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Dietary mannan oligosaccharide and *Bacillus subtilis* in diets for Nile tilapia (*Oreochromis niloticus*)

Rafael Vieira de Azevedo[1*], João Carlos Fosse Filho[2], Samuel Louzada Pereira[2], Leonardo Demier Cardoso[2], Dalcio Ricardo de Andrade[2] and Manuel Vazquez Vidal Júnior[2]

*[1]Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, Av. Conde D'Eu, 344, 29670-000, Ibiraçu, Espírito Santo, Brazil. [2]Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Ciências e Tecnologias Agropecuárias, Campos dos Goytacazes, Rio de Janeiro, Brazil. *Author for correspondence. E-mail: azevedorv84@gmail.com*

ABSTRACT. A six week study was conducted to investigate the supplementation of prebiotic (Mannan oligosaccharide – MOS, from yeast *Saccharomyces cerevisiae*), probiotic (*Bacillus subtilis* – BS, C-3102 strain) and their combination in diets for Nile tilapia. 192 fishes (4.03 ± 0.28 g) were distributed into 16 tanks (40-L), in a completely randomized design (n=4). The following treatments were evaluated: control; prebiotic - 2 g MOS kg^{-1}; probiotic - 2 g BS kg^{-1} and synbiotic - 1 g MOS kg^{-1} plus 1 g BS kg^{-1}. Fishes fed diets pre-, pro- and synbiotic supplemented performed better in average daily gain, feed conversion rate, specific growth rate, protein efficiency ratio, carcass yield, total and standard length and body height than those maintained on control diets. The probiotic supplementation resulted in higher villus height and intestinal perimeter ratio than the control diet while the pre- and synbiotic supplementation in diets resulted in higher intestinal perimeter ratio. Carcass protein and ether extract were, respectively, higher and lower in fish fed synbiotic diets than other fish. The results of this study indicated that the mannan oligosaccharide and *Bacillus subtilis* supplementation, isolated or combined (synbiotic), could improve growth, body index, intestine morphometry and carcass composition in Nile tilapia.

Keywords: Aquaculture, prebiotic, probiotic, synbiotic, tilapia farming.

Mananoligossacarídeo e *Bacillus subtilis* em dietas para tilápia do Nilo (*Oreochromis niloticus*)

RESUMO. Um estudo de seis semanas foi conduzido para investigar a suplementação de prebiótico (mananoligossacarídeo – MOS, oriundo da levedura *Saccharomyces cerevisiae*), probiótico (*Bacillus subtilis* – BS, cepa C-3102) e sua combinação em dietas para tilápia do Nilo. Foram distribuídos 192 peixes (4,03 ± 0,28 g) em 16 tanques (40-L) em delineamento inteiramente casualizado (n=4). Avaliaram-se os tratamentos: controle, 2 g MOS kg^{-1}, 2 g BS kg^{-1} e 1 g MOS kg^{-1} mais 1 g BS kg^{-1} (simbiótico). As rações prebiótico, probiótico e simbiótico resultaram em melhores média de ganho diário, conversão alimentar, taxa de crescimento específico, eficiência protéica, rendimento de carcaça, comprimentos total, padrão e altura comparando-se a ração controle. A ração probiótico resultou em maiores altura de vilosidades e relação entre perímetros intestinais que peixes alimentados com ração controle, enquanto rações prebiótico e simbiótico resultaram em peixes com maior relação entre perímetros intestinais que peixes alimentados com ração controle. A ração simbiótica resultou em peixes com, respectivamente, maiores e menores porcentagens de proteína e extrato etéreo na carcaça. Os resultados sugerem que a suplementação de mananoligossacarídeo e *Bacillus subtilis*, isolado ou combinado (simbiótico), melhorou o crescimento, os índices corporais, a morfometria intestinal e a composição da carcaça em tilápia do Nilo.

Palavras-chave: Aquicultura, cultivo de tilápias, prebiótico, probiótico, simbiótico.

Introduction

Tilapias are the second most farmed freshwater fish in the world after carps, mainly due to features such as ability to reproduce in captivity, rusticity, fast growth rate, feed on a low trophic and good flesh quality. The Nile tilapia (*Oreochromis niloticus*) occupies a prominent position in the Brazilian fish farming, representing around 43% of production in (Instituto Brasileiro de Geografia e Estatística [IBGE], 2014).

The rapid expansion and intensification of fish farming, combined with the increase in intensive production strategies at higher densities, have resulted in the emergence of diseases that cause considerable economic losses and hinder the sustainable development of the industry (Rico et al., 2014).

Antibiotics have been used to prevent and treat diseases in aquatic animals and sub-therapeutic dosages have often been used for promoting growth. However, the antibiotics use may result into the

development of resistant bacteria, presence of antibiotic residues in the flesh and destruction of the microbial population in the aquatic environment (Marques et al., 2005). As a result, various alternative strategies for the antibiotic use have been proposed, among them the use of pre- and probiotics.

Prebiotics are non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon selectively (Gibson & Roberfroid, 1995).

Probiotics are live microorganisms which when administered in suitable amounts; confer benefits to the health of the host by improving the balance of the intestine microbiota (Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000). Synbiotic are the combination of prebiotics and probiotics that beneficially affects the host by improving the survival rate and modulation of microbial community in the gastrointestinal tract, by selectively stimulating the growth or by activating the metabolism of one or a limited number of beneficial bacteria (Gibson & Roberfroid, 1995).

One of the most common prebiotics used in animal nutrition is the mannan oligosaccharide (MOS). In aquaculture, this prebiotic demonstrates to improve the growth performance, survival, feed utilization, non-specific immunity and disease resistance (Burr, Hume, Ricke, Nisbet, & Gatlin III, 2008; Liu et al., 2013; Safari, Shahsavani, Paolucci, & Atash, 2014). Among the probiotics commonly used, the genus *Bacillus* is one of the most extensively evaluated as aquaculture feed supplements and has been demonstrated to improve a number of attributes when supplemented in diets of aquatic organisms (Azevedo, Fosse Filho, Pereira, Andrade, & Júnior, 2016; Geng et al., 2012; Ghosh, Sinha, & Sahu, 2008; Giri, Sukumaran, Sen, & Jena, 2014; Gupta, Gupta, & Dhawan, 2014).

Despite much information on the use of single pre- and probiotics, less information is found on their combined effects. However, the results showed that might yield better results than the individual application of pre- and probiotics (Azevedo et al., 2015; Rodriguez-Estrada, Satoh, Haga, Fushimi, & Sweetman, 2009; Zhang et al., 2013).

This study was carried out to evaluate the effect of single or combined supplementation of MOS and *B. subtilis* on the growth, body indices, intestine morphometry and carcass composition in Nile tilapia (*Oreochromis niloticus*).

Material and methods

As prebiotic, a MOS derived from the cell wall of the yeast *Saccharomyces cerevisiae* was evaluated. As the probiotic microorganism, spores of BS C-3102 strain in powder were used (1×10^{10} CFU per gram of product). The synbiotic was formed from a mixture (1:1) of MOS and BS.

The study was conducted at the Sector of Aquaculture of the *Laboratório de Zootecnia e Nutrição Animal* at the *Universidade Estadual do Norte Fluminense Darcy Ribeiro* (Campos dos Goytacazes, Rio de Janeiro, Brazil). A total of 192 Nile tilapia juvenile GIFT strain (4.03 ± 0.28 g) were adapted to a commercial diet and experimental facilities before the beginning of feeding trial for one week. Fishes were randomly distributed into 16 plastic tanks (40-L), kept indoors in a recirculation water system, in a completely randomized design and four replications. The following treatments were evaluated: control; prebiotic - 2 g MOS kg^{-1}; probiotic - 2 g BS kg^{-1} and synbiotic - 1 g MOS kg^{-1} plus 1 g BS kg^{-1}.

Water quality parameters such as temperature, pH and dissolved oxygen were measured daily and weekly ammonia-N. During the experimental period, temperature ranged over 28.2 ± 1.3°C, pH was 6.8 ± 0.8, dissolved oxygen 4.2 ± 0.5 mg L^{-1} and ammonia-N remained below 0.1 mg L^{-1}. The tanks were daily cleaned through siphoning out the material residues (fish feces and uneaten food).

Four diets were formulated and supplemented or not with pre-, pro- and synbiotic (Table 1).

MOS and BS were added to the basal diet at the expense of wheat flour. Dietary ingredients were ground into powder (0.5 mm), thoroughly mixed and blended oil and water to form dough. The dough was passed through a pelletizer-making device to obtain 3-mm diameter pellets. Pellets were dried in an oven at 50°C and stored in freezer at -20°C until use.

The feeding trial was conducted for six weeks and fish were fed to apparent satiation four times daily (08.00, 11.00, 14.00 and 17.00 hours).

At the end of the experiment, fish were starved for 24 hours. All surviving fish were anesthetized with Benzocaine (10 mg L^{-1}), weighed (0.01 g) and measured (0.01 cm) as for the body total and standard lengths and height (Pires et al., 2011). Eight fish from each treatment were randomly selected and killed by Benzocaine overdose for the analysis of body indexes, intestine morphometric analysis and carcass composition.

The liver and viscera were removed, separated and weighed. Fish carcasses were weighed for calculation of the carcass yield and stored in freezer at -20°C until carcass composition analysis. Also, gut samples were fixed in 10% neutral-buffered formalin for 24 hours and then transferred into 70% ethanol until histological examination.

Table 1. Composition of experimental diets of natural basis

Ingredient (g kg^{-1})	Control	Prebiotic	Probiotic	Synbiotic
Soybean meal	410.0	410.0	410.0	410.0
Wheat flour	250.0	248.0	248.0	248.0
Corn meal	200.0	200.0	200.0	200.0
Fish meal	79.7	79.7	79.7	79.7
Corn flour	34.0	34.0	34.0	34.0
Soybean oil	16.1	16.1	16.1	16.1
Supplement mineral and vitamin[1]	10.0	10.0	10.0	10.0
Mannan oligosaccharide	-	2.0	0.0	1.0
Bacillus subtilis	-	-	2.0	1.0
Antioxidant BHT	0.2	0.2	0.2	0.2
Proximate composition				
Dry matter (g kg^{-1})[2]	864.66	867.52	867.52	865.71
Crude protein (g kg^{-1})[2]	276.64	276.61	272.32	275.58
Crude energy (kcal kg^{-1})[3]	4114	4106	4111	4098
Crude fiber (g kg^{-1})[2]	49.79	49.60	49.31	49.40
Crude lipid (g kg^{-1})[2]	44.69	44.62	44.33	44.55
Mineral matter (g kg^{-1})[2]	127.72	138.28	143.43	152.38

[1]Composition kg^{-1}: Mg – 2,600 mg; Zn – 14,000 mg; Fe – 10,000 mg; Cu – 1,400 mg; Co – 20 mg; I – 60 mg; Se – 60 mg; Vit. A – 1,000,000 UI; Vit. D3 – 400.00 UI; Vit. E – 10,000 mg; Vit. K3 – 500 mg; Vit. B1 – 2,500 mg; Vit. B2 – 2,500 mg; Vit. B6 – 2,500 mg; Vit. B12 – 3,000 mcg; Vit. C – 35,000 mg; Folic acid – 500 mg; Pantothenic acid – 5,000 mg; Niacin – 10,000 mg; Biotin – 80,000 mcg; Choline – 200,000 mg; Methionine – 130 g; Inositol – 5,000 mg; Etoxiquin – 15,000 mg. [2]Analyzed according AOAC (2005). [3]Value analyzed by calorimetric bomb (1341 Parr Instrument Company, IL, USA).

Growth, feed utilization parameters and survival rate were determined as follows: daily feed intake (DFI) = 100 x (total feed intake / experimental period); average daily gain (ADG) = 100 x (final body weight – initial body weight) / experimental period; feed conversion rate (FCR) = feed consumed / weight gain; specific growth rate (SGR) = 100 x (ln final weight – ln initial weight) / experimental period; protein efficiency ratio (PER) = weight gain / protein intake and survival rate (SUR) = 100 x (number of fish remaining / initial number of fish).

Besides the total length (TL), standard length (SL) and height (H), body indexes were determined as follows: carcass yield (CY) = 100 x (fish weight without viscera / total fish weight); hepatosomatic index (HSI) = 100 x (liver weight / total weight) and viscerosomatic index (VSI) = 100 x (viscera weight / total weight).

Segments of distal gut were embedded in paraffin, sliced transversely into 5-μm sections and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (E200, Nikon, Tokyo, Japan) equipped with a camera for image capture. Villus height (VH) and width (VW) and perimeter ratio (PR) = 100 x (internal perimeter / external perimeter of the intestinal lumen) (Sweetman et al., 2007) were measured with an analysis software package Infinity Analyze®. For each diet, twenty micrographs originating from eight fish were analyzed.

Final fish carcass samples were pooled by treatment, homogenized and oven-dried at 55ºC prior the carcass composition analysis according to the procedures of the AOAC (2005). Moisture (MO) was measured gravimetrically by oven-drying of homogenized samples at 105ºC by 24 hours; crude protein (CP) by the micro-Kjeldahl method (N x 6.25); ether extract (EE) of samples were extracted with ether using an automatic Soxtec system and mineral matter (MM) by incineration in a muffle at 550ºC for five hours. Crude energy (CE) was measured using a Calorimeter Bomb (1341 Parr Instrument Company, IL).

Date on growth, feed utilization, survival, body indices, intestine morphometry and carcass composition are presented as means ± SD. The SAS® software version 9.0 was used to conduct one-way ANOVA to determine the effects of pre-, pro- and synbiotic supplementation. When there were significant differences, the Tukey test was applied for the use or not of pre-, pro- and synbiotic. The data expressed as percentage were transformed using the formula y = arcsine $\sqrt{x}$ for later evaluation. The P-value was fixed at 0.05.

Results and Discussion

There was no effect of supplements on DFI and SUR ($p > 0.05$) (Table 2). Fishes fed diets pre-, pro- and synbiotic supplemented performed better in terms of ADG, FCR, SGR and PER than those maintained on control diets ($p < 0.05$).

In the case of body indexes, the supplements did not affected HSI and VSI values ($p > 0.05$) (Table 3). The pre-, pro- and synbiotic supplementation in diets resulted in better CY, TL, SL and H than control diet ($p < 0.05$).

Similar results have been reported for aquatic species fed MOS (Gültepe, Salnur, Hoşsu, & Hisar, 2011; Refstie, Baeverfjord, Seim, & Elvebø, 2010; Staykov, Spring, Denev, & Sweetman, 2007), BS (Essa et al., 2010; Giri et al., 2014) and MOS plus BS as synbiotic (Azevedo et al., 2016; Daniels, Merrifield, Ringø, & Davies, 2013) on growth, feed utilization and survival.

Table 2. Growth, feed utilization and survival of Nile tilapia

Parameter[1]	Control	Prebiotic	Probiotic	Synbiotic	p-value
DFI (g day^{-1})	0.48±0.02^{a}	0.48±0.02^{a}	0.48±0.02^{a}	0.49±0.01^{a}	0.4955
ADG (g day^{-1})	0.26±0.02^{b}	0.33±0.01^{a}	0.32±0.03^{a}	0.33±0.01^{a}	0.0194
FCR	1.87±0.15^{a}	1.49±0.05^{b}	1.52±0.08^{b}	1.44±0.11^{b}	0.0114
SGR (% day^{-1})	3.55±0.15^{b}	3.98±0.11^{a}	3.93±0.21^{a}	4.09±0.15^{a}	0.0142
PER (%)	1.68±0.14^{b}	2.10±0.07^{a}	1.93±0.11^{a}	2.03±0.06^{a}	0.0069
SUR (%)	93.75±4.17^{a}	95.83±4.81^{a}	97.92±4.17^{a}	95.83±4.81^{a}	0.6636

[1]DFI, daily feed intake; ADG, average daily gain; FCR, feed conversion rate; SGR, specific growth rate; PER, protein efficiency ratio; SUR, survival rate. Values followed by a different letter within the same line were different ($p < 0.05$).

Table 3. Body indices (%) in Nile tilapia

Parameter[1]	Control	Prebiotic	Probiotic	Synbiotic	p-value
TL (cm)	9.07±0.19^{b}	9.86±0.19^{a}	9.67±0.10^{a}	9.82±0.17^{a}	0.0234
SL (cm)	7.19±0.17^{b}	7.92±0.08^{a}	7.76±0.14^{a}	7.76±0.22^{a}	0.0221
H (cm)	2.70±0.13^{b}	3.05±0.04^{ab}	3.21±0.33^{a}	3.10±0.11^{a}	0.0248
CY (%)	55.93±5.13^{b}	64.28±1.79^{a}	66.13±0.95^{a}	63.31±4.19^{a}	0.0011
HSI (%)	1.77±0.49^{a}	1.43±0.44^{a}	1.42±0.69^{a}	1.56±0.56^{a}	0.5134
VSI (%)	2.51±0.56^{a}	2.52±0.68^{a}	2.37±0.25^{a}	2.62±0.65^{a}	0.4466

[1]TL, total length; SL, standard length; H, body height; CY, carcass yield; HSI, hepatosomatic index; VSI, viscerosomatic index. Values followed by a different letter within the same line were different ($p < 0.05$).

The mechanisms by which prebiotics and probiotics can improve the performance are still unclear. However, *Bacillus* sp. can synthesize various vitamins and extracellular enzymes (Azokpota, Hounhouigan, Nago, & Jakobsen, 2006), while MOS can selectively modulate the gut microbiota and improve the integrity of the intestinal villi (Safari et al., 2014). The greatest growth observed by the fish who received supplemented diets, may be due to better feed utilization, as observed in this research through improved FCR and PER.

In this study, there was no influence of pre-, pro- and synbiotic supplementation on the DFI. A common difficulty observed when new supplements or alternative food sources are used in fish feed is the acceptability, which is related to palatability (Carvalho, Azevedo, Ramos, & Braga, 2012). The similarity in feed intake values in this study suggests that the supplementation of the evaluated supplements did not alter the palatability of feed.

The results showed that FCR and PER can be improved by supplementation of pre-, pro- and synbiotic. Feed formulations accounts for more than 50% of the total production costs in intensive aquaculture. Increasing FCR by improving dietary nutrients assimilation would have a direct positive effect on profitability of aquaculture (Azevedo et al., 2015). Some factors may be responsible for improved feed utilization due to the inclusion of prebiotic and probiotic in diets for fish, including the enzymatic contribution by beneficial bacteria (Aly, Ahmed, Ghareeb, & Mohamed, 2008; Anguiano, Pohlenz, Buentello, & Gatlin, 2013; Bairagi, Ghosh, Sen, & Ray, 2002), increased maturation of the gastrointestinal tract (Anguiano et al., 2013; Mello et al., 2013; Salze, McLean, Schwarz, & Craig, 2008) and improved feed apparent digestibility coefficients of nutrients and energy (Burr et al., 2008; Grisdale-Helland, Helland, & Gatlin-III, 2008; Mohapatra et al., 2012).

There was no effect of supplementation on VW ($p > 0.05$). The probiotic supplementation in diets resulted in higher VH and PR than control diet while the pre- and synbiotic supplementation in diets resulted in higher PR than control diet ($p < 0.05$) (Table 4).

Table 4. Intestine morphometric analysis of Nile tilapia

Parameter[1]	Control	Prebiotic	Probiotic	Synbiotic	p-value
VH (μm)	188.75±10.14^{b}	268.50±37.75^{ab}	296.50±56.18^{a}	273.25±60.88^{ab}	0.0415
VW (μm)	50.00±8.12^{a}	55.00±12.91^{a}	53.75±10.31^{a}	57.50±11.90^{a}	0.3244
PR	1.59±0.28^{b}	2.47±0.41^{a}	2.79±0.14^{a}	2.61±0.18^{a}	0.0024

[1]VH, villus height; VW, villus width; PR, perimeter ratio. Values followed by a different letter within the same line were different ($p < 0.05$).

The structural knowledge of the intestinal mucosa may provide important information for studies on fish nutrition. Similarly to results obtained in this study, some authors observed structural improvement of intestine morphometry in fish fed diets MOS or BS supplemented (Mello et al., 2013; Schwarz, Furuya, Natali, Gaudezi, & Lima, 2011). Intestinal absorption capacity is related to the surface area available for absorption, which depends on the villi size and number. To maintain digestive and absorptive intestinal capacity there must be a balance in cell turnover (renewal and cell loss). However, when in response to any agent (anti-nutritional factor, microorganisms) may occur imbalance in the turnover and the consequent change in villus height.

MOS is a non digestible glucomannan derived from the cell wall of *Saccharomyces cerevisiae*, being rich source of mannose which is available for bacterial adhesion, which adsorbing pathogens prevents its binding to the intestinal wall (Newman, 1994). When the bacterial adhesion to the enterocytes is inhibited, there is no formation of colonies that can turn nutrients to the animal unavailable or infect their intestinal cells, thus there is an improvement in intestinal health, increase the integrity of the intestinal villi and, consequently, better utilization of nutrients (Pelicano et al., 2005).

According to Pelicano et al. (2005), the presence of undesirable microorganisms in contact with the

intestinal mucosa can lead to imbalance and interference in the cell renewal modifying the villus height, length and width. Thus, it can be inferred that the opposite (presence of beneficial microorganisms) can positively affect the rate of cell renewal, resulting in improvement in the structure of the villi intestinalis. The highest value in PR presented by fish fed supplemented diets suggests a better integrity of the intestinal mucosa, allowing its better development and therefore greater efficiency in the absorptive process, which may explain the improved FCR and PER.

Results of fish carcass composition show that MO, CE and MM values were unaffected by supplement in diets ($p > 0.05$), while CP and EE content were, respectively, higher and lower in fish fed synbiotic supplemented diets than other fish (Table 5).

Table 5. Proximate carcass composition of Nile tilapia

Parameter[1]	Control	Prebiotic	Probiotic	Synbiotic	p-value
MO (%)	75.09±3.26^a	74.49±2.56^a	74.65±2.75^a	74.80±2.92^a	0.6137
CP (%)	51.36±0.87^b	51.51±0.52^b	52.00±0.44^b	53.71±1.17^a	0.0132
EE (%)	16.86±0.26^a	16.82±0.14^a	16.20±0.69^a	15.02±0.82^b	0.0012
CE (kcal kg^{-1})	4925±51.12^a	4942±102.32^a	4908±65.15^a	4797±69.05^a	0.6788
MM (%)	17.26±0.33^a	18.05±0.39^a	17.06±0.27^a	17.71±0.80^a	0.8121

[1]MO, moisture; CP, crude protein; EE, ether extract; CE, crude energy; MM, mineral matter. Values followed by a different letter within the same line were different ($p < 0.05$).

The carcass composition of fish can be changed both by nutrient concentration in diets as the feed rate (Shearer, 1994). In this study, synbiotic supplementation resulted in an increase in CP and reduced EE content in carcass, agreeing with Ayce, Yilmaz, Genc and Aktas (2007) fed hybrid tilapia with diets MOS supplemented and Bagheri, Hedayati, Yavari, Alizade and Farzanfar (2008) fed rainbow trout diets BS and *B. licheniformis* supplemented. Changes in protein levels and fat in the carcass may be related to better relationship between synthesis and deposition rate in fish muscle. The increase in CP content and the reduction of EE in fish fed supplemented diets may indicate greater absorption of amino acids, improving the balance digestible energy: digestible protein of the diet, reducing carcass EE. According to Cerezuela, Meseguer and Esteban (2011), synbiotic refer to supplement combining prebiotics and probiotics in a form of synergism, increasing their isolated beneficial effects, which was observed for carcass composition in this study.

Conclusion

The results of this study indicated that the mannan oligosaccharide and *Bacillus subtilis* supplementation, isolated or combined (synbiotic), could improve growth, feed utilization, body indexes, intestine morphometry and carcass composition in Nile tilapia.

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