Microbial Control of the Culture of *Artemia* Juveniles through Preemptive Colonization by Selected Bacterial Strains

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The use of juvenile *Artemia* as feed in aquaculture and in the pet shop industry has been getting more attention during the last decade. In this study, the use of selected bacterial strains to improve the nutritional value of dry food for *Artemia* juveniles and to obtain control of the associated microbial community was examined. Nine bacterial strains were selected based on their positive effects on survival and/or growth of *Artemia* juveniles under monoxenic culture conditions, while other strains caused no significant effect, significantly lower rates of survival and/or growth, or even total mortality of the *Artemia*. The nine selected strains were used to preemptively colonize the culture water of *Artemia* juveniles. Xenic culture of *Artemia* under suboptimal conditions yielded better survival and/or growth rates when they were grown in the preemptively colonized culture medium than when grown in autoclaved seawater. The preemptive colonization of the culture water had a drastic influence on the microbial communities that developed in the culture water or that were associated with the *Artemia*, as determined with Biolog GN community-level physiological profiles. Chemotaxonomical characterization based on fatty acid methyl ester analysis of bacterial isolates recovered from the culture tanks was performed, and a comparison with the initially introduced strains was made. Finally, several modes of action for the beneficial effect of the bacterial strains are proposed.

Because of convenience in production and their suitable biochemical composition, the brine shrimp *Artemia* is the most frequently used live food in the larviculture of economically important crustaceans and fishes. Among *Artemia* at different life stages that are appropriate for use in aquaculture, the use of juvenile and adult *Artemia* has been getting more attention over the last decade.

It has been demonstrated that bacteria have a beneficial effect in the culture of the obligate suspension feeder *Artemia*, as the addition of bacterial strains to axenic cultures of *Artemia* fed other foods revealed that some bacterial strains may improve the survival and growth rates of *Artemia* (2). Similarly, the culture of *Artemia* under nonsterile conditions usually results in higher biomass production (BP) than that under axenic conditions, showing that the nutritional value of the food partly depends on the spontaneous colonization of the food particles by harmless bacteria (2, 4). Colonization by bacteria may even be essential when using inexpensive agricultural waste products like rice bran to support *Artemia* culture (2, 4). However, this may as well depend on the quality of the provided food, since other authors were able to culture *Artemia* axenically on autoclaved rice bran (5).

Several attempts to culture *Artemia* on a diet consisting solely of bacteria failed (2, 19). However, Gorospe and Nakamura (5) found a *Pseudomonas* sp. that was able to delay the death of the *Artemia* when no other food was given, and they assumed that it was used as a food. Rico-Mora and Voltolina (18) came to the same conclusions regarding the use of several strains isolated from a diatom culture. Yasuda and Taga (23) found an *Acinetobacter* strain which was by itself able to support the mass culture of *Artemia*. A *Flexibacter* strain, lnp3, provided as the only food source supported survival and growth (88% survival rate and 5-mm body length, respectively) of nauplii to preadults in 8 days, although seven times more bacterial biomass than algal biomass was required to yield similar growth (9).

Bacteria are reported to contribute to the nutritional value of foods by being a major source of protein and amino acids (6, 20). The results of Intriago and Jones (9) suggest that the bacteria also assisted in the digestion of the unicellular algae, although convincing evidence was not provided.

Agricultural byproducts, such as rice bran, corn bran, soybean pellets, lactoserum, etc., are used as cheap food sources for the intensive culture of *Artemia* up to the adult stage as a cost-effective alternative to algae (3). Under these intensive culture conditions, opportunistic bacteria develop, and unfavorable colonization of the culture medium and the *Artemia* may occur (22). As no further microbial control is usually performed, this may lead to a low production of *Artemia* biomass or result in the transfer of pathogenic bacteria via the *Artemia* to the predator (4, 13). In this perspective, Yasuda and Taga (23) anticipated that bacteria would be found to be useful not only as food for *Artemia* but also as biological controllers of fish disease and activators of the rate of nutrient regeneration.

The present study examines the application of bacterial strains in the culture of *Artemia* juveniles with a twofold goal, the improvement of the nutritional value of food for *Artemia*, leading to a higher biomass production of the culture and the control of deleterious bacteria associated with *Artemia* through preemptive colonization of the culture medium. In the first stage of this study, bacterial isolates were selected based on their positive effect on the *Artemia* culture under monoxenic conditions. In the second stage, xenic cultures were performed in media preemptively colonized by the selected bacterial
strains, and their effect on the zootechnical performance and the microbial community was assessed.

MATERIALS AND METHODS

Bacterial strains. Eighteen arbitrarily chosen bacterial strains were examined under monoxenic culture conditions. All the strains originated from previous well-performing Artemia cultures, except Pseudomonas fluorescens LMG1244 (ATCC 17571; isolated from polluted seawater in Denmark), Vibrio alginolyticus LMG44907 (ATCC 17749; isolated from spoiled horse mackerel), Vibrio proteolyticus Q113 (isolated from a well-performing culture of sea bass in Spain), and V. proteolyticus CW8T2 (isolated from the artificial feed used in a sea bass hatchery in Spain).

Monoxenic culture of Artemia juveniles. (i) Axenic hatching. As axenic Artemia nauplii were required for the experiments, high-quality cysts (EG grade; INVE Aquaculture Inc., Baasrode, Belgium) were disinfected according to a modification of the procedure of Provasoli and Shiraihi (16). About 0.5 g of cysts was suspended in 30 ml of autoclaved seawater and shaken for approximately 2 min. Floating cysts were removed from the surface and discarded. Subsequently, the seawater was removed and replaced by 30 ml of an aseptic solution of merthiolate (1 g/liter), and the tube was shaken for approximately 10 min. The liquid was replaced by merthiolate and the shaking was done two more times. The merthiolate was then discarded, and the cysts were rinsed five times with 30 ml of autoclaved artificial seawater containing 33 g of Instant Ocean synthetic sea salt (Aquarium Systems Inc., Sarrebourg, France)/liter. An aliquot of the disinfect cysts was subsequently transferred to test tubes containing 2 ml of marine broth 2216 (Difco Laboratories, Detroit, Mich.) and hatched for 24 h. If the disinfection procedure was not efficient or if any bacterial contamination had occurred, it manifested itself by an increased turbidity of the marine broth compared to that in uninoculated test tubes. In such cases, the hatched Artemia nauplii were not used further in the experiments.

(ii) Growth conditions. The hatched nauplii were diluted in autoclaved seawater, and 20 nauplii were transferred to sterile 50-ml Falcon tubes (Becton Dickinson Labware, Lincoln Park, N.J.) containing 30 ml of autoclaved seawater. The Artemia were fed daily with 4.9 mg (days 0 and 1) or 5.6 mg (days 2 to 6) of gamma-irradiated food/liter. This culture medium was incubated for 2 or 3 days at 28°C, and the selected bacterial strains were allowed to adapt to the conditions occurring in the Artemia culture. Before use, the food particles still in suspension on the agar plates were compared statistically to the corresponding control treatment with the t test (if the experiment was performed only once) or with analysis of variance (general factorial procedure) in which both the treatment and the experiments were considered fixed factors, taking into consideration the interaction between both factors whenever it occurred. The latter statistical comparison was performed with SPSS for Windows release 7.5.2 (SPSS, Inc.).

The xenic culture of Artemia in PCCM. To examine the effect of the bacterial colonization of the culture medium on the Artemia culture performance, seawater was preemptively colonized by opportunistic bacteria through a prolonged recirculation over a bioliter exposed to the ambient air. The seawater was allowed to be colonized spontaneously by bacteria incidentally present in the environment and able to proliferate under the prevailing conditions.

(i) Preparation of PCCM. Autoclaved natural seawater was inoculated with all nine strains that were selected based on the results of the monoxenic cultures (Table 1). They were individually grown in marine broth, centrifuged, resuspended, and quantified as described above. Each strain was added under sterile conditions at a density of approximately 10^6 cells/ml together with 0.1 g of the gamma-irradiated food/liter. This culture medium was incubated for 2 or 3 days at 28°C, and the selected bacterial strains were allowed to adapt to the conditions occurring in the Artemia culture. Before use, the food particles still in suspension were allowed to settle, and only the supernatant was used for the culture of the Artemia.

(ii) Preemptive colonization through a bioliter. Another aliquot of microfiltered (0.22-μm pore size) natural seawater was preemptively colonized through prolonged recirculation over a bioliter exposed to the ambient air. Seawater (approximately 13 liters) was recirculated for 3 weeks over 1.5 liters of aerated activated carbon at a flow rate of 9 liters/h. The activated carbon was not sterilized initially. The temperature was kept at 28°C, and 0.1 g of the gamma-irradiated food/liter was added daily to the seawater. Remaining food particles were also allowed to settle before use. Autoclaved natural seawater was used as a control culture medium for the control. At regular intervals, sterility controls of the axenic control treatment were performed by plating 100 μl of the undiluted culture medium on marine agar.

(iv) Evaluation of the addition of the individual strains. After 3 or 6 days, depending on the experiment, the surviving Artemia in the Falcon tubes were counted, and their body lengths were determined as described by Verschuere et al. (22). The individual dry weights (IDW [in micrograms]) were calculated from the body length (in millimeters) according to the method of Abreu-Grobois et al. (1). The BP in the Falcon tube was calculated based on the number of surviving Artemia (ranging from 0 to 20) and their IDW as follows:

\[
\text{IDW} = 10^{1.253 \times \log_{10}(\text{BP}) - 0.45 \times \log_{10}(\text{BP})^2} \times 1.000 (\mu g);
\]

\[
\text{BP} = \text{IDW} \times \text{no. of surviving Artemia (mg)}
\]

The survival rates, the body lengths, and the BP of the Artemia were compared statistically to the corresponding control treatment with the t test (if the experiment was performed only once) or with analysis of variance (general factorial procedure) in which both the treatment and the experiments were considered fixed factors, taking into consideration the interaction between both factors whenever it occurred. The latter statistical comparison was performed with SPSS for Windows release 7.5.2 (SPSS, Inc.).

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\]

\[
\text{BP} = \text{IDW} \times \text{no. of surviving Artemia (mg)}
\]
TABLE 1. Effects of the addition of the individual bacterial strains on survival, body length, and biomass production of Artemia juveniles in monoxenic culture

| Strain | No. of experiments | Effect of strain on Artemia
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Survival</td>
</tr>
<tr>
<td>LV58</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>LV57</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>LV56</td>
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<td>0</td>
</tr>
<tr>
<td>LV55</td>
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<td>0</td>
</tr>
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<td>LV51</td>
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</tr>
<tr>
<td>Kwestam3A</td>
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<td>0</td>
</tr>
<tr>
<td>KA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>T20kleinB</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>V. proteolyticus Q113</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>P. fluorescens LMG1244</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>V. alginolyticus LMG4409T</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>V. proteolyticus CW8T2</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>ArtemiaA1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Art8stem1B</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Raw seawatera</td>
<td>2</td>
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*a Total mortality resulted. b +, positive effect; 0, no effect; -, negative effect. Statistical significance: *, significant positive or negative effect at a 5% level; **, significant positive or negative effect at a 1% level.

RESULTS

Monoxenic culture of Artemia juveniles. In Fig. 1, survival, body length, and biomass production results are shown for the Artemia cultures treated with no bacteria (control); with the bacterial strains LV54, LV55, V. alginolyticus LMG4409T, and V. proteolyticus CW8T2; and with raw seawater (not autoclaved). There was no significant effect of the treatments on the survival of Artemia, except for V. proteolyticus CW8T2 and the raw seawater, for which total mortality occurred. The higher biomass production observed with LV54 and LV55 could be attributed to a better growth of the Artemia. V. alginolyticus LMG4409T affected the growth of the Artemia, causing a significantly lower biomass production than the control. Similar experiments were done with all the strains.

Table 1 presents an overview of the effects of the 18 tested bacterial strains and the raw seawater on survival, body length, and biomass production of the Artemia with an overall evaluation. The effect of a strain was considered significant when the P value was less than 0.05. Different categories of strains could be distinguished: nine strains showed a positive effect on the Artemia, either on the survival rate or on body length. All those strains gave rise to improved growth, while only three strains (LV53, LV58, and LV59) caused better survival rates. These nine strains were retained for further experiments. Four strains (Kwestam3A, KA, T20kleinB, and V. proteolyticus Q113) caused a significantly lower survival or growth rate, although the biomass production was not significantly affected. Two strains (P. fluorescens LMG1244 and V. alginolyticus LMG4409T) gave rise to a significantly lower rate of growth and/or survival, with a significantly lower biomass production as a consequence. Finally, three strains (V. proteolyticus CW8T2, Art8stem4A, and Art8stem1B) and the raw seawater—the latter allowing opportunistic bacteria to develop—caused total mortality of the Artemia. It should be noted, however, that on several occasions, a significant interaction between the experiment and the treatment occurred, showing each well was read with a biokinetix reader (EL312e) and the KinetiCalc enzyme immunoassay application software release 2.03 (Bio-Tek Instruments Inc., Winooski, Vt.) after 24, 30, 36, and 48 h of incubation. The results for the culture waters after 24 h and those for the Artemia after 48 h of incubation are given.
that the effect of the strain was not always of the same magnitude, although overall, a significantly positive or negative effect of the treatment could be found. This could be at least partially explained by the different harvesting times, as explained above.

The xenic culture of *Artemia* in PCCM. Subsequently, three identical test runs were performed. In Table 2, the initial *Artemia* densities and the zootechnical results of the cultures are given for the three test runs. At 36 h after transfer of the nauplii, a positive effect of the PCCM on the survival and/or the growth rate of the *Artemia* was observed in all test runs. A similar observation was made after 84 h. After 132 h, the only culture tanks that still contained living *Artemia* were those preemptively colonized with the selected bacterial strains.

![FIG. 2. Results of PCA on the Biolog profiles of the *Artemia* culture waters and the microbial communities associated with the *Artemia* for the third experiment, 12 and 84 h respectively, after the transfer of the nauplii to the culture tanks. ▼, control; ■, PCCM; ◆, biofilter-treated culture water. The Pearson correlation coefficients among the replicates of the treatment (within the outlining) and between the treatments (along the dashed lines) are given.](http://aem.asm.org/)
The plate counts of the culture waters revealed significant differences ($P < 0.05$) after 12 h between the control ($7.50 \pm 0.21$, $7.72 \pm 0.41$, and $7.153 \pm 0.090$ log CFU/ml for test runs 1, 2, and 3, respectively) and the other treatments, i.e., the PCCM ($8.00 \pm 0.31$, $8.06 \pm 0.17$, and $7.963 \pm 0.085$ log CFU/ml for runs 1, 2, and 3) and the biofilter-treated culture water ($7.88 \pm 0.15$, $8.10 \pm 0.24$, and $7.62 \pm 0.14$ log CFU/ml for runs 1, 2, and 3). The average bacterial density in the culture waters after 84 h amounted to $8.34$ log CFU/ml, but no significant differences among the different treatments could be observed.

After 12 h, no significant differences among the plate counts done for the *Artemia* were observed. On average, the bacterial colonization of the *Artemia* amounted to $5.52$ log CFU/*Artemia*. However, after 84 h, the surviving *Artemia* of the control treatment were significantly more colonized ($6.83 \pm 0.11$ and $5.74 \pm 0.22$ log CFU/ml for experiments 2 and 3, respectively) than the *Artemia* grown in PCCM ($5.52 \pm 0.19$ and $5.20 \pm 0.29$ log CFU/ml).

In Fig. 2, the comparison of the Biolog profiles with PCA is shown for the culture waters and the microbial communities associated with the *Artemia* for the third experiment. A clear difference among the results for the different treatments (control, biofilter, and PCCM) could be observed, for the *Artemia* as well as for the culture waters and for both sampling times (12 and 84 h). This shows that manipulation of the microbial communities of the *Artemia* culture water and those associated with the *Artemia* is possible through preemptive colonization of the culture water. A similar separation according to the treatments was found in the two other experiments (data not shown).

A chemotaxonomical comparison between all bacterial isolates recovered from the *Artemia* cultured in PCCM and the initially introduced nine strains based on FAME analysis was made. The results of the cluster analysis are shown in a dendrogram (Fig. 3). Several clusters can be distinguished (from top to bottom), as shown by the black bars. (i) A first cluster includes LVS7 and five recovered isolates. LVS7 has a very characteristic colony type that was not observed in any of the recovered isolates, suggesting that it is very improbable that the five recovered isolates were similar to LVS7. (ii) The second big cluster includes 42 isolates and LVS3, -8, and -9. In previous experiments, these three strains were shown to be chemotaxonomically quite closely related to each other, which may explain their appearance in the same cluster. (iii) A third cluster contains LVS4 and 15 related isolates. Furthermore, several recovered isolates had an orange colony type identical to that of LVS4. (iv) No isolates related to LVS5 were recovered from the *Artemia*. (v) A fifth cluster contains LVS1, LVS2, and one recovered isolate. (vi) The last cluster includes LVS6 and 13 recovered isolates. It was also visually observed that many recovered isolates showed the same characteristic yellow colony type as LVS6.

Considering the rather low reproducibility of the FAME analyses and the high chemotaxonomical similarity of some introduced strains, it is impossible to demonstrate unequivocally which of the initially introduced strains could dominate the microbiota associated with *Artemia* cultured in PCCM. However, some preliminary conclusions can be drawn. It seems clear that LVS1, -2, -5, and -7 are not able to colonize the...

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**FIG. 3.** FAME analysis of recovered bacterial isolates of *Artemia* grown in PCCM and of the originally introduced strains (LVS1 to LVS9). The code indicates the origin of the isolate: the number following E identifies the experiment (1 to 3), and the number following PCCM identifies the cone culture tank (numbered 1 to 4) and is followed by the incubation time at which the strain was isolated (12 or 84 h).
Artemia dominantly, as only one recovered isolate showed a high similarity to those strains. At least some of the isolates may be similar to LV53, -4, -6, -8, and -9. The large number of recovered isolates closely related to LV88 is remarkable. Although some conclusions could be made, more definitive information is needed before concluding which of the introduced strains can successfully colonize the Artemia.

DISCUSSION

Nine bacterial strains were selected based on their contribution to the growth and/or the survival of Artemia juveniles in xenic cultures (Table 1). As described in the introduction, it has been substantiated in the literature that bacteria can provide a nutritional contribution to the food for such cultures. This study shows that selected bacterial strains can increase the production, the growth rates, and the survival of the Artemia during the culture system. Further research is necessary to elucidate the exact mode of action of the observed beneficial effects and to understand the possibilities and the limitations of microbial control in aquaculture.

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