed significant differences with the control treatment (AfSc) ($P < 0.01$) (Figs. 2 and 5).

3.2. Larval development

Larvae under those treatments which resulted in a low survival, i.e. Af (Test 1), and AfB (Tests 1–5), showed stages from nauplii to metanauplii I with lengths of 570–720 μm. In

Table 1
Mean and standard deviation (μm) of the total length of *A. franciscana* larvae in challenge tests 1 (strain 8L: *Microbacterium* sp. A), 3 (strain 8N: *Exiguobacterium* sp.), and 4 (strains 8L + 8N)

Treatment	Test		
	1 (strain 8L)	3 (strain 8N)	4 (strain 8L + 8N)
AfSc	919.7 (60.6)	1083.6 (76.3)	1017.18 (63.4)
AfScB	942.8 (51.5)	1088.4 (82.0)	1067.4 (63.0) **

Treatments: *Artemia* with yeast solution (AfSc) and *Artemia* with yeast solution and bacteria (AfScB).
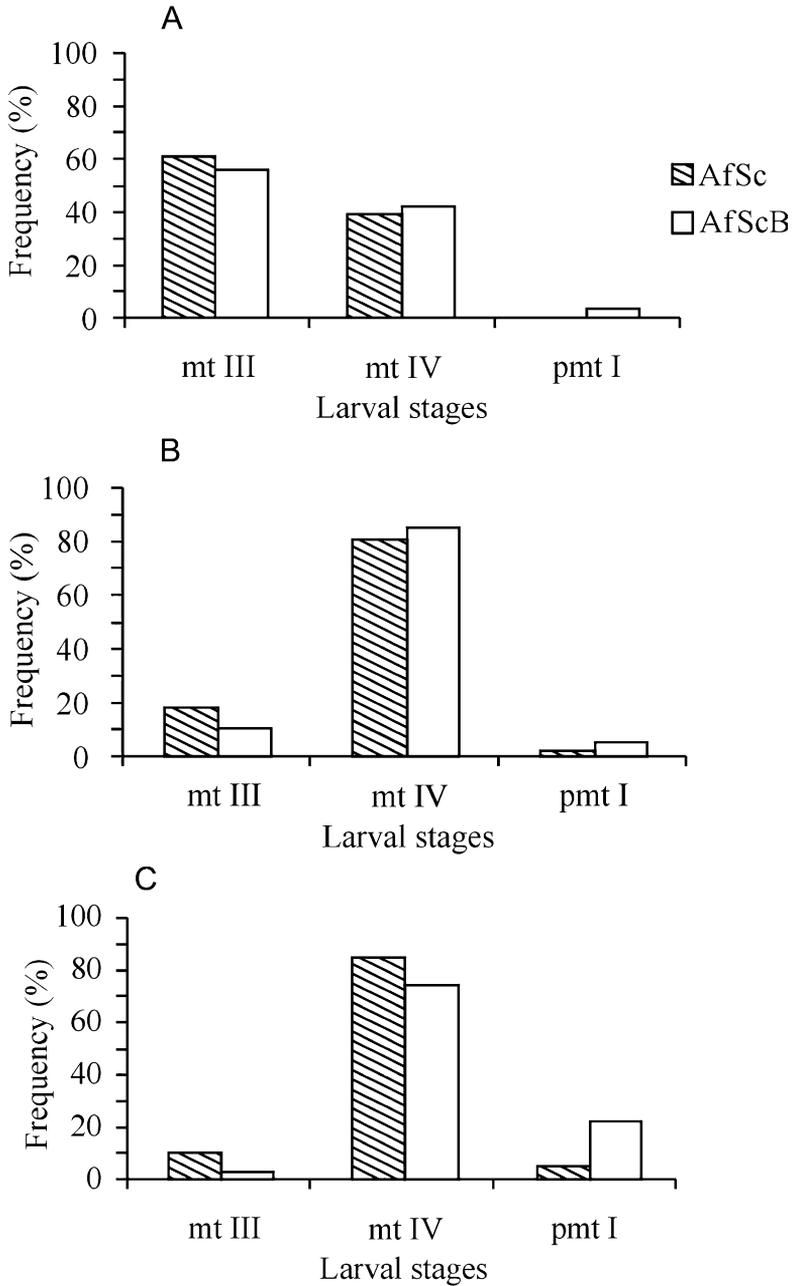   ** $P < 0.01$ (one-way ANOVA).

Fig. 6. Frequency of larval stages of *A. franciscana* after 6 days of culture. (A) Test 1 (strain 8L: *Microbacterium* sp. A); (B) Test 3 (strain 8N: *Exiguobacterium* sp.); and (C) Test 4 (strains 8L + 8N: *Microbacterium* sp. A + *Exiguobacterium* sp.). Treatments: *Artemia* with yeast solution (AfSc), and *Artemia* with yeast solution and bacteria (AfScB).

Test 2 (strain 8R), where the treatment AfScB resulted in low survival, the dead larvae occurred up to stage metanauplii III with lengths of 750–990 μm; and the surviving larvae occurred from metanuplii IV to postmetanauplii II with lengths of 1040–1790 μm. In Test 5 (*V. parahaemolyticus*), where, again, the treatment AfScB resulted in low survival, the dead larvae were from the nauplii to metanauplii IV stages with lengths of 360–960 μm, and the surviving larvae occurred from metanauplii IV to postmetanauplii I with lengths of 1040–1120 μm. Length and development stage of larvae in treatments that showed high survival at the end of culture day 6 (i.e. control treatment AfSc (Tests 1–5); and treatment AfScB using the strains 8L (Test 1), 8N (Test 3), and mixture of strains 8L + 8N (Test 4)) are shown in Table 1 and Fig. 6. No significant differences were found ($P > 0.01$) by comparing the effect of the treatment AfScB with the effect of the control treatment AfSc on larval length within Tests 1 and 3. However, there was a significant difference ($P < 0.01$) comparing these treatments within Test 4 (Table 1).

Larvae under treatments which presented a high survival at the end of culture day 6 (i.e. control treatment AfSc (Tests 1–5), and treatment AfScB using the strains 8L (Test 1), 8N (Test 3), and mixture of strains 8L + 8N (Test 4)) were in one of the three different developmental stages: metanauplii III, metanauplii IV, or postmetanauplii I (Fig. 6). No significant differences were found ($P > 0.05$) by comparing the effect of the treatment AfScB with the effect of the control treatment AfSc on the developmental stage within Tests 1 and 3 (Fig. 6). However, there was a significant difference between these treatments within Test 4 ($P < 0.05$) (Fig. 6).

Table 2
Mean and standard deviation (SD) of the number of colony-forming units (CFU) per milliliter determined in all the treatments of the five challenge tests on culture days 0 and 6

Test	Treatment	Culture day 0		Culture day 6	
		Mean	SD	Mean	SD
1	Af	0	0	0	0
	AfSc	0	0	0	0
	AfScB	5.8×10^7 [a]	1.9×10^7	5.5×10^8 [c]	1.6×10^8
	AfB	2.4×10^7 [a]	7.5×10^6	8.9×10^7 [a]	2.8×10^7
2	AfSc	0	0	0	0
	AfScB	1×10^5 [a]	6×10^4	1.9×10^5 [ab]	6.2×10^4
	AfB	1×10^5 [a]	6.4×10^4	4.6×10^5 [b]	2.3×10^5
3	AfSc	0	0	0	0
	AfScB	2.4×10^6 [a]	5.9×10^5	2.1×10^7 [b]	9.1×10^6
	AfB	1.5×10^5 [a]	7.6×10^4	1.1×10^5 [a]	5.2×10^4
4	AfSc	0	0	0	0
	AfScB	7.8×10^6 [b]	1.2×10^6	2.1×10^7 [c]	2.9×10^6
	AfB	4.7×10^6 [a]	7.4×10^5	4.8×10^6 [a]	1.1×10^6
5	AfSc	0	0	0	0
	AfScB	3.4×10^7 [a]	1×10^7	8.4×10^7 [b]	1.6×10^7
	AfB	3.2×10^7 [a]	1.1×10^7	4.9×10^7 [ab]	3.6×10^7

Test 1 (strain 8L: *Microbacterium* sp. A), Test 2 (strain 8R: *Microbacterium* sp. B), Test 3 (strain 8N: *Exiguobacterium* sp.), Test 4 (strains 8L + 8N: *Microbacterium* sp. A + *Exiguobacterium* sp.), and Test 5 (*V. parahaemolyticus*). Treatments: *Artemia* alone (Af), *Artemia* with yeast solution (AfSc), *Artemia* with yeast solution and bacteria (AfScB), and *Artemia* and bacteria (AfB).
Different letters indicate a significant difference: a < b < c at $P < 0.01$ (Tukey's test).

3.3. Bacteria in challenge tests

Mean and standard deviation of the CFU ml^{-1} determined in all the treatments of the challenge Tests 1–5 at culture days 0 (4 h) and 6 (144 h) are shown in Table 2. As expected, no bacteria were detected in the treatments Af (Test 1) and AfSc (control treatment in all the tests). At culture day 0 (4 h of inoculation), the number of CFU ml^{-1} was statistically the same in all the treatments within each test, except in Test 4 (strains 8L + 8N), where there was a significant difference ($P < 0.01$) between the treatments AfScB, and AfB. In treatment AfScB, the number of CFU ml^{-1} increased significantly from culture day 0 to culture day 6 in all the tests ($P < 0.01$), but not in Test 2 (strain 8R), where the numbers of CFU ml^{-1} showed no difference ($P > 0.01$). In treatment AfB, the number of CFU ml^{-1} showed no difference from culture day 0 to culture day 6 in all the tests ($P > 0.01$), but there was a difference in Test 2 (strain 8R), where the number of CFU ml^{-1} increased significantly ($P < 0.01$). On culture day 6, the number of CFU ml^{-1} in treatment AfScB compared with treatment AfB showed a significantly higher difference in Tests 1 (strain 8L), 3 (strain 8N), and 4 (strains 8L + 8R), but not in Tests 2 (strain 8R) and 5 (*V. parahaemolyticus*).

4. Discussion

Compared to the control treatment AfSc, challenge tests showed that the single bacterial strains 8L (*Microbacterium* sp. A) and 8N (*Exiguobacterium* sp.) used in the treatment "*Artemia* with yeast solution and bacteria" (AfScB) did not affect the survival and development of *Artemia* larvae (Figs. 1, 3 and 6; Table 1). Similarly, the mixture of strains 8L + 8N did not affect larval survival, but showed a positive effect on the growth and development of the larvae (Fig. 6C; Table 1). On the basis of these results, strains 8L and 8N may be potential candidates as probiotic bacteria for the culture of *Artemia* larvae when inert food, like autoclaved baker's yeast, is used. However, in what form(s) the mixture of the strains 8L + 8N produced a positive effect on the development of *Artemia* is difficult to establish and requires further studies. A positive effect of bacterial assemblages on larval development of *Artemia* has been reported by Douillet (1987) and Verschuere et al. (1997, 1999). In the interaction between *Artemia* and bacteria, a number of factors have been proposed: provision of additional digestive abilities in the intestine of *Artemia* with bacterial enzymes (Intriago and Jones, 1993; Verschuere et al., 1999); the use of bacterial biomass as food (Douillet, 1987; Rico-Mora and Voltolina, 1995; Intriago and Jones, 1993; Gorospe et al., 1996; Verschuere et al., 1999); the removal of toxic metabolic substances (Verschuere et al., 1999); and the synergistic relationship of the microbial community with positive effect on the growth of *Artemia* (Intriago and Jones, 1993).

Axenic *Artemia* nauplii without autoclaved baker's yeast (treatment "*Artemia* alone" (Af), Test 1) did not survive for more than 4 days (Fig. 1). The same result was obtained in the treatment "*Artemia* with bacteria" (AfB) in all the tests (Figs. 1–5). These data suggest that even the harmless bacterial strains 8L and 8N and their mixture were not an appropriate food for *Artemia* larvae at the concentrations tested. In contrast, the control treatment "*Artemia* with yeast solution" (AfSc) resulted in high survival in all the tests (survival >80%, larval stages metanauplii III, metanauplii IV, or postmetanauplii I). These results

demonstrate that *Artemia* larvae used autoclaved baker's yeast as food. Yeast has been extensively tested in the culture of *Artemia*. Under xenic conditions, Douillet (1987) used Fleishman's yeast with nine selected bacteria and obtained a survival of 60% and 3.2-mm total length of *Artemia* larvae after 192 h of culture. Also, under xenic conditions, Coutteau et al. (1990) used autoclaved baker's yeast and obtained a survival of 91.3% and 1.52-mm total length of *Artemia* larvae after 96 h of culture, although survival decreased to 36.3% after 192 h of culture. In this study, after 144 h, 80% or more survival with about 1.0-mm total length of larvae was obtained in the axenic test (control treatment) and in the mono-xenic (strains 8L or 8N) or dixenic (strains 8L + 8N) tests with autoclaved baker's yeast.

In the previous studies in general, evaluation of the growth of *Artemia* did not include the determination of the larval stage (Douillet, 1987; Coutteau et al., 1990; Abreu-Grobois et al., 1991; Hernandorena, 1991; Intriago and Jones, 1993; Gorospe et al., 1996; Verschuere et al., 1999). However, useful information on the condition and development of the organism is obtained when the larval stage is determined. We observed significant differences in larval growth when comparing the control treatment AfSc with the positive effect of the treatment AfScB using the mixture of strains (8L + 8N) (Table 1). This positive effect was further confirmed through the significant differences observed in the development of larvae (Fig. 6). Furthermore, no malformation could be detected. Hernandorena (1988, 1993) found anomalies in the pattern of *Artemia* larvae development because of either nutritional deficiencies or physical (temperature) culture conditions.

A method to obtain bacteria-free *Artemia* larvae was first published by Provasoli and Shiraishi (1959), which has been the basis for later works on axenic cultures (Douillet, 1987; Hernandorena, 1991; Verschuere et al., 1999). The method consists of immersing *Artemia* cysts in a Merthiolate solution and washing them three times in sterile sea water. Rico-Mora and Voltolina (1995) used another method, which includes the removal of the cyst shell (decapsulation) with sodium hypochlorite and the immersion of hatched nauplii in a Merthiolate solution. Gorospe et al. (1996) obtained bacteria-free larvae through the 24-h incubation of cysts in a mixture of antibiotics (kanamycin sulphate, streptomycin sulphate, and potassium penicillin-G). In our method, the cysts are decapsulated with sodium hypochlorite and then washed with sterile sea water six times, and the decapsulated cysts are then incubated in sterile sea water. Thus, experimental nauplii used in this study were never directly exposed to disinfectants. The results of the challenge tests demonstrate that our protocol to obtain and culture bacteria-free *Artemia* larvae, using autoclaved baker's yeast as food, is a useful standardized tool to evaluate the effect of bacteria on the survival and development of *Artemia* larvae. The method is an in vivo, small-scale (monoxenic and dixenic) test, which is in line with the rationale for the search for probiotics in aquaculture proposed by Verschuere et al. (2000b).

The strain 8R (*Microbacterium* sp. B) clearly had a negative effect on the survival of *Artemia* larvae (Fig. 2). Also, as expected, the *V. parahaemolyticus* strain used in this study had a negative impact on the survival of *Artemia* larvae (Fig. 5). A number of species and/or strains of *Vibrio* (*V. parahaemolyticus*, *V. alginolyticus*. *V. anguillarum*, and *V. proteoly-ticus*) have been reported as pathogenic to *Artemia* (Gunther and Catena, 1980; Rico-Mora and Voltolina, 1995; Verschuere et al., 1999, 2000a). Gunther and Catena (1980) demonstrated that cells of *V. alginolyticus* and *V. parahaemolyticus* attached to the body surface of *Artemia* larvae inhibit their normal swimming activities, thus causing a

deleterious effect. In our study, a similar action may have been caused by the strain 8R (*Microbacterium* sp. B). Observations with the light microscope showed cells of this strain forming irregular groups attached to the body surface of dead *Artemia* larvae in Test 3. Verschuere et al. (2000a), in cultures of *Artemia* nauplii exposed with *V. parahaemolyticus* CW8T2 (10^3 CFU ml^{-1}), found an infection process, where the bacterial cells penetrated between the ephitelial cells of the *Artemia* gut.

In treatment AfB, the number of CFU ml^{-1} showed no difference from culture day 0 to culture day 6 in all the tests, except in Test 2 (strain 8R), where the number of CFU ml^{-1} increased significantly. On the other hand, significantly higher values of CFU ml^{-1} on culture day 6 were observed in the treatment AfScB, when compared with the treatment AfB in all the tests except in the tests with strain 8R (Test 2) and the *V. parahaemolyticus* strain (Test 5). The increment of CFU numbers could be explained by the presence of organic matter derived from yeast. Although the estimated number of CFU of the strains 8R and *V. parahaemolyticus* did not increase with the presence of yeast, these bacteria were deleterious to *Artemia* larvae.

Acknowledgements

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