



Comparative effects of using a single strain probiotic and multi-strain probiotic on the productive performance and disease resistance in *Oreochromis niloticus*

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ABSTRACT

The study was carried out to evaluate the effect of dietary supplementation of three probiotic strains in isolation and a multi-strain formulation (mix) on the rearing of tilapia, based on the parameters of productive performance and resistance to infection. A total of 240 juveniles of *Oreochromis niloticus* (6.71 ± 0.93 g and 61.88 ± 1.44 mm) were fed for 90 days with fish food containing two varieties of *Enterococcus faecium* and one variety of *Bacillus cereus* following therapeutic concentrations reported in the literature, and one multi-strain formulation containing the three organisms. After the period of food supplementation, 45 specimens were injected intraperitoneally with 300 μ L of *Streptococcus agalactiae* at a lethal concentration of 1.7×10^7 CFU.g⁻¹ and their inflammatory response was monitored for 96 h to record the clinical signs of the disease, intensity of the infectious disease and accumulated mortality. The use of probiotics interfered ($p < 0.05$) in the productive performance of the animals. The fish treated with the multi-strain formulation (mix) of probiotics had higher survival rates ($97.9 \pm 4.1\%$), an absence of intestinal pathogenic strains, and greater hematocrit ($33.5 \pm 7.6 \times 10^6 \mu\text{L}^{-1}$), hemoglobin (13.8 ± 1.3 g.dL⁻¹) and total protein (5.3 ± 0.4 g. dL⁻¹) values. In the challenge assay, the more intense clinical signs and mortality rates were recorded in the positive control group (no supplementation and pathogen injection), while tilapia fed with the probiotics *E. faecium* (1), *B. cereus* and the mix recovered their normal coloration, had higher survival rates and had a significant increase ($p < 0.05$) in leukocytes. Finally, the dietary use of probiotics in single strain and multi-strain mix improved the productive and microbiological parameters besides the resistance to infection with *S. agalactiae* for 90 days of tilapia production.

1. Introduction

The world's aquaculture production is one of the main protein source activities that raises an average income of US\$243.5 billion per year. Among the species produced, the tilapia *Oreochromis niloticus* stands out,

representing the second most produced freshwater fish on the planet and present in more than 100 countries (Abarike et al., 2018; FAO, 2018). However, the intensification of production systems has led to an increase in the incidence and spread of diseases, especially those of bacterial origin, which mostly cause irreversible productive and economic

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losses (Shameena et al., 2020).

To avoid the use of antibiotics to control these diseases and to meet the sustainable aquaculture model, the use of probiotics as a prophylactic strategy has been studied. Probiotics can develop beneficial effects on the host, such as intestinal microbiological modulation, optimization of the absorption of nutrients contained in the diet and improved host growth and immune response, in addition to being a safe method to control of bacterial infections due to their broad bactericidal spectrum (Hoseinifar et al., 2018; Doan et al., 2018; Sousa et al., 2019).

Among the diversity of bacteria with cataloged probiotic purpose, the varieties of *Enterococcus faecium* stand out due to their safe handling, besides being non-invasive to the environment and for stimulating digestive enzyme production (Pirarat et al., 2011). Similarly, the species of *Bacillus cereus* demonstrate several probiotic benefits including the ability to sporulate, which is a notable feature among the groups for industrialization processes (Hoseinifar et al., 2018; Van Doan et al., 2019).

The administration of multi-strain probiotics in the production system can provide significant productive advantages by enhancing their actions in a symbiotic, additive, and consistent way in the host (Doulard et al., 2018; Ringø et al., 2020; Melo-Bolivar et al., 2020).

The current study investigated the probiotic effects of three bacterial strains, including two varieties of *E. faecium* and one strain of *B. cereus* on productive, microbiological and hematological parameters and the resistance to bacterial infection.

2. Material and methods

The tests were approved by the animal experimentation ethics committee of the Brazilian Agricultural Research Corporation - Embrapa (n° 0033/2020).

2.1. Experimental conditions

Twenty tanks with a capacity of 150 L were used, coupled to a water recirculation system, with mechanical, biological, and ultraviolet light filters. Healthy juveniles of *O. niloticus* ($n = 240$; weight 6.71 ± 0.93 g, total length 61.88 ± 1.44 mm) were randomly distributed in the tanks (12 individuals per tank, with a mean initial biomass of 80.03 ± 1.4 g). Water variables were monitored on alternate days during the 90 days of the experiment. The temperature (29.4 ± 1.2 °C), electrical conductivity (218.91 ± 3.76 $\mu\text{S}\cdot\text{cm}^{-1}$), dissolved oxygen (6.4 ± 0.5 $\text{mg}\cdot\text{L}^{-1}$), pH (6.1 ± 0.2), and total ammonia (0.3 ± 0.8 $\text{mg}\cdot\text{L}^{-1}$) were recorded aided by multiparameter ProfessionalPlus YSI.

2.2. Preparation of strains and diets

The probiotic strains applied in this study included two varieties of *E. faecium* (Dias et al., 2019; Sousa et al., 2019) and one variety of *B. cereus* (Dias et al., 2018). The strains used in this study were acquired from the laboratory of Health of Aquatic Organisms at The Brazilian Agriculture Research Corporation (EMBRAPA) in Aracaju, Sergipe/Brazil.

Prior to the supplementation assay, an *in vitro* study was performed to evaluate the antagonism between the probiotic strains and to mitigate the competitive effect between the bacteria. For this assay, the method of Ramírez et al. (2006) was applied. Initially, the bacteria under evaluation were added into the Man Rogosa & Sharpe (MRS) broth culture medium and incubated at 35 °C for 24 h. After microbiological growth, serial dilutions (10^{-2} , 10^{-3} and 10^{-4}) were performed, where 5 μL was aliquoted and inoculated onto filter paper discs (Qualy 250 μm). The discs of each probiotic and each dilution were then placed in MRS plates previously inoculated with 10^4 CFU.mL $^{-1}$ of each probiotic (*E. faecium* 1 and 2 or *B. cereus*) and incubated at 35 °C for 48 h. Finally, the analysis for the presence or absence of inhibitory halos and the evaluation of antagonism between probiotic strains were performed, and the

concentration (CFU.g $^{-1}$) of each strain in the multi-strain treatment was determined.

A completely randomized design with four treatments in quadruplicate was used, corresponding to two diets with probiotics isolated from continental fish species, *E. faecium* (1) 2×10^6 CFU.g $^{-1}$ (Dias et al., 2019) and *E. faecium* (2) 1×10^8 CFU.g $^{-1}$ (Sousa et al., 2019); a diet with probiotic *B. cereus* 2.8×10^6 CFU.g $^{-1}$ (Dias et al., 2018); and the multi-strain probiotic consisting of 1×10^8 CFU.g $^{-1}$, containing the concentrations of *E. faecium* (1 and 2) and *B. cereus* previously stipulated; plus a control group (without the inclusion of the probiotic).

The strains were kept in MRS liquid medium, grown at 35 °C for 24 h, and then centrifuged at 1800g for 15 min and resuspended in a sterile saline solution (SSE 0.65%) until the experimental concentrations, according to the methodology of Jatobá et al. (2008). Then, the respective suspensions were sprinkled on the extruded commercial feed with guaranteed levels of 36% crude protein, 12% moisture, 7% ethereal extract and 5% crude fiber. The rations were renewed every seven days and stored at 4 °C.

2.3. Productive parameters

The animals were fed daily for 90 days at an initial rate of 5% in relation to the biomass of the tanks and readjusted to the amount according to monthly biometric values (Marengoni et al., 2015).

Biometric evaluations were registered with all fish under study to observe the variables of total length, standard length, weight, feed intake, and subsequent determination of the parameters: Weight Gain = Final Weight – Initial Weight; Total Length Gain = Final Total Length – Initial Total Length; Standard Length Gain = Final Standard Length – Initial Standard Length; Specific Growth Rate (SGR) = $\ln(\text{Final Weight in grams}) - \ln(\text{Initial Weight in grams}) \times 100/t$ (days of experiment); Fulton's condition factor (K) = $\text{Final body weight}/\text{Total length (cm)}^3$; Feed conversion ratio (FCR) = $\text{Feed intake (kg)}/\text{Weight gain (g)}$; Uniformity (U) = $(N \pm 20\%)/Nt$, Nt = total number of fish in each experimental unit; and $N \pm 20\%$ = number of animals with the parameter weight/length within $\pm 20\%$ around the mean of the experimental unit, and Survival rate = $(\text{Final number of fish}/\text{initial number of fish}) \times 100$ (Furuya et al., 1998; Gonçalves Júnior et al., 2013).

2.4. Pathogen preparation

Prior to the induction of the infection process, an *in vitro* test was carried out to evaluate the inhibition of the probiotic strains and the multi-strain formulation against the pathogen *Streptococcus agalactiae*. The pathogen was acquired from symptomatic infected animals, in which the strain was isolated and purified according to the methodology of Jatobá and Mourão (2015), incubated at 37 °C for 48 h in BHI medium. The sample was centrifuged at 1800g for 30 min, the supernatant was discarded, the pellet was washed and resuspended twice with SSE 0.65%, and the strain was characterized by the Gram stain and identified by matrix assisted laser desorption/ionization and time-of-flight MALDI-TOF mass spectrometry (Angeletti, 2017).

2.5. In vitro test against *S. agalactiae*

An *in vitro* test was performed to evaluate the inhibition of the probiotic strains and their product mix against the pathogen *S. agalactiae*. For this purpose, the method of Vieira et al. (2013) and Paixão et al. (2019) was applied, in which the probiotics were cultivated in the MRS broth medium and then 100 μL of each strain and the mix were inoculated in MRS agar medium and incubated at 35 °C for 48 h. After bacterial growth, discs with diameters of 0.8 cm were removed from the agar plates and superimposed on plates with Mueller Hinton agar containing the pathogen *S. agalactiae*. This assay was performed in four replicates, and then incubated at 35 °C for 48 h for further analysis of the halo of inhibition (mm).

2.6. Infection with *S. agalactiae*

At the end of 13 weeks of feeding, nine animals from each treatment, three individuals per repetition, were injected intraperitoneally with 300 μL of *S. agalactiae* at a concentration of 1.7×10^7 CFU.g⁻¹. In addition to two controls, one fish without probiotic supplementation and injected with the pathogen ($n = 3$ per replicate, positive control) and the fish without probiotic supplementation and injected with sterile saline solution (0.65%) ($n = 3$ per replicate, negative control) were used. The design was completely randomized with 6 treatments and 3 replications.

The pathogen was activated for 24 h in BHI broth enriched with 10% sterile fish blood and incubated at 30 °C for 24 h according to Mouriño et al. (2017). After this period, the bacteria were centrifuged for 30 min at 1800g, the supernatant was discarded and the pellet was resuspended in a sterile NaCl (0.65%) solution in order to reach the lethal infection concentration, which was determined by serial dilution by factor of 1:10 and confirmed by Colony Forming Unit by plating on BHI agar medium (Silva et al., 2012; Angeletti, 2017; Mouriño et al., 2017).

The injected animals were monitored to record clinical signs of skin darkening, erratic swimming, exophthalmia, opercular hemorrhage, lethargy, ocular opacity, epidermal ulcerations and daily mortality for 96 h (Silva et al., 2012). The infectious intensity was classified on a scale from zero to five, adapted to the protocol from Fishbein et al. (2005), considering the estimated percentage of observed clinical signs: grade 0, < 1% of clinical signs; grade 1, 1–5%; grade 2, 6–10%; grade 3, 11–25%; grade 4, 26–50%; grade 5, > 50%.

For this experiment, the monitoring of water variables was performed were monitored daily, analyzing including temperature (29.8 ± 0.6 °C), electrical conductivity (202.5 ± 7.0 $\mu\text{S}\cdot\text{cm}^{-1}$), dissolved oxygen (6.4 ± 0.5 mg.L⁻¹), hydrogen potential (6.3 ± 0.4) and total ammonia values that varied between (0.2 ± 0.5 to 0.9 ± 0.6 mg.L⁻¹) were recorded aided by multiparameter ProfessionalPlus YSI.

2.7. Hematological analysis

At the end of the experimental period of productive performance, as well as, from the infection experiment, ten fish were randomly collected per treatment. The sick fish, from the infection experiment, and the survivors were taken for evaluation of the hematological parameters. Lastly, the animals were anesthetized using a solution of 60 mg.L⁻¹ eugenol, and then 1 mL of blood was collected from the caudal vessel with the aid of syringes moistened with EDTA (10%).

Additionally, an aliquot of 5 μL of the total volume was used immediately after collection to measure the blood glucose with an AccuCheck Active device. Aliquots of the same volume were also used for determining the triglycerides and cholesterol (Accutrend® Plus). Leukocyte and thrombocyte counts were manually performed using the blood extension method after staining with the NewProv panoptic hematological dye (Fontes et al., 2014).

For the total count of erythrocytes (cell $\times 10^6$ μL^{-1}) 10 μL of blood was added in 2.5 mL microtubes, filled with 1 mL of a sterile saline solution (0.65%) and homogenized for subsequent counting in a Neubauer chamber (Tavares-Dias and Moraes, 2004). The hematocrit was determined by applying the microcentrifugation methodology described by Goldenfarb et al. (1971).

The total plasma protein was measured by using a refractometer (QuimisRHC-200ATC). For the hemoglobin concentrations, an aliquot of 10 μL of blood was added to 2.5 mL of cyanide and measured with a ThermoPlate biochemical analyzer (Kamper and Zijlstra, 1961).

Finally, the hematimetric indices were calculated for mean corpuscular volume (MCV: Htx10/er), mean corpuscular hemoglobin (MCH: Hbx10/er) and mean corpuscular hemoglobin concentration (MCHC: Hbx100/Ht) (Vallada, 1999; Tavares-Dias and Moraes, 2006).

2.8. Collection of visceral material

For the evaluation of the hepatosomatic, splenosomatic, and viscerosomatic indices and organ sampling for microbiology analysis, euthanasia was performed by deepening anesthesia, followed by a medullary section. Subsequently, organs (liver, spleen, and viscera) were removed for determining the Hepatosomatic Index (HSI) = (weight of liver of fish/body weight of fish) $\times 100$; Splenosomatic Index (SSI) = (spleen weight/body weight) $\times 100$; and Viscerosomatic Index (VI) = (visceral weight/body weight) $\times 100$ followed by microbiological analysis of the liver, intestine and intraperitoneal cavity.

2.9. Bacteriological analyses

In an attempt to keep the concentration of the probiotic stable, one gram of each experimental fish feed (w/v) was weekly sampled, ground and diluted in a sterile saline solution (0.65%) (1:10). An aliquot of 100 μL was plated in MRS agar culture medium to aid in the count of the probiotic bacteria (CFU.g⁻¹) and the posterior identification through the Gram staining and MALDI-TOF methods, respectively (Jatobá and Mouriño, 2015; Angeletti, 2017).

To assess the intestinal bacterial microbiota (probiotic bacteria vs potentially pathogenic bacteria), samples of the foregut and midgut of the animals were collected after euthanasia and macerated with a 0.65% sterile saline (w/v%), for subsequent serial dilutions of 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} with a 1:10 ratio in 15 mL tubes.

In addition, an aliquot of 100 μL of each dilution was plated in Petri dishes containing MRS and Tryptone Soya Agar (TSA) to evaluate the growth of the probiotic and potentially pathogenic bacteria, respectively. These plates were then incubated at 35 °C for 48 h followed by a cell count (CFU.g⁻¹) in animal intestine (Jatobá et al., 2008) and the identification of the different colonies of bacteria by the MALDI-TOF technique (Angeletti, 2017).

For confirmation of pathogen infection, blood, liver, and intraperitoneal swab were collected for microbiological diagnosis and bacterial re-isolation in BHI agar culture medium and incubated at 35 °C for 48 h to confirm the Koch's postulate. After bacterial growth, colonies were identified by the MALDI-TOF method (Evans, 1976; Angeletti, 2017).

2.10. Statistical analysis

The data were acquired, and the microbiological counts were converted into square roots, before being submitted to statistical tests. The data were evaluated for normality and homoscedasticity by the Shapiro-Wilk and Bartlett tests, respectively, and when heterogeneity of variance was observed, they were transformed into $\log_{10}(x + 1)$. Concerning mortality, the results were transformed into arcsin root ($x \cdot 100^{-1}$). Accordingly, the data were subjected to Analysis of Variance (ANOVA), with the F value being significant, and the means were compared by the Tukey's test at 5% probability. Data that did not meet the ANOVA premises were subjected to the non-parametric Kruskal-Wallis test and subsequently to the DUNN test for comparison by rank.

3. Results

The results of the multi-species formulation showed concentrations of 1×10^2 CFU per mL for strains of *E. faecium* and 1×10^4 CFU per mL for *B. cereus*. Furthermore, there was no inhibition halo under these concentrations for the evaluated bacteria (Table 1), which led to a final product concentration of 1×10^8 CFU per mL. This value was equivalent to CFU.g⁻¹ in the fish food after 48 h of incubation.

For the productive performance, the highest means ($p < 0.05$) of weight and weight gain were demonstrated by the fish that received a ration with the inclusion of multi-strain probiotics (mix) at the first 30 days of supplementation. Lower apparent feed conversion values and higher specific growth rates were observed in the treatment with the

Table 1
Determination of the concentration of the probiotic strains for the formulation of a multi-strain probiotic product.

	Concentration (CFU. ml ⁻¹)	BC	EF1	EF2
<i>Bacillus cereus</i> (BC)	10 ²	-	-	-
	10 ³	-	-	-
	10 ⁴	-	-	-
<i>Enterococcus faecium</i> strain 1 (EF1)	10 ²	-	-	-
	10 ³	+	-	-
	10 ⁴	+	-	-
<i>E. faecium</i> strain 2 (EF2)	10 ²	-	-	-
	10 ³	+	-	-
	10 ⁴	+	-	-

- without inhibition halo; + with inhibition halo.

inclusion of the mix compared to the treatment with *E. faecium* (2) and the control group ($p < 0.05$, Table 2).

Similar responses remained during 60 and 90 experimental days, with greater gains in standard length, and survival rate ($p < 0.05$) being observed at 60 days of the experiment for the treatment with the mix.

Concerning the treatments with probiotics during the 90 days, no significant differences were observed among them ($p > 0.05$). Furthermore, they demonstrated the highest averages ($p < 0.05$) for TL (187.9 ± 6.9 mm), SL (150.7 ± 4.8 mm), weight (89.7 ± 6.0 g), TLG (50.5 ± 3.9 mm), SLG (40.4 ± 3.5 mm), WG (49.1 ± 7.9 g) and survival (81.7 ± 11.9%), compared to the control group (140.2 ± 0.9 mm; 127.4 ± 9.4

Table 2
Productive performance of juveniles of *Oreochromis niloticus* supplemented with the probiotics *Enterococcus faecium* (1) 2×10^6 CFU.g⁻¹ in the feed, *E. faecium* (2) 1×10^8 CFU.g⁻¹ in the feed, *Bacillus cereus* 2.8×10^6 CFU.g⁻¹ in the feed and multi-strain (mix) 1×10^8 CFU.g⁻¹.

30 Days												
Treatment	TL	TLG	SL	SLG	WEIGHT	WG	U	FCR	SGR	K	S	
Controls	94.20 ± 1.04A	32.3 ± 1.1A	77.5 ± 1.7A	27.4 ± 1.7A	14.3 ± 1.1B	7.9 ± 1.3B	36.9 ± 3.4A	1.3 ± 0.1B	2.5 ± 0.2B	1.7 ± 0.1A	95.8 ± 4.8A	
<i>E. faecium</i> 1	95.27 ± 0.72A	33.4 ± 0.7A	79.4 ± 0.1A	29.3 ± 1.0A	15.6 ± 1.1B	8.9 ± 1.1AB	35.5 ± 12.5A	1.1 ± 0.1AB	2.8 ± 0.2AB	1.7 ± 0.1A	97.9 ± 4.1A	
<i>E. faecium</i> 2	95.14 ± 1.73A	33.2 ± 1.7A	78.2 ± 1.7A	27.1 ± 3.6A	14.5 ± 1.2B	7.8 ± 1.2B	39.9 ± 2.5A	1.3 ± 0.1B	2.5 ± 0.2B	1.6 ± 0.1A	93.7 ± 7.9A	
<i>B. cereus</i>	97.79 ± 1.31A	35.9 ± 1.3A	79.6 ± 0.6A	29.5 ± 0.6A	15.3 ± 1.1B	8.6 ± 1.1AB	35.8 ± 17.9A	1.1 ± 0.1AB	2.7 ± 0.2AB	1.6 ± 0.1A	97.9 ± 4.1A	
Mix	96.89 ± 3.55A	35.0 ± 3.5A	79.4 ± 1.7A	29.4 ± 1.7A	16.9 ± 0.8A	10.2 ± 0.8A	34.6 ± 11.3A	0.9 ± 0.1A	3.1 ± 0.1A	1.8 ± 0.1A	95.8 ± 4.8A	
60 Days												
Treatment	TL	TLG	SL	SLG	WEIGHT	WG	U	FCR	SGR	K	S	
Controls	140.22 ± 0.94C	48.4 ± 3.7A	113.3 ± 0.8C	37.8 ± 1.2B	42.9 ± 1.7C	31.2 ± 2.2B	42.0 ± 17.8A	0.9 ± 0.21A	3.9 ± 0.1A	1.4 ± 0.26A	75.0 ± 6.8B	
<i>E. faecium</i> 1	145.72 ± 1.58AB	50.3 ± 2.6A	119.4 ± 1.07B	39.9 ± 0.8AB	47.0 ± 3.4B	34.6 ± 1.1AB	50.0 ± 6.8A	0.8 ± 0.06A	3.8 ± 0.4A	1.5 ± 0.04A	89.5 ± 7.9AB	
<i>E. faecium</i> 2	143.42 ± 2.49BC	50.3 ± 3.1A	117.6 ± 1.3B	37.6 ± 0.8B	47.3 ± 0.6B	32.5 ± 2.1AB	45.7 ± 10.1A	0.8 ± 0.07A	3.8 ± 0.3A	1.5 ± 0.05A	85.4 ± 12.5AB	
<i>B. cereus</i>	146.82 ± 2.14AB	49.0 ± 2.7A	119.8 ± 1.7AB	40.6 ± 2.1AB	51.3 ± 2.9AB	36.9 ± 2.6A	49.2 ± 7.9A	0.8 ± 0.03A	4.0 ± 0.4A	1.6 ± 0.07A	85.4 ± 7.9AB	
Mix	150.47 ± 2.11A	52.3 ± 1.6A	122.1 ± 0.2A	41.8 ± 1.1A	52.8 ± 1.5A	36.5 ± 0.8A	52.8 ± 31.8A	0.7 ± 0.08A	3.8 ± 0.1A	1.5 ± 0.04A	100.0 ± 0.0A	
90 Days												
Treatment	TL	TLG	SL	SLG	WEIGHT	WG	U	FCR	SGR	K	S	
Controls	140.22 ± 0.94B	19.0 ± 3.0B	127.3 ± 9.4B	17.4 ± 3.7B	72.5 ± 10.4B	37.9 ± 2.4B	87.5 ± 8.3A	0.9 ± 0.4A	2.4 ± 1.0A	2.0 ± 0.06D	52.0 ± 32.1B	
<i>E. faecium</i> 1	182.47 ± 1.39A	47.2 ± 4.1A	147.2 ± 12.1AB	37.5 ± 3.7A	85.8 ± 10.8AB	42.3 ± 2.2AB	53.4 ± 13.6A	0.8 ± 0.1A	2.5 ± 0.7A	1.3 ± 0.04	83.3 ± 9.6AB	
<i>E. faecium</i> 2	181.85 ± 0.73AB	47.0 ± 12.5AB	146.2 ± 6.0AB	38.5 ± 9.1A	83.9 ± 6.8AB	45.8 ± 8.2AB	62.7 ± 20.4A	0.9 ± 0.2A	2.6 ± 1.2A	1.3 ± 0.05C	70.8 ± 14.4AB	
<i>B. cereus</i>	191.49 ± 0.97A	54.5 ± 23.4A	153.1 ± 9.8A	45.3 ± 15.7A	92.1 ± 10.6AB	60.5 ± 16.3A	66.1 ± 15.3A	0.8 ± 0.2A	2.5 ± 1.7A	1.2 ± 0.03AB	75.0 ± 6.8AB	
Mix	195.90 ± 1.08A	53.3 ± 9.2A	156.3 ± 8.8A	40.1 ± 6.1A	97.0 ± 10.6A	47.6 ± 7.7AB	72.2 ± 14.3A	0.8 ± 0.3A	2.4 ± 0.7A	1.2 ± 0.01A	97.9 ± 4.1A	

TL - Total Length (mm); TLG - Total Length Gain (mm); SL - Standard Length (mm); SLG - Standard Length Gain (mm); WG - Weight gain (g); U - Weight Uniformity (%); FCR - Feed Conversion Rate; SGR - Specific Growth Rate (%.day⁻¹); K - Fulton's condition factor; S - Survival rate (%). Different letters within the same row differ by the Tukey's test ($p < 0.05$).

mm; 72.6 ± 10.4 g; 19.0 ± 3.0 mm; 17.5 ± 3.8 mm; 37.9 ± 2.5 g and 52.1 ± 32.2%, respectively). However, the treatment supplemented with the probiotic multi-strain (mix) resulted in a better Fulton condition factor (1.24 ± 0.01 , $p < 0.05$) compared to the other probiotics and the control.

For the results of the viscerosomatic (VSI), hepatosomatic (HSI) and splenosomatic (SSI) indices, there was no statistical difference between the experimental units that presented mean values of 9.45 ± 0.32 IVS, 1.49 ± 0.04 IHS and 0.065 ± 0.004 IES, respectively. Concerning the hematological analyses performed at the end of 90 days of the experiment, there was a decrease in blood glucose, cholesterol and triglyceride levels in tilapia juveniles fed diets containing probiotics (Table 3).

The erythrocyte count revealed that the animals subjected to diets containing the probiotic demonstrated the highest concentrations of these cells ($p < 0.05$; mean of treatments of $1.56 \pm 0.2 \times 10^6 \mu\text{L}^{-1}$), compared to the control treatment ($1.31 \pm 0.02 \times 10^6 \mu\text{L}^{-1}$). Regarding the multi-strain probiotic diet (mix), the hematocrit and hemoglobin values were higher than for the other treatments ($p < 0.05$), as well as the concentrations of total plasma protein, with a mean of $5.3 \pm 0.4 \text{ g.dL}^{-1}$, differing statistically from the treatments with the inclusion of *E. faecium* (1) and *B. cereus* in the diet, and from the control group without the inclusion of probiotics.

For the differential count of thrombocytes, the animals that were subjected to diets containing probiotics, regardless of their strain and mix, exhibited higher means compared to the control group (Table 3). In the differential count of leukocytes, a statistical difference was noted for lymphocytes and neutrophils, which presented higher concentrations in

Table 3

Biochemical, hematological, hematimetric parameters, thrombocyte and leukocyte counts in the blood of juveniles of *Oreochromis niloticus* supplemented with the probiotics *Enterococcus faecium* (1) 2×10^6 CFU.g⁻¹ in the feed, *E. faecium* (2) 1×10^8 CFU.g.feed⁻¹, *Bacillus cereus* 2.8×10^6 CFU.g⁻¹ in the feed and multi-strain (mix) 1×10^8 CFU.g⁻¹, for 90 days.

Treatment	Control	<i>E. faecium</i> 1	<i>E. faecium</i> 2	<i>B. cereus</i>	Mix
Glucose (mg.dL ⁻¹)	49.2 ± 1.1A	31.0 ± 3.2B	29.7 ± 3.2B	30.50 ± 3.4B	33.0 ± 2.2B
Cholesterol (mg.dL ⁻¹)	176.1 ± 33.8A	163.7 ± 18.7AB	131.1 ± 10.1B	140.1 ± 11.1B	128.1 ± 13.1B
Triglycerides (mg.dL ⁻¹)	299.3 ± 22.1A	256.1 ± 27.3A	230.1 ± 12.0AB	196.1 ± 12.0B	200.1 ± 11.16B
Erythrocytes (x10 ⁶ µL ⁻¹)	1.311 ± 0.02B	1.723 ± 0.04A	1.201 ± 0.02A	1.612 ± 0.02A	1.721 ± 0.06A
Hematocrit (%)	23.2 ± 3.7B	29.1 ± 4.2AB	24.4 ± 1.4B	27.7 ± 7.5AB	33.5 ± 7.6A
TP (g.dL ⁻¹)	4.6 ± 0.4B	4.9 ± 0.4AB	4.6 ± 0.2B	4.6 ± 0.1B	5.3 ± 0.4A
Hemoglobin (g.dL ⁻¹)	8.7 ± 2.0C	12.0 ± 2.6AB	10.3 ± 1.6BC	10.6 ± 1.9BC	13.8 ± 1.3A
MCV (fL)	167.9 ± 7.6A	176.9 ± 7.2A	187.5 ± 4.2A	177.9 ± 4.5A	177.9 ± 4.9A
MCH (g.dL ⁻¹)	64.3 ± 6.8A	62.1 ± 8.5A	60.3 ± 7.3A	60.2 ± 6.3A	62.1 ± 8.5A
MCHC (g.dL ⁻¹)	34.6 ± 6.3A	35.0 ± 6.2A	33.3 ± 7.9A	36.5 ± 7.1A	36.5 ± 7.0A
Thrombocytes (x10 ³ µL ⁻¹)	15.4 ± 3.3B	20.6 ± 1.6A	20.6 ± 1.2A	20.6 ± 1.5A	23.5 ± 3.3A
Leucocytes (x10 ³ µL ⁻¹)	35.4 ± 24.5A	52.8 ± 27.1A	54.3 ± 18.2A	54.3 ± 17.4A	55.9 ± 23.6A
Lymphocytes (x10 ³ µL ⁻¹)	29.6 ± 4.0B	44.4 ± 7.9A	32.8 ± 3.2B	43.2 ± 6.7A	44.4 ± 7.9A
Monocytes (x10 ³ µL ⁻¹)	1.39 ± 1.2A	2.82 ± 1.3A	3.42 ± 2.5A	3.58 ± 4.7A	3.55 ± 4.7A
Neutrophils (x10 ³ µL ⁻¹)	0.53 ± 1.2B	2.96 ± 0.4A	1.08 ± 1.1AB	2.13 ± 0.8A	2.56 ± 0.2A
Basophils (x10 ³ µL ⁻¹)	0.82 ± 0.1A	0.77 ± 0.4A	0.91 ± 0.3A	0.83 ± 0.3A	0.70 ± 0.4A

TP - total plasma protein; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin, MCHC - mean corpuscular hemoglobin concentration. Different letters within the same row differ by the Tukey's test ($p < 0.05$).

the groups that contained the probiotics in the diet (Table 3).

Before the experimental infection, it was noted (*in vitro*) that there was an antagonistic capacity between the probiotics and the product mix against the pathogen *S. agalactiae*, although there was no difference ($p >$

0.05) between the strains and the mix used, which had mean inhibitory diameters of 15.7 ± 0.46 mm.

During infection with the pathogen, the animals in the negative control group that were not fed probiotics and were injected with 0.65% SSE, demonstrated a 100% survival rate, ensuring that the design occurred safely, without interference from management and external factors during the survey period.

The fish from the positive control groups, injected with the pathogen, showed marked clinical signs after a 16 h period of infection, with a dark color of the epidermis and worsening symptoms during the experiment. After 22 h of infection, erratic swimming and exophthalmia were observed, increasing in severity from 29 h of infection. The appearance of hemorrhagic petechiae in the epidermis, lethargy, gill pallor, erosion of the fins and ulcerations on the back occurred at 43 h of infection. The maximum mortality rate reached a record 83%, as depicted in Fig. 1.

The tilapia that received the diet with the probiotic strains showed greater survival and resistance to the acute infection process with *S. agalactiae*. During the experimental period there was no mortality in the fish fed with rations containing *E. faecium* (1), *B. cereus*, and the treatment with the multi-strain (mix) (Fig. 1), even after 16 h of infection when the fish exhibited clinical symptoms such as dark coloration of the epidermis. However, this picture was reversed, with the animals recovering and returning to the normal color within 28 h after infection.

As far as the diet that contained the inclusion of the probiotic *E. faecium* (2) at intervals of 58 and 72 h, mortality rates of 33% were recorded by the end of the experiment (Fig. 1).

The animals with the treatment of *E. faecium* (2) and in the positive control group had the highest clinical records of infection by *S. agalactiae* of 15.38% and 92.30%, respectively. They obtained a pathological classification of grade 3 and 5, respectively.

For the hematological parameters during and after the infection, the animals that received the probiotics *E. faecium*, *B. cereus* and the multi-strain (mix) in the ration, presented the highest values of glycemia and total plasmatic proteins. The lowest values for erythrocytes, hemoglobin, hematocrit and MCV were observed in the infected fish from the positive control (Table 4).

At the end of the period of infection with *S. agalactiae*, there was a change in the number of thrombocytes in the positive control; it was being lower in relation to the other treatments, with the number of these cells in the probiotic treatments maintaining a similar value to that in the negative control. There was an increase in the numbers of monocytes and neutrophils in fish from the groups supplemented with the probiotic strains *E. faecium* (1), *B. cereus* and the multi-strain mix in the diet of tilapia juveniles (Table 4).

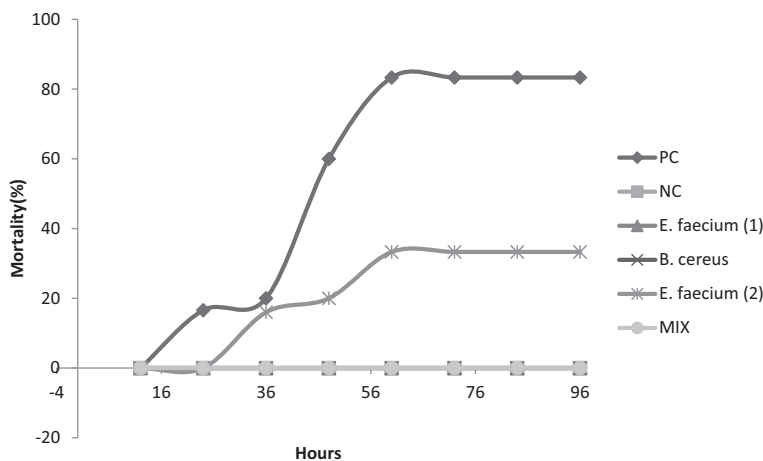


Fig. 1. Accumulated mortality up to 96 h after infection with *Streptococcus agalactiae* in tilapia juveniles (*Oreochromis niloticus*) fed for 90 days with diets supplemented with the probiotics *Enterococcus faecium* (1) 2×10^6 CFU.g⁻¹ in the feed, *E. faecium* (2) 1×10^8 CFU.g.feed⁻¹ in the feed, *Bacillus cereus* 2.8×10^6 CFU.g⁻¹ in the feed and multi-strain (mix) 1×10^8 CFU.g⁻¹. Dunn's test ($p < 0.05$) for every 12 h of observation. PC – Positive Control; NC - Negative Control.

Table 4

Biochemical, hematological, hematimetric parameters, thrombocytes and leukocyte counts in the blood of juveniles of *Oreochromis niloticus* infected with *Streptococcus agalactiae* after supplementation with the probiotics *Enterococcus faecium* (1) 2×10^6 CFU.g⁻¹, *E. faecium* (2) 1×10^8 CFU.g⁻¹, *Bacillus cereus* 2.8×10^6 CFU.g⁻¹ and multi-strain (mix) 1×10^8 CFU.g⁻¹, for 90 days.

Treatment	NC	PC	<i>E. faecium</i> 1	<i>E. faecium</i> 2	<i>B. cereus</i>	Mix
Glucose (mg.dL ⁻¹)	28.4 ± 1.1B	LoD	32.1 ± 2.1AB	31.92 ± 2.61AB	29.56 ± 1.9B	35.48 ± 1.1A
Erythrocytes (x10 ⁶ μL ⁻¹)	1.82 ± 0.02A	1.20 ± 0.06C	1.43 ± 0.11B	1.422 ± 0.12B	1.362 ± 0.12B	1.621 ± 0.26AB
Hematocrit (%)	35.1 ± 5.1A	15.31 ± 1.11B	33.9 ± 2.1A	33.14 ± 2.91A	32.40 ± 6.1A	34.11 ± 4.1A
TP (g.dL ⁻¹)	5.1 ± 0.32A	3.1 ± 0.63C	4.32 ± 0.09B	4.13 ± 0.12B	4.11 ± 0.10B	4.97 ± 0.4AB
Hemoglobin (g.dL ⁻¹)	7.6 ± 1.4A	3.2 ± 1.0B	6.4 ± 1.1A	5.81 ± 1.44A	6.66 ± 1.99A	6.95 ± 1.2A
MCV (fL)	192.8 ± 5.5D	122.2 ± 8.4C	237.2 ± 4.2A	234.4 ± 6.2AB	234.5 ± 6.4A	217.3 ± 7.9B
MCH (g.dL ⁻¹)	34.3 ± 3.1A	31.3 ± 2.8A	34.3 ± 2.9A	34.27 ± 3.21A	33.17 ± 4.2A	34.51 ± 4.1A
MCHC (g.dL ⁻¹)	24.6 ± 1.3A	23.3 ± 1.2A	24.1 ± 1.5A	23.85 ± 1.7A	24.41 ± 1.8A	24.54 ± 1.1A
Thrombocytes (x10 ³ μL ⁻¹)	11.2 ± 2.1A	5.5 ± 1.6B	10.9 ± 1.3A	10.7 ± 1.8A	10.8 ± 1.5A	11.2 ± 1.3A
Leucocytes (x10 ³ μL ⁻¹)	97.7 ± 14.2A	90.4 ± 11.5A	103.3 ± 10.4A	102.4 ± 12.1A	101.3 ± 17.8A	103.4 ± 13.9A
Lymphocytes (x10 ³ μL ⁻¹)	81.3 ± 2.0A	80.2 ± 1.9A	81.5 ± 1.7A	80.8 ± 1.8A	81.7 ± 1.5A	81.6 ± 1.9A
Monocytes (x10 ³ μL ⁻¹)	11.3 ± 1.4B	7.1 ± 1.1C	14.7 ± 1.1A	13.4 ± 1.7A	12.5 ± 1.8AB	14.9 ± 1.2A
Neutrophils (x10 ³ μL ⁻¹)	4.4 ± 0.4B	2.6 ± 0.6C	8.0 ± 0.6A	8.1 ± 0.8A	8.2 ± 1.1A	8.5 ± 0.3A

NC - Negative control; PC - Positive control; LD - Lower than detection limit; TP - total plasma protein; MCV - mean corpuscular volume; MCH - mean corpuscular hemoglobin and MCHC - mean corpuscular hemoglobin concentration. Different letters within the same row differ by the Tukey's test ($p < 0.05$).

In the bacteriological evaluation, the inclusion of probiotic bacteria in the diets was effective, with means of approximately $1.7 \pm 0.09 \times 10^6$, $1.2 \pm 0.13 \times 10^8$, $2.1 \pm 0.45 \times 10^6$ and $1.2 \pm 0.93 \times 10^8$ CFU.g⁻¹ for the strains of *E. faecium* (1 and 2), *B. cereus* and the mix of strains, respectively. The identification of colonies by the MALDI-TOF method was performed with a score greater than 2.0 at the level of genus and species, thus ensuring the levels stipulated in the design for up to seven days of storage under refrigeration at 4 °C. In the control diet, there was no presence of probiotic bacteria.

At the end of the 90 day period, it was possible to confirm the colonization of probiotic bacteria in the intestine of the animals. The colonies were identified at the genus and species level by the MALD-TOF method, and their concentrations were counted in plates at $1.2 \pm 0.10 \times 10^6$, $1.1 \pm 0.26 \times 10^6$, $1.0 \pm 0.15 \times 10^5$ and $1.2 \pm 0.93 \times 10^7$ CFU.g⁻¹, for diets with the inclusion of *E. faecium* (1 and 2), *B. cereus* and the mix of strains, respectively. For the treatment with the mix of strains 40% *E. faecium* and 60% *B. cereus* were identified.

For the group of pathogenic bacteria, the species of *Aeromonas jandaei*, *Pseudomonas aeruginosa*, *Vibrio* spp. and *Edwardsiella tarda* were identified. The microbiological diversity was influenced by the inclusion of probiotic bacteria in the feed. For the treatment with the inclusion of *E. faecium* (1), the occurrence of the bacteria *A. jandaei* $1.1 \pm 0.12 \times 10^3$ CFU.g⁻¹ was identified in the intestine, and in the *E. faecium* group (2) *A. jandaei* $2.1 \pm 0.03 \times 10^4$ CFU.g⁻¹ and *E. tarda* $1.2 \pm 0.32 \times 10^3$ CFU.g⁻¹ were found. Regarding the treatment with the probiotic *B. cereus*, the occurrence of *P. aeruginosa* $2.2 \pm 0.08 \times 10^3$ CFU.g⁻¹ was identified. Similarly, *P. aeruginosa* and *Vibrio* spp. $1.4 \pm 0.21 \times 10^6$ and $2.1 \pm 0.10 \times 10^5$ CFU.g⁻¹, respectively, were present in the control group. It is noteworthy that there was no growth of any potentially pathogenic bacteria in the intestinal fauna of the animals treated with the inclusion of the probiotic multi-strain (mix).

Lastly, for infection with the pathogen, Koch's postulate was confirmed, identifying the occurrence of *S. agalactiae* in the blood, intraperitoneal cavity and liver of animals dying and dead during the experimental period. The pathogenic re-isolation of the strains was confirmed by the MALDI-TOF method.

4. Discussion

The water quality parameters remained within the ideal range for the growth of tilapia juveniles (Kubitza, 2003; Ferreira et al., 2015), thus, they were not a limiting factor for growth or did not interfere with the results obtained. Therefore, these responses ensure that the mortality rates observed throughout the experiment were due to the infection caused by *S. agalactiae*.

For the concentration obtained in the formulation of the probiotic

multi-strain mix, the 1×10^8 CFU product corresponds to the concentration for prophylactic use, which is similar to the dosages used by Amir et al. (2018) and Ghori et al. (2018), with the inclusion of *E. faecium* and *B. cereus*, respectively, in the formulas of the probiotic mix in the production of *Labeo rohita*.

There was a growth of probiotics in the diet with the established experimental microbiological densities, which indicates an adequate manufacture diet process, with no contamination between treatments. Also, its microbiological density resisted the digestive processes of the host, which enabled its colonization in the intestinal microbiota of confined animals, as well as those obtained by Merrifield et al. (2010) and Dias et al. (2018).

As far as intestinal colonization, all strains were able to act in the animal's intestine, and at the end of 90 days it was possible to recover them from re-isolation, showing resistance, and modulation of gastrointestinal microbiota (Gatesoupe, 2008).

The treatments with *E. faecium* (1) and the mix, resulted in the smallest loss of bacteriological logarithmic density. This responses would be related to the strain of *E. faecium* (1) being isolated from the Cichlidae family, which includes the species *O. niloticus*. The smallest loss in the multi-strain mix group may be due to a synergistic effect in relation to a positive quorum sensing in the set of varieties used in addition to the inclusion of the variety of *E. faecium* (1) (Pompei et al., 2008; Dias et al., 2019).

The influence of probiotic strains on the concentration of potentially pathogenic bacteria observed in this work is a promising feature of the probiotic effect on the host, which is related to the competitive exclusion of these bacteria from the production of antimicrobials such as organic acids, bacteriocins, hydrogen peroxide and acetic acid (Miller and Bassler, 2001; Vieira et al., 2013).

Furthermore, bactericidal enzymes secreted mainly by the group of probiotic bacteria *Bacillus*, act against the invasion of pathogenic bacteria from the lysis of the cytoderm and hydrolysis of the chemical reactions between *N*-acetylglucosamine and *N*-acetylmuramic acid present in the glycan chain of bacteria leading to cell death of invading and native pathogenic microorganisms (Alexander and Ingram, 1992). *E. faecium*, which are part of the lactic acid bacteria group, also use enterocin P, A and B as their main bacteriocins as a strategy against infectious agents (Casaus et al., 1997). These facts may have contributed to the inhibition of growth of pathogenic bacteria in the treatment with the probiotic multi-strain (mix), after 90 days of supplementation.

These results are of great relevance because the pathogens *A. jandaei*, *P. aeruginosa*, *Vibrio* and *E. tarda* are found in the animal's intestine and an imbalance between environment-host-pathogen could promote one of the main pathways infection in fish. The control of these microorganisms is essential to prevent bacteriosis in the productive system (He

et al., 2009; Li and Cai, 2011). Studies indicate that the groups of *Bacillus* and *Enterococcus* naturally compose the intestinal microbiota of Nile tilapia (Del'Duca et al., 2013; Standen et al., 2016), and the dietary supplementation with the mixture of these bacteria would favor their bacterial density and also a synergistic probiotic effect, as the colonization of these strains in the animal was facilitated by their inclusion in the food.

The positive effect on the productive performance of fish in the treatments with probiotics may be related to the use of ingredients contained in the diet in addition to the production of short-chain fatty acids, which in the intestine act as a source of basic energy for epithelial cell growth, alter intracellular pH and lower the cell mortality rate during digestive metabolism (Doan et al., 2018).

Results that corroborate our findings were observed by Adeoye et al. (2016) and Liu et al. (2017), who worked with tilapia fed for 8 weeks, and obtained results of productive performance and feed efficiency that were optimized with the use of probiotics in the ration, resulting in greater length and weight, compared to a control group free of probiotics.

Additionally, the use of multi-strain probiotics favored the productive performance of confined animals, which corroborates the findings by Tanekhy et al. (2016), who observed the highest growth rates with the inclusion of a set of multi-strain (*Lactobacillus* sp., *Pediococcus* sp., *Gluconacetobacter* sp., and *Saccharomyces* sp.) compared to commercial probiotic based on *Saccharomyces cerevisiae*. These improvements in performance responses were attributed by the authors to the higher lysozyme rates of treatments that contained the mix of probiotic strains in the feed, which were able to colonize the host intestine and act synergistically from a positive quorum sensing.

Sutthi et al. (2018) and Ayyat et al. (2014) also worked with products from monostrains (*S. cerevisiae* and *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Bifidobacterium bifidum*, respectively) and multi-strain isolated from aquatic organisms (*B. subtilis*, *Bacillus megaterium*, and *Bacillus licheniformis* 1×10^6 CFU.g⁻¹ in the ration and *L. acidophilus*, *S. thermophilus*, *Bif. bifidum* and *S. cerevisiae* 3.2×10^7 CFU.g⁻¹, respectively). The animals that received the probiotic mix in the diet exhibited a greater weight gain compared to the control group. Their results corroborate those obtained in this study. The combination of strains isolated from aquatic organisms from the same habitat, can offer possible complementary, symbiotic, and additive effects on the host's intestine (Douillard et al., 2018).

Mohapatra et al. (2012) used a mix of *B. subtilis*, *L. lactis* and *S. cerevisiae*, at a concentration of 1×10^{11} CFU.kg⁻¹, and Khunrang et al. (2021) used a mix of *Lactobacillus* sp. and *S. cerevisiae* with a hyperconcentration of 7.7×10^{14} CFU.kg⁻¹, and they reported higher ($p < 0.05$) growth performance compared to control group. In contrast, the concentrations used are very high compared to the present work. These high concentrations of microorganisms and the lack of information on the minimum prophylactic concentration used can increase production costs.

Furthermore, *in vitro* studies of antagonism between the probiotic strains used in the formulation of the multi-strain treatment were not reported in previous studies. In contrast, in the current research, the antagonistic potential between all strains evaluated were considered, which allowed the use of a lower concentration than the ideal concentration recommended and possibly enabled a synergistic effect of the bacteria without competition between themselves. Besides enhancing the synergistic effect between probiotics, this lower concentration can reduce time and cost for the large-scale production of the probiotic mix.

At the end of 90 days, there were general improvements in the hematological parameters due to the feeding of tilapia containing probiotics in the feed. Even under the stressful conditions of confinement of a productive system, the glucose values for treatments with probiotics in the diet remained within normal levels for the species (Moreira et al., 2011), however the higher glucose levels in the control group ($p < 0.05$) indicate a possible response to the stressful situation of confinement

(Carneiro and Urbinati, 2001).

Probiotics can reduce cholesterol levels in the animal, promoting reduction of LDL concentrations, which provides a relevant nutritional status to the animals. However, this effect on the triglyceride and cholesterol parameters depends on the strains used and the feeding period (Holzapfel and Schillinger, 2002; Ziaei-nejad et al., 2021). This beneficial effect may have occurred in tilapia fed only with *E. faecium* (1 and 2) and *B. cereus* and for their multi-strain mixture (mix), especially when the lowest values of cholesterol are observed, when compared to the control units.

The highest concentrations for the total plasma protein in treatments containing *E. faecium* (1) and the multi-strain mix in the diet are related to the greater activity of plasma fluids and humoral defense, caused by the transport of nutrients and the blood osmotic balance in these treatments (Thomas, 2000; Satake et al., 2009). Results that corroborate those results were described by Abdel-Tawwab et al. (2008) with tilapia fed with *S. cerevisiae*.

The higher concentrations of erythrocytes, hematocrit, and hemoglobin for the treatments with *E. faecium* (1) and the multi-strain mix in the diet resulted in a greater transport of oxygen and nutrients to the host tissues (Tavares-Dias et al., 2002; Aly et al., 2008; Dawood et al., 2015), which is reflected in the excellent clinical condition of the confined animals.

As with the erythrogram, there were significant improvements ($p < 0.05$) in the differential count of defense cells, especially in thrombocytes, lymphocytes and neutrophils in all treatments that included probiotics in the diet, regardless of strains and mix. This may indicate a significant stimulation of immune responses, mainly due to the mode of action of these cells in the body during the processes of coagulation, phagocytosis, and migration to infectious sites (Tavares-Dias and Moraes, 2006; Nakandakare et al., 2018), in addition to the promotion of greater adaptation to the stress of confinement (Nayak, 2010).

In vitro analyses against *S. agalactiae* demonstrated the ability of probiotic strains to inhibit the action of the pathogen. This antagonistic effect of the probiotic strains and the mix formulation is probably related to the production of antibacterial compounds, including lactic acid and bacteriocins. The latter is considered the most important inhibitor for Gram-positive pathogenic species (Gillor et al., 2008).

In the acute infection of the evaluated animals, the survival responses and degree of pathological severity found in the positive control of the present study are equivalent to those obtained by Tanekhy et al. (2016). Considering the acute infection with *A. hydrophila* in tilapia, the best responses of pathogen resistance were observed in treatments that contained the probiotic multi-strain (*Lactobacillus* sp., *Pediococcus* sp., *Gluconacetobacter* sp., and *Saccharomyces* sp.) similar to the results in this investigation. The authors highlighted that the effect of the mix of probiotics improved the resistance to the pathogen when compared to the isolated use of *S. cerevisiae*, which had a lower performance because the strain was not isolated from aquatic animals.

The mix of probiotic strains in this study did not differ in relation to the effects of the isolated strains, this may be because all strains were isolated from aquatic organisms, demonstrating the importance of the isolation and proper selection of bacteria for fish farming.

The tilapia that received the inclusion of the probiotics *E. faecium* (1), *B. cereus*, and its mix in the feed did not present severe clinical signs, only dark pigmentation in the epidermis, after infection by *S. agalactiae*. In addition, there was no record of mortality in these treatments, a scenario that reflected an improvement in the immune system, especially cellular immunity with an increase in neutrophils and monocytes. This fact corroborates the immunostimulating potential of these strains reported in other species (Balcázar et al., 2006; Jatobá et al., 2018).

These improvements in cellular responses may be due to the effectiveness of proteins, such as toll-like receptors (TLRs), in which the cell wall of probiotic bacteria can activate the production of TLR2, which is responsible for identifying pathogenic agents in the body of these animals, especially when related to bacterial infections. This may have

altered the expression of inflammatory cytokines (NF κ B) (Lee et al., 2001; Kim and Austin, 2006; Wells, 2011). Furthermore, they can produce hydrolytic enzymes (chitinase, proteases, cellulases and β -1,3-glucanase), which have antimicrobial properties that degrade cell wall components of pathogenic microorganisms (Urdaci and Pinchuk, 2004).

Probiotic strains of *E. faecium* and the *Bacillus* genus also enhance the intestinal immunity of their host through the higher concentration of pro-inflammatory cytokines (TNF α and IL β) as already reported in tilapia (Standen et al., 2016). Moreover, they can act on pattern recognition receptors (PRRs), expressed in macrophages, such as dectin-1 and β -glucan, activating the transduction of immune system molecules, as observed by Hoseinifar et al. (2018) and Brown et al. (2002).

In treatments that received the inclusion of probiotics in the diet, regardless of the strain and administration strategy, the highest values of total plasma protein are related to the physiological control of globulin and albumin fractions, which contribute to the great sanitary and nutritional status of the animal (Thomas, 2000). During the inflammatory process caused by *S. agalactiae* the reduction in the concentrations of total plasma proteins, resulting from the increase in vascular permeability is common, thus causing its effusion (Paixão et al., 2017).

The stress caused by the infection with the increase in energy demand was minimized by adding probiotics to the feed. The increase in hematological parameters of glucose, hematocrit, erythrocytes, and hemoglobin in treatments that contained probiotics in the diet, indicate an improvement in the oxygen transport by red blood cells in addition to glycogen metabolism, which increases blood glucose levels and promotes greater availability of energy during the infectious process. This, consequently controls the stressful action of the pathogen (Moreira et al., 2015).

These facts are contrary to those observed in the positive control group, where the animals presented hemolytic conditions, due to the production of degradative proteases for the tissues and erythrocytes of the host, thus causing hemorrhagic conditions in the animals and changes in the hematimetric parameters, as observed for the volume corpuscular media (Garcia et al., 2009).

Considering that streptococcosis is one of the main diseases that reduce productive performance and survival in tilapia productions in the world (Pretto-Giordano et al., 2010), this research brings positive health perspectives for the sustainable production of the species, which are similar to those found by Wing-Keong et al. (2014) and Shelby et al. (2006). They demonstrated the isolated use of *B. subtilis* and *E. faecium* in feeding tilapia to control *S. agalactiae* and *Streptococcus iniae*. Nonetheless, these authors questioned the use of probiotic multi-strain of *B. subtilis* and *B. licheniformis* or *Bacillus* spp. and *Pediococcus* spp. against infection with *S. agalactiae*. This reported ineffectiveness of the multi-strain probiotic could be related to the origin of the probiotic strains (commercial, not isolated from aquatic organisms), as well as the non synergistic properties of these bacteria, which could have competed with each other for space and nutrients in the host organism, thus preventing its beneficial probiotic effects. This fact demonstrates the importance of tests using different multi-strains (mix), which allied the specialties from each probiotic variety (Wing-Keong et al., 2014).

Therefore, maintaining the health and well-being of animals through feeding with immunostimulants and probiotics is essential for sustainable fish farming because the use of these diets during the stages of productive performance of the species and the breeding system can improve the nutritional status of the animal and influence its physiology, immune system, and resistance to disease (Kiron, 2012; Chen et al., 2019).

5. Conclusion

The inclusion of the probiotics *E. faecium* (1 and 2), *B. cereus* and its multi-strain mix favored the productive, hematological, survival and rehabilitation parameters against infection with *S. agalactiae* in Nile tilapia juveniles after 90 days of feeding. In addition, their synergy when

used together (mix) was able to inhibit the growth of potentially pathogenic bacteria in the intestine after 90 days of supplementation, which makes this a promising feeding method for the species during the growth phase.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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