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Performance of jundiá larvae, *Rhamdia quelen*, fed on probiotic supplemented diets

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ABSTRACT. Since probiotics have proved to be a viable alternative to antibiotics as enhancers of animal growth, the performance, uniformity and mortality rates of the jundiá (*Rhamdia quelen*) larvae fed on diets with different probiotics were evaluated. Jundiá larvae, aged four days post hatching, were fed during 21 days with the following diets, in four replicates, namely, CO: control feed, without probiotics; PP: feed with *Pichia pastoris*; SB: feed with *Saccharomyces boulardii*; BT: feed with *Bacillus cereus* var. *toyoi*. Among the tested probiotic, *Bacillus cereus* var. *toyoi* had the best results due to the fact that the larvae were 25% heavier than CO at the end of the first week; the difference increased to 28% by the end of the trial. Further, BT also improved uniformity and Fulton's condition factor. Larvae fed on *Saccharomyces boulardii* had the lowest body weight, whereas those fed on *Pichia pastoris* grew similarly to the control diet. Mortality rate was not affected by treatments. *Bacillus cereus* var. *toyoi* improves the performance and uniformity of the larvae, but does not affect mortality rate.

Keywords: fish, uniformity, *Bacillus cereus* var. *toyoi*, *Saccharomyces boulardii*, *Pichia pastoris*.

Desempenho de larvas de jundiá, *Rhamdia quelen*, alimentadas com dietas contendo probióticos

RESUMO. Devido aos probióticos terem se mostrado uma alternativa viável ao uso de antibióticos como promotores de crescimento para animais, foi avaliado o desempenho, uniformidade e taxa de mortalidade de larvas de jundiá alimentadas com dietas contendo diferentes probióticos. Larvas de jundiá, com quatro dias pós-eclosão, foram alimentadas durante 21 dias com as seguintes dietas em quatro repetições: CO: ração controle, sem adição de probiótico; PP: ração com *Pichia pastoris*; SB: ração com *Saccharomyces boulardii* e BT: ração com *Bacillus cereus* var. *toyoi*. Dentre os probióticos testados, *Bacillus cereus* var. *toyoi* apresentou os melhores resultados, pois ao final da primeira semana as larvas desse tratamento estavam 25% mais pesadas que as do controle e ao final do experimento a diferença aumentou para 28%. Além disso, BT também melhorou a uniformidade e o fator de condição. As larvas alimentadas com *Saccharomyces boulardii* apresentaram menor peso dentre todos os tratamentos. *Pichia pastoris* proporcionou às larvas um crescimento semelhante ao tratamento controle. Portanto, conclui-se que *Bacillus cereus* var. *toyoi* melhora o desempenho e a uniformidade de larvas de jundiá, porém não afeta a taxa de mortalidade.

Palavras-chave: peixe, uniformidade, *Bacillus cereus* var. *toyoi*, *Saccharomyces boulardii*, *Pichia pastoris*.

Introduction

Although antibiotics have been used as growth promoters during several decades, alternatives were sought due to the development of bacterial resistance to active ingredients, residues in meat and accumulation of metabolites in the water and soil (Kesarcodi-Watson et al., 2008; Mandal et al., 2014; Manzetti & Ghisi, 2014; McPhearson et al., 1991).

According to Fuller (1989), probiotics are live microorganisms that benefit the host animal by a balance improvement of its intestinal microbiota when supplemented in feed. Im and Pothoulakis (2010) report that the yeast *Saccharomyces boulardii* is highly beneficial to the host because it alters bacterial virulence, releases toxin-breaking enzymes and decreases inflammatory response. Further, *Pichia pastoris* is the most studied and dynamic yeast because it may produce several protein types by combining the genetic material of the other organisms Potvin et al. (2012). Nevertheless, no registers are extant with regard to its use as a probiotic agent in fish, nor is it cited in the latest bibliographic reviews on the subject (Balcázar et al., 2006; Dimitroglou et al., 2011; Vine et al., 2006; Wang et al., 2008).

On the other hand, the understudied bacterium *Bacillus cereus* var. *toyoi* has been efficient to stimulate the immune system of piglets (Schierack et al., 2007) and broilers (Gil de los Santos et al., 2012), with growth improvement.

Although these microorganisms do not belong to the intestinal microbiota, on ingestion, they protect the intestinal mucosal barrier by nutrient competition with pathogenic bacteria and produce inhibitory substances (Kesarcodi-Watson et al., 2008). Moreover, by stimuli of the immune system, the response from the nonspecific immune system is faster if the organism is invaded by pathogens (Nayak, 2010; Picchietti et al., 2009).

When reared in ponds, fish larvae undergo many risks since the locomotion, digestive, nervous and immune systems are not fully developed (Pittman et al., 2013). Consequently, at the same time that probiotics decrease intestinal disorders and stimulate the immune system, they trigger the nutrients to complete ontogeny and increase survival chances.

The jundiá (*Rhamdia quelen*) is a South American catfish with good performance in subtropical climates and resistance to handling. However, its larval stage is highly problematic due to high heterogeneity that leads towards cannibalism and low survival (Carneiro et al., 2003).

Current study evaluates the performance, mortality and uniformity of jundiá larvae fed on different probiotics in the diet.

Material and methods

Ethical note

Current assay, registered under the number 23110.004309/2014-31, was evaluated and approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas.

Larvae

The larvae were obtained by induced spawning at the Laboratory of Ichthyology of the Federal University of Pelotas, Brazil. Four days after hatching (mouth opening at 22°C of water temperature), 1,600 jundiá larvae weighing 1.00 ± 0.12 mg and measuring 4.75 ± 0.46 mm in length were randomly collected.

Husbandry conditions

Sixteen plastic trays, 190 × 275 × 70 mm (W × L × H) with meshed bottom (200 microns), were submerged 40 mm in 10 glass aquariums (300 × 500 × 270 mm, W × L × H) with 40 L each. The aquariums were connected to a water-recirculating system with one reservoir (500 L) and one biologic filter (350 L).

Water inlets were fitted at the top of the trays and outlets at the bottom of the aquariums. The water flow was adjusted weekly in each tray to keep the same water quality. Temperature was measured daily in the morning and in the afternoon with a digital thermometer (25.2 ± 1.6ºC) and dissolved oxygen was measured weekly with a digital oximeter (7.18 ± 0.51 ppm) and pH (7.50 ± 0.00), ammonia (0.11 ± 0.05 ppm), and nitrite (0.01 ± 0.01ppm) were measured with colorimetric kits. The natural photoperiod was kept at approximately 14h of light with indirect incident on the aquariums.

Treatments and feeding

A base diet (Table 1) was prepared following Cardoso et al. (2004), consisting of bovine liver and inactivated sugarcane yeast. Fresh liver was crushed in a blender and sifted (100 microns) to remove the veins, arteries and connective tissue. Four portions of 1 kg of the sifted liver were then separated and the different probiotics were added to three portions at a concentration of 1×10^9 Colony Forming Units (CFU) per gram of ration. Probiotics in liquid form were obtained from the Department of Biotechnology of the Federal University of Pelotas.

Table 1. Ingredients in the experimental diet.

Ingredients	Inclusion (%)
Raw bovine liver[1]	30
Sugarcane yeast	57
Soy lecithin	2
Vitamin/mineral mix[2]	11

[1]Expressed in dry matter. [2]Composition per kg of product: Folic acid: 1,200 mg; Nicotinic acid: 24,000 mg; Pantothenic acid: 12,000 mg; Biotin: 48 mg; Vit. A: 1,200,000 UI; Vit. B1: 4,800 mg; Vit. B2: 4,800 mg; Vit. B6: 4,800 mg; Vit. B12: 4,800 mcg, Vit. C: 48 g, Vit. D3: 200,000 UI, Vit. E: 12,000 mg, Vit. K3: 2,400 mg, Cobalt: 10 mg, Copper: 3,000 mg; Iron: 50,000 mg; Iodine: 100 mg; Manganese: 20,000 mg; Selenium: 100 mg; Zinc: 3,000 mg.

After the introduction of probiotics, other ingredients were manually mixed following pelleting in a meat grinder. The following treatments were prepared: CO: control diet, without probiotic; PP: diet with *Pichia pastoris*; SB: diet with *Saccharomyces boulardii*; BT: diet with *Bacillus cereus* var. *toyoi*.

CO and BT diets were then dried at 55ºC for 24 hours in an air circulating buffer, whereas PP and SB diets were dried at 37ºC for 72 hours due to their high mortality of the probiotics above this temperature (Nwaka et al., 1994; Shahsavarani et al., 2012).

Thereafter, all diets were grounded, the 200-400 microns particles were selected and stored in a freezer (-18ºC).

Feeding was provided *ad libitum* every 2 hours, from 8h00 am to 10h00 pm manually and from 12h00 pm to 6h00 am by automatic feeders (BOYU ZW-82).

Handling

Trays were changed daily to remove food remains and feces, to allow water renewal and count the dead larvae.

Weight and length were measured with 10 larvae of each aquarium at days 7 and 14 and with 20 larvae at day 21. All larvae were randomly collected and anesthetized with eugenol (20 $mg \cdot L^{-1}$). Measured larvae at day 7 and 14 were not replaced into the aquariums due to high probability of death.

Measurements and calculations

The following were calculated from body weight (BW, mg) and total length (TL, mm):

WGp, % = percentage of weight gain between two consecutive measurements;

TLGp, % = percentage of total length gain between two consecutive measurements;

K = Fulton's condition factor = $100 \times BW\ TL^{-3}$

SGRp, $\%\cdot day^{-1}$ = specific growth rate between two consecutive measurements = $100 \times [(LnWi - LnWf)\ days^{-1}$ among measurements] when Wi and Wf are respectively initial and final weights;

M, % = Mortality = 100 × (dead + removed for measurements)/initial number of larvae;

WU, % = weight uniformity = 100 − (100 × standard deviation of mean BW/BW);

TLU, % = total length uniformity = 100 − (100 × standard deviation of mean TL/TL).

Statistics

The experimental design was completely randomized with 4 treatments and 4 replicates. After outlier exclusion (mean ± 2 × SD), variables were subjected to Shapiro-Wilk normality test and differences were considered significant when $p < 0.05$ by analysis of variance (ANOVA); means were compared by Duncan's test. All data were analyzed by SPSS (2005).

Results

In the first week, the larvae fed on BT diet showed a higher BW, TL, SGR, WGp and TLGp rate when compared to other probiotic diets ($p < 0.05$), although they did not differ from CO larvae ($p > 0.05$; Table 2).

At day 14, larvae fed on BT diet had higher BW, TL and K when compared to PP larvae ($p < 0.05$), but equal to CO and SB. SGR, WGp and TLGp were similar among the treatments ($p > 0.05$; Table 2).

In the third week, larvae fed on BT diet had higher BW, K and TLU ($p < 0.05$) when compared to other treatments. BT diet also promoted higher TL among probiotic diets and WU when compared to CO diet ($p < 0.05$). The parameters with growth rhythm in the last week (SGRp, WGp and TLGp) were similar ($p > 0.05$; Table 2), whilst highest K was caused by BT diet. Mortality rate was not affected by different treatments ($p > 0.05$).

Table 2. Performance of jundiá larvae fed on different probiotics at 7, 14 and 21 days.

Variables[1]	Treatments[2]			
	CO	PP	SB	BT
	7 days			
BW	7.87 ± 2.00^{ab}	5.80 ± 1.70^{b}	5.71 ± 1.86^{b}	9.84 ± 1.38^{a}
TL	9.29 ± 0.69^{ab}	8.31 ± 0.62^{bc}	8.08 ± 0.96^{c}	9.64 ± 0.25^{a}
K	0.96 ± 0.10	0.99 ± 0.14	1.06 ± 0.16	1.03 ± 0.09
SGRp	29.07 ± 4.03^{ab}	24.58 ± 4.68^{b}	24.20 ± 5.29^{b}	32.56 ± 1.88^{a}
WGp	686.74 ± 200.10^{ab}	480.00 ± 170.52^{b}	470.71 ± 186.88^{b}	883.61 ± 137.88^{a}
TLGp	95.56 ± 14.62^{ab}	75.03 ± 13.20^{bc}	70.01 ± 20.21^{c}	102.98 ± 5.41^{a}
	14 days			
BW	25.31 ± 4.54^{ab}	20.13 ± 4.72^{b}	24.61 ± 2.06^{ab}	31.69 ± 4.03^{a}
TL	13.14 ± 0.69^{ab}	12.36 ± 0.96^{b}	13.03 ± 0.48^{ab}	13.72 ± 0.60^{a}
K	1.11 ± 0.03^{ab}	1.05 ± 0.04^{b}	1.11 ± 0.07^{ab}	1.23 ± 0.11^{a}
SGRp	16.91 ± 5.47	17.97 ± 7.16	21.52 ± 6.20	16.73 ± 0.98
WGp	247.24 ± 153.46	287.60 ± 203.13	385.34 ± 222.49	223.12 ± 22.88
TLGp	42.04 ± 13.70	49.75 ± 20.69	63.49 ± 24.14	42.48 ± 9.12
	21 days			
BW	76.92 ± 1.80^{b}	61.72 ± 6.55^{bc}	60.00 ± 14.79^{c}	98.97 ± 13.22^{a}
TL	19.26 ± 0.30^{ab}	18.09 ± 0.66^{b}	17.95 ± 1.41^{b}	20.11 ± 1.00^{a}
K	1.08 ± 0.05^{b}	1.04 ± 0.02^{b}	1.02 ± 0.04^{b}	1.21 ± 0.06^{a}
SGRp	16.05 ± 2.50	16.28 ± 4.75	12.41 ± 3.05	16.25 ± 1.51
WGp	211.23 ± 54.76	226.02 ± 111.74	142.31 ± 46.95	213.33 ± 34.56
TLGp	47.04 ± 9.88	47.22 ± 15.97	37.65 ± 6.72	46.60 ± 4.20
WU	42.64 ± 11.51^{b}	51.11 ± 9.27^{ab}	51.45 ± 4.50^{ab}	57.77 ± 7.50^{a}
TLU	82.75 ± 0.67^{b}	83.30 ± 1.09^{b}	82.10 ± 0.66^{b}	85.41 ± 2.17^{a}
M	23.50 ± 2.88	23.25 ± 2.21	23.75 ± 1.70	23.25 ± 0.50

[1]BW = body weight (mg); TL = total length (mm); K = Fulton's condition factor; SGRp = specific growth rate in the period ($\%\cdot dia^{-1}$); WGp = weight gain in the period (%); TLGp = total length gain in the period (%); WU = uniformity of weights (%); TLU = uniformity of total lengths (%); M = mortality (%). [2]CO = control diet, without probiotic; PP = diet with Pichia pastoris; SB = diet with Saccharomyces boulardii; BT = diet with *Bacillus cereus* var. *toyoi*. Note: each rate comprises mean ± SD (n = 4). Rates in the same row with different superscript letters are significantly different ($p < 0.05$) by Duncan's test.

Discussion

Nutrition begins at the yolk sac during the initial phase of fish development. At this moment, intestine is only a short straight undifferentiated tube that develops simultaneously through yolk and first feeding.

According to Nayak (2010), gastrointestinal tract is colonized significantly from first feeding and, depending of the microorganisms in the environment, weight loss may occur due to intestinal dysfunction. In current study, during the first week, the larvae fed on BT diet were heavier than those on PP and SB diets. Further, although statistically similar, they were 25% heavier than those on CO diet.

It is important to note that the initial performance was determinant for BT larvae to be 28% heavier than those of the CO diet at the end of the test, since WGp was similar among treatments at 14 and 21 days (Figure 1). Since 89% of weight increment occurred in the first seven days, the importance of microbiota modulation in this period may be highlighted.

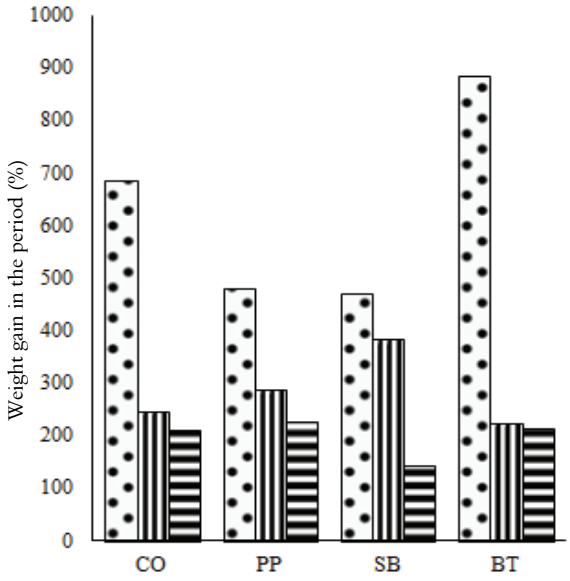

Figure 1. Weight gain of larvae fed on different probiotics at 7, 14 and 21 days (dots, vertical and horizontal bars, respectively). CO: control diet, without probiotic; PP: diet with *Pichia pastoris*; SB: diet with *Saccharomyces boulardii*; BT: diet with *Bacillus cereus* var. *toyoi*.

Although environmental microorganisms affect intestinal microbiota composition, Souza et al. (2012) reported no differences in the growth of the jundiá larvae (160 mg of initial weight) when *Bacillus cereus* var. *toyoi* (5×10^8 CFU·mL^{-1}) or *Saccharomyces boulardii* (2×10^9 CFU·mL^{-1}) were added in cultivation water for 30 days. However, the same authors reported an *in vitro* inhibitory effect of the probiotics on *Vibrio carchariae*.

Larvae performance of the CO treatment was higher than that reported by Cardoso et al. (2004) which reached 38.90 mg after 21 days, with the same species, after feeding on a diet based on bovine liver and sugarcane yeast.

The evaluation of K of the treatments showed that all maintained values close to 1 in the first week, indicating that weight and length grew synchronously.

During the second and third weeks, BT larvae maintained K close to 1.2 due to weight gain was more significant than length gain. The above indicated that the ontogeny of internal organs was more advanced in this treatment. In fact, when the bacterium *Enterococcus faecium* was used as probiotic to *Solea solea* larvae, the cortisol level increased in its bloodstream (Palermo et al., 2011). It is well-known that the hormone enhances thyroid hormones on metamorphosis (Yamano et al., 1991).

When WU and TLU were taken into account, the best results occurred with BT treatment. Similar data were registered by Gisbert et al. (2013) who verified lower heterogeneity in rainbow trout (*Oncorhynchus mykiss*) fingerlings (2.8 g) fed on *B. cereus* var. *toyoi* supplemented diet for 93 days. The authors still suggest that probiotic inclusion had a practical importance due to decreased dominance situations caused by larger individuals.

Two tested yeasts showed the poorest results. However, Abu-Elala et al. (2013) reported that *S. boulardii* increased the growth and non-specific immune response of Nile tilapia (*Oreochromis niloticus*) juveniles (80 g). Similarly, Waché et al. (2006) also reported that this yeast improved the digestive system maturation of the rainbow trout (*O. mykiss*) when compared to control group.

On the other hand, Gil de los Santos et al. (2012) compared the performance of broilers fed on control diet or on diet containing *P. pastoris*, *P. pastoris* recombinant *Clostridium perfringens* gene or *B. cereus* var. *toyoi* as probiotic. The authors reported that animals fed on non-modified *P. pastoris* had the same performance of the control group while the others were better.

Abu-Elala et al. (2013) and Waché et al. (2006) used gelatin and cod liver oil, respectively, to yeasts inclusion in the prepared diet. Thus, the hypothesis that larvae fed on PP and SB diets showed, respectively, 24 and 28% less weight than those fed on CO treatment, may be due to the probable death of the yeasts since temperature increased considerably when processing. Moreover, the association of ration moisture and drying temperature (37°C) may have developed other bacteria that depreciated its quality.

Therefore, the application of yeasts in the industrial processing is difficult due to the survival difficulties, with extrusion temperature process at 195°C (Benchaar et al., 1994). Contrastingly, bacteria such as *B. cereus* var. *toyoi* sporulate when subjected to high temperatures and return to their active form when in favorable conditions (Hong & Cutting, 2005).

Avella et al. (2011) evaluated the effect of *E. faecium* as probiotic in *S. solea* larvicultura and reported growth increase, although survival rate did not change. Similarly, Lobo et al. (2014) reported that the probiotic *Shewanella putrefaciens* improved the growth and homogeneity and did not affect survival of *S. senegalensis*. In general, current results corroborate those reported for other species.

Conclusion

Bacillus cereus var. *toyoi* improved growth and homogeneity but did not affect mortality rate of the jundiá (*Rhamdia quelen*) larvae. Further studies are

required to evaluate level and methods of inclusion of the probiotics in fish larvae diet.

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