



## Pathogenicity and colonization of *Litopenaeus vannamei* larvae by luminescent vibrios

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### Abstract

The virulence of the bacterial strains CAIM 333 and CAIM 372 identified as *Vibrio campbellii* and strain CAIM 79, identified as *V. harveyi*, were evaluated using bioassays with *Litopenaeus vannamei* larvae at bacterial densities of  $10^5$  CFU ml<sup>-1</sup>. There was no difference in virulence observed between the two strains of *V. campbellii*. The shrimp zoea stages were more susceptible to the CAIM 333 strain. *L. vannamei* larvae did not show any susceptibility to the bacterial strain CAIM 79. No statistical correlation ( $P > 0.05$ ) was found between the number of CAIM 333 and CAIM 79 bacteria inoculated and ingested (inside the digestive tract), and no statistical correlation ( $P > 0.05$ ) was found between number of CAIM 333 bacteria ingested and shrimp larval mortality. The colonization process begins in the oral region, followed by the cephalotorax and then the inside the MGT. A 2 h exposure experiment (Exp-2 h) proved to be better to evaluate the colonization process when compared to a 24 h exposure method. © 2006 Elsevier B.V. All rights reserved.

**Keywords:** Pathogenicity; Experimental infection; Luminescent vibrios; Shrimp larvae

### 1. Introduction

Reports on the epizootic luminescent bacterial diseases, especially in the shrimp farms of Asian countries, have been connected to an increase in the shrimp production and intensive rearing systems (Karunasagar et al., 1994). Luminescent vibriosis is mainly caused by *V. harveyi*, *Vibrio campbellii* and occasionally *V. splendidus*, which can infect larval, juveniles and adult stages of penaeid shrimp (Gómez Gil et al., 1998b; Lavilla Pitogo et al., 1998). In the

Philippines, virulent *V. harveyi* strains have caused 100% losses in the larval production of *Penaeus monodon* with bacterial cell densities as low as  $10^2$  cells ml<sup>-1</sup> (Lavilla Pitogo et al., 1990). Bacterial infections related to *V. harveyi* luminescent strains have also been reported to cause major losses in the shrimp larviculture in Australia (Pizzutto and Hirst, 1995), South America (Álvarez et al., 1998; Robertson et al., 1998) and Mexico (Vandenberghe et al., 1999). Most of these studies have focused on the isolation, characterization and identification of the bacterial strains present in tissues and cultured water at the time of infection. Additionally, there has been an effort to develop a consistent and repeatable experimental infection protocol for shrimp larvae (Robertson et al., 1998). However,

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at this time, the infection studies with shrimp have had limited success, mainly to the intrinsic variability and lack of reproducibility (Saulnier et al., 2000). There is an urgent need to develop a standardized protocol in order to evaluate the virulence of different *Vibrio* strains associated with shrimp mortality.

Histopathological studies of infected shrimp larvae with luminescent vibriosis reveal a massive bacterial colonization of the oral region (Lavilla Pitogo et al., 1990), as well as atrophy and necrosis of the hepatopancreatic tubules (Robertson et al., 1998). These observations strongly suggest an oral infection route; however, there are no descriptions of the process of gut colonization in vivo. The first step in the development of a bacterial infection is the colonization of a specific host tissue surface through adhesion mechanisms and initial multiplication (Finlay and Falkow, 1997). In penaeid shrimp, the intestinal microflora is dominated by members of the *Vibrio* genus, and it is suggested that the large majority of bacteria are associated to the digestive tract lining and not to the undigested particulate matter inside the tract (Gómez Gil et al., 1998b; Moss et al., 2000). Many studies on intestinal bacteria in aquatic organisms are time consuming, labor intensive in vitro tests and neither of these methodologies enables in vivo and real time observation of the infectious process, nor do they guarantee that the observed bacteria are those that were inoculated or of interest. A method that makes use of fluorescent stain and enables real time observations has been described enabling tracking studies of bacterial colonization inside the digestive tract of crustacean larvae (Soto Rodríguez et al., 2003b).

The present study evaluates the pathogenicity of luminescent *Vibrio* strains in different stages of *Litopenaeus vannamei* larvae, the reproducibility of the trials and colonization sites of these bacteria in the larval shrimp digestive tract by observations of fluorescent labeled bacteria (FLB).

## 2. Materials and methods

### 2.1. Larval culture system

*L. vannamei* nauplii (stages IV–V) were acquired from a hatchery, in Sinaloa, Mexico and maintained in a 60 l glass aquarium at 27–28 °C with 0.45 µm filtered natural seawater (pH 7.8–8.2, 33–34‰). From zoea<sub>1</sub>, they were fed with a mixture of *Isochrysis galbana* (30%) and *Chaetoceros muelleri* (70%) at a minimum density of 75,000 cells ml<sup>-1</sup>. The microalgae came from the Microalgae Strain Collection (CICESE, Ensenada,

Mexico) and were cultivated under *Vibrio* spp.-free condition. The water was exchanged at a 30% daily rate. Larvae were kept in a 12 h light–dark photoperiod. Before each experiment, *L. vannamei* nauplii were observed with a microscope to evaluate activity, deformities, yolk sac condition, parasites and debris adherence.

### 2.2. Bacterial strains and inoculum preparation

Bacterial strains CAIM 333 and CAIM 372 (*V. campbellii*) and CAIM 79 (*V. harveyi*) all identified by molecular methods (Gomez-Gil et al., 2004) were obtained from the Collection of Aquatic Important Microorganisms (CAIM, CIAD Mazatlan, [www.ciad.mx/caim](http://www.ciad.mx/caim)) kept in glass beads in cryovials at –70 °C (Gherna, 1994). Strain CAIM 333 was isolated from a *Litopenaeus stylirostris* broodstock tank water, while CAIM 79 was isolated from the water of a hatching tank. Both strains came from a shrimp hatchery in the Gulf of Santa Clara, Sonora, Mexico. Strain CAIM 372 was isolated from the lymphoid organ of diseased juvenile shrimp (*P. monodon*), from the Philippines. The virulence of the strains CAIM 333, CAIM 372 and CAIM 79 was tested in another study with *Artemia* nauplii (Soto Rodríguez et al., 2003a).

Experimental strains were recovered from the cryovials and inoculated in 10 ml of tryptic soy broth (TSB)+2.0% NaCl (Bioxon, Mexico), incubated and plated in tryptic soy agar (TSA)+2.0% NaCl (used in all trials), and incubated overnight at 30 °C. Colonies were suspended in sterile saline (2.5% NaCl) and centrifuged at 5724×g for 10 min at 15 °C. The bacterial suspension was adjusted to an optical density of 1.0 at 610 nm wavelength, similar to 0.5 MacFarland Standard (equal to 10<sup>8</sup> CFU ml<sup>-1</sup>, tested in previous trials) (Soto Rodríguez et al., 2003a) and serially diluted to achieve a density of 10<sup>4</sup> to 10<sup>6</sup> CFU ml<sup>-1</sup>. This suspension was inoculated onto thiosulfate citrate bile salts sucrose agar (TCBS) plates to determine the real density of *Vibrio*-type bacteria used in each experiment.

### 2.3. Susceptibility of different larval stages to pathogenic strains

Two experiments were run under the same conditions. In Experiment 1, CAIM 333 and CAIM 372 were tested because they were the most virulent strain to *Artemia* nauplii (M1 and PN9801, respectively, Soto Rodríguez et al., 2003a). In Experiment 2, we decide to test CAIM 333 and CAIM 79 (Ea) to compare a virulent versus no-virulent strains. To test reproducibility, the

third experiment was repeated again under the same experimental conditions (Experiment 3). Six replicates were done for each treatment and control (without the addition of any bacteria). Each experiment consisted of independent trials run consecutively and including various stages from nauplius<sub>5</sub>–zoea<sub>1</sub>, zoea<sub>1</sub>–zoea<sub>2</sub>, zoea<sub>2</sub>–zoea<sub>3</sub>, zoea<sub>3</sub>–mysis<sub>1</sub>, mysis<sub>1</sub>–mysis<sub>2</sub>, and mysis<sub>2</sub>–mysis<sub>3</sub> and misis<sub>3</sub>–postlarvae<sub>1</sub>. Each trial was run for 48 h with no water exchange, but in all trials, 10 ml of filtered (0.45 µm) and sterile seawater were added daily.

The experimental system consisted of 500 ml glass round-bottom flasks filled with 400 ml of 0.45 µm filtered and autoclaved (121 °C/30 min) natural seawater, aerated through hydrophobic 0.22 µm filters. Flasks were partially immersed in a thermostatically controlled water bath at 30 °C. Sixty *L. vannamei* larvae were counted individually and transferred into each flask and fed with *I. galvana* and *C. muelleri* (3:7 ratio), both grown without vibrios. The final microalgae density was maintained at 75,000 cells ml<sup>-1</sup> throughout the experiment. Before adding the microalgae, 100 µl of the culture were plated directly onto TCBS to confirm that no cultivable vibrios were present in the microalgae cultures. The experimental bacterial suspension was inoculated once, immediately after the microalgae were fed to the larvae. At the end of each trial, the surviving larvae were counted using a stereo microscope. Ten live larvae from each treatment (including the control) were used to estimate the internal bacterial load by maceration after a thorough wash with sterile saline solution; 0.1 ml of the supernatant was plated onto TCBS, incubated at 30 °C and the CFU counted.

#### 2.4. Larval colonization at two different exposure times with a pathogenic strain

The methodology of Soto Rodríguez et al. (2003b) was used to stain individual bacteria with 5-(4,6-dichlorotriazin-2-yl)aminofluorescein, Sigma® (DTAF). 0.5 ml from a 0.5 mg ml<sup>-1</sup> DTAF solution was added to 9.5 ml of the bacterial suspension to prepare the fluorescent labeled bacteria (FLB) suspension. The final bacterial density was accurately estimated from this suspension. A fresh FLB suspension was used for each independent trial. Two independent experiments with *L. vannamei* larvae were done using 2 (Exp-2 h) and 48 h exposure (Exp-48 h) times to the fluorescent labeled strain CAIM 333 (FLB-CAIM 333); each treatment and the control had six replicates with experimental units under the same conditions. Experiments were performed in 500 ml spherical flasks, under the same conditions

previously described. Each flask was stocked with 50 larvae in zoea<sub>3</sub>–mysis<sub>1</sub> stage and inoculated with 1.0 ml of freshly prepared FLB-CAIM 333, between 10<sup>5</sup> and 10<sup>6</sup> CFU ml<sup>-1</sup>; sterile seawater was used in the control units. In the Exp-48 h, random samples of larvae were collected at *t*<sub>0</sub> (beginning of experiment), *t*<sub>1</sub>=15 min, *t*<sub>2</sub>=30 min, *t*<sub>3</sub>=2 h, *t*<sub>4</sub>=4 h, *t*<sub>5</sub>=16 h, *t*<sub>6</sub>=24 h and *t*<sub>7</sub>=48 h. In the Exp-2 h, after the 2 h exposure, the larvae were washed with sterile seawater using a 150 µm sterile mesh and transferred to 500 ml spherical flasks filled with sterile seawater. Immediately (*t*=0), the larvae were collected and after which the larvae were sampled at the same time intervals as Exp-48 h.

Sampled larvae were stored in complete darkness until the animals were observed within the next 3 days with an Olympus® BX60 epi-fluorescence microscope. Excitation filter was BP 450–490 nm, dichroic filter was DM500 nm and the barrier filter was BA 515 nm.

#### 2.5. Statistical analysis

A Kolmogorov–Smirnov normality test was run for each group of data and depending of the results, a one-way ANOVA or a one-way ANOVA on ranks (Kruskal–Wallis) was run to determine statistical differences between treatments. Where differences were statistically significant, individual treatments were then compared against each other using a posteriori multiple Holm–Sidak or Student’s *t*-test. Statistical analyses were carried out at 0.05 significance level using the program Sigma Stat v3.02®.

### 3. Results

#### 3.1. Susceptibility of different larval stages to pathogenic strains

Five different larval stages were challenged with strains CAIM 333 and CAIM 372 at bacterial density that ranged from 10<sup>4</sup> to 10<sup>6</sup> CFU ml<sup>-1</sup> (Experiment 1, Table 1). The mortality observed was highest in zoea<sub>2</sub>–zoea<sub>3</sub> stage for both strains: 31.1% and 28.3%, respectively. In all challenges, both strains produced significant higher mortalities than the un-inoculated controls (*P*<0.05), but no significant differences could be observed between both strains (*P*>0.05). No significant correlation was observed between the density of inoculated bacteria of both strains and the mean larval mortality; Spearman coefficient (SC) correlation between the numbers of inoculated bacteria and mean

Table 1  
Experiment 1: mean mortality of *L. vannamei* larval stages bath exposed to CAIM 333 and CAIM 372 *V. campbellii* strains for 48 h

Larval stage	Strain	Inoculation density of bacteria (log CFU ml <sup>-1</sup> )	Mean mortality (%)	S.E.
N5–Z1	Control	0.0	74	2.58
	CAIM 333	6.30	87 <sup>*.a</sup>	0.86
	CAIM 372	6.31	85 <sup>*.a</sup>	0.81
Z2–Z3	Control	0.0	8	5.74
	CAIM 333	6.11	31 <sup>*.a</sup>	9.82
	CAIM 372	5.88	28 <sup>*.a</sup>	5.40
Z3–M1	Control	0.0	10	2.02
	CAIM 333	5.48	21 <sup>*.a</sup>	1.96
	CAIM 372	4.73	15 <sup>a</sup>	1.82
M1–M2	Control	0.0	18	2.48
	CAIM 333	4.75	29 <sup>*.a</sup>	1.26
	CAIM 372	5.45	27 <sup>*.a</sup>	2.67
M2–M3	Control	0.0	12	1.30
	CAIM 333	4.23	21 <sup>*.a</sup>	3.11
	CAIM 372	4.63	15 <sup>a</sup>	2.14

N5–Z1=nauplius<sub>5</sub>–zoea<sub>1</sub>, Z2–Z3=zoea<sub>2</sub>–zoea<sub>3</sub>, Z3–M1=zoea<sub>3</sub>–mysis<sub>1</sub>, M1–M2=mysis<sub>1</sub>–mysis<sub>2</sub>, M2–M3=mysis<sub>2</sub>–mysis<sub>3</sub>. Treatments per stage with the same letter are not statistically different ( $P>0.05$ ). S.E.=standard error.

\* Statistically different from respective control ( $P<0.05$ ).

mortality was  $r=0.900$ ,  $P=0.080$ ,  $n=5$ , for CAIM 333, and  $r=0.700$ ,  $P=0.233$ ,  $n=5$ , for CAIM 372.

During the second experiment, the strain CAIM 333 induced mortalities between 23.3% and 66.4%, and the significant differences observed were on the nauplius<sub>5</sub>–zoea<sub>1</sub> and zoea<sub>3</sub>–mysis<sub>1</sub> stages (Table 2), while for the CAIM 79 strain it varied between 6.1% and 65.0% no significant differences were found at any stage.

No significant correlation was found between the number of inoculated and ingested bacteria for all larval stages tested, the SC correlation for CAIM 333 was  $r=-0.41$ ,  $P=0.49$ ,  $n=5$ , and for CAIM 79,  $r=0.37$ ,  $P=0.47$ ,  $n=6$ . Strain CAIM 333 did not show any significant correlation between the numbers of ingested bacteria and mean mortality for all larval stages tested. In contrast, strain CAIM 79 showed a positive correlation between these two variables, the SC correlation was  $r=0.379$ ,  $P=0.53$ ,  $n=5$ , for CAIM 333, and  $r=0.903$ ,  $P=0.014$ ,  $n=6$ , for CAIM 79.

In the third experiment, significant differences were obtained in the mortalities of larvae inoculated with the strain CAIM 333; the mortality was highest at the nauplius<sub>5</sub>–zoea<sub>1</sub> stage (70.5%, Table 3). No significant differences were observed between the mortalities caused with the strain CAIM 79 and the control at any larval stage. No significant correlation was found between the number of inoculated and ingested bacteria for both strains and all larval stages tested; the Pearson

coefficient correlation for CAIM 333 was  $r=0.582$ ,  $P=0.17$ ,  $n=7$ , and for CAIM 79,  $r=-0.738$ ,  $P=0.058$ ,  $n=7$ . During the third experiment inoculating the strain CAIM 333, no bacterial load could be obtained during the nauplius<sub>5</sub>–zoea<sub>1</sub> and zoea<sub>1</sub>–zoea<sub>2</sub> stages.

### 3.2. Reproducibility

In order to evaluate the reproducibility of results, the mean mortalities of each treatment in the three experiments were compared for each larval stage. No significant differences were observed in the control treatments from each experiment ( $P=0.557$ ,  $n=15$ ), neither in the CAIM 333 treatments ( $P=0.225$ ,  $n=15$ ) nor between the CAIM 79 treatments ( $P=0.693$ ,  $n=12$ ).

### 3.3. Larval colonization at two different exposure times with a pathogenic strain

In the Exp-48 h, at a test density of  $5.7 \times 10^5$  CFU ml<sup>-1</sup>, monodispersed FLB (mFLB) were observed attached to the feeding appendages setae of the *L. vannamei* larvae between 0 and 2 h. Around the mouth

Table 2  
Experiment 2: mean mortality of *L. vannamei* larval stages bath exposed to CAIM 333 and CAIM 372 (*V. campbellii* strain) and CAIM 79 (*V. harveyi* strain) for 48 h

Larval stage	Strain	Inoculation density of bacteria (log CFU ml <sup>-1</sup> )	Density of bacteria ingested (log CFU larvae <sup>-1</sup> )	Mean mortality (%)	S.E.
N5–Z1	Control	0.0	3.15	79	1.17
	CAIM333	5.91	$>10^8$	88 <sup>*.a</sup>	1.26
	CAIM 79	6.11	$>10^8$	83 <sup>b</sup>	1.40
Z1–Z2	Control		2.96	17	3.71
	CAIM333	5.0	3.03	24 <sup>a</sup>	1.8
	CAIM 79	5.0	3.32	16 <sup>a</sup>	2.30
Z2–Z3	Control	0.0	3.47	5	2.04
	CAIM333	n.t.	n.t.	n.t.	
	CAIM 79	6.07	4.16	6	1.86
Z3–M1	Control	0.0	2.56	11	2.15
	CAIM333	3.30	$>10^8$	23 <sup>*.a</sup>	3.51
	CAIM 79	5.52	4.20	17 <sup>a</sup>	2.63
M1–M2	Control	0.0	4.07	60	1.31
	CAIM333	4.34	$>10^8$	66 <sup>a</sup>	2.17
	CAIM 79	5.11	$>10^8$	65 <sup>a</sup>	2.40
M2–M3	Control	0.0	2.48	52	0.81
	CAIM333	5.99	4.25	58 <sup>a</sup>	2.44
	CAIM 79	6.26	$>10^8$	46 <sup>a</sup>	4.18

N5–Z1=nauplius<sub>5</sub>–zoea<sub>1</sub>, Z1–Z2=zoea<sub>1</sub>–zoea<sub>2</sub>, Z2–Z3=zoea<sub>2</sub>–zoea<sub>3</sub>, Z3–M1=zoea<sub>3</sub>–mysis<sub>1</sub>, M1–M2=mysis<sub>1</sub>–mysis<sub>2</sub>, M2–M3=mysis<sub>2</sub>–mysis<sub>3</sub>. Treatments per stage with the same letter are not statistically different ( $P>0.05$ ). S.E.=standard error. n.t.=not tested.

\* Statistically different from respective controls ( $P<0.05$ ).

Table 3  
Experiment 3: mean mortality of *L. vannamei* larval stages bath exposed to *V. campbellii* and *V. harveyi* strains for 48 h

Larval stage	Strain	Inoculation density of bacteria (log CFU ml <sup>-1</sup> )	Density of bacteria ingested (log CFU larvae <sup>-1</sup> )	Mean mortality (%)	S.E.
N5-Z1	Control	0.0	2.65	39	5.74
	CAIM 333	6.10	NG	70 <sup>*,a</sup>	2.82
	CAIM 79	6.70	NG	37 <sup>b</sup>	2.44
Z1-Z2	Control	0.0	1.30	68	6.6
	CAIM 333	6.36	NG	83 <sup>*,a</sup>	7.1
	CAIM 79	6.36	4.13	59 <sup>b</sup>	7.4
Z2-Z3	Control	0.0	>10 <sup>8</sup>	14	2.97
	CAIM 333	6.26	>10 <sup>8</sup>	33 <sup>*,a</sup>	4.20
	CAIM 79	6.32	3.98	19 <sup>b</sup>	2.54
Z3-M1	Control	0.0	4.34	12	2.98
	CAIM 333	6.27	>10 <sup>8</sup>	39 <sup>*,a</sup>	3.79
	CAIM 79	6.08	3.35	12 <sup>b</sup>	4.34
M1-M2	Control	0.0	3.41	32	1.89
	CAIM 333	5.99	2.98	33	1.99
	CAIM 79	5.85	>10 <sup>8</sup>	33	1.92
M2-M3	Control	0.0	2.81	24	3.33
	CAIM 333	5.23	2.98	34	4.95
	CAIM 79	5.97	>10 <sup>8</sup>	37	3.10
M3-PL1	Control	0.0	3.96	19	3.01
	CAIM 333	6.41	4.21	22	4.90
	CAIM 79	6.04	3.93	13	3.58

N5-Z1 = nauplius<sub>5</sub>-zoea<sub>1</sub>, Z1-Z2 = zoea<sub>1</sub>-zoea<sub>2</sub>, Z2-Z3 = zoea<sub>2</sub>-zoea<sub>3</sub>, Z3-M1 = zoea<sub>3</sub>-mysis<sub>1</sub>, M1-M2 = mysis<sub>1</sub>-mysis<sub>2</sub>, M2-M3 = mysis<sub>2</sub>-mysis<sub>3</sub>, M3-PL1 = mysis<sub>3</sub>-postlarvae<sub>1</sub>. Treatments per stage with the same letter are not statistically different ( $P > 0.05$ ). NG = no growth. S.E. = standard error.

\* Statistically different from respective controls ( $P < 0.05$ ).

region, bacteria formed clumps causing fluorescence was too strong to enable observation of single bacteria cells. After 3–4 h exposure, individual cells could be observed inside the larval MGT. After 16 h, the number of bacteria attached to the oral region decreased, while it could be observed the presence of bacterial clumps inside the MGT and in the central region of the cephalotorax (bacterial density was  $3.57 \times 10^5$  CFU ml<sup>-1</sup>). At 18 h, the hindgut was filled with fluorescent bacteria and, at 24 h, the entire digestive tract was stained and mFLB could hardly be observed. At the end of the experiment (48 h), most larval tissues were fluorescently stained.

During the Exp-2 h, a FLB-CAIM 333 bacterial density of  $1.73 \times 10^6$  CFU ml<sup>-1</sup> was used. At the beginning of the trial, FLB could be seen surrounding the *L. vannamei* larvae and, at 15 min, the majority of the larvae had mFLB attached to the oral region and some larvae already had some bacteria inside the MGT. At 30 min, bacteria clumps could be observed in the oral

region. After 2 h, only mFLB were observed in the oral region; at 13 h, there are few bacteria attached to the oral region and no bacteria were present in the MGT. Bacteria were observed in the oral region, while in the MGT several FLB clumps were formed after 22 h. After this time, there was a generalized decrease of fluorescence.

#### 4. Discussion

There is yet no reproducible experimental infection method to induce vibriosis in *L. vannamei* larvae. The challenge of penaeid shrimp larvae with pathogenic luminescent strains, isolated from diseased organisms and identified phenotypically as *V. harveyi*, has provided mixed results. Doses below  $10^2$  CFU ml<sup>-1</sup> can cause more than 50% mortality, while other strains do not cause mortality at doses larger than  $10^6$  CFU ml<sup>-1</sup> (Prayitno and Latchford, 1995; Abraham et al., 1997). In general terms, the mean mortalities were between 15% and 80% for the strains CAIM 333 and CAIM 372 at tested densities of  $10^4$ – $10^6$  CFU ml<sup>-1</sup>. Previous trials with CAIM 416 identified also as luminescent *V. campbellii* strain caused 52.6% mortality in shrimp larvae (data not shown); in *L. vannamei* juveniles, the same strain caused 100% mortality (Hernandez and Olmos, 2004). In the present study, the stages of zoea were more susceptible to infection by the CAIM 333 and CAIM 372 strains, while mysis stages were more resistance to infection. In contrast, the CAIM 79 strain did not show any relation between mortality and larval stages, with mortalities sometimes below the control, similar results were found with *Artemia* nauplii (Soto Rodríguez et al., 2003a).

In the present work, no statistical correlation was found between the number of inoculated and ingested bacteria by shrimp larvae. Challenges with *Artemia franciscana* nauplii showed similar results independent of the bacterial density to which they were exposed (Gómez Gil et al., 1998a; Makridis et al., 2000; Soto Rodríguez et al., 2003a). We suggest that the larvae of the marine crustaceans have a limited capacity of ingest bacteria in the interval of  $10^2$ – $10^4$  CFU nauplius<sup>-1</sup>. It is understood that the ingested bacteria colonized the digestive tract of the larvae or were a part of the transit microbiota, and that a successful colonization must include the bacterial load capacity of the digestive tract.

In some trial, high mortalities in the control treatment were observed, possibly due to a poor nauplii quality. In commercial shrimp hatcheries, from which the nauplii were obtained, are common to mix nauplii from different females and the quality decreases considerably

in females with multiple broods or sick organisms (Palacios et al., 1999). Although there were not significant differences of the controls and treatments at any larval stage in the three experiments, there was high variability in the larvae mortality. It is very difficult to reproduce an experimental infection with shrimp larvae; it is necessary to standardize each step of the pathogenicity test because many environmental parameters, the bacterial strain and biological factors may influence in the results (Saulnier et al., 2000).

#### 4.1. Larval colonization

There are few studies on the interaction between the microflora in the crustacean gut and the hosts, despite their importance. When comparing the results between Exp-48 h and Exp-2 h trials, the latter provided a better option to study bacterial colonization processes in the gut of shrimp larvae, as bacteria were in contact with the potential host for a shorter period. The short gut retention time of zoea and mysis stages (12 and 20 min, respectively, Le Vay et al., 2001) allows only to remain in the digestive tract, those bacteria with a capacity to adhere to the epithelial tissue. In contrast, when using a longer infectious bath challenge, larvae are continuously exposed to the bacteria and thus a wider range of bacteria can colonize. From both experiments, it can be concluded that bacterial colonization of shrimp larval gut is established after 13 h in the cephalothorax region and anterior MGT. Pathogenicity studies using adult penaeid shrimps have suggested the stomach as the region where the initial multiplication began followed for the hepatopancreas (de la Peña et al., 1995), although in the first minutes to hours of a bacterial infection, many bacteria can be cleared by the shrimp (Alday-Sanz et al., 2002).

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