Aerobic Gram-positive heterotrophic bacteria *Exiguobacterium mexicanum* and *Microbacterium* sp. in the gut lumen of *Artemia franciscana* larvae under gnotobiotic conditions

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In a previous study, *Exiguobacterium mexicanum* strain 8N (DSM 16483^T) and *Microbacterium* sp. strain 8L (DSM 16485) in gnotobiotic cultures of the brine shrimp, *Artemia franciscana* indicated beneficial effects on the development of *Artemia* larvae. In this study, *Artemia* larvae with and without autoclaved yeast as food, were challenged with strains 8N and 8L under monoxenic condition to determine ingestion, location and viability of bacterial cells in *Artemia* gut lumen. The results of light and scanning electron microscopy demonstrated that the bacterial cells of both strains were ingested by *Artemia* metanauplii. Furthermore, both strains were detected by fluorescence microscopy as live or dead cells along the *Artemia* gut lumen. However, the band profile in denaturing-gradient gel electrophoresis of the 16S rDNA sequence, retrieved from *Artemia* gut lumen exposed to both bacteria in monoxenic and dixenic conditions, showed only a band corresponding to strain 8N, suggesting that a differential bacterial ingestion or a differential generation of PCR products occurred. In all *Artemia* experiments, the cells of the strains 8N and 8L were confined within the chyme in the space limited by the perithrophic membrane. No evidence was found that the bacteria adhered or colonized the intestinal epithelium.

Keywords: *Artemia* larvae, cultures, gnotobiotic, gut lumen, peritrophic membrane.

Introduction

THE bacterial communities in cultures of *Artemia* are mainly studied to improve larval survival and development, and to increase disease resistance[1–4]. Harris[5] reviewed the types of interactions between aquatic invertebrates and microbes, and stressed that communities of bacteria in the gut require a careful examination of individual populations. According to Gatesoupe[6], beneficial microbial cells (probiotics) are expected to function through antagonism with pathogens, intestinal colonization (with possible adhesion to gut mucus), and increased resistance of the host to pathogens. Some researchers have proposed that beneficial effects of bacteria in *Artemia* are a result of contributing nutritional supplements, or competition by excluding pathogenic bacteria[7–9]. Marques *et al.*[2] proposed the use of gnotobiotic systems as a standard tool for research on interactions between aquatic animals and microbes. Several culture methods for the production of *Artemia* under gnotobiotic conditions have already been developed[2,10,11].

Aerobic, Gram-positive, heterotrophic bacteria *Exiguobacterium mexicanum* (strain 8N)[12] and *Microbacterium* sp. (strain 8L) in dixenic bioassays with *Artemia* resulted in beneficial effects on development and larval growth[11]. A probable mode of action of these bacteria to provide beneficial effects for *Artemia* may be acting at the intestinal tract level. However, there is no study on the interaction of these particular bacteria and the *Artemia* gut lumen. It has long been accepted that *Artemia* is a nonselective filter-feeding organism[13,14]. However, a study on the selectivity of food in brine shrimp demonstrated the importance of particle size in the filtration process at different life-stages[15]. Gomez-Gil *et al.*[16] reported that the ingestion of bacteria strongly depends on the type of bacteria, time of exposure and the status (live or dead) of the bacteria. Thus, the aim of this study was to investigate the interaction among *E. mexicanum* 8N and *Microbacterium* sp. 8L and the gut lumen of *Artemia franciscana* in gnotobiotic cultures, with and without autoclaved baker's yeast as food, and to determine if these bacteria maintained their viability inside the intestinal tract. We focused on the following questions: Are these particular bacteria ingested? If so, where are they

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located in the intestinal tract? Are the bacteria alive or dead in the intestinal tract?

Materials and methods

Bacterial strains and culture conditions

E. mexicanum strain 8N (DSM 16483^T)[12] and *Microbacterium* sp. strain 8L (DSM 16485^T) were isolated from cysts of *A. franciscana*[11] and preserved with 5% (v/v) glycerol in a marine broth at –196°C in liquid nitrogen. Pure cultures of the strains were obtained in marine broth 2216 (Difco Corp.) at 30°C with agitation at 120 rpm and harvested in the exponential growth phase[11]. The bacteria were separated from the culture medium by centrifugation at 6000 *g* for 5 min at 25°C, and suspended in 20 ml of sterile artificial sea water (ASW, Instant Ocean®, 35 g l^{-1}) (source of inoculums).

Ingestion and location of bacteria in the Artemia gut lumen

Test I lasted 24 h and included the following treatments: IA, axenic *Artemia*; IB, monoxenic *Artemia* with bacteria strains 8N or 8L; IC, monoxenic *Artemia* with strains 8N or 8L plus 0.05 g l^{-1} autoclaved commercial baker's yeast *Saccharomyces cerevisae* (Nevada SAF-MEX, México) as food. The treatments were made in duplicate using 40 bacteria-free *Artemia* nauplii, each cultured in test tubes containing 40-ml ASW (Instant Ocean®, 35 g l^{-1}), autoclaved, and filtered on a 0.2 µm Whatman filter #7182-004 at 28°C in a water bath. All experiments had two replicates. According to the treatment, a bacterial density of 1×10^6 cells ml^{-1} of each strain was inoculated in the corresponding test tube. The volume of inoculums in each test tube was calculated using the formula $V_2 = (C_1)(V_1)/C_2$, where V_2 is the volume to inoculate, C_1 is the cellular concentration (1×10^6 cells ml^{-1}), V_1 is the ASW, volume in each test tube (40 ml), and C_2 is the cellular concentration in the inoculum source as determined by three total counts with a Petroff–Hausser counting chamber.

The bacteria-free *Artemia* nauplii were obtained using the method described by Orozco-Medina *et al.*[11]. Cysts were decapsulated with sodium hypochlorite and incubated in autoclaved ASW. The hydration solution and the cyst decapsulating solution were prepared with distilled water. The experiments were done under sterile conditions in a laminar flow chamber and all glassware were autoclaved at 120°C for 20 min. At the beginning of the experiments, the *Artemia* nauplii, yeast, and bacterial inoculum (if tested) were introduced in that order into all the test tubes with ASW as culture medium. To suspend the yeast in the medium, the test tubes with *Artemia* were agitated by hand for 1 min, every 8 h. Monitoring of bacteria from the ASW of all *Artemia* test tubes was carried out by total counts (cells ml^{-1}) in a Petroff–Hausser counting chamber under a phase-contrast microscope (Nikon Labophot) and by counting live bacteria (CFU ml^{-1}) in marine agar 2216 plating (Difco Corp.). Assays were made at 0 and 24 h from the ASW with and without yeast in the larval culture. Results of bacterial counts were analysed by one-way ANOVA with multiple comparisons (Tukey's test)[17] using Statistica™ version 6.0 software.

Scanning electron microscopy of Artemia intestinal tract

The location of the bacteria in the *Artemia* intestinal tract was determined by scanning electron microscopy (SEM). Larvae from the axenic and monoxenic cultures were processed according to Felgenhauer[18], with some modifications. Larvae were fixed in a solution of 200 µl 2.5% glutaraldehyde in 0.1 M sodium cocadylate and 3.5% (w/v) artificial marine salt (Instant Ocean®) with three changes of solution every hour, and incubated at 4°C. Before fixation, live larvae were anaesthetized by immersion in a solution of chloroform–ASW (SIGMA C-2432) 1 : 100 for 30 s to prevent foecal excretion during the fixation process. The larvae were then washed three times with 500 µl ASW at 25°C, with each wash for 10 min. Postfixation was done in the dark using a solution of 2% OsO_4 in water (SIGMA #75633) for 8 h at 4°C. The samples were then washed three times with 1000-µL sterilized deionized water with 30 min for each wash. The gradual dehydration process was at 25°C with acetone in deionized water at 10, 20, 30, 45, 65, 80, 90 and 100%, three times at each concentration and for 5 min each. The critical dry point of the specimens was achieved with CO_2 in a critical point dryer (Samdri-PUT-3B) at 33.4°C for 30 min. The specimens were dissected with a sterilized blade to expose the contents of the *Artemia* gut lumen. The samples were mounted on aluminum stubs with adhesive tape (EMS #77100), coated with palladium (Denton Vacuum Chamber, DESK II at 45 mA for 35 s), and analysed with a scanning microscope (HITACHI S3000-N) at 5–20 kV accelerating voltage.

Light microscopy of Artemia intestinal tract

The location of bacteria in the *Artemia* intestinal tract was also studied by light microscopy. Larvae from axenic and monoxenic cultures were fixed in 100% Davidson's solution[19] for 24 h, and then immersed in 70% ethanol for 24 h. Fixed larvae were then dehydrated in 80, 90, 96, and 100% ethanol, for 30 min at each concentration, immersed in paraffin, sectioned (4 µm) and stained with hematoxiline–eosine, according to standard procedures[20]. Finally, the samples were embedded in styrene-monomer polyester resin (Entellan®, Merck #1.07960) and viewed under a phase contrast microscope (Olympus BX41).

Viability of bacteria in the Artemia gut lumen

Test II lasted 24 h with treatments as in test I, using fluorescence microscopy, with treatments IIA, IIB and IIC to study ingestion and viability of the bacteria in the *Artemia* gut lumen. To show that autoflorescence did not occur, an additional treatment (IID) consisting of axenic *Artemia* culture with autoclaved *S. cerevisiae* with two replicates was also made. For the microscopic analyses, five *Artemia* larvae were taken from each treatment at 0 and 24 h of culture and placed in 500 µl of the same larval culture medium. To this medium 30 µl stock solution of two fluorescent dyes (SYTO 9 and propidium iodine) prepared as described in a commercial fluorescent stain product (LIVE/DEAD® BacLight™ bacterial viability kit L13152) was added. The fluorochrome SYTO 9 gives a fluorescent green colour to the live bacterial cells (at 480–500 nm, excitation-emission) and propidium iodine gives a fluorescent red colour to the dead bacterial cells (at 490–635 nm). The mixture of specimens, culturing medium, and staining solution was incubated for 30 min at 25°C in the dark and then the larvae were anaesthetized with 10 µl 10% eugenol for 15 min to avoid excretion of the gut contents. The stained larvae were viewed under a fluorescence microscope (Olympus BX41) with a U-MD/F/TXRD 470–490 nm filter using a 50 W mercury lamp as light source. Photomicrographs were obtained using a digital camera (CoolSNAP-Pro Media Cybernetics) and edited with Image-Pro Plus 4.5.0.1.9 software.

Denaturing-gradient gel electrophoresis to detect bacterial strains

Test III: Axenic *Artemia* nauplii were maintained for 24 h to obtain the larval-stage metanauplius I. In this stage the filtration and ingestion of food particles began[21]. Thus, the metanauplii I were used to detect ingested bacteria under two conditions, active and passive. In the active condition the larvae swim, filter and feed. To obtain larvae in the passive condition (nonmobile), the metanauplii were anaesthetized with a solution of chloroform–ASW 1 : 100 for 30 s. Groups of metanauplii I, both passive and active, were challenged under monoxenic conditions with the strains 8N and 8L, and the mixed strains. The experiment was designed to study if the bacteria were able to enter into the gut lumen in the larval passive condition and to corroborate that they were able to enter into the gut lumen in the active condition. The treatments were as follows: One group (control group 1) of axenic metanauplii I in the active condition was not exposed to bacteria. Three additional groups in active conditions and three groups in passive conditions were exposed to a monoxenic condition with 8N and 8L strains, and a third to a dixenic condition with a mixture of both bacterial strains. Exposure of metanauplii I to the bacteria was done by immersion for 1 h in ASW, with aliquots of each bacterial strain added at a concentration of 1×10^6 cells ml^{-1}. The inactivation of the larvae was by anesthesia with 0.25% chloroform solution for 1 h. Denaturing-gradient gel electrophoresis (DGGE) analysis of each treatment was done with 160 *Artemia* metanauplii I distributed in four test tubes with 40 larvae each. This experiment was repeated four times. In all treatments external bacteria were eliminated by immersing the larvae for 30 s in a solution with 1% sodium hypochlorite (final concentration 0.06% active chlorine) prepared with ASW. Larvae were washed three times in 15 ml sterile ASW, washed with 0.05% surfactant solution (Tween 80, SIGMA, catalog #P4780, dissolved in sterile ASW), and finally washed three times in sterile ASW. To corroborate the elimination of external bacteria, ten complete and ten homogenized larvae were placed separately in a plate with Marine Agar 2216 (Difco Corp.), with three replicates and incubated at least 72 h at 30°C. Total DNA was extracted[22] from three kinds of samples: (1) pure bacterial cultures (control samples), (2) total DNA bulk samples of *Artemia* culture, and (3) total DNA bulk samples of *Artemia* larvae exposed to bacteria. A fragment of the gene that codes for 16S rRNA was amplified by PCR[23] using primers 341F and 907R, which are universal to eubacteria[24]. A GC clamp was attached to the 341F primer in the 5′ position. The primer sequence of GC-clamp-341F was 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCT ACG GGA GGC AGC AG-3′ and for primer 907R it was 5′-CCG TCA ATT CCT TTG AGT TT-3′ (ref. 24). The PCR conditions were an initial denaturing step of 5 min at 94°C, 35 consecutive cycles consisting of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, and a final cycle of 5 min at 72°C. Partial sequences of 16S rDNA from bacteria ingested by *Artemia* were detected by DGGE, which was done on 6% polyacrylamide gel with a gradient of 30–70% denaturant of 7 M urea and 40% formamide. DNA bands were observed[25] on gels with silver staining after electrophoresis[26] at 50 V and 60°C for 16 h.

Results

Bacterial ingestion and location

Cultures from treatment IA during the 24 h incubation were maintained in axenic conditions as shown by the absence of viable and total bacterial counts in the culture medium (ASW; Table 1). Treatments IB and IC began with viable bacteria counts in the culture medium close to 1×10^6 cells/ml ($P > 0.05$). Comparisons between total and viable bacteria counts of both strains 8N and 8L after 24 h culture of treatments IB and IC indicated that suspended bacteria remained viable ($P > 0.05$). In treatment IC, a significant increase in the total number and viable

Table 1. Bacterial counts from *Artemia* culture medium (artificial sea water) of test I expressed as base-10 log values of the mean of total cell counts (cells ml^{-1}) and viable cells (CFU ml^{-1})

Treatment	Units	Test with strain 8N		Test with strain 8L	
		0 h	24 h	0 h	24 h
IA	Cells ml^{-1}	0	0	0	0
	CFU ml^{-1}	0	0	0	0
IB	Cells ml^{-1}	5.92^{a} (± 0.18)[1]	6.05^{a} (± 0.08)	5.74^{a} (± 0.29)	6.99^{a} (± 0.12)
	CFU ml^{-1}	5.65^{a} (± 0.22)	6.08^{a} (± 0.08)	6.33^{a} (± 0.32)	7.33^{a} (± 0.27)
IC	Cells ml^{-1}	6.40^{b} (± 0.39)	6.44^{b} (± 0.09)	6.71^{a} (± 0.20)	8.09^{b} (± 0.14)
	CFU ml^{-1}	5.64^{a} (± 0.13)	6.51^{b} (± 0.06)	5.98^{a} (± 0.24)	7.96^{b} (± 0.23)

IA, Axenic culture of *Artemia*; IB, *Artemia* and bacterium; IC, *Artemia*, bacterium, and yeast (*Saccharomyces cerevisiae*). Different letters indicate significant difference among averages within each test with strain 8N or with strain 8L: a < b at $P < 0.05$ (Tukey's test).

[1]Values in parentheses are the standard deviation of $N = 16$. Treatments were done in duplicate with two replicates.

count of 8L ($P < 0.05$) was observed in *Artemia* cultures after 24 h, but with 8N a significant increase was evident only in viable cells (Table 1). At the beginning of the experiments in all treatments (IA, IB, IC), *Artemia* larvae showed an empty gut lumen with microvilli on the epithelial surface and abundant yolk bodies in the epithelial cells. The microvilli in the apical zone suggest an ability to feed on an external source, but no bacteria were observed (Figures 1 *a* and 2 *a*). Larvae of all treatments after 24 h culture showed three regions of the alimentary tract: stomodeum or esophagum (Figure 1 *b*), mesenteron or midgut, and proctodeum or hindgut (Figure 1 *c*).

After 24 h of culture, the axenic *Artemia* larvae (treatment IA) contained dark and light intestinal–epithelial cells (Figure 1 *b*). In the intestinal hepatic caecum, dark cells partially opened and released material to the lumen. Light cells had a fibrillar matrix in the lumen of the foregut (Figure 1 *b*). The digestive system had a mesenteric region with no differentiated epithelial cells and the lumen had an empty, achromatic peritrophic envelope (Figure 2 *b*). The peritrophic membrane had a fibrous texture (Figure 2 *b*). No bacteria were observed in the intestinal tract of larvae in treatment IA of tests with strains 8N and 8L.

In treatment IB with strain 8N, *Artemia* larvae had gut lumen with chyme consisting of particulate material and, as expected, only one type of bacteria was present with the cell morphology of 8N (Figure 1 *c* and *d*). Under light microscopy, bacteria were not strikingly evident; under SEM observations, bacteria were clearly present. *Artemia* larvae in treatment IB, with strains 8N and 8L, had gut lumen with chyme covered with the peritrophic membrane (Figures 1 *c* and 2 *c*), which is a thin film along the alimentary canal that limited the contact between chyme and the intestinal–epithelial surface along the mesenteric region. *Artemia* larvae with strain 8L showed a peritrophic membrane with two zones, granular and fibrous (Figure 2 *c*). The granular zones had predominantly dark bodies in a homogeneous distribution and they were cells of strain 8L. In contrast, the fibrous zone had few dark bodies. SEM observations confirmed that the strains 8N and 8L were mixed with particulates that formed the chyme (Figures 1 *d* and 2 *d*).

At 24 h, *Artemia* larvae in treatment IC had gut lumen filled with food and the lumen was covered with the peritrophic membrane. In the endoperitrophic space, the food consisted of yeast cells mixed with cells of strain 8N (Figure 1 *e* and *f*), or with cells of strain 8L (Figure 2 *e* and *f*). The peritrophic membrane had an irregular surface (Figure 1 *f*). Cell walls of some yeast cells were broken or deformed (Figure 2 *f*).

Bacterial viability

The absence of fluorescent emission by the bacteria in the gut lumen of *Artemia* larvae under axenic conditions was confirmed at the beginning of the experiment for all treatments (IIA–IID) and after 24 h incubation for treatment IIA. In treatment IIB, larvae exposed to strain 8N had areas of green (live cells) and red fluorescent emission (Figure 3 *b*). In the same treatment, but with strain 8L, areas mainly with red fluorescence emission, interpreted as dead cells, were seen (Figure 4 *b*). Metanauplii in treatment IIC with strain 8N and yeast had areas with a green fluorescent emission along the middle gut (Figure 3 *d*), and with strain 8L and yeast had a mixture of red and green fluorescent bodies in the middle gut lumen (Figure 4 *d*). Observations under fluorescence microscopy of suspended bacteria from the culture medium of *Artemia* showed that strains 8N and 8L maintained their viability in experiment II.

Molecular detection of bacterial strains

Two separate bands in the bacterial marker lane (lane M, Figure 5) corresponded to a mixture of PCR products of cultures of both bacterial strains 8N and 8L. No signals

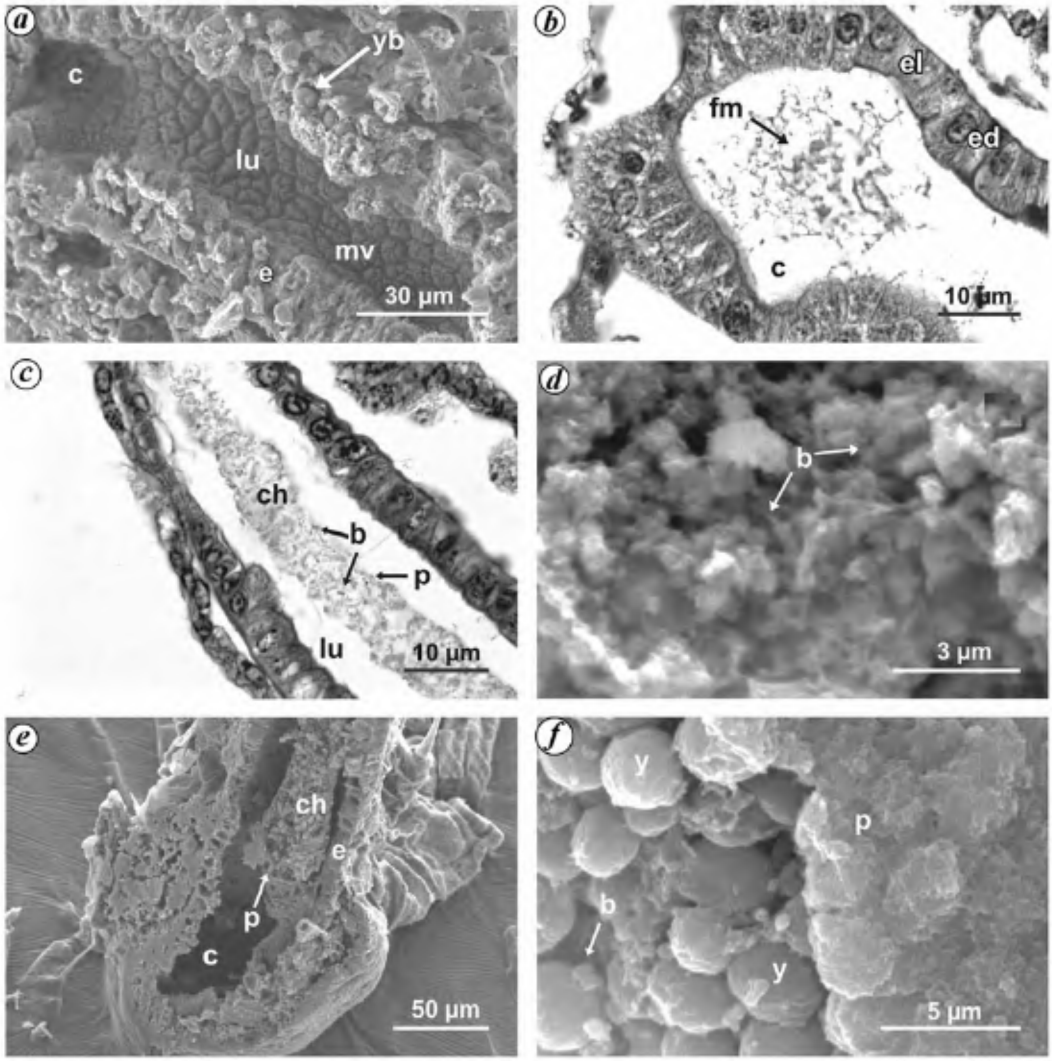

Figure 1. Photomicrographs of sagittal medial sections of *Artemia* larvae under test for ingestion and location of *Exiguobacterium mexicanum* strain 8N (***a***, ***d***–***f***, SEM; ***b*** and ***c***, Light microscopy). ***a***, Nauplius exposed to treatment IB at the beginning of the test, showing the inner surface of an empty gut lumen (lu), microvilli (mv) and yolk bodies (yb). ***b***, Cephalic region of metanauplius I exposed to treatment IA (axenic conditions) for 24 h culture showing the stomodeum region with dark (ed) and light epithelial (el) cells. A fibrillar matrix (fm) is visible in the lumen of the hepatopancreatic caecum with no bacteria. ***c***, The mesenteron–proctodeum region of metanauplius I exposed to treatment IB for 24 h culture showing the gut lumen (lu) containing chyme (ch) with bacterial cells (b) enveloped by the peritrophic membrane (p). ***d***, Magnification of chyme from the hindgut of metanauplius I exposed to treatment IB for 24 h culture showing bacteria (b). ***e***, Cephalic region of metanauplius I exposed to treatment IC (bacteria and yeast) for 24 h culture showing the hepatopancreatic caeca (c), epithelial cells (e) and chyme (ch) enveloped by the peritrophic membrane (p). ***f***, Magnification of chyme from the hindgut of metanauplius I exposed to treatment IC (bacteria and yeast) for 24 h culture and showing yeast cells (y) and bacteria (b) adhering to yeast cells enveloped by the peritrophic membrane (p).

from PCR products of bacterial strains 8N and 8L from the gut lumen of *Artemia* in the passive condition were recorded (Figure 5); the same was true for axenic metanauplii not exposed to bacteria (not included in Figure 5). The band profiles of lanes for the gut lumen of *Artemia* under active monoxenic and dixenic conditions (lanes 8N, 8L, and the mixture 8N + 8L, Figure 5) showed the signal of the 8N strain, but no signal for the 8L strain. Extraction and quantification of DNA showed a lower content from strain 8L than from strain 8N at the same concentration of bacteria in the culture medium (data not shown). Amplification of the same amount of template (50 ng) yielded an evident result when the PCR product bands were viewed after electrophoresis in agarose gel and DGGE gel, which were considered as band markers in the DGGE analysis (lane M, Figure 5).

Discussion

Ingestion and viability of bacteria in the Artemia gut lumen

Several workers have studied the beneficial effects of different types of bacteria on the growth and biomass production of *Artemia* assuming de facto their ingestion[7,9].

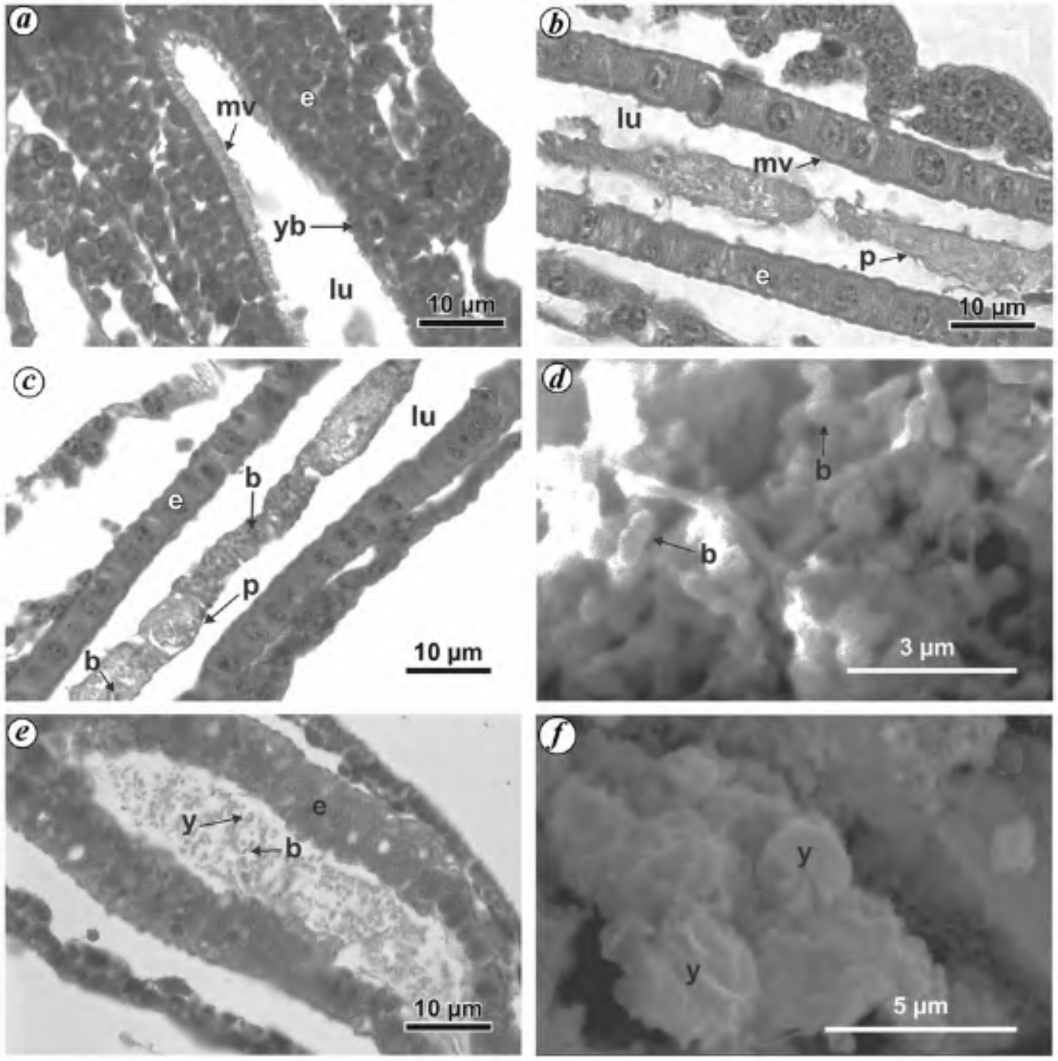

Figure 2. Photomicrographs of sagittal medial sections of *Artemia* larvae under test for ingestion and location of *Microbacterium* sp. strain 8L (***a–c***, and ***e***, Light microscopy; ***d*** and ***f***, SEM). ***a***, Metanauplius I exposed to treatment IA (axenic culture) at 0 h culture showing the foregut, ephitelial cells (e) with abundant yolk bodies (yb), and microvilli (mv) in formation. ***b***, Metanauplius I exposed to treatment IA (axenic culture) for 24 h culture showing the mesenteron lumen (lu) with an empty peritrophic membrane (p), epithelial cells (e), and microvilli (mv). ***c***, Metanauplius I exposed to treatment IB (strain 8L) for 24 h culture showing the mesenteron lumen (lu) with a peritrophic membrane (p) and enveloping bacteria (b). ***d***, Magnification of chyme from the hindgut of metanauplius I exposed to treatment IB (strain 8L) for 24 h culture showing bacteria (b) mixed with organic material. ***e***, Metanauplius I exposed to treatment IC (bacteria and yeast) for 24 h culture showing the mesenteron lumen with yeast cells (y) and bacteria (b). ***f***, Magnification of chyme from the mesenteron of metanauplius I exposed to treatment IC (bacteria and yeast) for 24 h culture showing yeast cells (y).

Our results of SEM, light, and fluorescence microscopy (Figures 1–4) have demonstrated that the bacterial cells of strains 8N and 8L were ingested by the *Artemia* metanauplii. Furthermore, both strains were detected by fluorescence microscopy as live or dead cells along the *Artemia* gut lumen fed with and without baker's yeast (Figures 3 *b*, 3 *d*, 4 *b* and 4 *d*). Some authors have studied the ingestion of bacteria by the brine shrimp *Artemia* using different approaches. Soto-Rodriguez *et al.*[27] proposed the use of the fluorochrome DTAF (fluorescence microscopy) and demonstrated the ingestion of three strains of *Vibrio harveyi*. However, this did not allow discrimination between live and dead bacteria, and often there was a diffusion of the fluorochrome stain into all *Artemia* tissues. Our previous assays with bacteria stained with BacLight™ showed individual bacterial cells inside the *Artemia* gut lumen. Staining bacteria lodged in the gut lumen (*in situ*) offered information about the physiological state of the ingested bacteria. However, the images of monodispersed bacterial cells were unclear and the dye SYTO 9 also stained epithelial cells emitting a light green colour (Figures 3 *b* and 4 *b*). Using transmission electronic microscopy, the infection of the pathogenic *Vibrio proteolyticus* CW8T2 in the *Artemia* intestinal epithelium was demonstrated. However, it was not determined how the bacterium entered into the *Artemia* gut lumen.

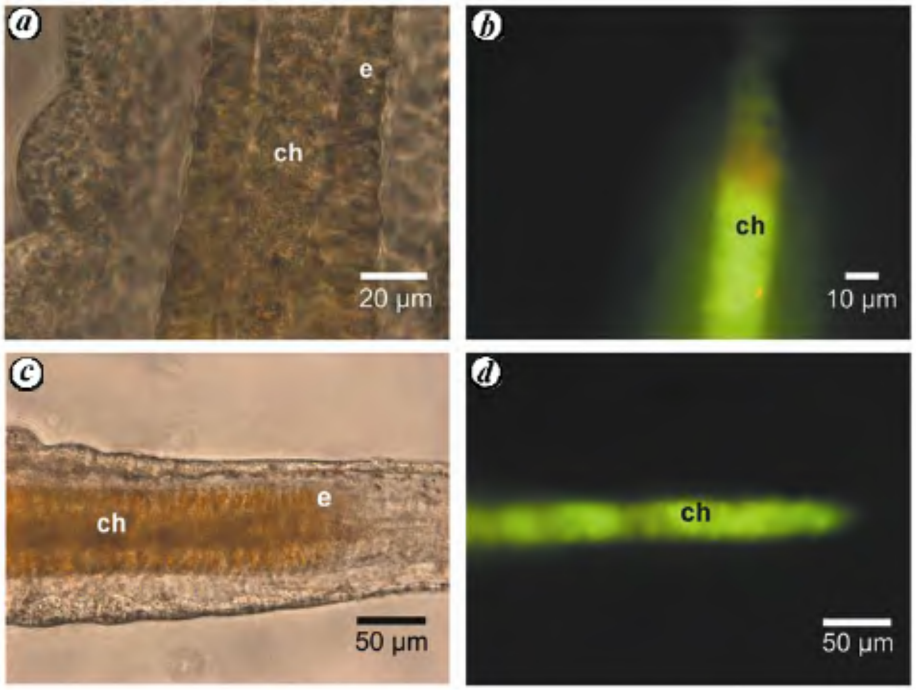

Figure 3. Photomicrographs of *Artemia* larvae under test for viability of *E. mexicanum* strain 8N in gut lumen (***a*** and ***c***, Phase contrast light microscopy; ***b*** and ***d***, Fluorescence microscopy). ***a***, ***b***, Metanauplius I under treatment IIB (larvae + bacteria) for 24 h culture showing the mesenteron of the epithelial gut with chyme (ch). ***c***, ***d***, Metanauplius I under treatment IIC (larvae + bacteria + yeast) for 24 h culture showing the content of the mesenteron. (e) Epithelial cells. The green colour corresponds to live cells, and the red colour to dead cells.

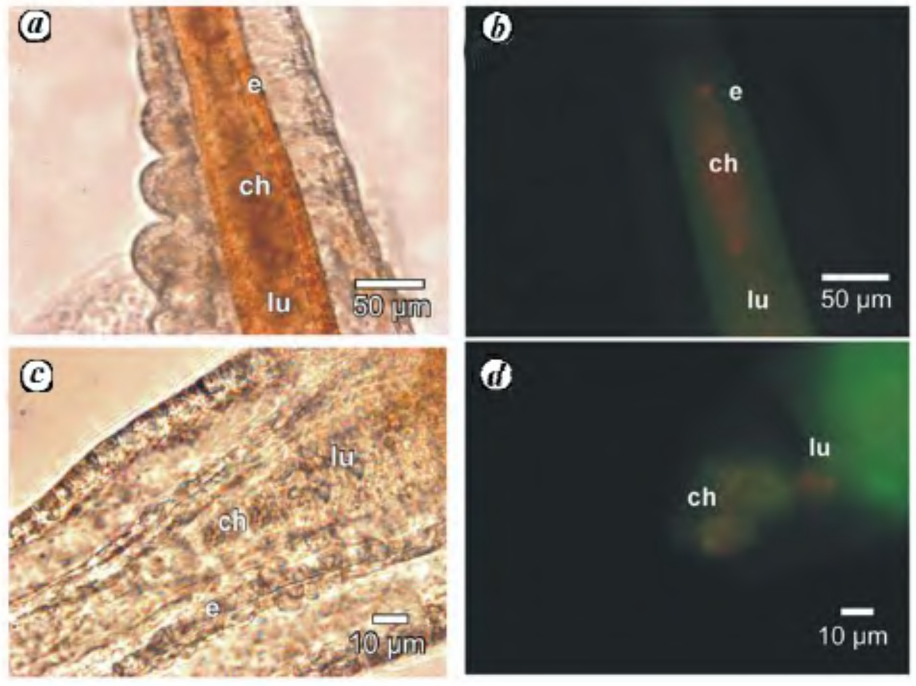

Figure 4. Photomicrographs of *Artemia* larvae under test for viability of *Microbacterium* sp. strain 8L in gut lumen (lu) (***a*** and ***c***, Phase contrast light microscopy; ***b*** and ***d***, Fluorescence microscopy). ***a***, ***b***, Metanauplius I exposed to bacteria (treatment IIB) for 24 h culture showing the middle gut with chyme (ch). ***c***, ***d***, Metanauplius I exposed to bacteria and yeast (treatment IIC) for 24 h culture showing chyme limited by the mesenteron. (e) Epithelial cells. The green colour corresponds to live cells, and the red colour to dead cells.

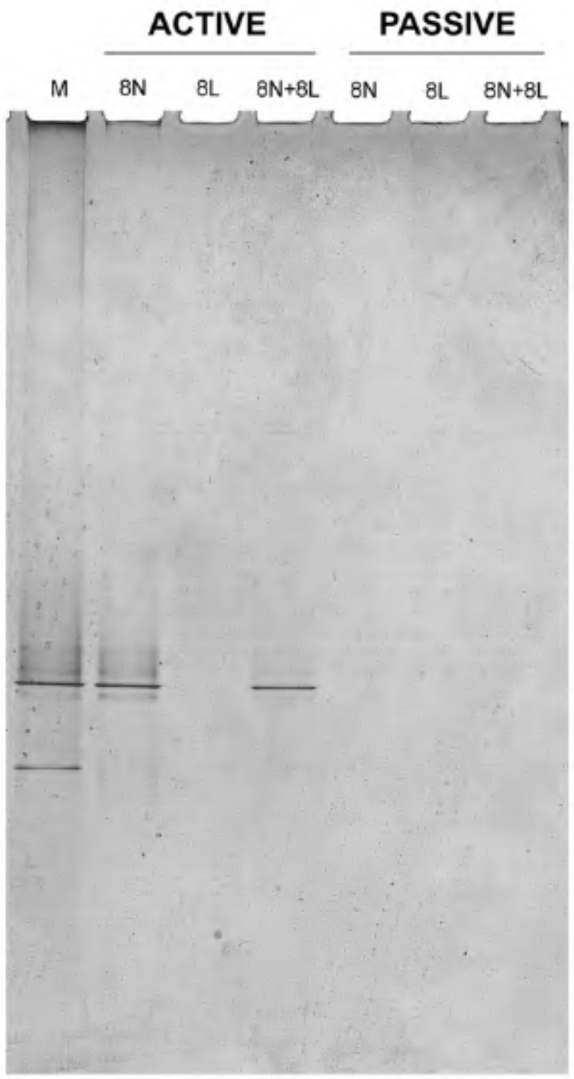

Figure 5. DGGE of PCR products of 16S rDNA of bacterial cultures and bacteria from the *Artemia* gut lumen. Lane M, Profile of bands of PCR products of 16S rDNA obtained from a mixture of pure cultures of *E. mexicanum* strain 8N and *Microbacterium* sp. strain 8L. Active lanes; Active *Artemia* larvae exposed to single bacterial strains 8N, 8L and their mixture (8N + 8L). Passive lanes, Passive *Artemia* larvae exposed to bacteria as above.

The concept that *Artemia* is a nonselective filter-feeding organism[13,14] should be re-evaluated because several studies have demonstrated the selective ability of *Artemia* depending on the size of suspended particles. Makridis and Vadstein[28] reported that *Artemia* metanauplii preferred particles of 4–8 μm, and Gelabert[15] determined a major filtration frequency for particles of 4–18 μm, with the highest capture for 10 μm particles by *Artemia* of 1–1.9 mm total length. It has been demonstrated that the preference of *Artemia* for a particular size range of suspended particles, including bacteria is influenced by the concentration of the particles[29], larval stage[15,28], exposure time[16,30], bacterial species and bacterial status (live or dead)[16]. In this study with *Artemia* metanauplii, we have demonstrated the ingestion of cells of the bacterial strain 8N with a 0.5–0.75 μm diameter and 0.8–3.0 μm length[12], and 8L with a 0.32–0.5 μm diameter and 0.79–1.27 μm length. Although we have no direct evidence that a differential filtration and- or ingestion of cells of these strains occurred, the DGGE analysis gave a positive signal for the 8N strain only, suggesting that a major ingestion of this strain had occurred. The differential ingestion of bacteria has been also reported in the marine filter-feeding clam *Argopecten purpuratus*, exposed to a mixture of bacteria[31]. According to Muyzer *et al.*[26], the sensitivity of the DGGE for a differential detection of specific strains within a bacterial community depends on the amount of strain-specific DNA template. Thus, another explanation for the absence of a signal for the strain 8L in the DGGE analysis is by a differential generation of PCR products. Farrelly *et al.*[32] suggested that differential yields in the PCR amplification of DNA could be caused by the genome size in bacterial cells and by the organization and number of 16S rRNA genes. Although we have no information on the genome and organization of genes of the strains 8N and 8L, we have observed that the amount of DNA template obtained from similar cellular concentrations was always higher for strain 8N. In a study of microbial populations present in the digestive tract of the fish *Oncorhynchus mykis*, comparing between the 16S rRNA gene clone libraries and the DGGE, the latter underestimated the diversity of the bacterial communities[33].

The evaluation of viability of suspended bacteria in *Artemia* cultures has been determined using plating and the tetrazolium salt, MTT[9]. In our study, the viability in test I was assessed by plating and in test II using the fluorescent indicator, BacLight^{MT}. The results showed that the bacteria were viable both at the beginning and the end of the tests (0 and 24 h; Table 1).

Location of bacteria in the Artemia gut lumen

For different developmental stages of *Artemia* in agnotobiotic conditions, Hansen and Peters[34] described the peritrophic membrane (PM) as a microfibrillar structure. In the present study the PM in *Artemia* metanauplii also showed a microfibrillar structure (Figure 2 *b*). Furthermore, we found that even metanauplii without food and without bacteria formed a PM. These results suggest that the formation of PM in *Artemia* is not promoted by contact with the bacteria nor by an intestinal distention, as proposed for some insects[35].

In all experiments with bacteria and *Artemia*, the former were confined into the space of the PM, i.e. no attachment of bacteria on the intestinal epithelium was observed. The PM of metanauplii separated the chyme (including 8N and 8L cells) from the intestinal epithelium. Thus we confirm that the PM functions as a barrier for direct

contact between the chyme and the epithelium[36–38]. For the establishment of a microbial community in the intestinal lumen of aquatic organisms, it is considered important that the alimentary tract be equipped with structures such as pouches, crevices and caecum[5]. However, *Artemia* larvae have a simple gut with an incipient hepatopancreatic caecum in the anterior region[36,39]. Thus, the colonization of ingested 8N and 8L bacteria to the *Artemia* intestinal epithelium seems to be limited because the PM is impermeable to particles up to 327 nm size[34]. Another possible entrance of bacteria is through the anus by antiperistaltic movements[39,40]. Some bacteria have been reported to adhere to the intestinal posterior region in aquatic crustaceans[5,41–43]. However, we did not observe the cells of strains 8N and 8L adhering to posterior intestinal epithelium of *Artemia* metanauplii.

The establishment of bacterial communities in crustaceans has been related to their ability to adhere to the chitinous surface. Such an ability is an important factor of the pathogenicity of *Vibrio* spp. for *Artemia* and other crustaceans[4,42,44]. Studies have reported that the bacteria *Vibro alginolyticus* and *V. parahaemolyticus* inhibited swimming activity by forming aggregates on the surface of external appendages, thus causing the death of *Artemia* larvae[44]. Although a few 8N and 8L cells were attached to the external surface of the *Artemia* metanauplii, no cell aggregates were observed on their appendages.

Modes of bacterial action

In our previous study, the bacterial strains 8N and 8L in dixenic bioassays with *Artemia* larvae yielded a positive effect on the larval development and growth[11]. The results of the present work suggest that beneficial influences of these strains occur in the endoperitrophic space limited by the PM. In contrast, other studies have suggested a symbiotic association in the ectoperitrophic space between the microorganisms and non-*Artemia* branchiopods[41] and insects[45]. One benefit of the strains 8N and 8L may be caused by the bacterial action on yeast cells, making nutrients bioavailable to *Artemia*. Strain 8N has the ability to oxidize the compound N-acetyl glucosamine[12], which is an important constituent of the cell wall of yeast cells[46]. Marques *et al.*[2] mentioned that the probable beneficial effect of the strains 8N and 8L was as a nutritional complement for *Artemia*, or as a positive influence in the culture conditions. The use of bacterial biomass as a food complement is probable because baker's yeast is a suboptimal diet for *Artemia*[9,47,48]. Using another suboptimal diet (rice bran) with the bacteria *Pseudomonas* sp. resulted in an improvement of the survival and growth of *Artemia*, with the bacteria contributing proteins and essential amino acids[7].

We conclude that the bacteria *E. mexicanum* strain 8N, and *Microbacterium* sp. strain 8L were ingested by *A. franciscana* metanauplii and these bacteria were observed both alive and dead inside the *Artemia* digestive tract. The bacteria were located within the chyme circumscribed by the PM and no evidence that the bacteria adhered to the intestinal epithelium was found.

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