**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 larval 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 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 an alternative disease treatment. **Lactobacillus plantarum** and **Bacillus** spp. spores have been reported to decrease the amount of **Vibronia** 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 enhanced 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 tryptose soy agar A. hydrophila on tryptose 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-64, cultures were grown overnight in TSB at R.T. After incubation, cultures were separated by centrifugation at 500 g x 30 min. The supernatants were collected (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 (106 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 106 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, 30 µg disk-1; nitrofurantoin, 300 µg disk-1; oxolinic acid, 2 µg disk-1; oxytetracycline, 30 µg disk-1; streptomycin, 10 µg disk-1; tetracycline, 30 µg disk-1; trimethoprim-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 107 CFU ml-1, 2: B. cereus at 107 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-
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 trimethoprim-sulfamethoxazole, ampicillin and oxolinic acid. Only B. cereus was resistant to ciprofloxaxine. B. cereus was susceptible to nitrofurantoín, 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. subtilis showed a higher rotifer number than the non-supplemented control culture (Figure 2).

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 (10^5 - 10^7 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-proteinaceous extracellular product of B. cereus with cyanoabacteriolytic 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 supplemented with B. pumilus, and B. clausii at 10^7 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 gillhead 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 Pedicoccus 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 be also 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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References

1. Munro PD, Henderson RJ, Barbourk A, Birbeck TH (1999) Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. Aquaculture 170: 229-244.
2. Skjermo J, Vadstein O (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177: 333-343.
3. Plante S, Pernet F, Hache R, Ritchie R, J L, B, et al. (2007) Ontogenetic variations in lipid class and fatty acid composition of haddock larval Melanogrammus aeglefinus in relation to changes in diet and microbial environment. Aquaculture 263: 167-171.
4. Prol-Garcia MJ, Planas M, Pintado J (2010) Different colonization and residence time of Listonella anguillarum and Vibrio splendidus in the rotifer Brachionus plicatilis determined by real-time PCR and DGGE. Aquaculture 302: 26-35.
5. Hagiwara A, Hamada K, Hori S, Hirayama K (1994) Increased sexual reproduction in Brachionus plicatilis (rotifier) with the addition of bacteria and rotifer extracts. J Exp Mar Biol Ecol 181: 1-8.
6. Douillet PA (2000) Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions. Evaluation of commercial products and pure isolates. Aquaculture 162: 249-260.
7. Tinh NT, Dierckx K, Sorgeloos P, Bossier P (2008) A review of the functionality of probiotics in the larviculture food chain. Mar Biotechnol (NY) 10: 1-12.
8. Munro PD, Barbourk A, Birbeck H (1995) Comparison of the growth and survival of larval turbot in absence of culturable bacteria with those in the presence of Vibrio anguillarum, Vibrio alginolyticus, or a marine Aeromonas sp. Appl Environ Microbiol 61: 4425-4428.
9. Hektoen H, Berge JA, Horanzabal V, Yndestad M (1995) Persistence of antibacterial agents in marine sediments. Aquaculture 133: 175-184.
10. Mulero I, Sepulcre MP, Fuentes I, Garcia-Alcazar I, Meseguer J, et al. (2008) Vaccination of larvae of the bony fish gillhead seabream reveals a lack of correlation between lymphocyte development and adaptive immuneocompetence. Mol Immunol 45: 2981-2989.
11. Gatesoupe FJ (1999) The use of probiotics in aquaculture. Aquaculture 180: 147-165.
12. Gatesoupe FJ (1991) Fish Nutrition in Practice, SJ Kaushik, P. Luquet (Eds.). Institut National de la Recherche Agronomique, Paris.
13. Gunther J, Jimenez-Montealegre R (2004) Efecto del probiótico Bacillus subtilis sobre el crecimiento y alimentación de tilapia (O. niloticus) y langostino (M. rosenbergii) en laboratorio. Rev Biol Trop 52: 937-943.
14. Villamil L, Novoa B (2009) Probiotics in Aquaculture. Probiotics: Production, Evaluation and Uses in Animal Feed, 91-118. Perez Guerra N & Pastrana Castro L (Eds). Research Signpost, Trivandrum, Kerala, India.
15. Queiroz JF, Boyd CE (1998) Effects of a bacterial inoculum in channel catfish ponds. J World Aquacult Soc 29: 67-73.
16. Balcazar JL, De Blas I, Zarzuela-Ruiz I, Cunningham D, Vendrell D, et al. (2008) The role of probiotics in aquaculture (Review). Vet Microbiol 114: 173-186.
17. Li K, Zheng T, Tian Y, Xi F, Yuan J, et al. (2007) Beneficial effects of Bacillus licheniformis on the intestinal microflora and immunity of the white shrimp, Litopenaeus vannamei. Biotechnol Lett 29: 525-530.
18. Aly SM, Mohamed MF, John G (2008) Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (Oreochromis niloticus). Aquacult Res 39: 647-656.
19. Ghosh K, Sen SK, Ray AK (2002) Characterization of bacilli isolated from the gut of rohu Labeo rohita fingerlings and its significance in digestion. J Appl Aquacul 12: 33-42.
