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Short Communication

Probiotic actions of *Bacillus cereus* var. *toyoi* and *Saccharomyces boulardii* in silver catfish (*Rhamdia quelen*) larvae culture

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ABSTRACT - The objective of this study was to evaluate the use of *Bacillus cereus* var. *toyoi* and *Saccharomyces boulardii* as probiotics to improve *Rhamdia quelen* culture. Six hundred larvaes (0.16±0.07 g) were divided in three replicate tanks (25-L recirculation, 20 ºC, photoperiod of 12 h light/12 h darkness) per treatment and were randomly assigned to the following treatments: *Bacillus cereus* var. *toyoi*; *Saccharomyces boulardii*; *B. toyoi* and *S. boulardii*; and control (without probiotic addition) for a period of 30 days. The fish were fed five times daily (56% crude protein – Supra alevino inicial®) and the probiotics were applied in water once a day. The doses of probiotics were ≅ 5×10^8 and ≅ 2×10^9 CFU (colony forming unit)/mL for *B. cereus* var. *toyoi* and *S. boulardii*, respectively. Both probiotics have an inhibitory effect *in vitro* against *Vibrio carchariae* and are able to grow in media prepared with fishery water; however, no effect was observed on growth parameters when they were administered to *Rhamdia quelen* larvae.

Key Words: aquaculture, feed additives, silver catfish, vibriosis

Introduction

Globally, aquaculture is expanding into new directions, intensifying and diversifying. This expansion has inevitably generated disease problems that are now a primary constraint to the culture of aquatic species, affecting both economic and social development (Bondad-Reantaso et al., 2005).

Silver catfish (*Rhamdia quelen*) (Quoy & Gaimard, 1824) culture is spread across from southern Mexico to central Argentina. This species is popular for consumption in the countries of Latin America, and the husbandry of *R. quelen* is increasing in Brazil, Uruguay and Argentina (Gomes et al., 2000; Salhi et al., 2004). As a consequence, nowadays Brazil is the main producer of this aquaculture incipient species, with 2500 tonnes in the year 2000 (Copatti et al., 2005).

Defining alternative strategies to support aquaculture productivity is extremely necessary. The use of probiotics is a strategy that has shown promising results as a complementary tool for the management and improvement of the nutrition of aquatic animals (Wang et al., 2008). In the last decade, the scientific community examined roles and effects of probiotics as an alternative to antimicrobial drugs, demonstrating positive effects on fish survival (Villamil et al., 2002), growth (Burr et al., 2005), stress resistance (Smith & Davey, 1993; Rollo et al., 2006), immune system enhancement (Erickson & Hubbard, 2000; Picchietti et al., 2007), and general welfare (Balcázar et al., 2006; Silvi et al., 2008).

Bacillus species are found in marine sediments and are naturally ingested by animals that feed in or on the sediment (Moriarty, 1999). *B. cereus* var. *toyoi* have been already exploited in probiotics products for human and animal health (Sanders, 2003). *Saccharomyces boulardii* is a non-pathogenic yeast widely used as probiotic in humans and animals for prevention and treatment of gastrointestinal diseases (Lourens-Hattingh & Viljoen, 2001), also, it resists feed peletization temperature, giving an extra advantage over lactobacilli in manufacturing and shelf product lives.

The objective of the present study was evaluate the use of *Bacillus cereus* var. *toyoi* and *Saccharomyces boulardii* as probiotics in the culture practices of the Silver catfish to verify the performance (final weight) of *Rhamdia quelen* larvae with the probiotic addition.

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Material and Methods

The probiotics, *Bacillus cereus* var. *toyoi* and *Saccharomyces boulardii*, were provided by microorganisms collection in the Department of Microbiology and Parasitology of Universidade Federal de Pelotas (UFPel).

For the purpose of evaluating the probiotic use, first, the microorganism viability in the water from the culture tank was examined, then its ability to inhibit *in vitro* the pathogenic bacteria *Vibrio carchariae* and *Vibrio anguillarum*.

The viability of probiotics was analyzed in two independent experiments. First, the capacity of these microorganisms to grow in water from fish culture tanks was studied. For *B. toyoi*, NYSM (Yousten medium) was used and for *S. boulardii*, YPD medium (Yeast Peptone Dextrose) was used, both media prepared with water from fish culture and control media were prepared with distilled water. The cultures were incubated for 48h at 37 °C and 28 °C for *B. toyoi* and *S. boulardii*, respectively. The survival time of these microorganisms in fish culture tank water was also analyzed. Cultures of *B. toyoi* and *S. boulardii* contained, $\cong 1 \times 10^8$ and $\cong 6 \times 10^8$ CFU/mL, respectively. They were suspended in water from the fish culture then keep at 20 °C for 12 weeks. Every two weeks, a plate counting was performed to evaluate their concentration. Control microorganism suspensions were prepared using distilled water.

Pathogenic strains of *Vibrio carchariae* (=*V. harveyi;* Thaithongnum et al., 2006), and *Vibrio anguillarum*, were obtained from the culture collection from Laboratory of Phytoplankton and Marine Microorganisms Ecology (Universidade Federal do Rio Grande - Brazil). The *in vitro* inhibitory activity of *B. toyoi* and *S. boulardii* against these pathogenic strains was evaluated using cross-streak (adapted from Decamp et al., 2008) and disc-diffusion methods using Müller-Hinton agar (Difco®) observing the results of inhibitory activity at 48 h after incubation at 28 °C.

The probiotic effect of *B. toyoi* and *S. boulardii* was evaluated using five days after hatch of six hundred larvaes with initial weight of 0.16±0.07 g, which were divided in three replicate tanks per treatment (25-L recirculation, 20 °C photoperiod, 12 h light/12 h darkness) which were randomly assigned to probiotics and control for a period of 30 days.

The treatments were: *B. toyoi* (5×10^8 CFU/mL); *S. boulardii* (2×10^9 CFU/mL); *B. toyoi* and *S. boulardii*; and a control group without probiotic addition. The probiotics were applied in water once daily at 14 h, and the fish were fed five times a day (8, 11, 14, 17 and 20 h) with 56% crude protein (Supra® - Alevino inicial – Brazil).

The experiment was performed for 30 days, and at the end of this period, final weight was evaluated. During the experimental period, the water quality was assessed by pH (8.29±0.25), temperature (23.54±1.41°C) and dissolved oxygen (7.92±0.62 mg/L). In order to evaluate the presence of probiotics in the gastrointestinal tract of the fish, a total of six fish per treatment were collected (two from each tank), dissected and a gastrointestinal tract macerate was plated onto NYSM and YPD to identify, by biochemical tests, the presence of *B. toyoi* and *S. boulardii*, according to the methodology described by Bergey (2001) and De Hoog et al. (2000), respectively.

One-way ANOVA was used to determine significant differences ($P<0.05$) on fish total weight under probiotics effect. The *in vitro* probiotics inhibition against *Vibrio carchariae* was analysed by the student "t" test. All analysis considered a significance level of 5%. Tukey test was applied when significant differences were detected.

Results

The results demonstrated that the two microorganisms were able to grow in media prepared with water from the fish culture, and their growths were similar to the control media prepared with distilled water. Subsequently, when the time survival of these microorganisms in water from the culture was evaluated, it was observed that the fish culture water had an influence in the microorganism viability (Figure 1). *B. toyoi* suspended in water from the culture was able to keep the original concentration up to the 4th week, reducing one log (8×10^6 to 8×10^5 CFU/mL) by the end of the third month (Figure 1), whereas the control maintained its concentration. The effect of water from the culture in *S. boulardii* survival was more drastic. This micro-

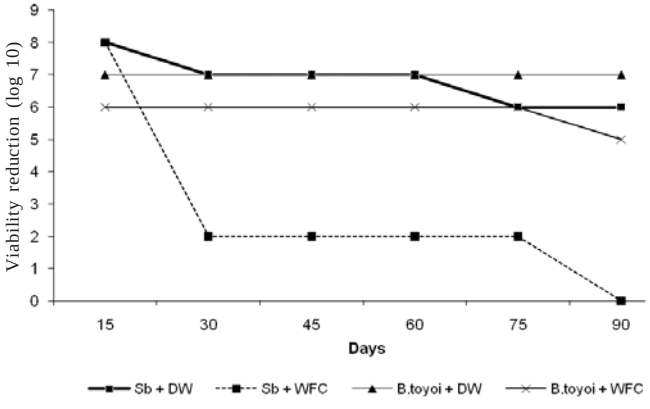
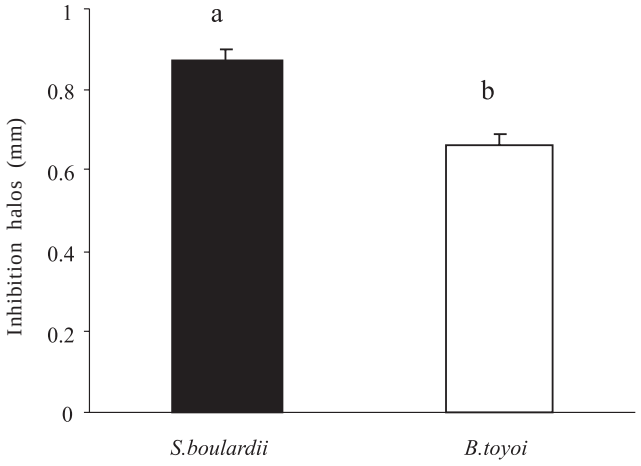


Figure 1 - Probiotic viability of *S. boulardii* (Sb) and *B. cereus* var. *toyoi* (B.toyoi) in water of fish culture (WFC) and distilled water (DW), time points (two weeks of interval) during three months.

organism showed a significant drop in the concentration by the 3rd week from 10^8 to 10^2 CFU/mL, maintaining this concentration until the 5th week, and no viable cell was detected at the last time evaluated (90 days). Moreover, the control (*S. boulardii* suspended in distilled water) had a reduction of one log at 30 days of evaluation and two logs in the beginning of the 3rd month; however, this concentration (1×10^6 CFU/mL) was maintained until the end of the period tested (Figure 1).

The inhibitory effect of probiotics microorganism was analyzed against the fish pathogens *Vibrio carchariae* (Figure 2) and *V. anguillarum*. The results demonstrated that both probiotics had an inhibitory effect against *V. carchariae* on disc-diffusion method, although *S. boulardii* presented an inhibition zone significantly wider than *B. toyoi*. The inhibition against *V. anguillarum* was not observed by these two probiotic microorganisms.

The probiotics did not enhance fish total weight (Table 1). However, the *B. toyoi* was isolated in the gastrointestinal tract of fish and identified by biochemical analysis, showing that the animal ingested the probiotic.



Different superscript letters indicate significant differences of means.

Figure 2 - Probiotic inhibitory activity against *V. carchariae* by *S. boulardii* and *B. cereus* var. *toyoi*.

Discussion

In this study, the results showed that probiotic can not only grow, but also survive in media prepared with water from the fish culture. Besides, the *B. cereus* var. *toyoi* was ingested and survived in the larvae gastrointestinal tract for certain period of time. More promising were the results demonstrating the inhibitory effect against *Vibrio carchariae*.

The observation on viability of *B. toyoi* in water from the fish culture confirmed that these bacteria have ideal resistance characteristics for a promising probiotic for aquaculture use. Sufficient evidence suggests that adding *Bacillus* as spores to rearing ponds has a beneficial effect due to the improvement of water quality by reduction of *Vibrio* spp. (Hong et al. 2005). The *B. toyoi* inhibitory effect observed to *V. carchariae* represents a very important step in order to utilize it as probiotic. This pathogen is an important agent causing gastroenteritis in fish and can lead them to death (Yii et al., 1997; Liu et al., 2003). Thus, it is very important reduce its concentration in culture of aquatic microorganisms. Decamp et al. (2008), working with strains of *B. subtilis*, *B. licheniformis* and other *Bacillus* species also showed strong inhibitory activity of this strains against a variety of *Vibrio* spp., and suggests that this effect was by the improvement of water quality and reduction of *Vibrio* spp. in the water.

Although most probiotics used in aquaculture are bacteria, the yeast, *S. boulardii*, was found to be an effective probiotic (Czerucka et al., 2007). *S. boulardii* possesses many properties that make it a potential probiotic agent, i.e., it survives transit through the gastrointestinal tract, and it inhibits the growth of a number of microbial pathogens (Czerucka et al., 2007). Lara-flores et al. (2003) found positive effect of *S. cerevisiae* as feed additive on growth performance and survival of Nile tilapia fed with different crude protein levels (40 and 27%). However, Meurer et al. (2004) found no effect of the addition of *S. cerevisiae* on the performance of Nile tilapia during the sex reversal and attributed this result to the absence of potentially pathogenic microorganisms in the laboratory environment.

Table 1 - Effect of probiotics on mean weight (g) of *Rhamdia quelen* (Mean ± SE)

	Treatments			
	Control	*S. boulardii*	*B. cereus* var *toyoi*	S.b + B.c
Mean weight (g)	0.170±0.004; 114	0.161±0.008; 108	0.169±0.009; 138	0.149±0.005; 140

S.b - *Saccharomyces boulardii*; B.c - *Bacillus cereus* var. *Toyoi*; SE - standard error.

It should be noted that in animals kept in good management (density, nutrition and health), often no effects of adding probiotics on performance are observed (Lima et al., 2003). In those situations, the possibility of contact with pathogens is lower (Loddi et al., 2000; Zuanon et al., 1998). Also, the short experimental period made it difficult to observe the probiotic effect. Probably, the same occurred in this experiment, the safe management did not allow the establishment of potentially pathogenic microorganisms and the probiotic effects were not observed in the parameters studied.

The probiotics dose could be limiting factor for achieving optimum beneficial effects in any host (Donnet-Hughes et al., 1999; Kishi et al., 1996). The optimum concentration of probiotics is not only required for establishment and subsequent proliferation in the gastrointestinal tract, but also needs to exert various beneficial effects including immunostimulatory activity. The optimum dose of probiotics may vary with respect to host and also type of immune parameters. Song et al. (2006) found that a higher dose (10^{10} CFU/kg feed) of *Lactobacillus plantarum* failed to protect fish on a challenge study, despite the enhancement of certain immune parameters at the particular dose. Nikoskelainen et al. (2001) also recorded higher percentage of mortality in *Oncorhynchus mykiss* fed at high dose of *Lactobacillus rhamnosus* (10^{12} CFU/g feed), compared with lower dose (10^{9} CFU/g feed). Brunt et al. (2007) determined that the effective dose of the probiotic to *Bacillus* species must be 2×10^{8} cells, at which they have recorded the least percentage mortality in rainbow trout (*O. mykiss*) during challenge study. In our study, the probiotic dose was kept at 5×10^{4}/tank, a dose lower than effective reported; however, until the present, the optimal dose has not been determined for any probiotics in silver catfish. Therefore, according to Nayak (2010), the dose of the individual probiotics needs to be determined for a particular host.

Moreover, the fact that it was tested with this species of fish that presents a large disparity in the size of animals of the same age made it difficult to observe the probiotic effect. Barton & Iwama (1991) postulate that it is difficult to determine whether the impaired growth is the result of metabolism changes (food consumption and social interaction) or direct factors such as levels of hormones and enzymes. Previous studies analyzing the growth of silver catfish have pointed to agonistic interactions promoting effects of reduction and heterogeneity in growth due to increased aggressiveness, cannibalism and/or competition for food when the fingerlings are kept at low stocking density (Piaia & Baldisserotto, 2000) as used in this study (≅ 1larva/L). In addition, the improvement of fish growth was not observed. Further studies need to be carried out in order to establish the effective probiotic dose to best evaluate a possible beneficial effect for this species.

Conclusions

Probiotics based on *Bacillus cereus* var. *toyoi* and/or *Saccharomyces boulardii* do not enhance fish performance, but demonstrate viability in the water of fish culture and present an inhibitory effect against *V. carchariae*.

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