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Growth performance and intestinal histomorphology of Nile tilapia juveniles fed probiotics

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ABSTRACT. The objective of this study was to assess the growth performance and the histomorphometric characterization of the middle intestine of GIFT strain Nile tilapia, *Oreochromis niloticus* juveniles fed probiotics, added either before or after the feed processing. The experimental design was completely randomized, with five treatments and five replications: pelleted feed without any probiotic; pelleted feed with inclusion of probiotic before the processing; pelleted feed with inclusion of probiotic after the processing; extruded feed without any probiotic and extruded feed with inclusion of probiotic after the processing. Two hundred and fifty juveniles were distributed in 25 aquaria (20L) and fed for 63 days. Differences in the mean values of total weight were found at the end of the experiment. After 42 days of feed intake, significant differences in feed conversion were verified for treatments with extruded diets when compared to the pelleted ones. The fishes fed diets supplemented with probiotic presented increase in the thickness of the epithelium of the intestine. The best zootechnical performance was observed in the extruded diet supplemented with probiotic added after the feed processing. The inclusion of 4.0 x 10^8 CFU g^{-1} of *Bacillus subtilis* and 4.0 x 10^8 CFU g^{-1} of *Bacillus toyoi* in both the pelleted and the extruded diets promoted an increase in the thickness of the epithelial layer of the middle intestine.

Keywords: *Bacillus*, intestine, feed processing, supplement.

Desempenho produtivo e histomorfologia intestinal de juvenis de tilápia do Nilo alimentados com probióticos

RESUMO. Objetivou-se avaliar o desempenho zootécnico e a caracterização histomorfométrica do intestino médio de juvenis de tilápias do Nilo, *Oreochromis niloticus*, variedade GIFT, alimentadas com probiótico, incluído antes e após o processamento de ração. O delineamento experimental foi inteiramente casualizado com cinco tratamentos e cinco repetições composto pelos seguintes tratamentos: ração peletizada sem probiótico; ração peletizada com inclusão do probiótico antes e após o processamento; ração extrusada sem probiótico e ração extrusada com inclusão do probiótico após o processamento. Duzentos e cinqüenta juvenis foram distribuídos em 25 aquários (20L) e alimentados durante 63 dias. Foram constatadas diferenças para os valores médios do peso total no final do experimento. Após 42 dias de alimentação foram verificadas diferenças significativas na conversão alimentar para tratamentos com as dietas extrusadas comparadas com as peletizadas. Os peixes alimentados com as dietas suplementados com o probiótico apresentaram aumento na espessura da camada epitelial do intestino. O melhor desempenho zootécnico foi observado, na dieta extrusada suplementada de probiótico incorporado após o processamento de ração, e a inclusão de 4.0 x 10^8 CFU g^{-1} of *Bacillus subtilis* e 4.0 x 10^8 CFU g^{-1} of *Bacillus toyoi*, em dietas peletizadas e extrusadas, promoveu o aumento na espessura da camada epitelial do intestino médio.

Palavras-chave: *Bacillus,* intestino, processamento de ração, suplemento.

Introduction

Professionals of the private sector connected to educational and research institutions strive to improve the viability of functional food such as probiotics. Probiotics are live microorganisms that act beneficially in the host, promoting the balance of the intestinal microbiota, favoring the health of the animals (FULLER, 1989). Nayak (2010) stated that an ideal

probiotic, irrespective of its source must be able to colonize and multiply in the intestine of the host.

Several studies have exhibited promising results of the use of probiotics in fish, mollusk, crustacean and amphibian farming (VERSCHUERE et al., 2000; RINGO; GATESOUPE, 1998; IRIANTO; AUSTIN, 2002; BALCÁZAR et al., 2006; DIAS et al., 2008; KESARCODI-WATSON et al., 2008; EL-RHMAN et al., 2009; ZHOU et al., 2009), which enables the probiotics to substitute the antibiotics as growth promoters. In fish, they are usually administered orally in order to improve the microbial flora of the intestine (NAGESWARA; BABU, 2006; SAHU et al., 2008).

The genera of probiotics most frequently used in the culture of aquatic organisms are the bacteria *Lactobacillus*, *Bacillus*, *Enterococcus* and the yeast *Saccharomyces* (DIAS et al., 2008, 2009; ALY et al., 2008; EL-RHMAN et al., 2009; MEURER et al., 2006; WANG et al., 2008).

Bacillus toyoi and *B. subtilis,* whose main advantage is the ability to sporulate, providing higher survival rates during the intestinal transit, were used in the present study (HOA et al., 2000). Sweeteman et al. (2008) demonstrated that the function of the intestinal tract and its efficiency are fundamental for a successful commercial production of fish species, and also that the histological study of the intestine is important in establishing the status of structural integrity. After administration, there are ways to assess the effects of probiotics given to fish that colonize the intestinal epithelium: the nutritional contributions of the bacteria, stimulating immune function (TINH et al., 2008), animal welfare and growth performance (CARNEVALI et al 2004; MERRIFIELD et al., 2010).

The objective of this experiment was to assess the influence of the different ways of inclusion of probiotic in the feed processing on the growth performances and the histomorphology of the intestine of Nile tilapia, *Oreochromis niloticus*, juveniles kept under laboratorial conditions.

Material and methods

The experiment was carried out at the Laboratory of Pathology of Aquatic Organisms of the Instituto de Pesca, APTA, S. A. A., São Paulo, São Paulo State, Brazil. One thousand reversed Nile tilapia, *Oreochromis niloticus* juveniles (batch 100% male) belonging to the GIFT strain were placed in two 500 L tanks. A completely randomized design (CRD) was established, with five treatments and five replications, totalizing 25 plots. The experimental unit consisted of 10 fish.

After 15 days of acclimation, 250 fish fed commercial feed containing 36% of gross energy, with mean initial weight and total length of 5.23 ± 0.40 g and 6.90 ± 0.23 cm, respectively, were selected and distributed randomly in twenty-five 20 L aquaria, equipped with continuous aeration and an individual filtering system.

The water quality parameters: temperature, dissolved oxygen and pH were monitored daily with a multiparameter Hanna HI 9828. Ammonia was monitored weekly by chemical analysis.

The probiotic used was a lyophilized product of *Bacillus* supplied by IMEVE Biotecnology Ltd, Brazil. It is composed of *Bacillus subtilis* 4.0 x 10^8 CFU g^{-1} and *Bacillus toyoi* 4.0 x 10^8 CFU g^{-1} and the dose recommended dose by the producer is 4 g kg^{-1} of feed, the commercial name is PAS TR®.

The probiotic was added to the diet for the experimental test during the process of pelletization. After the processing (pelletization and extrusion), and oily vehicle (2% soybean oil) was added. The dietary formulations and proximate composition of the experimental diets can be found in Table 1. The rations were formulated based on the data presented in (NRC, 1993). The treatments followed the experimental design shown in Table 2.

The fish were fed experimental diets three times a day (KUBITZA, 1999), at 8 a.m., 2 p.m. and 6 p.m. for 63 days, at a proportion of 1% of live weight.

Table 1. Dietary formulations and proximate composition of the experimental diets of Nile tilapia juveniles after 63 days of experiment.

Ingredients (%)	Basal diet	
	Without probiotic	With probiotic
Soybean Meal	45.10	45.10
Wheat Meal	21.24	21.24
Corn Meal	10.45	10.05
Fish Meal	10.00	10.00
Corn Gluten	4.00	4.00
Dicalcium Phosphate	3.80	3.80
Vitamin and Mineral Premix	0.50	0.50
L – Lysine	0.40	0.40
DL – Methionine	0.41	0.41
Vitamin C	0.08	0.08
BHT	0.02	0.02
Probiotic	0.00	0.40
Total	100.00	100.00
Chemical composition		
Dry Matter (%)		86.52
Crude Protein (%)		36.00
Crude Protein (%)		4.58
Crude Fiber (%)		8.43
Mineral Matter (%)		13.48
Crude Energy (kg cal^{-1})		3,524.00

Table 2. Treatments used of Nile tilapia juveniles after 63 days of experiment.

Treatments	Processing	Addition of probiotic	Addition of oil
T1	Pelleted control	-	4 g kg^{-1}
T2	Pelleted	Before	4 g kg^{-1}
T3	Pelleted	After	4 g kg^{-1}
T4	Extruded control	-	4 g kg^{-1}

T5	Extruded	After	4 g kg^{-1}

The fish were anesthetized in solution of benzocaine 100 mg L^{-1} for the evaluation of the growth performance according to the ethical principles of animal manipulation established by the Brazilian School of the Animal Experimentation (COBEA, 2010). The fish were weighed and measured every 21 days for the determination of the following growth performance parameters: Weight (W) = Total weight; Length (L) = Total length; Daily Feed Consumption (DC) = Final consumption – Initial consumption/number of days; Weight gain (WG) = Final weight – Initial weight; Apparent feed-conversion (AFC) = Feed consumption/Weight gain; Survival (S) = 100 * (initial number of fishes – final number of fishes)/initial number of fishes.

In order to analyze the histomorphological structure of the intestine, one fish from each replication was used, totalizing five Nile tilapia juveniles per treatment. The fish were anesthetized, euthanized by anesthetic deepening, had their bone marrow dissected, and were then submitted to a longitudinal abdominal incision for organ exposure.

Fragments of the middle portion of the intestines were removed with sterilized surgical instruments and fixed in 10% buffered formalin solution for 48 hours. The samples were then transferred to alcohol 70% G.L., and remained thus conserved until the moment of inclusion. At the Laboratory of Fish Endocrinology of the University of São Paulo (USP), the fragments were submitted to gradual dehydration in a graded alcohol series, diafanized in xylene and embedded in paraffin. The blocks obtained were then cut in 5.0 μm-thick sections, placed in histological slides stained with hematoxylin-eosin (HE) (MAIA, 1979), and analyzed under light microscope.

In order to determine the height of the intestinal epithelium, the villi were measured according to the structural characteristics. Thus, two villi per histological section were selected in 75 slides and 30 measurements per treatment were performed. The images were obtained with digital camera attached to a photomicroscope and analyzed by the program Image J (40X). Image J is an image processing and analysis software, developed by Wayne Rasband at the National Institute of Mental Health, USA.

In order to verify the results of the growth performance, histomorphological paremters of the intestine and water quality parameters in the different treatments, an analysis of variance was used, followed by the Bartlett's test to verify the homogeneity of the data, and the Tukey test to establish differences between the treatments. The results were considered significant when $p < 0.05$ (ZAR, 2009).

Results and discussion

The means and standard deviation of the values of the water quality parameters during the experimental period were: temperature = 27.56 ± 0.54°C; pH = 7.81 ± 0.41; and dissolved oxygen = 5.10 ± 0.39 mg L^{-1}. Those parameters did not show significant differences $p < 0.05$, indicating that water quality remained constant throughout the 63 days, and were within the optimum levels recommended for fishes (SIPAÚBA-TAVARES, 1995). The levels of ammonia (NH_3) were kept at ≤ 1.0 ppm due to the constant water renewal and biological filtering, as recommended by Kubitza (2003).

The indices of growth performance of the Nile tilapia juveniles are presented in Table 3. Baccarin and Pezzato (2001), as well as Castro and Cervon (2004), working with Nile tilapia fries and juveniles fed diets supplemented with yeast *Saccharomyces cerevisiae* showed increase in the values of weight. Therefore, it may be stated that the product was effective and consequently, that the fish presented better development than in the other treatments, indicating that the use of probiotic is beneficial for the production of the species.

The results presented by Rodrígues and Fernandes (2006) differed from the ones found in the present study because those authors evaluated different feed processing for the freshwater angelfish, *Pterophilum scalare,* where the mean values of consumption means were higher in fishes fed the extruded diet.

Pezzato et al. (2002) studying the diet processing for Nile tilapia, observed higher feed-conversion and productive performance in fish that had received extruded feed when compared to the pelleted one. According to Andriguetto et al. (1981) the extrusion of the feed increases the alimentary efficiency of the fish and improves the palatability of the ingredients, generating a satisfactory performance of the animals. Although the results are different from the ones found in literature, it has been proven that the use of the product is favorable to the culture of aquatic organisms.

The data of weight gain were similar to the ones found by Meurer et al. (2006), who did not observe increase in this parameter for tilapia fed *Saccharomyces cerevisiae* for 30 days. The same was described by Suzer et al. (2008) with gilthead seabream, *Sparus aurata* larvae fed *Lactobacillus* ssp. Lara-Flores et al. (2003), on the other hand,

emphasized an improvement in the zootechnical performance in studies with tilapia using probiotics in different ways, included in the feed and in the water, respectively. In the present study, the product did not influence weight gain.

The mean values of survival rates did not show significant differences ($p < 0.05$) between the biometries, ranging between 80 and 100%. Similar data were described by Meurer et al. (2006) in studies with the inclusion of live yeast, demonstrating that there was no influence on the Nile tilapia larvae, and also by Makridis et al., (2000), who observed differences in the survival rates with the addition of two bacterial strains in the diet during the larviculture of *Scophthalmus maximus*.

Table 3. Parameters of growth performance of Nile tilapia juveniles after 63 days of experiment.

Treatments	W (g)	L (cm)	DC (g)	WG (g)	AFC	S (%)
T1	18.50 ± 6.54^{a}	10.16 ± 1.10^{a}	3.21 ± 0.16^{a}	2.23 ± 0.10^{a}	1.43 ± 0.02^{ab}	100.00 ± 0.00^{a}
T2	15.66 ± 6.00^{a}	9.55 ± 1.14^{a}	2.43 ± 0.67^{a}	1.68 ± 0.48^{a}	1.45 ± 0.04^{ab}	97.14 ± 6.39^{a}
T3	19.19 ± 6.10^{a}	10.11 ± 1.10^{a}	2.84 ± 0.14^{a}	1.98 ± 0.10^{a}	1.43 ± 0.02^{ab}	97.14 ± 6.39^{a}
T4	23.46 ± 8.92^{b}	10.79 ± 1.22^{a}	3.37 ± 0.81^{a}	2.42 ± 0.59^{a}	1.40 ± 0.02^{bc}	100.00 ± 0.00^{a}
T5	25.60 ± 8.37^{b}	11.26 ± 1.37^{a}	3.12 ± 1.47^{a}	2.29 ± 1.10^{a}	1.37 ± 0.03^{c}	97.14 ± 6.39^{a}

Different letters in the column are statistically different according to the Tukey test ($p < 0.05$). Total weight (W); Total length (L); Daily Consumption (DC); Weight gain (WG); Apparent Feed-Conversion (AFC) and Survival (S)

However, these values are different from the ones presented by Carnevali et al. (2004) for the survival of *Sparus aurata* larvae, where a positive effect of the addition of probiotics was verified in an association between *Lactobacillus plantarum* and *L. fructivorans*.

Due to those high indices, it can be stated that the product was efficient under the conditions of the present experiment.

As for the histomorphology of the middle portion of the intestine, in the present study, the control treatments did not show significant differences ($p < 0.05$) when compared to each other (Table 4). The fish fed diets with probiotics (T2, T3 and T5) showed significant differences in the height of the epithelial layer of the villi of the middle portion of the intestine, when compared to the control treatments (Table 4). That fact shows that the probiotic promoted an increase in tha epithelial layer of the middle intestine of those fis, coinciding with the studies described by Medri et al. (1999) for the same species, but fed yeast. The same observations were confirmed by Silva et al. (2005) with *Steindachnerina notonota*.

Table 4. Mean thickness ($\bar{x}$) and standard deviation (s) of the epithelial layer (μm) of the middle portion of the intestine of Nile tilapia juveniles after 21, 42 and 63 days.

Treatments	Biometries (days)		
	21st	42nd	63rd
T1	26.92±3.44^{a}	28.55±2.77^{a}	27.91±2.89^{a}
T2	31.66±4.33^{b}	32.04 ±3.32^{b}	31.36±4.71^{b}
T3	32.01±4.54^{b}	31.53 ±4.09^{b}	32.63±4.17^{b}
T4	26.37±3.17^{a}	28.56 ±3.01^{a}	27.43±3.36^{a}
T5	32.09±3.76^{b}	32.69±3.50^{b}	32.94±3.37^{b}

Different letters are different to each other in the column according to the Tukey test ($p < 0.05$).

Figure 1 represents the cross sections where it is possible to identify the villi of the intestine of fish fed diets supplemented with probiotic or without it.

According to Hisano et al. (2006), when studying whole yeast and its by-products, the morphological changes in the intestine occurred due to the nucleotides present in the diet, influencing the intestinal tract and its microbiota. It is important to notice that the alterations show a beneficial nature to the morphological characteristics of the intestinal tract, promoting an increase in the surface area for absorption in the mucosa of Nile tilapias fed diet supplemented with yeast.

The rainbow trout also exhibited an increase in the height and thickness of the intestinal villi when fed soybean protein, a vegetal nutrient rich in structural polysaccharides (ESCAFFRE et al., 2007).

Burrells et al. (2001) studying the Atlantic salmon, *Salmo salar* emphasized the differentiated morphological responses in the intestines of fish fed diets supplemented with nucleotides. The authors reported that the mean values of length of the proximal, middle and distal portion of the intestine, as well as the total surface of the intestine were statistically higher than in the fishes that had received the control feed.

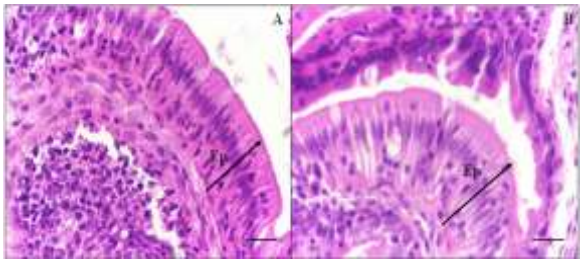

Figure 1. Photomicrographs of the epithelial layer of the villi of the middle intestine of Nile tilapia juveniles: (A) Control treatment; (B) Treatment supplemented with probiotic; (Ep) Epithelium; (→)

Thickness. (Bar = 10 μm). Stain: HE.

Salinas et al. (2008), studying the supplementation of *Lactobacillus delbrueckii*, and Ringo et al. (2010) working with lactic acid bacteria, evaluated the effect of the probiotics on the intestinal epithelium of the salmonides. Merrifield et al. (2010) showed improvements in the morphology of the intestinal microvilli of the salmonides when fed probiotics, and Sweetman et al. (2008) attested that the interactions between the intestinal microflora, morphology of the intestine, immune system and absorption of nutrients may influence on the health and performance of the fishes.

Conclusion

The inclusion of 4 g kg^{-1} of probiotic in both pelleted and extruded diets promoted an increase in the thickness of the epithelial layer of the middle intestine of tilapia juveniles. The best zootechnical performance was observed in the extruded diet supplemented with probiotic added after the feed processing.

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