

# EFFECTS OF BAC-TRAT® PROBIOTIC COMPLEX ON GROWTH, HEMATOLOGICAL AND INTESTINAL PARAMETERS OF NILE TILAPIA, REARED AT LOW TEMPERATURES\*

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## ABSTRACT

The aim of this study was to evaluate the effects of the BAC-TRAT® probiotic complex applied on diet and in the rearing water of Nile tilapia, maintained at low temperatures. Three treatments were evaluated: control (absence of the probiotic: T0), probiotic applied to the water (T1) and a diet with probiotic (T2). Productive performance of Nile tilapia was not affected by the probiotic inclusion ( $p>0.05$ ). The hematological parameters were influenced by treatments, with leukocytes and lymphocytes presenting lower concentrations in T2. Regarding to the whole-fish body composition, the inclusion of the probiotic in the water led to significant increases in the ether extract concentration, whilst the ash contents was higher in the treatments T1 and T2. Regarding histomorphometry parameters of the intestine, was observed that villi height and thickness of the muscle tunic were higher in T1 in comparison to T0. For the hepatic tissue, T2 determined the lowest values of perimeter, area and volume, and the qualitative evaluation revealed little and intermediate vacuolization in all treatments. It was concluded that despite contributing for the increase of intestinal villi area, the use of BAC-TRAT® probiotic complex at low temperatures does not have beneficial effects on Nile tilapia growth.

**Keywords:** productive performance; leukocytes; histology; morphology; vacuolization

## EFEITOS DO COMPLEXO PROBIÓTICO BAC-TRAT® SOBRE O CRESCIMENTO, PARÂMETROS HEMATOLÓGICOS E INTESTINAIS DA TILÁPIA DO NILO, MANTIDA A BAIXAS TEMPERATURAS

## RESUMO

O objetivo desse trabalho foi avaliar os efeitos do complexo probiótico BAC-TRAT® aplicado na ração e na água de criação de tilápias-do-Nilo, mantidas em condições de baixa temperatura. Foram avaliados três tratamentos: controle (ausência de probiótico: T0), adição de probiótico na água (T1) e ração com probiótico (T2). O desempenho zootécnico da tilápias-do-Nilo não foi influenciado pela inclusão do probiótico ( $p>0,05$ ). Os parâmetros hematológicos foram influenciados pelos tratamentos, com leucócitos e linfócitos apresentando menores concentrações no T2. Para composição corporal do peixe inteiro, a aplicação do probiótico na água determinou aumentos significativos nos teores de extrato etéreo, enquanto o conteúdo de cinzas foi superior nos peixes dos tratamentos T1 e T2. Em relação aos parâmetros histomorfométricos do intestino, observou-se que a altura dos vilos e a espessura da túnica muscular foram maiores no T1 em comparação ao T0. Para o tecido hepático, T2 determinou os menores valores para perímetro, área e volume, e a avaliação qualitativa demonstrou pouca e média vacuolização para todos os tratamentos. Conclui-se que, apesar de contribuir para o aumento da área dos vilos intestinais, o uso do complexo probiótico BAC-TRAT® em baixas temperaturas não apresenta efeitos benéficos ao crescimento da tilápias-do-Nilo.

**Palavras-chave:** desempenho; leucócitos; histologia; morfologia; vacuolização

## INTRODUCTION

As fish farming activities increased, diseases began to emerge (Khati et al., 2018), resulting in economic losses (Jahangiri and Esteban, 2018). Then, antibiotics were widely used in the attempt of controlling bacterial diseases, but its misuse caused a

thoroughly selection of more resistant microorganisms, leading to an imbalance in the environment (Jatobá et al., 2008; Amarante et al., 2018). For this reason, antibiotic use was reduced (Elsabagh et al., 2018), with a share of these products being replaced by additives such as probiotics, to which the potential of reducing diseases is attributed, as well as to the reduction of pathogenic bacteria and to the stimulation of the animals' immunological system (Wang et al., 2017), besides improving digestion (Verschuere et al., 2000; Ibrahim, 2015).

The application of probiotics in aquaculture has been increasingly practiced by fish farmers worldwide, including in Brazil, where several products are already found in the market containing different strains of Gram-positive bacteria, like species of the genus *Bacillus* and *Lactococcus*, with great potential for fish nutrition and nutrients cycling in ponds (Ibrahim, 2015; Vieira and Pereira, 2016).

Probiotics administration in aquaculture is performed through feeding, either by incorporating the product in the diets or by adding it directly to the cultivation water (Jahangiri and Esteban, 2018). In both forms of application, positive effects on animal's growth are reported, as well as increasing tolerance to stress and improving their immunity, which is considered essential to the animals' welfare (Nakandakare et al., 2013; Noffs et al., 2015; Wang et al., 2017).

In addition, several probiotic products are found containing different bacterial species, which have been gaining attention, e.g. species of the genus *Bacillus* and *Lactococcus*. These products are important for achieving good productive performances, for producing high quality food, and to strengthen the immunity of Nile tilapia (Round and Mazmanian, 2009; Adeoye et al., 2016; Elsabagh et al., 2018).

Some widely used species in aquaculture are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus* and *Lactococcus lactis*, which have an important nutritional role in increasing the activity of digestive enzymes, causing beneficial effects to the host as better immune and stress responses, in addition to improving water quality (Ibrahim, 2015; Banerjee and Ray, 2017; Elsabagh et al., 2018).

Southern Brazil is a region of low temperatures and oscillations, especially during winter, however, low temperatures are of concern for tilapia farming, as such oscillations cause fish to stop eating and becoming vulnerable to diseases (Rebouças et al., 2014). Given this scenario, probiotics are used by a few fish farmers during winter, aiming to improve productive performance and the animals' welfare. However, the indicated doses and method of application are still factors that require investigation, in order to obtain better zootechnical results.

Besides displaying lower growth at suboptimal temperatures, the Nile tilapia (*Oreochromis niloticus*) is more vulnerable to disease due to low immunity triggered by stress responses and by low temperature (Mazeaud et al., 1977; Sardella et al., 2004). As a consequence, increasing mortality rates are registered throughout periods of critical temperatures. For tilapia, severe mortalities may occur at 12°C in conditions of longer exposure, while feeding is severely reduced below 20°C (Balarin, 1982;

Chervinski, 1982; El-Sayed, 2006). According to Signor et al. (2010), these conditions may lead to osmoregulation disturbances and physiological changes that alter the animal's hematological profile. Therefore, studies evaluating the effects of probiotics on animal health during periods of exposure to low temperatures become relevant, as they may provide important information regarding fish handling and preparation of the animal to face such adverse conditions.

Due to the aforementioned issues associated with the exposure to temperatures below the comfort, the aim of this study was to evaluate the effects of probiotic use, regarding two methods of application (in the water and in the diet), on productive performance, hematological, intestinal and hepatic alterations of Nile tilapia, maintained at low temperatures.

## MATERIAL AND METHODS

### Experimental procedure

A total of 108 Nile tilapia juveniles ( $8.4 \pm 0.7$  g and  $8.09 \pm 0.5$  cm length) were randomly distributed in 18 tanks (18 L), which received six fish each. These experimental units were supported with aeration and constant water renewal. The experiment was performed in the Laboratory of Aquaculture of the State University of West Paraná (UNIOESTE), campus Toledo, during the season of low temperatures in this region (July - August 2018). The study was approved by the Ethics Committee on Animal Use (CEUA) of UNIOESTE, according to the protocol 65-17/2018-CEUA.

The diet used in the experiment consisted of defatted corn bran, milled whole corn, soy bran, wheat bran, meat and bone meal, calcitic lime, sodium chloride (common salt), methionine, lysine, antifungal, and a premix of vitamins and minerals. The guarantee levels of the commercial feed are presented in Table 1.

The BAC-TRAT® probiotic complex (lyophilized probiotic) is a blend of different bacteria (*Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. cereus* and *Lactococcus lactis*), with a total concentration of  $1.25 \times 10^8$  CFU g<sup>-1</sup>, according with the manufacturer, which was mixed in 2% of soybean oil, referring to the diet's weight, and manually added in the commercial feed, followed by a 10 min mix until complete homogenization, prior to the beginning of the experiment. The amount of probiotic added in the diet (g kg<sup>-1</sup> ration) was established according to the manufacturer recommendation, by means of the calculation: quantity of feed (kg) x factor 0.17.

Fish were fed three times a day until apparent satiety, with an extruded commercial diet (pellet 2.0 mm and 35% crude protein) during five days of acclimatization and 40 days of experiment. The experimental design was completely randomized composed by three treatments and six replicates: a control without the addition of probiotic (T0), one containing probiotic in the water (T1) and one containing the product in the feed (T2). On a daily basis, 10 minutes before each meal, the water flow of the system was turned off and 1.0 g of the BAC-TRAT® probiotic complex was added in the tanks of the group T1. During feeding, the diet was offered for all groups of animals until apparent satiety, when the

**Table 1.** Guarantee levels presented on the label of the commercial feed used in the experiment, offered for Nile tilapia juveniles.

Composition	Guarantee Levels
Moisture (g kg <sup>-1</sup> )	120.0
Crude protein (g kg <sup>-1</sup> )	350.0
Ether extract (g kg <sup>-1</sup> )	50.0
Fibers (g kg <sup>-1</sup> )	50.0
Ash (g kg <sup>-1</sup> )	80.0
*Crude energy (kcal kg <sup>-1</sup> )	4,127.5
Calcium (minimum) (g kg <sup>-1</sup> )	10.5
Calcium (maximum) (g kg <sup>-1</sup> )	15.0
Phosphorus (mg kg <sup>-1</sup> )	5,000.0
Vitamin C (mg kg <sup>-1</sup> )	450.0
Vitamin A (UI kg <sup>-1</sup> )	8,000.0
Vitamin D3 (UI kg <sup>-1</sup> )	2,100.0
Vitamin E (UI kg <sup>-1</sup> )	100.0
Vitamin K3 (mg kg <sup>-1</sup> )	3.0
Vitamin B1 (mg kg <sup>-1</sup> )	2.0
Vitamin B2 (mg kg <sup>-1</sup> )	4.0
Vitamin B6 (mg kg <sup>-1</sup> )	6.0
Vitamin B12 (mg kg <sup>-1</sup> )	10.0
Biotin (mg kg <sup>-1</sup> )	0.5
Antioxidant (mg kg <sup>-1</sup> )	120.0
Nicotinic acid (mg kg <sup>-1</sup> )	30.0
Pantothenic acid (mg kg <sup>-1</sup> )	10.0
Folic acid (mg kg <sup>-1</sup> )	0.5
Iron (mg kg <sup>-1</sup> )	40.0
Copper (mg kg <sup>-1</sup> )	8.0
Manganese (mg kg <sup>-1</sup> )	70.0
Zinc (mg kg <sup>-1</sup> )	50.0
Iodine (mg kg <sup>-1</sup> )	1.2
Selenium (mg kg <sup>-1</sup> )	0.12
Choline (mg kg <sup>-1</sup> )	500.0

**Source:** Kowalski: fish feed. \*Crude energy calculated according to the value of combustion heat of 9.44, 4.11 and 5.64 kcal g<sup>-1</sup> for lipids, carbohydrates and proteins, respectively (Blaxter, 1989).

water flow was turned on once again. Different treatments were equipped with independent recirculation systems.

Water temperature (°C) inside the experimental tanks was daily measured, twice a day in the morning and afternoon, whilst other quality parameters such as pH, dissolved oxygen (mg L<sup>-1</sup>), electrical conductivity (µS cm<sup>-1</sup>) and total dissolved solids (mg L<sup>-1</sup>) were daily measured in the afternoon, with the aid of a multiparameter probe (Hanna HI98196), prior to cleaning the tanks by siphoning.

At the beginning and end of the experiment, biometrics were performed in order to obtain the individual weight and length of the fish, which were used to assess the zootechnical performance. The following indexes were calculated: survival [SV (%)] = (final number of fish/initial number of fish) x 100;

weight gain [WG (g)] = final weight (g) - initial weight (g); apparent feed conversion (AFC) = (feed consumption (g)/weight gain (g)); feed efficiency (FE) = [weight gain (g)/feed consumption (g)]/100; specific growth rate [SGR (% dia<sup>-1</sup>)] = [(ln final weight - ln initial weight)/time of experiment (days)] x 100; viscerosomatic index [VSI (%)] = [(weight of the viscera (g)/body weight (g)) x 100]; hepatosomatic index [HIS (%)] = [(weight of the liver (g)/body weight (g)) x 100]; and visceral fat [VIF (%)] = [(weight of the fat (g)/body weight (g)) x 100].

### Sampling of biological material

At the end of the experiment, one fish from each tank was anesthetized with a eugenol solution (100 mg L<sup>-1</sup>), for the collection of blood, using syringes of 1.0 mL containing the anticoagulant EDTA (3.0%). Blood collected by puncture of the caudal vessel was stored in individual Eppendorf tubes and two cytological slides per fish were used for blood smear for further analysis. Total length and total weight of each fish were acquired using a measuring tape (cm) and a precision scale (0.001 g), then the intestines, liver and visceral fat were sampled and disposed in Petri dishes in order to obtain the desired data for the evaluation of somatic indexes. Both intestines and liver were preserved in Alfac for 24h according to Caputo et al. (2010) and then transferred to a 70% alcohol solution for further histological processing.

### Proximate analysis

Regarding body composition, 23 fish were sampled at the beginning of the experiment, and one fish from each tank was euthanized at the end of the experiment. Before analysis, a pre-drying of the samples was performed at 55 °C for 72h in an oven with forced ventilation. Subsequently, the whole-fish body samples were milled and the following parameters were evaluated: moisture (%), ether extract (%), crude protein (%) and ash content (%), according to AOAC (2012). For carbohydrate (%) determination, the methodology proposed by Silva and Queiroz (2002) was employed.

### Hematological analyses

Erythrocyte counts were performed in Neubauer chambers by the hemocytometer method, using Hayem liquid (1:200) in an automatic pipette, with the obtained values (cells µL<sup>-1</sup>) being employed in the calculation of the total number of red blood cells (Ranzani-Paiva et al., 2013).

Regarding the differential and total leukocytes count, two slides were prepared per fish in each treatment. Blood smears were made and subsequently stained with May-Grünwald Giemsa. Counting was performed with the aid of a light microscope using 100x lens, running the entire slide in a “zig-zag” motion as suggested by Tavares-Dias and Moraes (2004). In order to determine the differential count, 200 cells were counted, thus establishing the percentage of lymphocytes, neutrophils and monocytes. As for total counts (cells µL<sup>-1</sup>), 2000 cells were considered by observing the number of leukocytes. Posteriorly, a biochemical analysis was performed to evaluate plasma glucose (mg dL<sup>-1</sup>). For this

purpose, samples were centrifuged at 2500 rpm for 5 min and then were conserved in a freezer (-5 °C) for posterior analysis, with the aid of a “glucose kit” (Gold Analisa Diagnóstica Ltda, Belo Horizonte - MG, Brazil), by means of the colorimetric-enzyme method with the reading being performed in a spectrophotometer (Femto, 600-Plus) at 505 nm, according to the manufacturer recommendations.

## Histological analyses

Both the intestines and livers were prepared in the Laboratory of Histology of UNIOESTE/Campus Toledo. Samples of the midgut and liver were collected from each experimental unit. These were serially dehydrated in alcohol solutions (70%, 80%, 90%, 100% I, 100% II, and 100% III), diaphanized in xylol and placed in histological paraffin, for further acquisition of semi-serial histological sections of 7 µm. One slide per animal was prepared, being stained with hematoxylin-eosin (HE). The analyses of histological sections were performed in an optical microscope under 40x and 100x lenses (P1 Olympus BX 50, Manila, Philippines), coupled to a BEL Capture camera to capture images of the intestine and liver, respectively. The image analysis system Eureka 3.0 Plus was used, in order to assist in the measurements of villi and hepatocyte nuclei.

Intestinal villi histomorphometry was performed by measuring ten villi's height (from base to top) and width of the folds (close to apex region) per sample, and the thickness of the muscle tunic (smooth muscle) in ten points per sample, using 40x and 100x lens. Histomorphometry was performed according to Rodrigues et al. (2017), from five randomly selected pictures with a 1000x magnification. The area (µm<sup>2</sup>), perimeter and diameter (µm) of the hepatocytes nuclei were measured in 50 cells per fish, aiming to calculate the volume of the nucleus  $V_{nh} (\mu\text{m}^3) = 4/3 \pi \cdot r^3$ , where  $r$  is the nuclear ray. A quantitative evaluation of the hepatic tissue

was performed to verify the presence of cytoplasmic vacuoles, applying a vacuolization score, as suggested by Caballero et al. (2004) and Tessaro et al. (2014), where the following scores were used: 0 = not observed, 1 = few vacuoles, 2 = average vacuolization, 3 = severe vacuolization.

## Statistical analysis

The obtained data was submitted to a normality test, and when a normal distribution was observed, a one-way analysis of variance was performed (*one-way* ANOVA), followed by the multiple comparison of means test of Tukey. Regarding data that did not follow a normal distribution, data transformations were carried out and, in case normality premises were not attended, the non-parametric test of Kruskal-Wallis was applied. The adopted significance level in all tests was of 5%. All results were presented as mean ± standard deviation. The analyses were performed with the aid of the software STATISTICA 7.0®.

## RESULTS

Water temperature varied throughout the experiment presenting morning and afternoon values of 19.6 ± 0.8 °C and 21.3 ± 1.5 °C, respectively. Regarding the other measured parameters of water quality, the following results were obtained: pH = 8.0 ± 0.6; dissolved oxygen = 6.6 ± 0.6 mg L<sup>-1</sup>; total dissolved solids = 64.3 ± 0.6 mg L<sup>-1</sup>; and electrical conductivity = 94.2 ± 0.8 µS cm<sup>-1</sup>.

The evaluated zootechnical parameters did not present significant variation ( $p > 0.05$ ) (Table 2), whilst centesimal composition parameters displayed significant differences ( $p < 0.05$ ) (Table 3). Moisture was lower in T2 in comparison to the other treatments, whilst ether extract was higher in T1, while ash content was higher

**Table 2.** Zootechnical performance of Nile tilapia juveniles submitted to probiotic addition in the feed and in the rearing water at low temperatures.

Variables	Treatment			P value
	T0	T1	T2	
Initial MW (g)	8.6±0.7	8.8±0.5	8.7±0.7	0.8
Final MW (g)	17.3±5.2	19.6±5.7	23.4±6.0	0.2
Intestinal length (cm)	41.8±8.7	35.1±5.4	37.9±11.3	0.4
SV (%)	41.6±27.3 (33.3)	25.0±17.4 (25.0)	13.8±6.8 (16.6)	0.1
WG (g)	8.7±5.7	10.8±5.8	14.6±5.4	0.2
AFC	5.2±4.9	4.0±2.1	6.1±3.0	0.6
FE	0.13±0.07	0.19±0.1	0.19±0.08	0.4
SGR (%)	1.6±0.8	1.9±0.7	2.4±0.5	0.2
VSI (%)	3.7±2.2	2.7±0.9	2.5±1.5	0.4
HSI (%)	2.2±0.6	1.9±0.4	1.6±0.6	0.2
VIF (%)	0.60±0.4	1.3±1.3	0.49±0.5	0.2

Values presented as mean ± SD for normally distributed data, and mean ± SD (median) for data not normally distributed. T0: control treatment; T1: probiotic addition in the rearing water; T2: probiotic addition in the feed. MW: mean weight; SV: survival; WG: weight gain; AFC: apparent feed conversion. FE: feed efficiency; SGR: specific growth rate; VSI: viscerosomatic index; HSI: hepatosomatic index; VIF: visceral fat.

in T2. On the other hand, crude protein and carbohydrates were statistically similar ( $p>0.05$ ) among treatments.

Hematological parameters displayed significant effects ( $p<0.05$ ) regarding the number of leukocytes and lymphocytes, with fish of treatment T2 showing reduced values in comparison to the control treatment. No effect was verified in the number of neutrophils and monocytes after 40 days of experiment, as well as regarding the concentration of glucose ( $p>0.05$ ) (Table 4).

The histomorphometric measurement of the intestine revealed significant differences ( $p<0.05$ ) regarding villi height and tunic thickness, which were higher in T1 and T2 in comparison to the control. However, no differences were observed ( $p>0.05$ ) for villi's width among treatments (Table 5).

Histomorphometric variables of the hepatocytes nuclei are presented in Table 6. The area, perimeter and volume of the nuclei presented significant differences ( $p<0.05$ ), as T2 presented the

**Table 3.** Proximate composition of Nile tilapia juveniles after 40 days submitted to probiotic addition in the feed and in the rearing water at low temperatures.

Variables	Treatment				P value
	Initial	T0	T1	T2	
Moisture (%)	74.08±0.01	75.05±0.01 (75.05 <sup>a</sup> )	74.04±0.01 (74.04 <sup>b</sup> )	73.83±0.04 (73.83 <sup>c</sup> )	0.02
Crude protein (%)	14.19±0.21	14.27±0.19	14.89±0.18	14.33±0.35	0.09
Ether extract (%)	5.33±0.13	4.32±0.09 (4.30 <sup>b</sup> )	4.52±0.02 (4.53 <sup>a</sup> )	4.17±0.10 (4.18 <sup>b</sup> )	0.03
Ash (%)	4.58±0.01	4.48±0.03 (4.49 <sup>b</sup> )	4.72±0.05 (4.73 <sup>a</sup> )	4.79±0.04 (4.80 <sup>a</sup> )	0.03
Carbohydrates (%)	1.80±0.27	1.86±0.22 (1.80)	1.81±0.17 (1.88)	2.86±0.43 (3.03)	0.06

Values presented as mean ± SD for normally distributed data, and mean ± SD (median) for data not normally distributed. Means in the same row with distinct letters indicate significant differences ( $p<0.05$ ) according to the Mann-Whitney's test. T0: control treatment; T1: probiotic addition in the rearing water; T2: probiotic addition in the feed.

**Table 4.** Hematological parameters of Nile tilapia juveniles submitted to probiotic addition in the feed and in the rearing water at low temperatures.

Variables	Treatments			P value
	T0	T1	T2	
Erythrocytes ( $10^6 \mu\text{L}^{-1}$ )	1.50±0.33	1.34±0.53	1.47±0.16	0.49
Leukocytes ( $10^4 \mu\text{L}^{-1}$ )	8.35±3.76 <sup>a</sup>	5.55±3.30 <sup>ab</sup>	5.26±2.05 <sup>b</sup>	0.04
Lymphocytes ( $10^4 \mu\text{L}^{-1}$ )	7.54±3.48 <sup>a</sup>	4.84±3.23 <sup>ab</sup>	4.95±2.10 <sup>b</sup>	0.03
Monocytes ( $10^3 \mu\text{L}^{-1}$ )	4.35±4.30	5.77±2.58	1.62±1.29	0.05
Neutrophils ( $10^3 \mu\text{L}^{-1}$ )	3.79±3.61	1.34±0.77	1.46±0.83	0.18
Glucose (mg dL <sup>-1</sup> )	93.1±24.8	73.7±7.4	117.1±41.6	0.27

Values presented as mean ± SD. Means within the same row with distinct letters indicate significant differences ( $p<0.05$ ) according to the Tukey's test. T0: control treatment; T1: probiotic addition in the rearing water; T2: probiotic addition in the feed.

**Table 5.** Intestinal morphometry of Nile tilapia juveniles submitted to probiotic addition in the feed and in the rearing water at low temperatures.

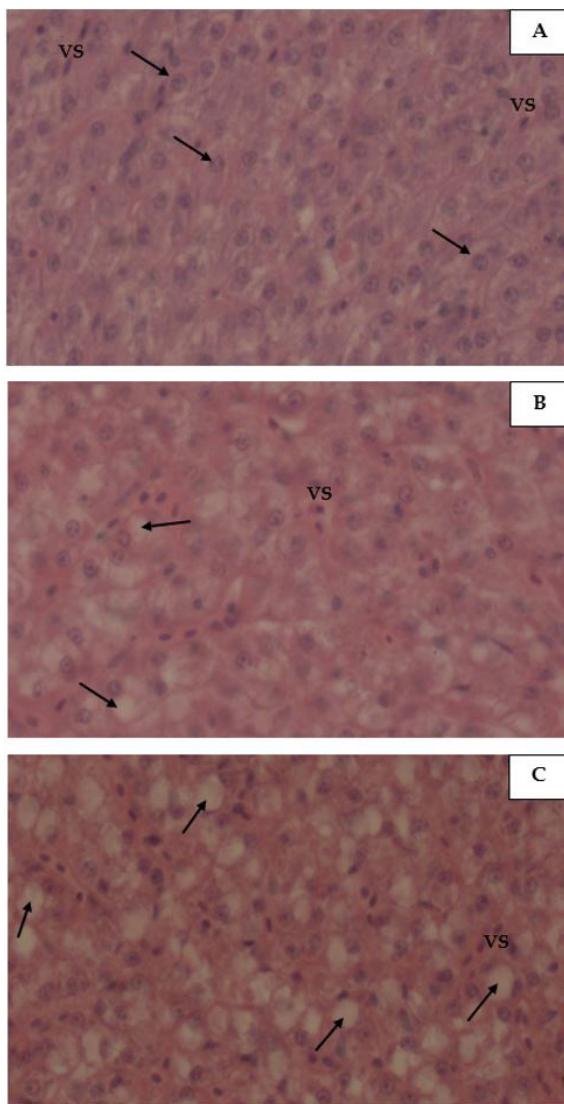
Variables	Initial	Treatments			P value
		T0	T1	T2	
Villi height ( $\mu\text{m}$ )	80.58±22.70	144.75±36.15 (139.46 <sup>b</sup> )	236.39±42.08 (235.35 <sup>a</sup> )	224.62±71.87 (222.80 <sup>a</sup> )	0.00
Villi width ( $\mu\text{m}$ )	52.38±12.21	85.31±18.92	88.64±8.04	81.07±16.88	0.49
Tunic thickness ( $\mu\text{m}$ )	22.40±9.18	17.46±3.56 (16.67 <sup>b</sup> )	25.56±6.79 (26.53 <sup>a</sup> )	27.49±8.07 (27.75 <sup>a</sup> )	0.00

Values presented as mean ± SD for normally distributed data, and mean ± SD (median) for data not normally distributed. Means in the same row with distinct letters indicate significant differences ( $p<0.05$ ) according to the Tukey's test. Means in the same row with distinct letters differ ( $p<0.05$ ) according to the Mann-Whitney's test. T0: control treatment; T1: probiotic addition in the rearing water; T2: probiotic addition in the feed.

**Table 6.** Morphometric characteristics of hepatocytes nuclei of Nile tilapia submitted to probiotic addition in the feed and in the rearing water at low temperatures.

Variables	Initial	Treatments			P value
		T0	T1	T2	
Nucleus perimeter ( $\mu\text{m}$ )	4.97 $\pm$ 0.31	10.49 $\pm$ 0.44 (10.46 <sup>a</sup> )	10.54 $\pm$ 0.49 (10.55 <sup>a</sup> )	9.85 $\pm$ 0.39 (9.81 <sup>b</sup> )	0.00
Nucleus area ( $\mu\text{m}^2$ )	6.08 $\pm$ 0.66	27.07 $\pm$ 2.68 (27.42 <sup>a</sup> )	27.65 $\pm$ 3.18 (27.82 <sup>a</sup> )	24.06 $\pm$ 2.47 (24.10 <sup>b</sup> )	0.00
Nucleus volume ( $\mu\text{m}^3$ )	7.92 $\pm$ 1.27	76.07 $\pm$ 9.63 (74.97 <sup>a</sup> )	77.07 $\pm$ 10.56 (76.92 <sup>a</sup> )	62.96 $\pm$ 7.72 (61.89 <sup>b</sup> )	0.00
Vacuolization score	0-1-2	0-1-2	0-1-2	0-1-2	-

Values presented as mean  $\pm$  SD for normally distributed data, and mean  $\pm$  SD (median) for data not normally distributed. Means in the same row with distinct letters indicate significant differences ( $p < 0.05$ ) according to the Tukey's test. Means in the same row with distinct letters differ ( $p < 0.05$ ) according to the Mann-Whitney's test. T0: control treatment; T1: probiotic addition in the rearing water; T2: probiotic addition in the feed.



**Figure 1.** Livers with hepatic tissue presenting different degrees of the vacuolization score (H.E. 40X) **A:** not observed (0); **B:** few vacuoles (1); **C:** average vacuolization (2); **VS:** blood vessel; **→:** vacuoles.

lowest values of the evaluated parameters among treatments. In addition, all treatments demonstrated consistent presence of vacuolization 1 and 2 in the hepatic tissue.

The evaluation of the liver of the fish submitted to probiotic addition in the water and in the diet presented low and middle vacuolization scores (Table 6). The normal hepatic tissue with score 0 (vacuolization not observed) have well-defined vessels and hepatopancreas area, with homogeneity of the tissues, larger hepatocytes with irregular shape (Figure 1A). Conversely, the score 1 (few vacuoles) shows little presence of intracellular vacuoles, observed in both types of probiotic inclusion (Figure 1B), whilst score 2 (average vacuolization) was attributed to the presence of easily visible vacuoles with larger areas (Figure 1C).

## DISCUSSION

At the end of the experiment, no morphological abnormalities were observed in the fish submitted to probiotic inclusion in the rearing water (T1) or in the feed (T2) at low temperatures. Water quality variables were daily monitored throughout the experimental period, where the dissolved oxygen content remained above 4 mg L<sup>-1</sup>, being considered adequate for fish rearing, the pH were found to be close to neutrality, and both the total dissolved solids and electrical conductivity remained within the recommended levels for the species (Popma and Lovshin, 1996; Kubitzka, 2000). On the other hand, water temperature remained below the recommended for the species' satisfactory growth (27-30 °C) (El-Sayed, 2006). Therefore, considering that temperature is one of the most relevant factors affecting growth and metabolism of fish (El-Sayed, 2006), the exposure of tilapia to low temperatures could impair the physiologic condition and consequently, the production efficiency. Effects on hematological parameters and immunosuppression have been demonstrated for tilapia in relation to stress caused by low temperature (Barros et al., 2014; Guimarães et al., 2016).

In this study, the use of probiotic in the water or in the feed did not influence the productive performance of the fish at low temperatures. Therefore, the BAC-TRAC® probiotic complex was not able to improve the physiologic condition of the fish and a reduced

growth was observed, which was associated to the low temperature ( $19.6 \pm 0.8$  °C in the morning and  $21.3 \pm 1.5$  °C in the afternoon). According to Sardella et al. (2004), suboptimal temperatures may lead to osmoregulatory disturbances and physiological changes. In addition, it may lead to the onset of diseases and increased mortality (Zhou et al., 2018). By another hand, studies conducted with Nile tilapia has been demonstrated satisfactory results when the probiotics were provided in appropriate thermal conditions (Reda and Selim, 2015; Elsabagh et al., 2018; Gobi et al., 2018). Xia et al. (2020) when using *B. cereus* and a blend of *B. subtilis* and *B. cereus* NY5 ( $1 \times 10^8$  CFU g<sup>-1</sup>), obtained higher weight gain and feed conversion rates in diets offered for *O. niloticus*, using a mean temperature around 28 and 29°C. Abarike et al. (2018) also demonstrated satisfactory results using the blend of *B. subtilis* and *B. licheniformis*, with higher weight gain, specific growth rate and feed conversion in Nile Tilapia (*O. niloticus*), at  $28 \pm 2$ °C.

Similarly, the observed lower feed efficiency might be related to the low consumption at low temperatures, which lead to negative impacts on survival rates. Other studies with Nile tilapia also revealed these effects (Araujo et al., 2011; Zhou et al., 2018). In the study developed by Corrêa et al. (2017), the authors observed reduced feeding, weight gain and survival of Nile tilapia reared at suboptimal temperatures.

The obtained results in the present study evidence that the use of the BAC-TRAC® probiotic complex in both water or feed under suboptimal temperatures did not display a positive action in the rearing of Nile tilapia juveniles, seen that the fish did not adequately feed and grew satisfactorily. Several studies using rainbow trout (Bagheri et al., 2008; Merrifield et al., 2010; Azari et al., 2011) revealed that the use of probiotic for this cold-water species resulted in good growth and survival, besides improving the animals' welfare.

Differences in carcass composition were observed among treatments, which was possibly related to alterations in the metabolic rates, as the increasing of digestibility and absorption of nutrients, that occurred throughout the experiment by the supply of BAC-TRAT® and impacted moisture, ether extract and ash contents. Volkoff and Butt (2019) highlighted that the intestinal microbiota influences the fish's metabolism, leading to alterations in ingestion and nutrients digestibility. Silva (2014) evaluated different probiotic concentrations in a diet for Nile tilapia, and did not observe differences in moisture, ether extract, protein and ash contents, whilst Reda and Selim (2015) found significant differences for protein and lipid in the carcasses, when testing different concentrations of *B. amyloliquefaciens* in diets for Nile tilapia. Both Sekirov and Finlay (2009) and Volkoff and Butt (2019) commented that stress may alter the structure of the intestinal mucosa and lead to alterations of the intestinal mucous, thus affecting nutrient absorption. In this sense, it is possible that in the present study, the alterations observed in mineral matter and lipid deposition may be related to the effects of microbiota modulation and/or to the intestinal enzyme activity by the probiotic, resulting in greater nutrient accumulation in comparison to the treatment that did not receive the probiotic. However, such effects may not have been sufficient to determine significant alterations in the animals' performance.

Temperature seemed to cause alterations in the leukocyte and lymphocyte count, corroborating the results of Araújo et al. (2011) and Fernandes Junior et al. (2010); which performed a challenge with Nile tilapia at 17°C for 54 hours and seven days, respectively; Falcon et al. (2008) and Signor et al. (2010) which studied the Nile tilapia maintained at 18-15°C for 36 days and 13°C for seven days, respectively. Freitas (2015) also highlighted that such reductions are called leukopenia and lymphopenia. Martins et al. (2004) and Barros et al. (2014) observed that a reduction in the number of lymphocytes and the consequent reduction of leukocytes are typically a stress response. Therefore, the responses observed in this study probably characterize a low temperature stress response.

Freitas (2015) demonstrated that under stressful situations and low temperature, Nile tilapia juveniles displayed leukopenia, lymphopenia and neutrophilia. However, according to Hrubec et al. (2000), hematological counts are within the standard determined for the species. Still in relation to stress conditions, high glucose concentrations in the animals' blood represent a restauration function of the organism during critical situations, whilst the highest values observed in this study were obtained in the control treatment and also in T2, where the probiotic was included in the feed, however not being significant. Freitas (2015) also observed increased glucose concentrations in fish that underwent stress at low temperatures. It is possible that such increased glucose concentrations refers to a physiologic response to adjust the higher energy demand during stress (El-Khalidi, 2010; Costas et al., 2011).

Regarding the analysis of the intestine, an increase in the villi's height was observed in T1, resulting in a larger nutrient absorption area. According to Jesus (2014), increased intestinal morphometry might be related to the capacity of probiotics in reducing the adhesion of pathogenic bacteria of the intestinal epithelium. On the other hand, the increase of the muscle tunic observed in T2 may be related to the adaptation of the intestinal structure in response to the probiotic addition (Rotta, 2003), as the intestine may not be in optimal health conditions.

When adding probiotic in the feed, Mello et al. (2013) observed an increase in the villi structures, which led to a greater absorption and retention of nutrients, besides an increase in the zootechnical performance and body composition. In this sense, this study shows a positive action of the probiotics in the feed on villi morphology and consequently a better nutrient absorption in low-temperature conditions. In studies with other fish species analyzing the influence of gut microbiome on absorption and metabolism, such as the Japanese sole (*Paralichthys olivaceus*) (Ye et al., 2011), zebrafish (*Danio rerio*) (Semova et al., 2012), and the grass carp (*Ctenopharyngodon idella*) (Ni et al., 2014), several changes in the morphometry of the villi were observed.

The analyses of histomorphometric measurements of the area of hepatocyte nuclei are used as biomarkers, aiming to observe the state and metabolic activity of cells (Rodrigues et al., 2017).

Measurements of perimeter, area and volume of the nuclei were higher in T1 in comparison to T2. In the studies developed by Cavalier-Smith (1982) and Neumann and Nurse (2007) about the size of hepatocyte nuclei, the authors highlight that this parameter may be related to the quantity of DNA in the nucleus, which is proportionally adjusted to the size of the cell. However,

Webster et al. (2009) observed that cytoplasmic proteins and some nuclear proteins regulate the nucleus' size in comparison to the size of the cell. In addition, Ostaszewska et al. (2011) stated that under optimal nutritional conditions, the liver stores good amounts of glycogens or lipids in the cytoplasm, which consequently reflects in the area and volume of hepatocytes. The low histomorphometric values of hepatocyte nuclei found in treatment T2 may be related to a nutritional stress status, as suggested by Wold et al. (2009), who reported that fish submitted to nutritional stresses suffers reduction of the size of hepatocytes. Rodrigues et al. (2017), when studying the liver of a surubim hybrid (*Pseudoplatystoma reticulatum* × *Pseudoplatystoma corruscans*), observed that inadequate feeding reduces hepatocyte's metabolism, and consequently the nuclear area.

Regarding hepatic tissues, the scores 0, 1 and 2 were observed, whilst score 3 (severe vacuolization) was not revealed by any of the evaluated treatments. According to Tessaro et al. (2014), these aspects might be related to the quantity of lipids stored in the liver, which are removed in the H.E. staining technique during tissues processing, evidencing vacuoles. However, these qualitative parameters indicated the homogeneity among treatments, without the probiotic effect in the liver lipid deposition.

## CONCLUSION

The use of BAC-TRAT® probiotic complex for Nile tilapia, whether in the rearing water or in the feed, at low temperatures, had no effect on the animals' growth; however, it provided an increase on the intestinal villi height, and its addition in the water resulted in a larger area and volume of the hepatocytes nuclei, which can be considered as good indicators of the nutritional status of the fish, thus contributing for the animal's welfare in adverse periods.

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