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Short communication: *Bacillus subtilis* C-3102 improves biomass gain, innate defense, and intestinal absorption surface of native Brazilian hybrid Surubim (*Pseudoplatystoma corruscans* x *P. reticulatum*)

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Abstract

The aim was to evaluate the effect of a diet supplemented with *B. subtilis* for a period of 10 days and to verify its effects on the zootechnical performance, hematological parameters, and intestinal histomorphometry of fish. The present study was designed to verify the effects of a commercial product containing *Bacillus subtilis* C-3102 for short administration period in the diet of Brazilian catfish *Pseudoplatystoma* sp. The experimental design was completely randomized with five treatments (0, 10, 20, 30, and 40 g of CALSPORIN® kg feed^{-1}) and six replicates. Supplementation with *B. subtilis* did not influence ($p > 0.05$) on erythrogram and plasma glucose of fish. However, probiotic supplementation for 10 days was effective and caused beneficial changes in productive performance of the 30 g kg feed^{-1} group compared to the other groups. In addition, improvements in the immune system, such as an increase in the number of neutrophils, lymphocytes, thrombocytes, and total blood leukocytes were observed in the treated groups, besides increased height and width of the intestinal villi was possibly related with an improvement in nutrient absorption. This study shows the feasibility in to use short time of feeding in the *Pseudoplatystoma* sp. diet that improved the fish health.

Keywords Hematology · Intestine · Probiotic · Immune system

Highlights

-Short time of commercial probiotic feeding evaluation in the diet of Brazilian native catfish *Pseudoplatystoma* sp.
-Increased number of defense cells like neutrophils, lymphocytes, thrombocytes, and white blood cells was observed.
-Probiotic feeding did increase the height and width of the intestinal villi.
-This study shows the feasibility in to use the short time feeding in *Pseudoplatystoma* sp. production.

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Introduction

The catfish of the genus *Pseudoplatystoma* sp. in some cases it is known as surubim stand out as one of the main genus of native Brazilian fish raised in aquaculture systems. Brazilian aquaculture production has increased rapidly over the past two decades and native species in Brazil contribute to the development of the Brazilian aquaculture industry as a food supplier (Pincinato and Asche 2016). The State of Mato Grosso do Sul, Central Western Brazil stands out in the national scenario of fish farming for pioneering the commercial production of these species (Campos 2005; Inoue et al. 2009). The genus *Pseudoplatystoma* and their hybrids, originated from the crossbreeding of the *Pseudoplatystoma corruscans* (male) x *P. reticulatum* (female) breeds, is among the most cultivated species in Brazil, with production near 16 thousand tons in 2016 (IBGE 2016; Tavares et al. 2018). However, sanitary challenges in the commercial production of catfish *Pseudoplatystoma* sp. are constantly related to bacterial outbreaks, which generate large economic losses (Tavares-Dias and Martins 2017; Tavares et al. 2018).

The growth of fish farming in Brazil, as in other countries, leads the activity to an intensive or super intensive farming regime, using systems with high stocking densities and supplying high amounts of feed daily. Thus, mistaken management procedures become prominent factors for the increased occurrence of diseases and infections, which are the main causes of mortality in *Pseudoplatystoma* farming, where the control of diseases usually performed through the administration of antibiotics (Tavares-Dias and Martins 2017).

Currently, it is known that fish nutrition and health are closely linked to the balance of the gastrointestinal microbial flora of the hosts. Thus, positive effects can be obtained by manipulating the intestinal microbiota when beneficial microorganisms incorporated into animal diets adhere to the surface of the epithelial cells lining the interior of the intestine (Merrifield et al. 2010; Mouriño et al. 2012; Jesus et al. 2019; Owatari et al. 2019). The use of probiotic bacteria that benefits host health is a widespread and desirable alternative to the sustainability of world aquaculture as it may be an option for antibiotic use on fish farming. (Gatesoupe 1999; Pereira et al. 2017; Kuebutornye et al. 2019; Zhou et al. 2019).

Among the several beneficial actions of probiotics we highlight its inclusion in the die in order to improve the zootechnical performance, inhibitory action on growth of pathogenic bacteria, production of metabolites with bactericidal or bacteriostatic effects, longer survival against some pathogens, production of B vitamins, stimulation of the immune system by macrophage activation and restoration of the intestinal microbiota (Castro 2003; Raida et al. 2003; Korenblum et al. 2005; Panigrahi et al. 2005; Panigrahi et al. 2007; Kumar et al. 2008; Panigrahi et al. 2010; Mouriño et al. 2012; Zokaeifar et al. 2012; Kuebutornye et al. 2019).

Gram-positive bacteria of the genus *Bacillus* are a diverse group and are frequently tested in aquaculture research due to their sporulation ability, which allows them to survive under adverse environmental conditions. In addition, they are not pathogenic or toxic when added to fish diets, making them strong candidates for probiotic status (Hong et al. 2005; Ninawe and Selvin 2009; Kuebutornye et al. 2019). *Bacillus subtilis* produces bacteriocins and subtilines and these metabolites can be used to improve health and immune status, increasing disease resistance in other fish species, improving growth performance (Aly et al. 2008; Desriac et al. 2010; Kuebutornye et al. 2019). Even so, the costs involved and the long periods of administration of probiotics are still barriers imposed by the productive sector to use them.

Given this information, this study was strategically designed to verify, in an unprecedented way, the effects of administration of the commercial product CALSPORIN®, probiotic food additive containing viable spores of a strain of *Bacillus subtilis* C-3102, on the hematological parameters and changes in the digestive system, as well as the zootechnical performance of juveniles *Pseudoplatystoma* sp. in aquaculture systems.

Material and methods

The experiment was developed in the fish farming sector of the State University of Mato Grosso do Sul, Aquidauana, MS. All fish handling and slaughtering procedures in this study were approved by the CEUA / UEMS Animal Use Ethics Committee under protocol 014/ 2013, using eugenol (75 mgL^{-1}) for anesthetic procedure.

The experimental design was completely randomized design with five treatments with six replications. 180 hybrid surubins with total weight 25.52 g ± 1.10 and total length 17.54 cm ± 0.46 were used. Firstly, the juveniles were submitted to macroscopic evaluation to verify the sanitary aspects. Overall, no animals showed clinical signs of any apparent illness. The fish were distributed in 80 L tanks, 6 animals per experimental unit. The system was arranged with continuous water flow and the fish were fed with experimental food containing *B. subtilis*. Four levels of inclusion of the commercial probiotic, CALSPORIN® containing *B. subtilis* C-3102 (1×10^9 *B. subtilis* g product $^{-1}$ spores) were added to the experimental feed: 0 g CALSPORIN® kg $feed^{-1}$; 10 g CALSPORIN® kg $feed^{-1}$; 20 g CALSPORIN® kg $feed^{-1}$; 30 g CALSPORIN® kg $feed^{-1}$; 40 g CALSPORIN® kg $feed^{-1}$.

The feed was offered twice a day until the satiety of the fish. The control group consisted of probiotic-free treatment. Probiotic was added to the diet through oily medium (2% soybean oil) to incorporate bacteria into the pellets. Diets were provided daily in two periods (morning and afternoon) for 10 days.

After included the probiotic a sample of the diet was microbiologically analyzed at the Microbiology Laboratory of Empresa Comércio e Indústria Uniquímica Ltda - Diadema, São Paulo, in order to quantify *B. subtilis* concentration in the diet. To verify the concentration of the probiotic in the diet, after inoculation, 1 g of feed was macerated in 1 mL of 0.65% sterile saline solution and then serially diluted nine times in 1:10 factor test tubes. Dilutions from 10^{-4} to 10^{-9} were seeded in Petri dishes containing culture medium. The plates were incubated at 30 °C for 24 h. The values found were: control = not detected; in 10 g kg $feed^{-1} = 8{,}3 \times 10^6$ CFU; in 20 g kg $feed^{-1} = 1{,}5 \times 10^7$ CFU; in 30 g kg $feed^{-1} = 2{,}3 \times 10^7$ CFU; in 40 g kg $feed^{-1} = 3{,}2 \times 10^7$ CFU.

Water quality monitoring was performed daily at 08:00 with the aid of a handheld YSI Professional Plus multiparameter equipment. The environmental conditions during the experiment remained at 26.2 ± 2.9 °C; dissolved oxygen 4.6 ± 0.4 mg L^{-1}; pH 6.3 ± 0.3. Ammonia, nitrite and nitrate were not detected in water. To evaluate the zootechnical performance were performed biometrics at the beginning and the end of the experiment, to obtain the weight and length data of the animals. The zootechnical parameters evaluated were: Weight gain (g) = final weight (g) - initial weight (g); Apparent feed conversion = feed intake (g) / weight gain (g); Biomass gain (g) = final biomass (g) - initial biomass (g); Specific growth rate (% day) = (ln final weight - ln initial weight) ×100.

For hematological analyzes, blood samples were collected from six animals of each treatment by caudal vessel puncture. Blood collected with anticoagulant emulsified syringes was used to perform erythrocyte counting in a Neubauer chamber; hematocrit determination by the microhematocrit method according to Goldenfarb et al. (1971), and hemoglobin, by the cyanometahemoglobin method according to Collier (1944). Blood extensions were made and stained with May Grünwald-Giemsa-Wright (Tavares-Dias and de MORAES 2003) for differential leukocyte count, total leukocyte, and thrombocyte count.

With the values obtained from the erythrocyte count, hematocrit, and hemoglobin rate, the absolute hematimetric indices were calculated according to Wintrobe (1934). For intestine collection the fish ($n = 4$) were euthanized and a 3.0 cm segment was collected (4 cm below the junction of the stomach with the intestine). After removal the segment was sectioned longitudinally, and the attached ends were opened to keep the villi exposed. Each gut fragment was placed in Bouin's solution, where it was fixed for 6 h. The posterior preservation was done in 70° alcohol until the time of histological processing. The intestines were cut into 0.5 cm sections and prepared according to the usual histological techniques to obtain 3 μm thick paraffin sections that were stained with hematoxylin-eosin (HE). Five intestinal villi per animal were measured for height and width (20 villi/treatment) in a computer program Motic Images Plus 2.0 ML.

The experiment was structured presenting a logical ordering between the treatments that can be expressed as a function of each other. Regression analysis was used to verify the variation of y as a function of x. For the villus height parameter regression analysis was performed. For the results of hematology, productive performance, the analysis of variance (ANOVA) with a significance level of 5% was used. When the means showed significant differences, they were compared by the Tukey test ($p < 0.05$).

Results

In the present study the productive performance of surubins *Pseudoplatystoma* sp. was influenced by dietary supplementation with the probiotic containing *B. subtillis* (Table 1). Overall, biomass gain was higher in the 30 g kg feed^{-1} group (20.06 ± 0.92 g) when compared to the other groups.

In addition, no significant effect ($p > 0.05$) of supplementation was observed on all erythrogram parameters between all supplemented levels (Table 2).

However, in the present study a significant effect ($p < 0.05$) of the treatments was observed on the absolute values of lymphocytes, neutrophils, total leukocytes, and thrombocytes (Table 3). Higher values were observed for fish submitted to 40 g kg feed $^{-1}$ compared to other treatments.

Table 1 Growth performance of juveniles *Pseudoplatystoma* sp. fed for 10 days with probiotic *B. subtilis*

Indexes	Treatments (g kg feed^{-1})				
	0	10	20	30	40
WG (g)	3.38 ± 0.92	4.73 ± 3.75	4.62 ± 2.12	2.91 ± 1.70	2.29 ± 0.40
AFC	1.89 ± 0.90	2.68 ± 1.95	1.50 ± 0.53	2.34 ± 1.37	2.99 ± 0.70
BG (g)	13.15 ± 4.08^{b}	15.50 ± 1.24^{b}	16.67 ± 2.02^{b}	20.06 ± 0.92^{a}	8.83 ± 2.00^{b}
PER	1.50 ± 0.34	1.20 ± 0.23	1.49 ± 0.77	0.95 ± 0.62	0.83 ± 0.15
SGR	0.62 ± 0.17	0.52 ± 0.10	0.43 ± 0.18	0.43 ± 0.18	0.39 ± 0.03

Mean values and standard errors of weight gain (WG); apparent feed conversion (AFC); biomass gain (BG); protein efficiency ratio (PER), and specific growth rate (SGR). Initial weight 25.75 ± 1.1 g

Table 2 Erythrogram and glucose (mean ± standard error) of juveniles of hybrid surubim *Pseudoplatystoma* sp. fed different concentrations of *B. subtilis*-containing probiotics

Parameters	Treatments (g kg feed^{-1})				
	0	10	20	30	40
Htc (%)	24.67 ± 1.83	22.75 ± 4.10	21.67 ± 3.11	22.70 ± 5.96	23.08 ± 1.16
Hb (g dL^{-1})	4.48 ± 1.07	4.85 ± 1.10	4.65 ± 0.53	5.05 ± 0.70	4.85 ± 0.27
Er (x10^{6}μL^{-1})	2.68 ± 0.53	2.38 ± 0.84	2.27 ± 0.45	2.69 ± 0.52	2.84 ± 0.27
MCV (fL)	94.19 ± 13.55	101.80 ± 24.06	88.38 ± 11.78	84.89 ± 1.89	82.13 ± 10.52
MCHC (gdL^{-1})	18.15 ± 4.23	21.31 ± 2.52	21.54 ± 1.31	22.40 ± 3.50	21.01 ± 1.06
Glucose (mgdL^{-1})	33.28 ± 7.54	34.69 ± 5.11	27.54 ± 7.70	29.80 ± 8.35	33.46 ± 5.85

Htc = hematocrit; Hb = hemoglobin; Er = erythrocyte; MCV = mean corpuscular volume; MCHC = Mean corpuscular hemoglobin concentration

Histomorphometric analysis of the anterior portion of the intestine showed significant differences in the height and width values of the intestinal villi of fish fed diets containing the probiotic (Table 4). Probiotic use in surubin diets had positive influence on villi width and height. It was found that supplementation with *B. subtilis* differed in treatment 10 g kg feed^{-1} (343.08 ± 4.37 μm) compared to treatment 40 g kg feed^{-1} (325.72 ± 9.08 μm) for villi height, as well as treatment 30 g kg feed^{-1} (204.06 ± 80.67 μm) compared to treatment 20 g kg feed^{-1} (74.53 ± 9.91 μm) for villi width.

Discussion

The present study verified the use of a viable spore-based probiotic food additive from a strain of *Bacillus subtilis* C-3102 for the first time for *Pseudoplatystoma* sp. Research in the field of aquaculture around the world has shown the positive effects of probiotic bacteria *B. subtilis* on animal performance, with consistent improvements, in some cases in growth, and feed conversion, as well as increased fish innate defense mechanisms (Galagarza et al. 2018; Hassaan et al. 2018; Di et al. 2019; Kuebutornye et al. 2019).

In the present research, we observed significant differences in the productive indexes after 10 days of supplementation with probiotics. Positive responses to the zootechnical performance of the animals with probiotic supplementation were observed in the study by El-Haroun et al. (2006), where animals that received a diet with Biogen® probiotic containing *B. subtilis* Natto (not less than 6 × 10^7 g^{-1}) presented higher weight gain and better specific growth rates and efficiency.

Dias et al. (2012), when evaluating *Brycon amazonicus* breeders, observed after 83 days of supplementation with *B. subtilis* (5 g kg^{-1} and 10 g kg^{-1}), improvement in performance parameters such as better apparent feed conversion, higher specific growth rate, and weight gain. A relevant factor that should be taken into consideration is the time of administration of the probiotic diet. In the present study, a short period of 10 days of supplementation was enough to show a significant improvement in zootechnical performance between group 30 g kg feed^{-1} and the other treatments. This can be considered a good strategy for *Pseudoplatystoma* sp. production, since a shorter probiotic administration period may be more financially advantageous compared to longer supplementation periods.

Table 3 Absolute values (mean ± standard error) of blood leukocytes of surubim juveniles *Pseudoplatystoma* sp. fed for 10 days with *B. subtilis* and control group

	Treatments (g kg feed^{-1})				
	0	10	20	30	40
Monocytes	160.87 ± 40.87	244.12 ± 198.65	99.78 ± 27.27	214.90 ± 135.06	294.93 ± 56.83
Lymphocytes	32,709.8 ± 10,281.42^{b}	27,585.05 ± 14,203.84^{b}	27,162.52 ± 5610.84^{b}	37,163.93 ± 6683.90^{ab}	52,322.99 ± 15,708.77^{a}
Basophils	184.71 ± 11.16	180.52 ± 142.06	119.06 ± 0,00	150.70 ± 36.27	236.07 ± 86.51
Eosinophils	353.63 ± 0,00	263.16 ± 145.71	213.86 ± 123.04	237.95 ± 104.14	236.13 ± 61.17
Neutrophils	20,274.18 ± 6888.38^{ab}	21,346.14 ± 8634.86^{b}	21,629.97 ± 5729.15^{b}	26,749.41 ± 8380.53^{b}	40,658.10 ± 8238.24^{a}
EGC	506.53 ± 150.74	343.45 ± 286.40	262.72 ± 204.86	390.31 ± 212.24	773.65 ± 630.94
IL	566.04 ± 397.70	443.28 ± 240.86	467.66 ± 547.47	717.33 ± 607.05	849.60 ± 409.18
TL	63,105.63 ± 16,488.22^{b}	50,008.13 ± 23,178.84^{b}	49,317.71 ± 10,830.17^{b}	64,978.33 ± 13,812.49^{b}	94,690.42 ± 18,547.37^{a}
Thrombocytes	79,748.13 ± 23,040.27^{b}	88,457.92 ± 44,479.21^{b}	96,885.21 ± 32,828.01^{ab}	124,576.88 ± 33,153.01^{ab}	141,133.96 ± 21,364.89^{a}

EGC: Special Granulocytic Cell; IL = Immature Leukocytes; TL = Total leukocytes. Different letters indicate significant differences between the groups ($p < 0.05$)

Table 4 Histomorphometric parameters (mean ± standard error) of the midgut portion of surubins *Pseudoplatystoma* sp. after 10 days of feeding with probiotic *Bacillus subtillis*

Intestine morphometry	Treatments (g kg feed^{-1})				
	0	10	20	30	40
Villi height (μm)	334.91 ± 7.36^{ab}	343.08 ± 4.37^{a}	339.94 ± 7.65^{ab}	334.43 ± 9.09^{ab}	325.72 ± 9.08^{b}
Villi width (μm)	135.79 ± 98^{ab}	181.19 ± 91.2^{ab}	74.53 ± 9.91^{b}	204.06 ± 80.67^{a}	158.81 ± 97.57^{ab}

Different letters indicate significant differences between the groups ($p < 0.05$)

Probiotic supplementation in fish feed may determine different positive blood responses as well as different immune responses, according to target species (Azarin et al. 2015; Zaineldin et al. 2018; Kuebutornye et al. 2019). However, in the present study, feeding for 10 days with *B. subtilis* did not influence the amount of red blood cells. The hematological parameters of the red series are commonly related to the general health of the fish and, since they did not present significant alterations, indicated that the animals were healthy, corroborating the studies by Barros et al. (2009) and Harikrishnan et al. (2010), in which no alterations related to erythropoiesis were observed. Nevertheless, in the present study, we observed a significant effect of probiotic *B. subtilis* C-3102 on the leukocyte profile of animals.

Although, hematological responses may vary in each experimental situation and according to the species used (Kumar et al. 2008; Merrifield et al. 2010; Dias et al. 2012; Nakandakare et al. 2018; Kuebutornye et al. 2019), in the present study, thrombocytes increased, as probiotic concentration was increased, as well as total neutrophils, lymphocytes, and leukocytes, resulting in a better supplementation with 40 g CALSPORIN® kg ration^{-1}. This may be related to the higher amount of *B. subtilis* in contact with the intestinal lumen and enterocytes, causing the secretion of antibacterial factors and a consequent innate response.

Pereira et al. (2016) found an increase in circulating thrombocytes in animals supplemented with the *W. ciberia* probiotic strain (1×10^8 CFU L^{-1}) when compared to animals not receiving supplementation. The authors report that the different results may be related to the experimental period, fish species, and type of bacteria used in the studies. Likewise, the authors of this study share this reasoning. Each experimental situation has intrinsic characteristics. Thus, it is possible that similar experimental situations may show different results or different experimental designs may have similar results.

Thrombocytes, as well as other leukocyte lineages, play an important role in the defense mechanism of fish, being responsible for coagulation, and inflammatory response, as well as having phagocytic activity during infections (Kiron 2012; Zachary et al. 2018). The increase in leukocyte amounts observed in the present study indicates a possible improvement in animal defense mechanisms due to the higher concentration of these defense cells circulating in the blood. There is a strong indication of increased innate immunity of the fish, indicating that these animals were better prepared for eventual confrontation with pathogenic microorganisms.

The gut is an organ involved in important physiological functions and the main site of food digestion and nutrient absorption (Kiron 2012; Kuebutornye et al. 2019). Therefore, the increase in villi height and width observed in the present study can be understood as the improvement in intestinal mucosa integrity. This condition may possibly allow better development of the animal and favor the digestion of food and absorption of nutrients. Dietary probiotics may induce an increase in villus epithelium thickness, which may suggest hypertrophy as a positive response to stimulation (Carvalho et al. 2011; Galagarza et al. 2018; Kuebutornye et al. 2019)

Beneficial changes in intestinal morphometry of fish when fed diets supplemented with probiotics, as observed in the present study, are strong indications that it can be inferred that interactions between intestinal microflora and intestinal morphology, together with the immune system, and the absorption of nutrients can positively influence fish health and performance (Sweetman et al. 2008; Merrifield et al. 2010; Mouriño et al. 2012; Galagarza et al. 2018; Brum et al. 2018; Owatari et al. 2019; Jesus et al. 2019).

Conclusion

The *B. subtilis* C-3102-containing probiotic administered at different doses for 10 days provided zootechnical benefits and was enough to cause an immunomodulatory effect, influencing the hematological parameters in the animals, which increased the defense blood cells as the inclusion of the probiotic was increased in the feed. In addition, it was found that supplementation significantly interfered with villi height and width, possibly improving nutrient absorption. Thus, we indicate that the CALSPORIN® probiotic product can be used in strategic periods, where predictable epidemic events may occur in *Pseudoplatystoma* farming.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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