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Inhibitory activity and adhesive ability of potential probiotic *Bacillus* species to confer protection for *Artemia* gnotobiotic culture against pathogenic *Vibrio* spp.

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Abstract: To evaluate the potential probiotic effect of *B. coagulans* and *B. subtilis* strains toward *Artemia* cultured in different gnotobiotic conditions, antibacterial and adherence assays were investigated and challenge tests with *Artemia* larvae were performed. Virulent *V. alginolyticus* (IFL), isolated from infected fish, was tested for comparative purposes. The bacterial strains *B. coagulans* and *B. subtilis* produced antimicrobial activity against the pathogenic *Vibrio* species, including *V. alginolyticus* isolated from infected fish, and inhibitory zones that ranged from 12 to 20 mm in diameter. An adherence assay revealed that these potential probiotic bacteria are fairly adherent, with a values ranging from 0.10 to 0.32 at 595 nm. Challenge tests with *Artemia* larvae demonstrated that *B. coagulans* and *B. subtilis* have no impact on survival, but do enhance growth and protection against the pathogenic *Vibrio*. On the basis of these results, it is suggested that the tested *Bacillus* strains in this study show tremendous potential for use as a probiotic in *Artemia* cultures, particularly with regard to improving the survival and growth of *Artemia* larvae and their ability to inhibit the pathogen in in vivo and in vitro conditions.

Key words: *Artemia*, probiotics, *Bacillus*, pathogenic control, adhesion

Introduction

The incidence of microbial diseases has increased dramatically in accordance with the growth of fish larvae production, and high mortalities occur during the larval first feeding due to the incidence of pathogenic and opportunistic bacteria (1). Different authors have suggested that this infection happens mainly through the food chain, and especially during feeding with *Artemia* (2). Live feed sources such as *Artemia* play an important role in the dietary regime of fish and shellfish larvae produced in industrial hatcheries because of their nutritional profiles (3). However, live feed can be a source of pathogenic bacteria, such as *Vibrio*, responsible for fish and aquatic animal pathologies in hatchery environments. For this reason, the prevention of disease spreading through the feed animal is essential in aquaculture (4-6). Products that can enhance host immunity and disease resistance, such as immunostimulants and probiotics, are being used in shrimp disease prevention and have garnered much interest in recent years (7). Probiotics are harmless bacteria that

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promote the wellbeing of a host animal and contribute to the direct and/or indirect protection of the host animals against harmful bacteria. The positive effects of applying certain beneficial bacteria in aquaculture have been well documented, and treatments with probiotics have also rapidly increased. Several research articles regarding the benefits of using *Bacillus* to improve shrimp growth performance, survival, immunity, and disease resistance in aquaculture have been published (7,8). Vaseeharan and Ramasamy (4) reported on the antagonistic effect of *B. subtilis* BT23 against pathogenic vibrios in tiger shrimp (*Penaeus monodon*), which showed a 90% reduction in cumulative mortality. Other microbiological studies have demonstrated that *Bacillus sp*. and *Aeromonas hydrophila* can protect *Artemia* cultures against pathogenic *Vibrio* (9-11). The addition of *Bacillus coagulans* SC8168, as a water additive for shrimp larvae, significantly increases their survival rate and some digestive enzyme activities (10). In the search for more effective and environmentally friendly treatments, these previous studies concluded that it is necessary for other research activities to be developed with the aim of finding other probionts as biocontrol agents against pathogenic species in shrimp larval cultures. The present study aimed to find a possible probiotic for *Artemia* cultures through the investigation of the probiotic properties of 2 *Bacillus* strains, their effect on the *Artemia* culture, and their role in the enhancement of *Artemia* larvae resistance against pathogenic *Vibrio*.

Materials and methods

Bacterial strains

The tested bacterial strains used in this study were isolated aseptically from an *Artemia* culture recovered from Tunisian hypersaline environments (saltworks of Sfax, 34°43′N, 10°44′E; saltworks of Sahline, 35°45′N, 10°42′E). Water samples (1 mL of *Artemia* culture) were enriched for 24 h at 37 °C in nutrient broth sea water (NBSW) (salinity 34‰, pH 7.99), smeared on a nutrient agar plate, and incubated at 37 °C. The colonies that appeared were passaged on nutrient agar plates. Gram- and catalase-positive rods were retained. These were identified using standard morphological and physiological characteristics and the API 50 CHB and Api 20 E systems (BioMérieux, Marcy-l'Etoile, France). Results were read using an automated microbiological mini-API (BioMérieux). Virulent *V. alginolyticus* (IFL) isolated from infected fish, previously described by Ben Kahla-Nakbi et al. (12), was used in the infectivity experiments. *Bacillus* strains were preserved at −80 °C and were routinely checked for purity during this investigation prior to use. There were 3 other reference pathogenic bacterial strains used for the antimicrobial activity assay: *V. parahaemolyticus* ATCC17802, *V. alginolyticus* ATCC17749, and *V. alginolyticus* (IFL) (12).

Antimicrobial activity using well diffusion agar assay (WDAA) and adherence assay

Potential probiotic strains were tested for their antagonistic activity using the well diffusion agar assay (WDAA) (4) against 3 target strains: *V. parahaemolyticus* ATCC17802, *V. alginolyticus* ATCC17749, and *V. alginolyticus* (IFL) (12). Pathogenic bacterial strains were grown in 10 mL of nutrient broth and cultured for 24 h on nutrient agar at 30 °C. The colonies from pure culture were suspended in 10 mL of physiological medium and well mixed for 5 min, and 1 mL of this was spread over the Mueller-Hinton agar (MHA) plates and incubated for 30 min at 37 °C. Previously isolated *Bacillus* strains were incubated in marine broth (24 h, 30 °C) and incorporated into pour plates. Wells were cut into the agar and filled with 100 µL of the marine broth isolate. The presence of antimicrobial metabolites produced by the isolate inhibited the growth of the pathogen, producing a zone of inhibition around the well (4).

The adhesion ability of *Bacillus* strains grown in Trypticase soy broth (TSB, Bio-Rad, France) was determined using a semiquantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark), as described previously (13,14). Briefly, following overnight incubation at 37 °C, the optical density at 595 nm (OD_{595}) of the bacteria was measured. An overnight culture grown in TSB at 37 °C was diluted to 1:100 in TSB with 2% (w/v) glucose. A total of 200 µL of these cell suspensions were transferred to a U-bottomed 96-well microtiter plate (Nunc). Each strain was tested in triplicate. The plates were incubated aerobically at 37 °C for 24 h. The cultures were removed, and the microtiter wells were washed twice with phosphate-buffered saline (PBS) (7 mmol/L Na_2HPO_4, 3 mmol/L NaH_2PO_4,

and 130 mmol/L NaCl at a pH of 7.4) to remove nonadherent cells and were dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µL of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed 3 times with 300 µL of sterile distilled water. The water was then cleared and the microplates were air dried. The optical density of each well was measured at 595 nm (OD_{595}) using an automated Multiskan reader (GIO DE VITA E C, Rome, Italy). Adhesion ability was interpreted as high ($OD_{595} \geq 1$), fair ($0.1 \leq OD_{595} < 1$), or slight ($OD_{595} < 0.1$).

Artemia gnotobiotic culture

Experiments were performed with *Artemia salina* cysts collected from the saltworks of Sfax. Bacteria-free cysts and nauplii were obtained via decapsulation, as described by Sorgeloos et al. (15). Decapsulated cysts were washed with filtered and autoclaved sea water (FASW) over a 50-µm sterile filter net. This procedure was repeated 9 times, using new FASW. After this step, washed decapsulated cysts were transferred to a sterile Falcon-brand culture tube containing 30 mL of FASW. The Falcon tube was capped, placed on a shaking incubator (28 °C, 120 rpm), and exposed to a constant incandescent light. After 18-20 h, 10 axenic nauplii second instars were picked and transferred to sterile Falcon tubes containing 30 mL of FASW, together with the amount of feed scheduled for day 1. All manipulations were carried out under a laminar flow hood and all necessary tools were previously autoclaved at 120 °C for 20 min.

Inoculation of bacterial suspensions

Sterile 60-mL Falcon tubes containing 10 axenic nauplii second instars were inoculated with beneficial bacterial strains (10^7-10^8 cfu/mL) and pathogenic *Vibrio* (10^6 cfu/mL) from pure culture in the exponential growth phase. The concentration of each bacterial strain was estimated through a regression analysis of the optical density of the pure culture. The number of culture forming units per milliliter was determined using petri plates with marine agar.

Experimental design

To evaluate the effect of the bacterial strains on the *Artemia* culture, 8 challenge tests were performed: *Artemia* with commercial food (red pepper, BERN AQUA) (A + AL), *Artemia* axenic (A. axe), *Artemia* with *Bacillus coagulans* (A + BC), *Artemia* with commercial food and *Bacillus coagulans* (A + AL + BC), *Artemia* with *Bacillus subtilis* (A + BS), *Artemia* with commercial food and *Bacillus subtilis* (A + AL + BS), *Artemia* with beneficial bacteria and pathogenic *Vibrio* (A + BC + VA, A + BS + VA), and *Artemia* with commercial food, beneficial bacteria, and pathogenic *Vibrio* (A + AL + BC + VA, A + AL + BS + VA). Treatment of *Artemia* with commercial food (A + AL) was chosen as a control treatment because larvae survival showed a higher percentage at the end of the experimentation period. The duration of the tests was 6 days. Commercial food and potential probiotic bacterial strains were added during the first 3 days. The pathogenic bacterial strain was provided only at day 3.

Survival and growth of Artemia and methods used to verify axenity

During the challenge tests, the number of swimming larvae (survival percentage) was determined daily and the percentage of survival was calculated. At the end of each experiment, live *Artemia* were fixed with Lugol's solution to measure their individual length (IL) under a binocular magnifying glass containing a graduated micrometer (Nikon Eclipse 50i, Japan).

At the beginning and end of each run of the procedure, the absence of bacteria was monitored by transferring larvae and 100 µL of water to petri plates in triplicate with marine agar.

Statistical analysis

The values of survival larvae (percentage) were arcsine transformed, while the values of IL were square-root transformed to satisfy normal distribution and homoscedasticity requirements. Differences in survival, individual length, and total length of the *Artemia* larvae under different treatments were examined by analysis of variances (ANOVA) and Duncan's test using Statistica 5.5 software.

Results

Bacterial strain identification, antimicrobial activity, and adherence assay

Bacterial strains isolated from the *Artemia* culture were identified as *B. subtilis* and *B. coagulans* using the Api 50 CHB and 20 E systems.

Potential probiotic strains have an inhibitory effect against pathogenic *Vibrio* strains *V. parahaemolyticus* ATCC17802, *V. alginolyticus* ATCC17749, and *V. alginolyticus* (IFL) isolated from infected fish (Table). The inhibitory zones were about 12-20 mm in diameter.

Estimation of biofilm formation on a polystyrene surface revealed that the tested *Bacillus* strains were fairly adhesive, with values ranging from 0.10 to 0.32 at 595 nm (Table).

Probiotic treatment and infection of Artemia culture

Larval survival

Larval survival rates are shown in Figure 1. Treatment "*Artemia* axenic" (A. axe) resulted in 0% on culture day 4 (Figure 1). Furthermore, treatment of *Artemia* with beneficial bacteria (A + BC, A + BS) resulted in a low survival rate at the end of treatment (13% with *B. coagulans* and 25% with *B. subtilis*) (Figure 1). As expected, the control treatment of *Artemia* with commercial food (A + AL) resulted in a high survival rate (80%) at the end of culture day 6 (Figure 1). Treatment of *Artemia* with commercial food and beneficial bacteria (A + AL + BC, A + AL + BS) resulted in a high survival rate (77.5% to 90%) and showed no significant differences with the control treatment (P > 0.05) (Figure 1). The addition of *B. subtilis* in the presence of commercial food in the culture medium challenged pathogenic *Vibrio*, significantly increasing the survival rate of the *Artemia* larvae compared to the treatment of *Artemia* with commercial food and pathogenic *Vibrio* (A + AL + VA) (P < 0.05). This positive effect is justified by the nonsignificant difference between the treatment of *Artemia* with commercial food, *B. subtilis*, and pathogenic *Vibrio* (A + AL + BS + VA) and the treatment of the control (P > 0.05). An increase in the survival rate was found when *B. coagulans* was used with commercial food in the presence of the pathogen. However, a significant difference in comparison with the control treatment was noted (P < 0.05) (Figure 1).

Larval development

Under axenic conditions, *Artemia* nauplii have an individual length ranging between 0.45 and 0.50 mm. In the treatment of *Artemia* with commercial food (A + AL), larvae reached a length that varied between 1.2 and 1.4 mm. When *Artemia* was treated with pathogenic *Vibrio* in the presence or absence of food, the length of dead larvae was between 0.6 and 0.8 mm, and a significant difference was found in comparison with the control (P < 0.05) (Figure 2). The introduction of BS or BC alone in *Artemia* culture increased the development from 0.4 to 1.3 mm. When beneficial strains were used in the presence of commercial food, the larvae had a length of 1.57 and 1.66 mm, respectively, and a significant difference was found in comparison with the control treatment (A + AL) (P < 0.05) (Figure 2). The results of interaction between probiotics associated with commercial food and pathogenic *Vibrio* showed that the tested strains confer protection to the *Artemia* culture against the pathogen. In fact, the *Artemia* larvae had an individual length that ranged from 1.3 to 1.5 mm and 1.4– to 1.6 mm when BC and BS were added to the culture medium. No significant difference was assessed between these 2 treatments (P > 0.05) (Figure 2).

Table. Antagonistic activity of *Bacillus* strains against pathogens, mm, and adhesion ability value.

Strains	Pathogens			Adhesion value OD_{595}
	V. alginolyticus IFL	*V. alginolyticus* ATCC 17749	*V. parahaemolyticus* ATCC 17802	
B. coagulans	13.6 ± 0.05	16.3± 0.05	17.5 ± 0.07	0.12 ± 0.02
B. subtilis	14.5 ± 0.5	18.6 ± 0.11	15 ± 0.05	0.30 ± 0.28

For each average, the respective standard deviation is added (mean ± S.D).
$OD_{595} \geq 1$: highly adherent, $0.1 \leq OD_{595} < 1$: fairly adherent, $OD_{595} < 0.1$: slightly adherent.

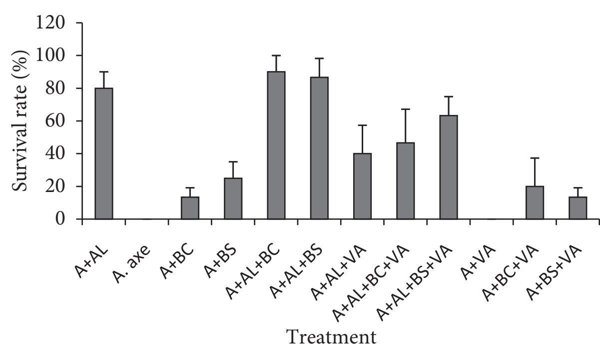

Figure 1. Survival of *Artemia* larvae after various treatments: *Artemia* with commercial food (A + AL), *Artemia* axenic (A. axe), *Artemia* with *Bacillus coagulans* (A + BC), *Artemia* with commercial food and *Bacillus coagulans* (A + AL + BC), *Artemia* with *Bacillus subtilis* (A + BS), *Artemia* with commercial food and *Bacillus subtilis* (A + AL + BS), *Artemia* with commercial food and pathogenic *Vibrio* (A + AL + VA), *Artemia* with pathgenic bacteria (A + VA), *Artemia* with beneficial bacteria and pathogenic *Vibrio* (A + BC + VA, A + BS + VA), and *Artemia* with commercial food, beneficial bacteria, and pathogenic *Vibrio* (A + AL + BC + VA, A + AL + BS + VA).

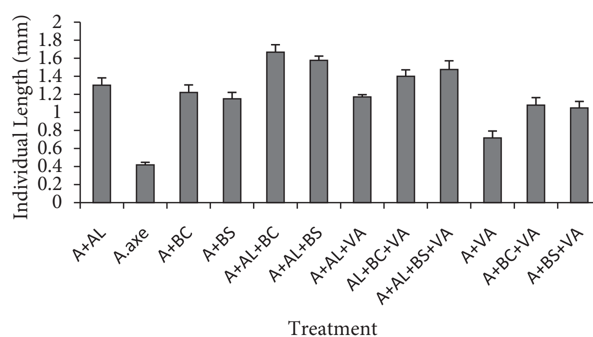

Figure 2. Total length of *Artemia* larvae: *Artemia* with commercial food (A + AL), *Artemia* axenic (A. axe), *Artemia* with *Bacillus coagulans* (A + BC), *Artemia* with commercial food and *Bacillus coagulans* (A + AL + BC), *Artemia* with *Bacillus subtilis* (A + BS), *Artemia* with commercial food and *Bacillus subtilis* (A + AL + BS), *Artemia* with commercial food and pathogenic *Vibrio* (A + AL + VA), *Artemia* with pathgenic bacteria (A + VA), *Artemia* with beneficial bacteria and pathogenic *Vibrio* (A + BC + VA, A + BS + VA), and *Artemia* with commercial food, beneficial bacteria, and pathogenic *Vibrio* (A + AL + BC + VA, A + AL + BS + VA).

Discussion

The present study demonstrates the ability of potential probiotic bacteria to control the pathogenic *Vibrio* in in vitro and in vivo conditions. On diffusion agar plates, *B. coagulans* and *B. subtilis* exhibited a zone of clearance against pathogenic *Vibrio*. The inhibitory mechanism of the interaction was not characterized in this study. However, this result can be related to the ability of *Bacillus* to produce antibacterial compounds such as bacitracin, gramicidin S, polymyxin, and tyrothricin, which are active against a wide range of gram-positive and gram-negative bacteria (16,17). A previous study suggested that the inhibitory effects of *Bacillus* might be due to the alteration of pH in the growth medium, utilization of essential nutrients, or the production of volatile compounds (18). The addition of potential probionts to the *Artemia* culture enriched with commercial food did not affect survival and showed a positive effect on the development of the larvae. Similarly, these strains enhanced protection against the target pathogenic strain. These results can be explained by their great antagonistic ability to inhibit pathogenic bacteria, possibly due to high doses that can induce artificial and temporary dominance. Furthermore, the ability of some microorganisms to adhere to the intestinal tract and their effect in the digestive processes of aquatic animals can participate in the improvement of the *Artemia* culture and protection against the pathogen (11). The positive effect of these bacteria on the *Artemia* culture can be due to the capacity of the bacteria to provide, either directly or indirectly, nutritional elements like vitamins, fatty acids, polyamines, and enzymes (19). Adhesion to an abiotic surface partly explains the observed positive effect of the tested strains during this study and several others. In fact, this property can help *Bacillus* strains to remain present in the bacterial flora of the gut for several days, be active during intestinal transit, participate in the digestion process, aid in the elimination of potential pathogens, and create a healthy environment (14). Several microbiological studies have demonstrated the beneficial effect of *Bacillus* spp. on shrimp cultures. Balcazar and Luna-Rojas (20) demonstrated that probiotics such as *Bacillus* have potential applications for controlling pathogenic *Vibrio* in shrimp aquaculture. Vaseeharan and Ramasamy (4) found that growth of pathogenic

V. harveyi in tiger shrimp was controlled by the probiotic effect of *B. subtilis* BT23 in vitro and in vivo. Disease resistance was improved and accumulated mortality was reduced by 90% when juvenile *Penaeus monodon* specimens were exposed to *B. subtilis* BT23 isolated from shrimp culture ponds before a challenge with *V. harveyi*. Similarly, the introduction of probiotic *Bacillus* as a water additive for larval shrimp resulted in the enhancement of immunity and disease resistance (9,10,21). It has been also demonstrated that *Bacillus* spp. and *Aeromonas hydrophila* can protect *Artemia* cultures against pathogenic *Vibrio* and have a probiotic effect on the survival and growth of *Artemia* nauplii (9,11,22). This protection is significantly improved by the quantity and quality of feed provided to *Artemia* and the different combinations of the feed sources (9,11,14). In conclusion, the studied *B. coagulans* and *B. subtilis* strains can be considered as promising probiotic candidates for *Artemia* cultures and possibly other shellfish or fish larvae. More studies are required in order to clarify the exact contribution of the potential candidate probiotics used in this study, using a host gene expression to overcome disease in aquaculture farms.

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