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Response of *Artemia franciscana* fed with *Aeromonas hydrophilla* and challenged with *Vibrio campbellii*

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Abstract: Gnotobiotically grown *Artemia* is already proved useful in providing better understanding of host-microbial interactions. The aim of the present study is to make use of gnotobiotically grown *Artemia* in understanding the effect of *Vibrio campbellii* on *Artemia franciscana* fed with *Aeromonas hydrophilla*. In the first instance, the optimization of *V. campbellii* concentration was done to establish the challenge dose to *Artemia* up to 48 h. It was observed that *Artemia* when challenged with concentration of 10^7 cells /ml of *V. campbellii* showed lowest survival, hence this concentration has been used for the next experiments. In the other part, the optimization of concentration of dead LVS3 as feed was done up to 96 h. Four different concentrations of dead LVS3 were used in the same ratio together while animals having no feed and challenged with *V.campbellii* were taken as controls. The lowest survival was found with full concentration of LVS3 ($10.5x10^9$ cells/glass tube).

Keywords: *Artemia franciscana, Aeromonas hydrophilla, Vibrio campbellii,* gnotobiotic, host-microbial interaction.
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INTRODUCTION

The aquaculture production sector has been growing at an average of 8.8% annually, faster than any other animal food producing sectors[1]. Despite all technological improvements, that allowed the expansion of aquaculture over the years, diseases are still a major constraint[1]. To overcome the problem of diseases many strategies had been applied to understand the host-microbial interactions, out of them the basic is to define the animal functioning in the absence of all micro-organisms (i.e. under germ-free or gnotobiotic conditions) and then to observe the effects of adding a define microbes or compounds[2].

Several studies were performed on host-microbe interactions using gnotobiotic aquatic animals like fresh water zebra fish (Danio rerio)[3], marine fish cod and halibut[4], Pacific oyster larvae[5], abalone larvae[6], rotifers[7], *Hydra viridis* and *Hydra vulgaris*[8], while most studies performed so far on host-microbe interactions of crustaceans used germ free *Artemia* as test organism[8-17].

Artemia is used as live food in the aquarium trade and for marine finfish and crustacean larviculture. So far, *Artemia* has been used as food source for more than 85% of cultivated marine animals, either as a sole diet or together with other food ingredients[18]. *Artemia* possess several characteristics and advantages that make them a useful model organism for research in animal biology[19] i) such as they can be cultured under axenic / gnotobiotic conditions, ii) have short generation time of 2-3 weeks and high reproduction rate, iii) cysts from different species and strains are available, iv) can be cultured easily at high densities with a wide range of feed sources, and v) has the ability to tolerate adverse environmental conditions.

Artemia had been used as a model organism for numerous physiological, biochemical, ecological, and genetic studies in aquaculture. It has also been useful for studying the biology of infections, the effect of chemotherapeutic agent on diseases in crustaceans[9, 10, 15, 20 21], and probiont testing by using *Artemia* as a vector for transferring probionts to the larvae of target species[15, 19].

MATERIALS AND METHOD

Bacterial strains and their culture

Isolates of the bacterial strain, *Vibrio campbellii* (LMG21363) and *Aeromonas hydrophila* (LVS3) previously stored in 40% glycerol at –80°C, were aseptically inoculated and grown in petri dishes containing marine agar 2216 (Difco Marine Broth 2216 BD Becton, Dickinson and Company, USA). A colony of *A. hydrophila* was subsequently transferred and grown to stationary phase in marine broth 2216 (Difco Laboratories, Detroit, Mich., USA) by incubation overnight at 28°C with constant shaking while a colony of *V. campbellii* was transferred and grown to growth phase in marine broth 2216 by incubation for 5-7 h to obtain bioluminescence. The LVS3 is autoclaved at 121°C for 20 min before washing. The bacterial densities were determined spectrophotometrically at an optical density of 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), it is assumed that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml.

Axenic Artemia hatching

To obtain axenic *Artemia*, decapsulation procedure[22] and hatching procedures[13] were followed. High-hatching cysts of *Artemia*

franciscana, originating from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium) were used. About 2 g of cysts were hydrated in 90 ml tap water for 1 h with strong aeration under laminar flow hood. All equipments were previously sterilized and autoclaved at 120ºC for 20 min prior to use. Cysts were exposed to constant incandescent light (2000 lux) and temperature (28°C) for 18 - 20 h. Consequently, hatched nauplii that developed into stage II within 4-6 h after hatching were used in the experiments (mouth opens at stage II and nauplii became capable of ingestion).

Axenity verification

Methods used to verify axenity of the *Artemia* culture were conducted[13] by using plating techniques. Bacterial contamination in the control tubes was checked by plating 100 µl of the culture medium in marine agar. Plates were incubated for 5 days at 28°C. If contaminated control tubes were found, all results of that experiment were discarded.

Experimental design

The first experiment was conducted to optimize the concentration of *V. campbellii* for *Artemia* challenge test. In order to do so, the *Artemia* nauplii were challenged with a 10-fold dilution series of *V. campbellii* culture. The final concentration in the *Artemia* culture water was 10^0, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cells/ml. Each treatment was carried out in pentuplicate and average of them was obsereved. The survival of *Artemia* was scored 48 h after challenging with *V. campbellii*. The concentration of *V. campbellii* caused maximum mortality of *Artemia*, was used as a challenged dose in the next experiment.

The second experiment was conducted to optimize the feeding schedule for *Artemia* challenge study. In this experiment, we aimed to determine the best concentration of autoclaved LVS3 needed to obtain maximum mortality of *Artemia* after challenge with *V. campbellii* for a period of 96 h. Four different concentrations of LVS3 were used: $10.5x10^9$ (full concentration), $5.25x10^9$ (1/2 of full concentration), $2.62x10^9$ (1/4 of full concentration) and $1.31x10^9$ (1/8 of full concentration) cells/glass tubes containing 30 ml filtered autoclaved seawater (FASW). Each of these concentrations of feed was fed to the *Artemia* in the ratio of 10:10:15:15:25:25 at 0, 6, 12, 24, 48, and 72 h, respectively (Table 1). The feeding schedule used in our present study was adapted[23] with slight modification. Feeding schedule with highest mortality was used for the rest of the experiments. In this experiment, non-fed *Artemia* nauplii that were either not challenged or challenged with *Vibrio* were used as controls. The survival of *Artemia* was determined at 6, 12, 24, 48, 72 and 96 hours.

Artemia survival

The number of live *Artemia* was registered before feeding or adding bacteria by counting with the naked eye while exposing each transparent glass tubes to an incandescent light without opening the tube to maintain the axenity, while in the last counting larvae were sacrificed.

Larval survival in each replica was calculated by the following formula

Larval survival (%) = (Total number of live *Artemia* larvae/Initial number of *Artemia* larvae stocked) x 100.

Table 1: Experimental design of experiment 2. A-J the treatments performed. F - dead LVS3 as feed, 1F- feed at $10.5x10^9$ cells/glass tube, ½ F- feed at $5.25x10^9$ cells/glass tube, ¼ F - feed at $2.62x10^9$ cells/glass tube and 1/8 F-feed at $1.31x10^9$ cells /glass tube. VC-*Vibrio campbellii* added at 6 h. NF- no feed.

Treatment	Time (h)					
	0	6	12	24	48	72
A	NF	NF	NF	NF	NF	NF
B	NF	NF+VC	NF	NF	NF	NF
C	1F	1F	1F	1F	1F	1F
D	1F	1F+VC	1F	1F	1F	1F
E	½ F	½ F	½ F	½ F	½ F	½ F
F	½ F	½ F+VC	½ F	½ F	½ F	½ F
G	¼ F	¼ F	¼ F	¼ F	¼ F	¼ F
H	¼ F	¼ F +VC	¼ F	¼ F	¼ F	¼ F
I	1/8 F	1/8 F	1/8 F	1/8 F	1/8 F	1/8 F
J	1/8 F	1/8 F +P	1/8 F	1/8 F	1/8 F	1/8 F

Statistical analysis

Survival data (%) were arcSin transformed to satisfy normality and homocedasticity requirements as necessary. Data were then subjected to one-way analysis of variances (ANOVA) followed by Duncan's multiple range tests using the statistical software Statistical Package for the Social Sciences (SPSS) version 14.0. to determine significant differences among the treatments. Significance level was set at $P<0.05$.

RESULTS AND DISCUSSION

Optimization of V. campbellii dose for Artemia challenge study

Different concentrations of *V. campbellii* were used to determine their effect on *Artemia* larvae survival. The test was done as a preliminary trial to determine the optimum dose of *V. campbellii* as a

pathogen that could have maximum negative effect on *Artemia.*

Figure 1 showed the percentage survival of *Artemia* nauplii after 48 h of challenge with different concentrations of *V.campbellii* (10^0, 10^2, 10^4, 10^5, 10^6, 10^7 cells/ml) and fed once with autoclaved LVS3 bacteria at the concentration of 10^7 cells/ml. After 48 h *V. campbellii* had a significant effect ($P<0.05$) on the survival of *Artemia*, lowest value being observed in group challenged with a concentration of 10^7 cells/ml, which, however, was not significantly ($P>0.05$) different from groups challenged with *V. campbellii* dose of 10^4, 10^5, and 10^6 cells/ml. This dose of 10^7 cells/ml was used for the challenge study in the next experiment.

The results showed that *Artemia* challenged with *V. campbellii* at 10^7 cells / ml had the highest mortality (about 57%). Our results are almost in agreement with the findings[16]. In the next experiment, this dose (10^7cells/ml) is used for *Artemia* challenge study.

Table 2: Percentage survival of *Artemia* nauplii (mean ± standard error of 5 replicates) after challenge with different concentrations of *V. campbellii* LMG21363. Mean with different alphabet letters indicate significant difference (*P*<0.05)

***Vibrio. Campbellii* cell conc./ml**	**Survival (%)**
10^0	70 ± 1.2^{a}
10^2	63 ± 0.8^{ab}
10^4	57 ± 1.6^{bc}
10^5	50 ± 1.2^{bc}
10^6	45 ± 1.2^{c}
10^7	42 ± 0.9^{c}

Optimization of feeding schedule for Artemia challenge study

In experiment 2, we aimed to determine the best concentration of LVS3 needed to obtain maximum mortality of *Artemia* after challenge with *V. campbellii* for a period of 96 h. *Artemia* nauplii were fed with four different concentrations of autoclaved LVS3 which were distributed in six different feeding portions (in %)-10:10:15:15:25:25 fed at 0, 6, 12, 24, 48 and 72 h, respectively. The challenge test was performed with *V. campbellii* added at the time of first feeding. Nauplii not supplied with dead LVS3 and challenged with *V. campbellii* were used as controls.

Figure 2 indicates significant differences ($P<0.05$) in the percent survival among the different groups. Maximum mortality was observed in (1+VC) group fed LVS3 at full concentration ($10.5x10^9$ cells/falcon tube in the ratio of 10:10:15:15:25:25) and challenged with *V. campbellii*. The group 1+VC, however, did not differ significantly ($P<0.05$) from the group ½+VC. Surprisingly, there was also no significant difference ($P>0.05$) between 1/8–VC and 1/8+VC groups.

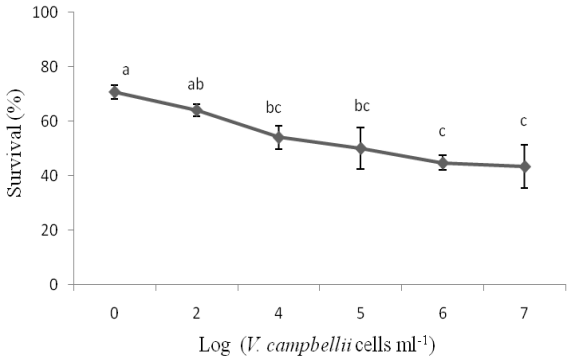

Figure 1: Percentage survival of *Artemia* nauplii (mean±SE of 5 replicates) after challenge with different concentrations of *V. campbellii* LMG21363. Error bars with different alphabet letters indicate significant difference (*P*<0.05).

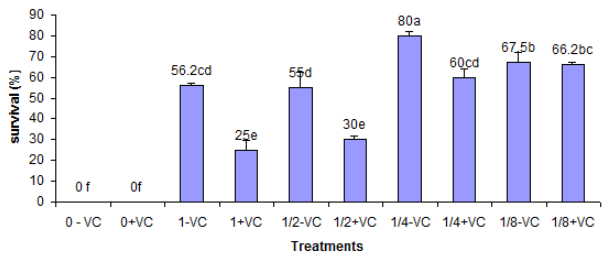

Figure 2: Average survival (%) of *Artemia* nauplii fed different concentrations of LVS3 and challenged with *V. campbellii* for 96 h. Error bars indicate standard error of 5 replicates. Different alphabet letters denote significant differences (P<0.05).

The strain LVS3 was selected as a feed source because it has been demonstrated previously as harmless to *Artemia*[9]. The LVS3 bacteria were killed by autoclaving before added to the *Artemia* cultures in order to eliminate any possible interactions between the live LVS3 bacteria and *V. campbellii* which were used to challenge. In this study, it was found that *Artemia* fed autoclaved LVS3 at ¼ and 1/8 of the full concentration of $10.5x10^9$ cells/glass tube performed better after challenged with *V. campbellii* and showed significant increase in survival as compare to the *Artemia* fed with full and half concentration of $10.5x\ 10^9$ cells/glass tube. This indicated that less feed provide better protection to *Artemia* challenge with *V. Campbellii*, however, *Artemia* fed autoclaved LVS3 at concentration of $10.5x10^9$ cells/glass tube in the ratio of 10:10:15:15:25:25 and challenged with *V. campbellii* had the maximum mortality. This could possibly be due to feeding LVS3 in such a high concentration that animal could not digest the ingested food and hence weakened.

The effect of concentration and feeding frequency of LVS3 on host-microbe interaction has been studied in the gnotobitic *Artemia*. Investigation revealed that less but continuous feeding to *Artemia* with dead LVS3 provides protection to animal for longer duration and seems to found protective against *V. campbellii* challenge however, the animals having more feed were less resistant, more susceptible to *V. campbellii* and have a high mortality rate.

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