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Bacillus cereus and *Bacillus subtilis* used as probiotics in rotifer (*Brachionus plicatilis*) cultures

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Abstract

Since live food is a key factor in fish and invertebrate larvae rearing, the objective of this study was to evaluate the probiotic potential of two Gram positive *Bacillus* species: *Bacillus cereus* (CCBM-2) and *Bacillus subtilis* (CCBM-64), originally isolated from marine sediments in the Colombian Caribbean, in rotifer *Brachionus plicatilis* cultures. The enzymatic activity and antibacterial activity against pathogens of *B. cereus* and *B. subtilis* were measured *in vitro*. Direct supplementation of the rotifer culture water with both bacteria was also carried out and the effect was measured *in vivo* by estimating the density of rotifers and bacterial counts in the culture. The results indicated that extracellular products of both bacteria strongly inhibited the growth of *Aeromonas hydrophila* and *Vibrio alginolyticus* isolated from diseased fish. Enzyme API ZYM assays showed that both bacteria have esterase lipase, leucine arylamidase, acid phosphatase, lipase, and Naphthol-AS-BI- phosphohydrolase activities. Furthermore, it was observed that the addition of *B. subtilis* to the rotifer culture water resulted in a significant increase in rotifer numbers and a reduction of Vibrio levels. *B. subtilis* appears to be a promising probiotic for rotifer cultures, but further research is required to determine the capacity in controlling *A. hydrophila* and *V. alginolyticus* when administered via rotifers to fish larvae.

Keywords: Probiotic; Rotifers; *Brachionus plicatilis*; *Bacillus cereus*; *Bacillus subtilis*

Introduction

Rotifers are widely used as first food in the culture of several marine species, although they represent a significant vector for disease transmission since they carry a large bacterial load in their digestive tract [1,2]. Bacteria in rotifers could be transferred by ingestion [2,3], but could also be transferred through contaminated seawater [4]. It has also been shown that selected bacterial inoculation plays a major role in the productivity of rotifer cultures, affecting mainly rotifer abundance and variability in their numbers within replicate cultures [5,6]. Fish larvae are susceptible to infection and high mortalities caused by opportunistic and pathogenic bacteria present in live prey [7]. This fact was demonstrated when survival was increased in fish larvae fed with axenic rotifers [8]. In order to treat and even prevent diseases in aquaculture, wide spectrum antibiotics have been frequently applied. However, there is great concern regarding this approach since it could favour the appearance of resistant pathogenic bacteria, and may cause alterations of aquatic environments and microbial communities due to the long half-life in the water of some chemicals, such as oxytetracycline [9].

A disease preventive method that could improve the host non-specific immune defenses and at the same time, inhibit or control pathogenic bacteria is desirable. Prophylactic methods such as the use of vaccines have been limited since at early larval stages, fish rely mostly on the non-specific immune responses [10]. Probiotics are live microbial additives that confer a variety of beneficial effects on the host principally by modifying its enteric microbial community to increase food utilization and/or enhancing its nutritional value. Probiotics also appear to enhance the host response towards disease, and improve the quality of the rearing environment [11].

The introduction of selected bacterial strains into the food chain has been proposed as a alternative disease treatment. *Lactobacillus plantarum* and *Bacillus* spp. spores have been reported to decrease the amount of Vibrionaceae in rotifers fed with these additives, and subsequently increase weight and survival of turbot larvae [12]. Also, dietary *Bacillus subtilis* has been reported to cause an increase in the growth of tilapia *Oreochromis niloticus* [13], and to enhance the immune responses of fish [14]. Furthermore, the addition of *Bacillus* spp. to the rearing water can increase survival and net production of channel catfish improving water quality [15]. In shrimp, other studies suggest stimulation of the immune response in response to *Bacillus* treatment as elevated *L. vannamei* survival levels were observed during *Vibrio harveyi* and White Spot Syndrome [16], and enhancemed of haemocyte counts and superoxide dismutase activities have also been reported [17].

It is well known that bacteria of the *Bacillus* genus have strong adaptability to diverse conditions and that several species produce highly resistant spores; they have been isolated from fish [18-20], soft corals and sponges [21] as well as other marine invertebrates [22,23]. One of the advantages of using *Bacillus* spp. as probiotics in aquaculture is that they are unlikely to use genes from Gram negative bacteria (e.g., *Vibrio*) that may confer antibiotic resistance [24]. Since rotifers are an important and costly live food for rearing larval fish and invertebrates, and could also be vectors for pathogenic bacteria entry, the present study was designed to assess the probiotic potential of *Bacillus cereus* and *Bacillus subtilis,* previously isolated from marine sediments, to increase rotifer production and control bacterial growth in rotifer cultures. Their antibacterial capacity and enzymatic activity of the probiotics were also studied *in vitro*.

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Materials and Methods

Bacterial strains

B. cereus (CCBM-2) and *B. subtilis* (CCBM-64) were obtained from the Natural History Museum of the Marine Research Institute INVEMAR (Santa Marta, Colombia) and originally isolated from marine sediments of the Colombian Caribbean. The *Aeromonas hydrophila* strain was originally isolated from tilapia (*Oreochromis* spp.) and kindly donated by Dr. Carlos Iregui, National University of Colombia. *Vibrio alginolyticus* was isolated from diseased Jack fish (*Caranx hippos*) housed at a local aquarium.

Bacillus strains were grown on trypticase soy agar *A. hydrophila* on trypticase soy broth (TSA and TSB, respectively, Oxoid, Cambridge, UK), and *V. alginolyticus* in TSB supplemented with 1% of NaCl, all at room temperature (R.T.). For long-term preservation, bacteria were frozen (- 80 C) in TSB with 15% glycerol.

In vitro antibacterial activity of extracellular products (ECPs) from *B. cereus* and *B. Subtilis*

The extraction of extracellular products (ECPs) from probiotic bacteria was performed according to Cabo et al. [25]. Briefly, *B. cereus* (CCBM-2) and *B. subtilis* (CCBM- cultures were grown overnight in TSB at R.T.. After incubation, cultures were separated by centrifugation at 500 x g for 30 min. The supernatants were filtered (0.45 µm filter), buffered at pH 6.0, and stored in aliquots at –80 °C. *A. hydrophila* was grown overnight at R.T. in TSB and *V. alginolyticus* in TSB+ 1% NaCl. The assays were performed in triplicate in a 96 well plate by dispensing 50 µl of the bacterial suspension (10^8 colony forming units (CFU) ml^{-1}) per well with 50 µl of the corresponding ECP treatment. Controls consisted of incubating the bacteria with TSB instead of ECPs. After 24 h incubation, changes in optical density (600 nm) of the culture aliquots were measured and the percentage of bacterial survival determined.

Analysis of enzyme activity

Enzyme production and activity were studied in *B. cereus* (CCBM-2) and *B. subtilis* (CCBM-64) using the API ZYM kit (BioMerieux Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, bacteria were grown overnight on TSA at R.T. and re-suspended in API suspension medium at 10^6 CFU ml^{-1}. Then, 65 µl of each sample was dispensed into each cupule and incubated for 4 h. After incubation, the strips were read placing a surface-active agent (ZYM A reagent) in the cupules which facilitated solubilization of the ZYM B reagent in the medium. Color was allowed to develop for at least 5 min, and graded on a scale from 0 to 5: 0, negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol and 5, 40 nmol or higher of hydrolyzed substrate.

Antibiotic sensitivity test

Antibiotic resistance patterns of *B. cereus* (CCBM-2) and *B. subtilis* (CCBM-64) were determined by the disk diffusion method on Mueller-Hinton agar (Oxoid, Cambridge, UK) supplemented with 1% NaCl. The following concentrations of antibiotics were used: ampicillin, 10 µg $disk^{-1}$; chloramphenicol, 30µg $disk^{-1}$; nitrofurantoin, 300µg $disk^{-1}$; oxolinic acid, 2µg $disk^{-1}$; oxytetracycline, 30 µg $disk^{-1}$; streptomycin, 10 µg $disk^{-1}$; tetracycline, 30µg $disk^{-1}$; trimethroprim-sulfamethoxazole, 25 µg $disk^{-1}$ and ciprofloxacin 5 µg $disk^{-1}$.

Rotifers and culture supplementation with *B. subtilis* and *B. cereus*

Rotifers (*Brachionus plicatilis*) were hatched from cysts following the supplier's instructions (Aquatic Eco-Systems, Florida, USA) and cultured at 25°C in 2 l conical tanks with seawater (20 ppt salinity) with gentle aeration. All rotifer cultures were fed Roti-Rich® (Florida Aqua Farms, Florida, USA) at 0.2 mg ml^{-1}; this product is based on yeast, microalgae, vitamins and specific essential trace elements.

Rotifer cultures were maintained in 250 ml Erlenmeyer flasks when supplemented with *B. subtilis* and *B. cereus*. The experiments started with an initial density of 40 rotifers ml^{-1} and three different treatments were assayed; 1: *B. subtilis* at 10^7 CFU ml^{-1}, 2: *B. cereus* at 10^7 CFU ml^{-1} and 3: a control group with no bacterial supplementation. In order to evaluate rotifer growth, an optical microscope was used to count the number of individuals in Sedgwick-Rafter plates at day 1, 3 and 6, reporting data as rotifers ml^{-1}. Experiments were conducted in triplicate.

Characterization of bacteria associated with the rotifer culture

Samples of rotifers were filtered (50 µm mesh), rinsed twice with 10 ml of sterile seawater (SSW), resuspended in 1 ml of SSW, homogenized aseptically in a glass homogenizer and transferred to sterile tubes. In order to determine the bacterial load, samples were serially diluted and 100 µl of each dilution was spread plated. Total viable counts were performed on marine agar media (M.A.; Difco, BD, Franklin Lakes, NJ, USA) and presumptive *Vibrio* counts on Thiosulfate Citrate Bile Sucrose Agar (TCBS). Plates were incubated aerobically for five days at 26°C, after this time the colonies were counted and the number of bacteria was calculated as CFU ml^{-1}.

Statistical analysis

Results were expressed as means ± standard error. All statistical analyses were evaluated using the Excel 2007 (Microsoft ®) software. The data were compared using Mann–Whitney and the multiple sample comparison Kruskal Wallis tests (Statgraphics Centurion XV). Differences among treatments were considered significant at $P < 0.05$.

Results

In vitro antibacterial activity of extracellular products (ECPs) from *B. cereus* and *B. Subtilis*

It was found that both *B. cereus* (CCBM-2) and *B. subtilis* (CCBM-

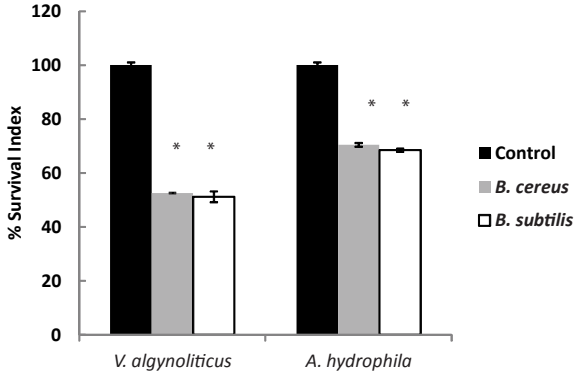

Figure 1: Growth inhibition of *V. algynoliticus* and *A. hydrophila* by incubation with ECPs of *Bacillus cereus* (CCBM-2) and *Bacillus subtilis* (CCBM-64), after 24 h. Data are presented as the mean percentage of bacterial survival index and standard deviation of three different experiments. *Significantly lower growth than the mean growth observed in controls incubated with MRS ($P<0.05$).

64) ECPs were able to significantly inhibit the growth of *V. alginolyticus* and *A. hydrophila* after 24 h of incubation, and reduced *V. alginolyticus* and *A. hydrophila* survival by approximately 50% and 30% of the initial levels, respectively (Figure 1).

Analysis of the enzyme activity

API ZYM assays revealed that *B. cereus* and *B. subtilis* produced esterase lipase (C8), leucine arylamidase, acid phosphatase, lipase, and naphthol-AS-BI-phosphohydrolase, while alkaline phosphatase and esterase (C4) were only produced by *B. cereus* and valine arylamidase by *B. subtilis* (Table 1).

Antibiotic sensitivity test

From the nine antibiotics tested, both *B. cereus* and *B. subtilis* appeared to be resistant to trimethroprim-sulfamethoxazole, ampicillin and oxolinic acid. Only *B. cereus* was resistant to ciprofloxaxine. *B. cereus* was susceptible to nitrofurantoin, tetracycline, oxytetracycline and highly susceptible to streptomycin, and chloramphenicol. *B. subtilis* was highly susceptible to ciprofloxaxine, oxytetracycline and tetracycline.

B. subtilis and *B. cereus* administration to rotifer cultures

Bacillus addition to rotifer cultures significantly affected the rotifer population dynamics. After 3 days of supplementation with *B. subtilis*, the rotifer numbers more than doubled in comparison with the control group. In the case of *B. cereus*, a decrease in the number of rotifers was observed (Figure 2). After 6 days of incubation, the culture supplemented with *B. subtilils* showed a higher rotifer number than the non-supplemented control culture (Figure 2).

Enzyme	Substrate	*B. cereus* (nmol)	*B. subtilis* (nmol)
Alkaline phosphatase	2-naphthyl phosphate	5	0
Esterase (C4)	2-naphthyl butyrate	5	0
Esterase Lipase (C8)	2-naphthyl caprylate	20	20
Lipase (C 14)	2-naphthyl myristrate	5	5
Leucinearylamidase	L-leucyl-2-naphthylamine	20	20
Valinearylamidase	L-leucyl-2-naphthylamine	0	5
Acidphosphatase	2-naphthyl phosphate	30	20
Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5	10

Table 1: Enzymatic activity of viable *Bacillus cereus* (CCBM-2) and *Bacillus subtilis* (CCBM-64).

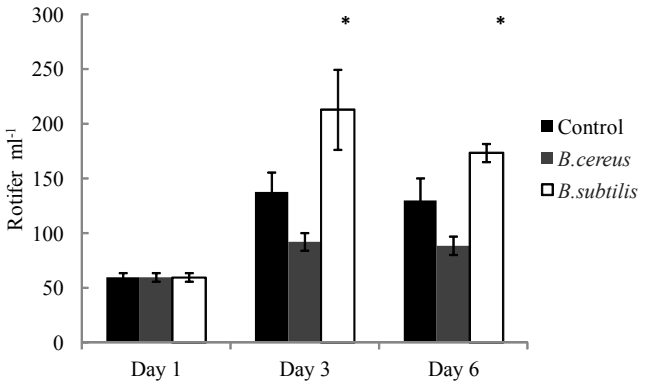

Figure 2: Rotifer counts after 3 and 6 days supplementation of *B. cereus* (CCBM-2) and *B. subtilis* (CCBM-64) at 10^7 CFU ml^{-1}. A control group without bacteria is also included. Data are presented as the mean number of rotifer per ml and standard error of three different experiments. * Significant differences in rotifer number among treatments (P<0.05).

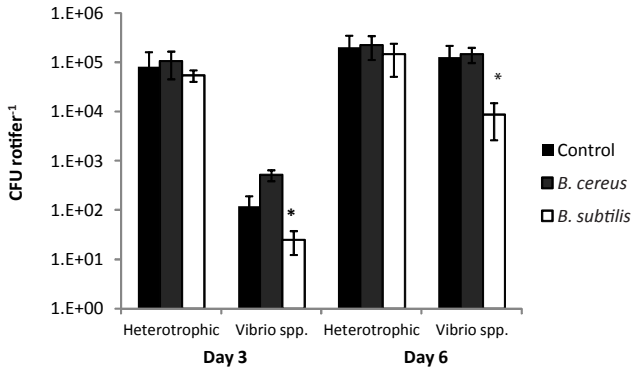

Figure 3: Total heretotrophic bacteria (M.A.) and *Vibrio* spp.(TCBS)from rotifer *B. plicatilis* cultures supplemented with *Bacillus cereus* (CCBM-2)and *Bacillus subtilis* (CCBM-64) at10^7 CFU ml^{-1}. A control group without bacteria supplentation is also included. Data are presented as the mean CFU $rotifer^{-1}$ and standard error of three different experiments. * Significant differences in bacterial number among treatments (P<0.05).

Characterization of bacteria associated to the rotifer culture

In the case of samples plated on the marine agar media (a medium that contains nutrients necessary to cultivate a broad spectrum of heterotrophic marine bacteria) no statistically significant differences were found in the number of CFU ml^{-1} among the three treatments studied (Figure 3). On the other hand, the number of CFUs grown on TCBS, which is an agar used for the selective isolation of *Vibrio* spp., was significantly lower than the number of bacteria grown on marine agar media. A significant reduction of presumptive *Vibrio* spp. levels at both day 3 and day 6 was observed in the *B. subtilis* treated rotifers in comparison to control group (Figure 3).

Discussion

Bacillus strains are suitable as probiotics for aquaculture as they are commonly found as part of the microbiota in fresh and marine water, as well as in the gastrointestinal tract of animals [26]. The control of bacterial load by the addition of probiotic bacteria, most frequently sought for the elimination of pathogenic bacteria, has been shown to be an effective treatment to increase the productivity and health status of cultured fish [27-29].

Here we report, a strong antibacterial activity of *B. cereus* (CCBM-2) and *B. subtilis* (CCBM-64) ECPs against *V. alginolyticus* and *A. hydrophila*; however this strong inhibitory activity was not clearly found *in vivo* since only a slight decrease in heterotrophic bacterial levels was observed when rotifer cultures were supplemented with the selected *Bacillus* strains. Nevertheless, *B. subtilis* supplementation of rotifer cultures caused a significant decrease in *Vibrio* levels after 3 and 6 days of treatment. The results presented here also indicate that *B. cereus* and *B. subtilis* were susceptible to most antibiotics tested, which is a desirable characteristic in the selection of probiotic bacteria.

The production of antimicrobial agents and inhibitory substances has been described previously in other *Bacillus* species. For example, *B. subtilis* BT23, isolated from a shrimp culture pond significantly

inhibited *V. harveyi* under *in vivo* and *in vitro* conditions, however, the antagonistic activity was only effective when the probiotic was used at high concentrations (10^7 - 10^9 UFC ml^{-1}) [30]. Additionally, *B. subtilis* found in the probiotic Biosporin was reported to inhibit *Helicobacter pylori* growth [31]. In the same manner, a non-proteinaceus extracellular product of *B. cereus* with cyanobacteriolytic activity has previously been described [32]. Other studies have shown that *Bacillus* pumilus significantly inhibited fish pathogens such as *V. harveyi*, *Vibrio metschnikovi* and *V. alginolyticus* and that *Bacillus clausii* has a strong antibacterial activity against *Vibrio parahaemolyticus*, *V. metschnikovi* and *V. alginolyticus* [33].

Furthermore, the addition of *B. subtilis* to the rotifer culture water induced an increase in rotifer numbers. This fact could be related to the production of bacteriocins by the probiotic bacteria that inhibit or regulate growth of harmful bacteria, and enzymes that could improve rotifer digestion and utilization of nutrients. It has been previously shown that *B. subtilis* produce large levels of extracellular proteases (exoproteases), which degrade proteins from the environment and are mainly encoded by two genes; aprE (subtilisin) and bpr (bacillopeptidase) [34]. Both *B. cereus* and *B. subtilis* showed an important enzymatic activity of esterase lipase (C8), leucine arylamidase, and acid phosphatase that may have a positive effect in the digestion of lipids in the gut [35]. Also, the production of β 1,3 glucanases by other *Bacillus* species, such as *B. clausii*, providing the potential to hydrolyze β 1-3 glucans has been described [36]. The production of these enzymes may increase rotifer nutrient uptake efficiency since they are important for the digestion of algae [37,38]. It is known that *B. pumilus*, isolated from fish gut, produce extracellular compounds with protease, amylase and cellulose activity that could play an important role in the fish nutrition [19].

Moreover, it was recently reported that fish diets supplementated with *B. pumilus*, and *B. clausii* at 10^8 cells g^{-1} significantly improved the feed conversion ratio and allowed more efficient use of nutrients by the grouper *Epinephelus coioides* [39]. In the same manner, it has been demonstrated that *B. subtilis* supplied as a food complement in rainbow trout diets, reduced mortalities caused by pathogenic bacteria [40,41]. Also, *B. pumilus* have been shown to improve immune functions in gilthead bream *Sparus aurata* [21] and Nile tilapia *Oreochromis niloticus* [18].

Other probiotics such as *Lactobacillus plantarum*, *Lactobacillus helveticus* and *Lactococcus lactis* have also been shown to increase rotifer growth [12,42]. The addition of other lactic acid bacteria such as *Pediococcus acidilactici*, has been also found to enhance rotifer population numbers, possibly associated to the production of vitamin B12 [43,44].

The results presented here indicated that *B. subtilis* (CCBM-64) was able to enhance rotifer growth, which was possibly associated with digestive enzyme production and microbial regulation. In the case of *B. cereus* (CCBM-2) use, care must be taken since even though *B. cereus* has never been associated with fish disease, there are some isolates that have been described as producers of two types of toxins that cause diarrhea and vomiting syndrome in humans [45]. Potential probiotic *Bacillus* species that enhance growth and resistance in food fishes could also be evaluated in ornamental fish culture since these fish stocks are routinely treated with antibiotics, and the larval stages of many of the species, as well as juveniles and adults rely on live food diets [46,47]. Further studies need to be carried out in order to purify and characterize the compounds that are responsible for the antibacterial activity of *B. subtilis*, and to determine the suitability of *B. subtilis* supplemented rotifers for the rearing of larval fish.

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