*Research Article*

# Effects of the probiotic *Bacillus amyloliquefaciens* on growth performance, hematology and intestinal morphometry in cage-reared Nile tilapia

**Thiago Fernandes A. Silva[1], Thalita R. Petrillo[2], Jefferson Yunis-Aguinaga[1], Paulo Fernandes Marcusso[2], Gustavo da Silva Claudiano[2], Flávio Ruas de Moraes[2] & Julieta R. Engrácia de Moraes[1,2]**
[1]Aquaculture Center of Unesp, São Paulo State University (Unesp), Jaboticabal, Brazil
Via Prof. Paulo Donato Castellane, km 05, Jaboticabal, SP. 14.884-900
[2]Department of Veterinary Pathology, São Paulo State University (Unesp), Jaboticabal, Brazil
Corresponding author: Julieta Engrácia de Moraes (julietaengracia@gmail.com)

**ABSTRACT.** The aim of this study was to evaluate the effect of probiotic *Bacillus amyloliquefaciens* on the growth performance, blood profile and intestinal morphometry in Nile tilapia (*Oreochromis niloticus*) reared in cages. 936 Nile tilapias were distributed in 12 cages (1.5 m$^3$). Fish were fed for 90 days on basal diets containing 0 (control); $1 \times 10^6$ CFU g$^{-1}$; $5 \times 10^6$ CFU g$^{-1}$; and $1 \times 10^7$ CFU g$^{-1}$ of the probiotic. The results showed no significant difference on performance and proximal composition of fish. Blood glucose and hemoglobin were lower in $1 \times 10^7$ CFU g$^{-1}$ group, suggesting improves of the homeostatic state of the fish. Other hematimetric indices did not differ between groups. It was observed significant increase of villi height and in the number of goblet cells of the intestine in fish supplemented $5 \times 10^6$ CFU g$^{-1}$ and $1 \times 10^7$ CFU g$^{-1}$ of food suggesting that fish improved the digestion and absorption of nutrients. However, more studies are needed to determine the efficacy of this probiotic in field conditions in Nile tilapia.

**Keywords:** *Oreochromis niloticus*, homeostatic state, intestinal morphology, blood glucose, aquaculture.

## Efectos del probiótico *Bacillus amyloliquefaciens* en el crecimiento, hematología y morfometría intestinal en tilapias del Nilo criadas en balsa jaula

**RESUMEN.** El objetivo de este estudio fue evaluar el efecto del probiótico *Bacillus amyloliquefaciens* en el desempeño del crecimiento, hemograma y morfometría intestinal en tilapia del Nilo (*Oreochromis niloticus*) criadas en balsas jaulas. 936 tilapias del Nilo se distribuyeron en 12 balsas jaulas (1,5 m$^3$). Los peces fueron alimentados durante 90 días con dietas que contenían 0 (control); $1 \times 10^6$ UFC g$^{-1}$; $5 \times 10^6$ UFC g$^{-1}$ y $1 \times 10^7$ UFC g$^{-1}$ del probiótico. Los resultados no mostraron diferencias significativas en el rendimiento y composición proximal de los peces. La glucosa en sangre y hemoglobina fue menor en el grupo $1 \times 10^7$ UFC g$^{-1}$, lo que sugiere un mejor estado homeostático de los peces. Otros parámetros hematimétricos no difirieron entre los grupos. Se observó aumento significativo de la altura de las vellosidades y número de células caliciformes del intestino en los peces suplementados con $5 \times 10^6$ UFC g$^{-1}$ y $1 \times 10^7$ UFC g$^{-1}$ de alimento que sugieren que los peces mejoraron la digestión y absorción de nutrientes. Sin embargo, se necesitan más estudios para determinar la eficacia de este probiótico en condiciones de campo en tilapia del Nilo.

**Palabras clave:** *Oreochromis niloticus*, homeostasis, morfología intestinal, glucosa sanguínea, acuicultura.

## INTRODUCTION

Nile tilapia reared in cages presents advantages over traditional pond and tank systems due to the easier handling, high volume of production, and use of areas unsuitable for other purposes, and the biological characteristics of this species (García *et al.*, 2014). Al-though the productivity advantages (Kim *et al.*, 2014), fish at high stocking densities may present stress and depress the immune system, increasing susceptibility to bacterial and parasitic diseases (Telli *et al.*, 2014).

The use of antibiotics as prophylactic is questioned for being a source of environmental pollution (Ayandiran *et al.*, 2014), accumulating in the fillet, and promoting

resistance to drugs with risks to human and animal health (Kersarcodi-Watson *et al.*, 2008).

Probiotics are live microorganisms able to establish, multiply and colonize the intestine of the host in order to promote a beneficial balance of microorganisms. These benefits are explain due to these microorganisms inhibit the proliferation of harmful agents in the intestinal mucosa, improve the digestibility and absorption of nutrients (Nayak, 2010), promote the synthesis of vitamins (Lee *et al.*, 2013), and improve the growth performance of animals (Mohapatra *et al.*, 2014) by increasing the survival percentage (Wu *et al.*, 2014) and water quality (Chi *et al.*, 2014).

Bacteria from genus *Bacillus* are one of the main probiotics used in aquaculture. These bacteria are ease to cultivate and form spores, which facilitates its conservation (Wang *et al.*, 2008; Nayak, 2010; Han *et al.*, 2015). In addition, several bacteria of this genus have the capacity to secrete antimicrobial compounds and different exoenzymes that aid digestion (Ziaei-Nejad *et al.*, 2006).

*Bacillus amyloliquefaciens* (ex Fukomoto, 1943) Priest *et al.*, 1987 is a non-pathogenic bacterium rod-shaped, aerobic, Gram-positive, and catalase positive (Loncar *et al.*, 2014). This bacterium is highly resistant to environmental changes (Mahdhi *et al.*, 2012; Das *et al.*, 2013), presents antibacterial activity of broad spectrum (Kaewklom *et al.*, 2013), and is an important producer of α-amylase, an enzyme that acts in the digestion of carbohydrates (Wang *et al.*, 2008).

Recently, in studies with *B. amyloliquefaciens* Reda & Selim (2015) observed in *O. niloticus* reared in laboratory conditions; improve in body composition and intestinal morphology. Ridha & Azad (2012) reported in the same fish improvements on growth performance and immune response. Cao *et al.* (2011) observed in *Anguilla anguilla*, antagonist activity of *B. amyloliquefaciens* and *Aeromonas hydrophila*. The same effect was observed by Ran *et al.* (2012) in *Ictalurus punctatus* against *A. hydrophila* and *Edwardsiella ictaluri.* Das *et al.* (2013) observed an increase in disease resistance in *Catla catla.*

The aim of this study was to evaluate the effect of diet containing three concentrations of the probiotic *B. amyloliquefaciens* on performance, blood profile and intestinal morphology in Nile tilapia, *O. niloticus*, reared in cages.

## MATERIALS AND METHODS

### Fish culture, diet and feeding

Masculinized Nile tilapia (*Oreochromis niloticus*) (35 ± 5 g) from the same spawning were obtained from commercial breeding. The cages (1.5 m³) were distri-

buted in four experimental groups (triplicate) in a completely randomized design (78 fish per cage). The experiment was conducted in the reservoir 3 of the Aquaculture Center of Sao Paulo State University, Jaboticabal (21°14.33'S, 48°17.55'W) in April-June 2014. This reservoir has approximately 10,000 surface m².

The animals were acclimated for 10 days and fed with basal diet (pellets used in this experiment without addition of probiotics). It was used the program "Super Crac Premium" to formulate the diet according the suggestions of Furuya (2010). Feed was extruded. The feed rate was 3% of body weight, 3 times a day for 90 days on basal diets containing 0 (control); $1\times10^6$ CFU $g^{-1}$; $5\times10^6$ CFU $g^{-1}$; and $1\times10^7$ CFU $g^{-1}$ of *Bacillus amyloliquefaciens*.

Diet composition is shown in Table 1. To achieve homogeneous final concentrations of bacteria in the diet and to facilitate the adhesion of the probiotic, the bacterial suspension was added and homogenized manually with commercial vegetable oil (4% by weight; 4.000 kcal $L^{-1}$). Thus, according to the recommendations of Das *et al.* (2013) food was prepared every 7 days to maintain optimal probiotic levels.

Fish were measured at 30 and 60 days for diet amount adjustment. During the experimental period, except temperature, there were no important changes in the water quality that could have interfered in the experiment results (Boyd & Massaut, 1999). The values were maintained as follows: the water temperature varied from 27.1 to 21.9°C (24.35 ± 2.43°C) and dissolved oxygen (7.31 ± 0.48 mg $L^{-1}$). Both were measured using an YSI 53 device (YSI Company, USA); pH (7.50 ± 0.30) and electrical conductivity (45.54 ± 10.40 µV) were measured using a 63 YSI device (YSI Company, USA). Water transparency (26.03 ± 2.10 cm) was measured using the Secchi disk at 11:00 h on alternate days.

### Isolation and identification of the probiotic bacterial strain

The bacterium was isolated from a commercial product containing *Bacillus amyloliquefaciens*. To confirm the presence of the bacteria in the product, 1.0 g of the probiotic was homogenized in 99 mL of phosphate buffered saline (145 mM NaCl, 1.4 mM $NaH_2PO_4$, 8 mM $Na_2HPO_4$, pH 7.4). Thus, it was performed serial dilutions with peptone solution (4%) (Sigma-Aldrich, USA), distributed into Petri dishes containing culture medium trypticase soy Agar (TSA), and incubated for 48 h under aerobic conditions at 26°C. Colonies of *Bacillus* sp. were identified according their morpho-logical, biochemical and dyeing features (Priest *et al.*,

**Table 1.** Ingredients and proximal composition of the basal diet.

| Ingredients | % |
|---|---|
| Corn flour | 30.000 |
| Soybean meal (38% CP) | 20.000 |
| Rice bran | 14.766 |
| Wheat flour | 13.000 |
| Corn gluten (20% CP) | 12.000 |
| Poultry by-product meal (65% CP) | 5.000 |
| Fish meal (60% CP) | 3.000 |
| DL-Methionine | 0.700 |
| Dicalcium phosphate | 0.610 |
| L-Tryptophan | 0.200 |
| L-Lysine | 0.164 |
| Antifungi | 0.030 |
| Antioxidant | 0.030 |
| Vitamin mix[1] | 0.500 |
| Proximal composition (%) | |
| Dry matter | 90.10 |
| Crude protein | 30.21 |
| Digestible energy (kcal kg$^{-1}$) | 2990.12 |
| Ether extract | 4.98 |
| Fiber | 4.67 |
| Ash | 4.78 |

[1]Composition of vitamin-mineral supplement (Fri-Ribe): vitamin A, 600,000 IU; Vitamin D3 600,000 IU; Vitamin E 12,000 IU; Vitamin K3, 1200 mg; vitamin B1, 1200 mg; vitamin B2, 1536 mg; vitamin B6, 1287 mg; B12, 4000 mg; folic acid, 198 mg; pantothenic acid, 3800 mg; Vitamin C, 48,000 mg; biotin, 20 mg; choline, 30,000 mg; niacin 19,800 mg; Fe, 25,714 mg; Cu, 1960 mg; Mn, 13,334 mg; Zn, 6000 mg; I, 948 mg; Co, 2 mg; Se, 30.10 mg.

1987). The sequencing of 16S rDNA gene was performed from isolated colonies by the method of Sanger *et al*. (1977).

The quantification of the probiotic bacteria in the feed was performed by homogenizing 1.0 g of diet in 99 mL of PBS. Then, it was performed serial dilutions as described above. Finally, they were evaluated the colonies that showed morphological, biochemical, and dyeing features compatible with *B. amyloliquefaciens*. Samples of all treatments were subjected to molecular identification of the bacterium as described previously.

## Growth performance

After 90 days, fish were measured and weighed to determinate performance indices:

• Weight gain (WG) = Final weight - initial weight

• Specific growth rate (SGR) = (In[final weight] - In[initial weight]) x 100/number of days

• Feed efficiency (FE) = weight gain x 100/food consumed

## Proximal composition

After the feeding period, 10 fish from each group were euthanized using benzocaine (0.5 g L$^{-1}$) (Wedemeyer, 1970) and stored at -18°C for later analysis of proximal composition. Then the samples were ground to obtain a homogenous product and dried in an oven at 55°C for 48 h. The humidity was determined by drying in an oven at 105°C for 24 h. The ash content was obtained after incineration in a muffle furnace at 550°C and the energy by bomb calorimetry. Protein was determined by Kjeldahl method and the ether extract by Soxhlet method (AOAC, 2005).

## Hematological profile and glucose

After anesthesia, blood of 10 fish per replicate were collected by puncture of the caudal vessel and distributed in tubes containing EDTA (10%) for determination of hematocrit (Goldenfarb *et al*., 1971), hemoglobin (Collier, 1944) and, red blood cell counts using the solution Natt & Herrick as diluent and dye. Then, they were calculated the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and, mean corpuscular hemoglobin concentration (MCHC).

Blood smears were stained with Panotic dye (Laborclin, PR, Brazil) to count leukocytes and thrombocytes. The determination of blood glucose was performed with the rapid glucose analyzer One Touch UltraMini® (Lifescan, CA, USA).

## Analysis of intestinal morphometry

The collection of the intestine was performed in the same fish used in the blood analysis. After deep anesthesia, it was performed a longitudinal incision in the abdomen and collected a fragment of the anterior portion of the intestine located two centimeters after the pylorus. The fragments were fixed in Bouin solution for 24 h. Then, they were dehydrated in 70% alcohol to histological processing (Honorato *et al*., 2011). The slides were stained with hematoxylin-eosin (HE) for morphometric evaluation of villi and with periodic acid-Schiff (PAS) for goblet cell count.

The measurement of the height and width of the villi, epithelial thickness, and counting of goblet cells were performed according to Mello *et al*. (2013). It was used a Zeiss Axio Vision image analyzer.

## Statistical analysis

Data were subjected to analysis of variance (ANOVA), differences between treatments were compared by Tukey

test ($P < 0.05$). Data were analyzed by statistic R software (version 0.98.1) and presented as mean ± standard deviation.

## RESULTS

### Performance and proximal composition

Under the conditions evaluated, there was no statistical difference in weight gain, feed conversion, specific growth rate, and feed efficiency (Table 2). Proximal analysis of fish carcass showed no significant difference between treatment and control groups (Table 3).

Hemoglobin and glucose levels showed differences between treatments. The group supplemented with the highest concentration of probiotic ($1\times10^7$ UFC g$^{-1}$) showed the lower values for these variables. Other red blood cell parameters (Table 4) and differential leukocyte count (Table 5) showed no statistical difference between treatments.

### Intestinal morphometry

Histomorphometric evaluation of the anterior portion of the intestine showed significant difference in the height of the villi in groups supplemented with $5\times10^6$ CFU g$^{-1}$ and $1\times10^7$ CFU g$^{-1}$ compared to $1\times10^6$ CFU g$^{-1}$ and control groups (Fig. 1). There were observed no lesion in the intestine and no differences in the width and thickness of the villi between control and treatment groups (Table 6). Fish that received the highest concentration of probiotic also had significantly greater

number of goblet cells comparing to control group (Table 6, Fig. 2).

## DISCUSSION

The use of *B. amyloliquefaciens* as a probiotic in fish feeding is recent and information is scarce. However, it has demonstrated that dietary supplementation with this bacterium improves growth performance in fish. In tilapia fingerlings reared in glass aquaria, supplementation with this bacterium improved weight gain, specific growth rate and feed conversion after supplement with $10^6$ CFU g$^{-1}$ of *B. amyloliquifaciens* for 60 days (Reda & Selim, 2015). However, no differences were observed when the probiotic was administered at $10^4$ CFU g$^{-1}$ of concentration. Similarly, Telli *et al*. (2014) found no effect of *Bacillus subtilis* ($5\times10^6$ CFU g$^{-1}$) on performance of Nile tilapia, even after 84 days of feeding supplemented diet.

In this study, there was no effect of the probiotic *B. amyloliquefaciens* on growth performance. This can be explained due to the low temperatures in the last 2 months. Marcusso *et al*. (2015) reported that the homeostasis of Nile tilapia rearing at temperatures below 24ºC could be affected, enhancing the susceptibility to bacterial infections and impairing the growth performance.

Ridha & Azad (2012) tested *B. amyloliquefaciens* ($10^8$ CFU g$^{-1}$) in Nile tilapia reared in tanks for 99 days and did not find any difference in performance indices either. However, they observed 60 days after that fish

**Table 2**. Growth performance indices of Nile tilapia fed with experimental diets. WG: weight gain, SGR: specific growth rate, FE: feed efficiency. Mean ± SD. There were no statistical differences ($P > 0.05$) between treatments.

| Treatments | WG (g) | SGR (%) | FE |
|---|---|---|---|
| Control | 86.84 ± 36.53 | 1.10 ± 0.51 | 0.842 ± 0.245 |
| $1\times10^6$ UFC g$^{-1}$ | 83.47 ± 30.84 | 1.09 ± 0.44 | 0.864 ± 0.245 |
| $5\times10^6$ UFC g$^{-1}$ | 90.44 ± 39.68 | 1.13 ± 0.53 | 0.791 ± 0.300 |
| $1\times10^7$ UFC g$^{-1}$ | 94.5 ± 39.66 | 1.18 ± 0.55 | 0.906 ± 0.206 |

**Table 3.** Body composition (%) and gross energy (kcal kg$^{-1}$) of Nile tilapia fed with experimental diets. CP: crude protein, EE: ether extract, M: moisture, Ash; GE: gross energy. Mean ± SD. There were no statistical differences ($P > 0.05$) between treatments. Values are in % of dry weight.

| Treatments | CP% | EE % | M % | Ash % | GE (kcal kg$^{-1}$) |
|---|---|---|---|---|---|
| Control | 43.1 ± 0.3 | 25.8 ± 0.8 | 77.5 ± 0.5 | 11.8 ± 0.7[a] | 5064 ± 21.1 |
| $1\times10^6$ UFC g$^{-1}$ | 44.6 ± 0.4 | 24.2 ± 0.9 | 76.2 ± 0.3 | 13.8 ± 0.4[a] | 5716 ± 13.5 |
| $5\times10^6$ UFC g$^{-1}$ | 39.9 ± 0.6 | 25.4 ± 0.7 | 76.8 ± 0.6 | 12.3 ± 0.4[a] | 5150 ± 16.1 |
| $1\times10^7$ UFCg$^{-1}$ | 42.8 ± 0.4 | 26.6 ± 0.8 | 77.3 ± 0.5 | 12.0 ± 0.6[a] | 5635 ± 18.3 |

**Table 4.** Hematological parameters. Hct: hematocrit, Hb: hemoglobin, RBC: red blood cells, MCHC: mean corpuscular hemoglobin concentration, MCH: mean corpuscular hemoglobin, MCV mean corpuscular volume, and G: plasma glucose of the Nile tilapia fed different probiotic concentrations in feed. Means ± SD. Values (mean ± SD) with different letter are significantly different ($P < 0.05$).

| Treatment | Hct (%) | Hb (g dL$^{-1}$) | RBC ($10^4$ μL$^{-1}$) | MCHC (g dL$^{-1}$) | MCH (pg) | MCV (fL) | G (mg dL$^{-1}$) |
|---|---|---|---|---|---|---|---|
| Control | 36.8 ± 2.9[a] | 10.75 ± 1.0[a] | 295.93 ± 49.6[a] | 27.01 ± 3.4[a] | 33.05 ± 5.8[a] | 125.96 ± 21.7[a] | 110.00 ± 34.9[a] |
| $1\times10^6$ UFC g$^{-1}$ | 38.5 ± 3.4[a] | 11.20 ± 1.6[a] | 292.33 ± 34.6[a] | 24.44 ± 3.2[a] | 32.53 ± 4.6[a] | 123.78 ± 11.0[a] | 66.80 ± 36.8[ab] |
| $5\times10^6$ UFC g$^{-1}$ | 39.2 ± 1.6[a] | 10.90 ± 1.3[a] | 303.06 ± 33.4[a] | 27.95 ± 4.9[a] | 35.72 ± 7.0[a] | 127.89 ± 11.8[a] | 41.40 ± 9.8[b] |
| $1\times10^7$ UFC g$^{-1}$ | 37.4 ± 1.9[a] | 8.53 ± 1.2[b] | 293.50 ± 36.5[a] | 26.36 ± 4.6[a] | 35.08 ± 7.9[a] | 133.31 ± 20.4[a] | 36.25 ± 5.8[b] |

**Table 5.** Differential leukocyte count in the circulating blood of Nile tilapia fed the experimental diets. Means ± SD. There were no statistical differences ($P > 0.05$) between treatments.

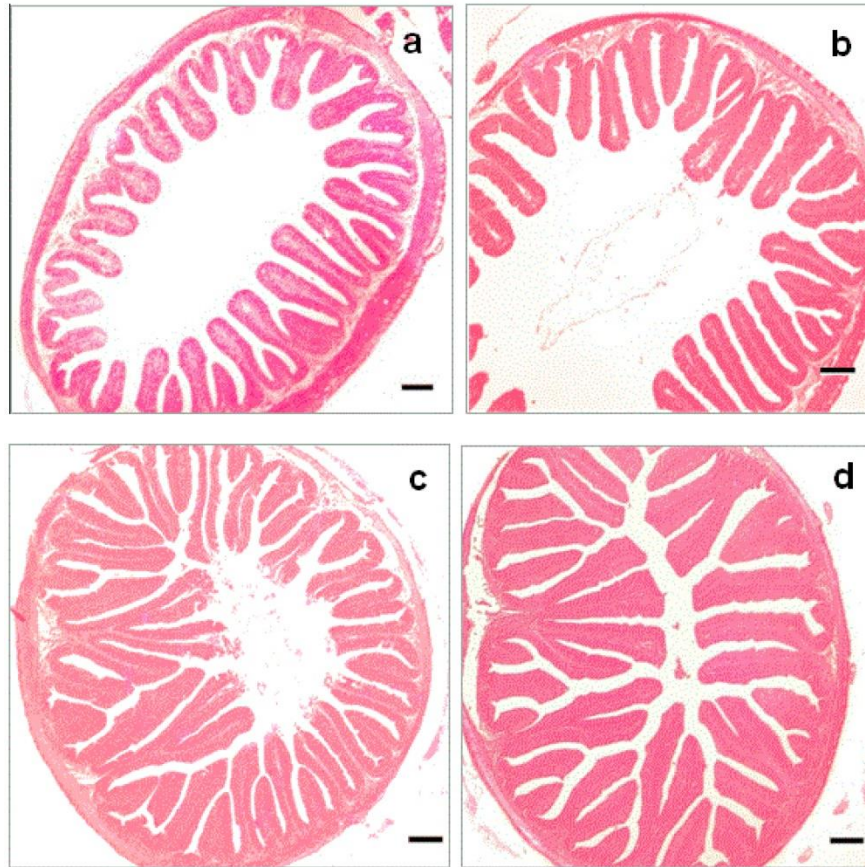| Treatment | Leukocyte (mm$^3$) | Lymphocytes ($10^3$ μL$^{-1}$) | Neutrophils ($10^3$ μL$^{-1}$) | Monocytes ($10^3$ μL$^{-1}$) | Basophils ($10^3$ μL$^{-1}$) | Eosinophils ($10^3$ μL$^{-1}$) |
|---|---|---|---|---|---|---|
| Control | 110.2 ± 20.5 | 31.44 ± 8.1 | 16.3 ± 4.92 | 16.02 ± 3.4 | 0 ± 0 | 0.3 ± 0.04 |
| $1\times10^6$ UFC g$^{-1}$ | 129.4 ± 27.8 | 26. 31 ± 13 | 19.55 ± 5.21 | 15.03 ± 4.7 | 0 ± 0 | 0.31 ± 0.2 |
| $5\times10^6$ UFC g$^{-1}$ | 105.92 ± 14.3 | 30. 72 ± 9.3 | 16.2 ± 4.16 | 17.46 ± 6.2 | 0 ± 0 | 0.27 ± 0.18 |
| $1\times10^7$ UFC g$^{-1}$ | 112 ± 13.4 | 28.11 ± 6.4 | 14.11 ± 3.1 | 14.6 ± 7.2 | 0 ± 0 | 0.32 ± 0.23 |



**Figure 1.** Comparative photomicrographs of the anterior portion of the intestine of Nile tilapia fed different probiotic concentrations in the diet. a) Control group, b) $1\times10^6$ CFU g$^{-1}$, c) $5\times10^6$ CFU g$^{-1}$, and d) $1\times10^7$ CFU g$^{-1}$. Villi height enhanced proportionally to the concentration of the probiotic. Scale bar: 200 μm. Stain: hematoxylin-eosin.
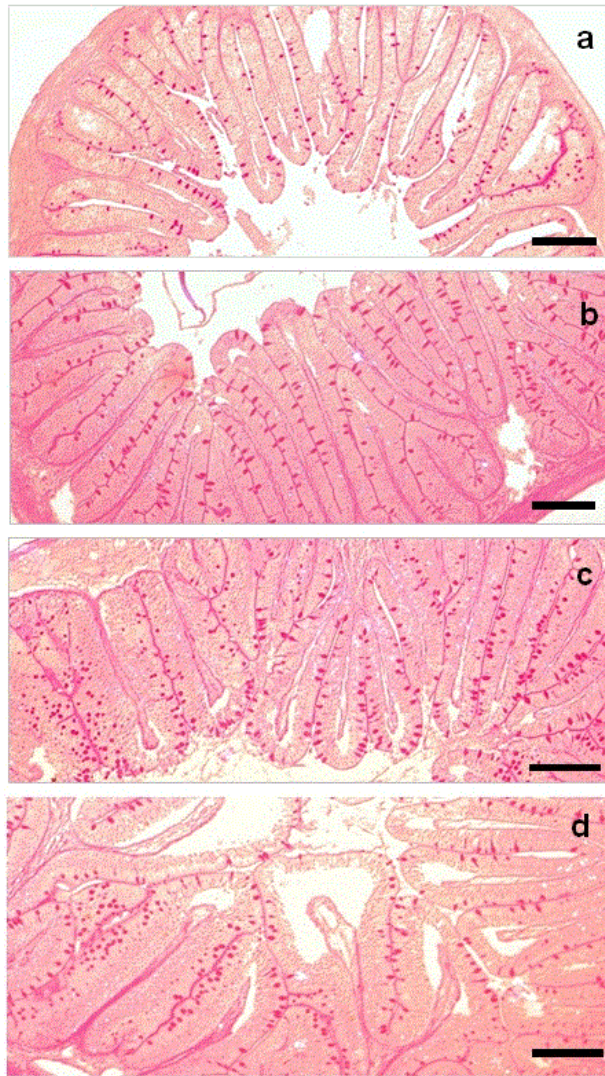
**Figure 2.** Comparative photomicrographs of the epithelial layer of the intestinal villi of Nile tilapia supplemented with different probiotic concentrations in the diet. a) Control group, b) $1\times10^6$ CFU g$^{-1}$, c) $5\times10^6$ CFU g$^{-1}$, and d) $1\times10^7$ CFU g$^{-1}$. Goblet cells (deep red colour -magenta-cells on the edge of the villi) increased proportionally to the concentration of the probiotic in the diet. Scale bar: 100 μm. Stain: Periodic acid-Schiff (PAS).

previously supplemented with the bacterium showed better growth performance than control group, suggesting that the effects of this probiotic occur over time. Unlike this report, in the present study fish were reared in cages in a reservoir with abundant algae (low transparency of water). This availability of food could influence the growth performance of fish, explaining why there was no difference between treatments.

The efficiency of nutrient transfer from food to the organism can be measured by the proximal composition analysis. This is of great importance due to it may

reflect the nutritional value of the fish and its organoleptic characteristics (Contreras, 1994). Several authors have reported improvements in body composition of Nile tilapia supplemented with probiotics (Mello *et al.*, 2013; Hassaan *et al.*, 2014; Reda & Selin, 2015). However, in this study, body composition analysis values showed no significant difference between treatment and control groups. Similar results were found by Telli *et al.*, (2014) in Nile tilapia fed diet supplemented with *B. subitilis*.

Hematological variables are commonly used as indicators of physiological condition in fish (Mohapatra *et al.*, 2014). Some studies reported that after feeding with *Bacillus* sp., Nile tilapia presented alterations on the hematological variables (Hassaan *et al.*, 2014; Telli *et al.*, 2014.) or not (Kumar *et al.*, 2006; Soltan & El-Laithy, 2008). In the present study, we did not observe differences in hematocrit, red blood cells count and hematological indices (MCHC, MCH and MCV). However, there was a higher hemoglobin levels in the fish fed with lower concentrations of probiotic. This could be related to the necessity of transport more oxygen in blood to meet the increasing energy demand of fish promoted by high levels of glucose (Nikinmaa *et al.*, 1983).

As in current study, Reda & Selim (2015) observed increase in hemoglobin levels in blood of Nile tilapia supplemented with *B. amyloliquefaciens*. However, the authors also observed an increase in the number of erythrocytes and leukocytes in blood, but without changes in blood glucose. They were reporter that probiotic bacteria enhance the iron absorption due to the release of organics acids in the gut. This would increase the availability of iron to produce hemoglobin in rats (Yadav *et al.*, 2007) and fish (Dahiya *et al.*, 2012).

Probiotics enhance fish tolerance to stress agents of environmental origin or related to handling (Tapia-Paniagua *et al.*, 2014). During stress, the release of catecholamines stimulates glycogenolysis (Martínez-Porchas *et al.*, 2009). The rapid increase of glucose in blood is one indicator of stress in fish (Simoes *et al.*, 2012). In the present study, the higher blood glucose observed in the control group compared to supplemented ones with the greater inclusion of probiotic in the diet may reflect the homeostatic state of the animals against adverse conditions. *B. amyloliquefaciens* seems to have positively influenced the glucose levels of fish supplemented with $5\times10^6$ CFU g$^{-1}$ and $1\times10^7$ CFU g$^{-1}$ of feed, enhancing the homeostatic state of fish. Similar findings have been reported by Mohapatra *et al.* (2014) in *Labeo rohita* stressed that presented lower glucose levels in fish fed with this probiotic.

**Table 6.** Histomorphometric parameters and goblet cells per villus in the intestine of Nile tilapia fed the experimental diets. Mean ± SD. Values (mean ± SD) with different letter are significantly different ($P < 0.05$).

| Treatments | Total height of villi (μm) | Height of villi (μm) | Width of villi (μm) | Epithelium thickness (μm) | Goblet cell count per villi |
|---|---|---|---|---|---|
| Control | $466.0 \pm 92.9^a$ | $378.1 \pm 99.7^b$ | $62.5 \pm 27.7^a$ | $113.3 \pm 83.4^a$ | $11.38 \pm 5.2^b$ |
| $1\times10^6$ UFC g$^{-1}$ | $407.2 \pm 121.1^a$ | $371.3 \pm 91.6^b$ | $51.4 \pm 8.7^a$ | $98.9 \pm 19.8^a$ | $16.45 \pm 6.63^{ab}$ |
| $5\times10^6$ UFC g$^{-1}$ | $438.4 \pm 105.5^a$ | $423.2 \pm 95.3^a$ | $54.2 \pm 11.4^a$ | $118.3 \pm 21.5^a$ | $25.24 \pm 8.32^a$ |
| $1\times10^7$ UFC g$^{-1}$ | $487.2 \pm 31.2^a$ | $469.4 \pm 25.1^a$ | $57.0 \pm 13.3^a$ | $107.3 \pm 23.9^a$ | $23.32 \pm 8.43^a$ |

Stress is one of the factors that contribute to the emergence of primary diseases and mortality of reared fish. Both food and environmental changes could promote stress modifying the gut microbiota and the establishment of pathogens in the gastrointestinal tract (Tannock & Savage, 1974). Thus, it is likely that an improvement in the homeostatic level promoted by the probiotic in this study may be related to increased stability of the gut microbiota with reduction of harmful bacteria in the epithelium (Rollo *et al.*, 2006).

The intestinal lumen of the fish is lined by a simple columnar epithelium interspersed with goblet cells (Reifel & Travill, 1979). The integrity of the intestinal mucosa is a consequence of the intensity of epithelial cell renewal that determines the uniformity of the villi and the number of goblet cells (Mello *et al.*, 2013).

The increased villi height observed in the supplemented groups in this study and the lower number of pathogenic bacteria in the enterocytes could be related to the increased availability of nutrients and reduced desquamation of the epithelium. Furthermore, it is known that some proteins produced by probiotic bacteria promote increased cellular survival time of the intestinal tract due to the inhibition of apoptosis signaling induced by cytokines (Sherman *et al.*, 2009).

Mello *et al.* (2013) observed in Nile tilapia fed diets containing *Bacillus cereus* and *B. subtilis* an increasing of the area of absorption, nutrient retention and consequent improvement in growth performance and body composition. In this study, although the intestinal absorption area has grown substantially, there was no difference in performance. This can be explained due to the short evaluation time, which was not enough to show its effects in performance and proximal analysis. It can also relate to the low concentration of the probiotic or due to the adverse weather conditions recorded in the last month of the experiment in which the temperature decreased.

The production of mucus by goblet cells is an important mechanism of protection against the entry of pathogens through the intestinal tract (Ellis, 2001). Furthermore, the mucus has bactericidal effect, protects against toxic substances and aids the transport between the luminal contents and epithelial cells (Smirnov *et al.*, 2005).

It is well documented that probiotics stimulate the development of goblet cells in the intestine (Nayak, 2010; Mello *et al.*, 2013). In this study, fish that received the highest level of inclusion of probiotic showed significantly higher number of goblet cells compared to control group. This result corroborates the observations of Reda & Selim (2015) that observed growth of the villi and increased goblet cell number in fish supplemented with *B. amyloliquefaciens*.

From the results obtained, it is possible to suggest that in the tested conditions, the use of *B. amyloliquefaciens* in the diet presented good indications, increasing the size of the intestinal villi, number of goblet cells and enhancing the homeostatic state of the fish. However, it is necessary longer studies with larger concentrations of the probiotic in field conditions to asseverate that the use of this product is suitable to Nile tilapia.

**ACKNOWLEDGEMENTS**

**REFERENCES**

Association of Official Analytical Chemists (AOAC). 2000. Official methods of analysis of the Association of Official Analytical Chemists. AOAC, Arlington, 1234 pp.

Ayandiran, T.A., A.A. Ayandele, S.O. Dahunsi & O.O. Ajala. 2014. Microbial assessment and prevalence of antibiotic resistance in polluted Oluwa River, Nigeria. Egypt. J. Aquat. Res., 40, 3: 291-299.

Boyd, C. & L. Massaut. 1999. Risks associated with the use of chemicals in pond aquaculture. Aquacult. Eng., 20: 113-132.

Cao, H., S. He, R. Wei, M. Diong & L. Lu. 2011. *Bacillus amyloliquefaciens* G1: a potential antagonistic bacterium against eel-pathogenic *Aeromonas hydrophila*. Evidence-based complement. Altern. Med., pp. 1-7.

Chi, C., B. Jiang, X.-B. Yu, T.-Q, Liu, L, Xia & G.-X Wang. 2014. Effects of three strains of intestinal autochthonous bacteria and their extracellular products on the immune response and disease resistance of common carp, *Cyprinus carpio*. Fish. Shellfish Immunol., 36: 9-18.

Collier, H.B. 1944. Standardization of blood haemoglobin determinations. Can. Med. Assoc. J., 50: 550-552.

Contreras, G.E.S. 1994. Bioquímica de pescados e derivados. Funep, Jaboticabal, 409 pp.

Dahiya, T., R.C. Sihag & S.K. Gahlawat, 2012. Effect of Probiotics on the Haematological Parameters of Indian Magur (*Clarius batrachus* L.). J. Fish Aquat. Sci., 7: 279-290.

Das, A., K, Nakhro, S. Chowdhury & D, Kamilya. 2013. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). Fish Shellfish Immunol., 35: 1547-1553.

Ellis, A.E. 2001. Innate host defense mechanisms of fish against viruses and bacteria. Dev. Comp. Immunol., 25: 827-839.

Furuya, W. 2010. Tabelas brasileiras para a nutrição de tilápias. Grafica & Editora, Toledo, 100 pp.

Garcia, F., J.M. Kimpara, W.C. Valenti & L.A Ambrosio. 2014. Emergy assessment of tilapia cage farming in a hydroelectric reservoir. Ecol. Eng., 68: 72-79.

Goldenfarb, P.B., F.P. Bowyer, E. Hall & E. Brosious. 1971. Reproducibility in the hematology laboratory: the microhematocrit determination. Am. J. Clin. Pathol., 56: 35-39.

Han, Y., E. Liu, L. Liu, B. Zhang, Y. Wang, M. Gui, R. Wu & P. Li. 2015. Rheological, emulsifying and thermostability properties of two exopolysaccharides produced by *Bacillus amyloliquefaciens* LPL061. Carbohyd. Polym., 115: 230-237.

Hassaan, M.S., M.A. Soltan & M.M.R. Ghonemy. 2014. Effect of synbiotics between *Bacillus licheniformis* and yeast extract on growth, hematological and biochemical indices of the Nile tilapia (*Oreochromis niloticus*). Egypt. J. Aquat. Res., 40: 199-208.

Honorato, C.A., C.D. Cruz, D.J. Carneiro & M.R.F. Machado. 2011. Histología e histoquímica do intestino anterior de tilápia do Nilo (*Oreochromis niloticus*) alimentadas com dietas contendo silagem de peixe. Braz. J. Vet. Res. Anim. Sci., 48: 4 pp.

Kaewklom, S., S. Lumlert, W. Kraikul & R. Aunpad. 2013. Control of *Listeria monocytogenes* on sliced bologna sausage using a novel bacteriocin, amysin,

produced by *B. amyloliquefaciens* isolated from Thai shrimp paste (Kapi). Food Control, 32: 552-557.

Kersarcodi-Watson, A., H. Kaspar, M.J. Lategan & L. Gibson. 2008. Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture, 274: 1-14.

Kim, T., J. Lee, D.W. Fredriksson, J. DeCew, A. Drach & K. Moon. 2014. Engineering analysis of a submersible abalone aquaculture cage system for deployment in exposed marine environments. Aquacult. Eng., 63: 72-88.

Kumar, R., S.C. Mukherjee, K.P. Prasad & A.K. Pal. 2006. Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). Aquacult. Res., 37: 1215-1221.

Lee, J., S. Rheem, B. Yun, Y. Ahn, J. Joung, S.J. Lee, S. Oh, T. Chun, I. Rheem, H.S. Yea, K.S. Lim, J.M. Cha & S. Kim. 2013. Effects of probiotic yoghurt on symptoms and intestinal microbiota in patients with irritable bowel syndrome. Int. J. Dairy Technol., 66: 243-255.

Loncar, N., N. Gligorijević, N.A. Božić & Z. Vujčić. 2014. Congo red degrading laccases from *Bacillus amyloliquefaciens* strains isolated from salt spring in Serbia. Int. Biodeter. Biodegr., 91: 18-23.

Mahdhi, A., M. Esteban, Z. Hmila, K. Bekir, F. Kamoun, A. Bakhrouf & B. Krifi. 2012. Survival and retention of the probiotic properties of *Bacillus* sp. strains under marine stress starvation conditions and their potential use as a probiotic in *Artemia* culture. Res. Vet. Sci., 93: 1151-1159.

Marcusso P., J. Yunis, G. Claudiano, S. Eto, D. Fernandes, H. Mello, F. Marinho Neto, R. Salvador, J.R. Moraes & F.R. Moraes. 2015. Influence of temperature on *Streptococcus agalactiae* infection in Nile tilapia. Braz. J. Vet. Res. Anim. Sci., (52)1: 57-62.

Martinez-Porchas, M., L.R. Martinez-Cordova & R. Ramos-Enriquez. 2009. Cortisol and glucose: reliable indicators of stress? Pan-Am. J. Aquat. Sci., 4: 158-178.

Mello, H.D., J.R.E. Moraes, I.G. Niza, F.R.D. Moraes, R.O.A. Ozório, M.T. Shimada, J.R. Engracia Filho & G.S. Claudiano. 2013. Efeitos benéficos de probióticos no intestino de juvenis de Tilápia-do-Nilo. Pesq. Vet. Brasil., 33: 724-730.

Mohapatra, S., T. Chakraborty, A. Prusty, K. PaniPrasad & K. Mohanta. 2014. Beneficial effects of dietary probiotics mixture on hemato-immunology and cell apoptosis of *Labeo rohita* fingerlings reared at higher water temperatures. PLoS ONE, 9(6): 1-9.

Nayak, S. 2010. Probiotics and immunity: a fish perspective. Fish Shellfish Immunol., 29: 2-14.

Nikinmaa, M., A. Soivio, T. Nakari & S. Lindgren. 1983. Hauling stress in brown trout (*Salmo-trutta*): physiological responses to transport in fresh-water or salt-water, and recovery in natural brackish water. Aquaculture, 34: 93-99.

Priest, F., M. Goodfellow, L. Shute & R. Berkeley. 1987. *Bacillus amyloliquefaciens* sp. nov., nom rev. Int. J. Syst. Bacteriol., 37: 69-71.

Ran, C., A. Carrias, M. Williams, N. Capps, B. Dan, J. Newton, J. Kloepper, E. Ooi, C. Browdy, J. Terhune & M. Liles. 2012. Identification of *Bacillus strains* for biological control of catfish pathogens. PLoS ONE, 7(9): 1-8.

Reda, R. & K. Selim. 2015. Evaluation of *Bacillus amyloliquefaciens* on the growth performance, intestinal morphology, hematology and body composition of Nile tilapia, *Oreochromis niloticus*. Aquacult. Int., 23: 203-217.

Reifel, C.W. & A.A. Travill. 1979. Structure and carbohydrate histochemistry of the intestine in ten teleostean species. J. Morphol., 162: 343-359.

Ridha, M. & I. Azad. 2012. Preliminary evaluation of growth performance and immune response of Nile tilapia *Oreochromis niloticus* supplemented with two putative probiotic bacteria. Aquacult. Res., 43: 843-852.

Rollo, A., R. Sulpizio, M. Nardi, S. Silvi, C. Orpianesi, M. Caggiano, A. Cresci & O. Carnevali. 2006. Live microbial feed supplement in aquaculture for improvement of stress tolerance. Fish. Physiol. Biochem., 32: 167-177.

Sanger, F., S. Nicklen & A. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. P. Natl. Acad. Sci. Usa., 74: 5463-5467.

Sherman, P., J. Ossa & K. Johnson-Henry. 2009. Unraveling mechanisms of action of probiotics. Nutr. Clin. Pract., 24: 10-14.

Simões, L.N., A.T.M. Gomide, V.M.F. Almeida-Val, A.L Val & L.C Gomes. 2012. O uso do óleo de cravo como anestésico em juvenis avançados de tilápia do Nilo (*Oreochromis niloticus*). Acta Sci. Anim. Sci., 34: 175-181.

Smirnov, A., R. Perez, E. Amit-Romach, D. Sklan & Z. Uni. 2005. Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. J. Nutr., 135: 187-192.

Soltan, M.A. & S.M.M. El-Laithy. 2008. Effect of probiotics and some spices as feed additives on the performance and behaviour of Nile tilapia, *Oreochromis niloticus*. Egypt. J. Aquat. Biol. Fish., 12(2): 63-80.

Tannock, G. & D. Savage. 1974. Influences of dietary and environmental stress on microbial populations in murine gastrointestinal-tract. Infect. Immunol., 9: 591-598.

Tapia-Paniagua, S.T., S. Vidal, C. Lobo, M.J. Prieto-Álamo, J. Jurado, H. Cordero, R. Cerezuela, I. García de la Banda, M.A. Esteban, M.C. Balebona & M.A. Moriñigo. 2014. The treatment with the probiotic *Shewanella putrefaciens* Pdp11 of specimens of *Solea senegalensis* exposed to high stocking densities to enhance their resistance to disease. Fish Shellfish Immunol., 41: 209-221.

Telli, G., M. Ranzani-Paiva, D. Dias, F. Sussel, C. Ishikawa & L. Tachibana. 2014. Dietary administration of *Bacillus subtilis* on hematology and non-specific immunity of Nile tilapia *Oreochromis niloticus* raised at different stocking densities. Fish Shellfish Immunol., 39: 305-311.

Wang, L., F. Lee, C. Tai & H. Kuo. 2008. *Bacillus velezensis* is a later heterotypic synonym of *Bacillus amyloliquefaciens*. Int. J. Syst. Evol. Microbiol. 58: 671-675.

Wedemeyer, G. 1970. Stress of anesthesia with ms-222 and benzocaine in rainbow trout (*Salmo gairdneri*). J. Fish. Res. Bd. Can., 27: 909 pp.

Wu, H-J., L-B. Sun, C-B. Li, Z-Z. Li, Z. Zhang, X-B. Wen, Z. Hu, Y-L. Zhang & S-K. Li. 2014. Enhancement of the immune response and protection against *Vibrio parahaemolyticus* by indigenous probiotic *Bacillus strains* in mud crab (*Scylla paramamosain*). Fish Shellfish Immunol., 41: 156-162.

Yadav H., J. Shalini & P.R. Sinha. 2007. Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. Nutrition, 23: 62-68.

Ziaei-Nejad, S., M. Rezaei, G. Takami, D. Lovett, A. Mirvaghefi & M. Shakouri. 2006. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture, 252: 516-524.