BOLETIM DO INSTITUTO DE PESCA
ISSN 1678-2305 online version
Scientific Article

PROBIOTIC *Bacillus subtilis* AND *Lactobacillus plantarum* IN DIET OF NILE TILAPIA

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Recebido em: Junho 06, 2017
Aceito em: Setembro 03, 2018

ABSTRACT

The aim of this study was to evaluate the effects of a probiotic, composed of *Bacillus subtilis* and *Lactobacillus plantarum* in Nile tilapia fry during the sex reversal phase under stress conditions caused by high stocking density. This experiment was conducted in the Fisheries Institute of São Paulo. The experiment design was completely randomized with four treatments: 1) probiotic added feed, 2) probiotic added to water, 3) probiotic added to feed and water and 4) control diet; with three replicates. The variables analyzed were: final weight, total length, specific growth rate, survival, intestinal microbiology and gene expression of TNF-α and HSP-70. The results of the zootechnical performance of growth and gene expression did not show significant differences between treatments in the parameters evaluated (P>0.05). In the intestinal tract of fry raised in water with added probiotic, $\log_{10}$ CFU (7.72 ± 0.51) count of *Bacillus* spp. was higher than with other treatments which themselves did not differ significantly. It was concluded that the addition of a probiotic of *Bacillus subtilis* and *Lactobacillus plantarum* in the feed or water of Nile tilapia fry during the sex reversal phase did not affect the zootechnical performance of growth or expression the genes studied, but modified the intestinal microbiota.

Keywords: microbiology; nutrition; production performance; gene expression; fry.

PROBIÓTICO *Bacillus subtilis* E *Lactobacillus plantarum* NA DIETA DE TILÁPIA-DO-NILO

RESUMO

Objetivou-se com este trabalho avaliar os efeitos da administração do probiótico composto de *Bacillus subtilis* e *Lactobacillus plantarum* em pós-larvas de tilápia-do-nilo, durante a fase de reversão sexual, em condições de estresse pela alta densidade de estocagem. O experimento foi realizado no Instituto de Pesca de São Paulo, e foi inteiramente casualizado com quatro tratamentos e cinco repetições. Sendo: 1) probiótico na ração; 2) probiótico na água; 3) probiótico na ração e água; 4) controle. As variáveis analisadas foram: peso final, comprimento total, taxa de crescimento específico, sobrevivência, microbiologia do trato intestinal e expressão dos genes TNF-α e HSP-70. Os resultados de desempenho zootécnico de crescimento e expressão gênica não apresentaram diferenças significativas entre os tratamentos nos parâmetros avaliados (P>0,05). No trato intestinal dos alevinos do tratamento com adição de probiótico na água, foi observado $\log_{10}$ (7,72±0,51) UFC, superior à contagem de *Bacillus* spp. em comparação aos demais tratamentos, e estes não apresentaram diferenças estatísticas entre si. Conclui-se que a adição dos probióticos *Bacillus subtilis* e *Lactobacillus plantarum,* na dieta ou na água de criação das pós-larvas de tilápia-do-nilo, durante fase de reversão sexual não causaram melhora no desempenho zootécnico de crescimento e expressão gênica, mas modificou a composição bacteriana intestinal.

Palavras-chave: microbiologia; nutrição; desempenho zootécnico; expressão gênica; pós-larva.

INTRODUCTION

In Brazil in 2017, production of Nile tilapia (*Oreochomis niloticus*) reached 357 thousand tons, representing 51.7% of total fish production (PEIXEBR, 2018), therefore being a species of great national importance.

Probiotics are defined as "microorganisms when administered in suitable amounts, conferring health benefits to the host" (Merrifield et al., 2010). Probiotics supplied in

feed or water can stimulate growth, inhibit pathogen development, improve immune system functioning, increase stress tolerance, and improve food digestibility (Kesarcodi-Watson et al., 2008; Ghazalah et al., 2010; He et al., 2013; Dawood et al., 2016). The mechanism of action of probiotics may involve competitive exclusion (Dawood et al., 2017), competition for adhesion sites in the digestive tract (Merrifield et al., 2010); (Dawood et al., 2017), stimulation of immune system and improvement in digestibility of food (Dawood et al., 2017), acidification of the intestinal tract through the release of short chain fatty acids and lactic acid, increasing the availability of feed minerals and not making the environment conducive to the proliferation of pathogenic organisms (Irianto and Austin, 2002; FAO, 2014).

In tilapia farming, the technique of sex reversal is common and widely used because males have a better growth performance than females. In the case of Nile tilapia, populations of monosex individuals are advantageous, since there is no energy expenditure with reproduction or risk of overpopulation (Meurer et al., 2006). The sex reversal phase of Nile tilapia is considered one of the most critical in relation to mortality (Farias et al., 2004). Therefore, the addition of probiotic in their diet can help increase growth rates and survival and decrease the occurrence of opportunistic diseases.

Special care is necessary in the choice of diet, since the diet must provide the animal's requirements, allowing it to grow, reproduce and have an active immune system (Araujo, 2015). The immune system plays an essential role in the defense of the animal, offering protection against the invasion of possible pathogens, preventing the proliferation of diseases (Araujo, 2015).

Gene expression has been used to evaluate growth, immune function and stress response in fish (Almeida et al., 2009; He et al., 2013; Ren et al., 2013). Tumor necrosis factor-alpha (TNF-α) is a cytokine that participates in the immune response mediated by cells of the immune system (Thomas, 2001). TNF-α is recognized as an important mediator in many cytokine-dependent inflammatory events. It is known that TNF-α is released in the allergic response by mast cells and macrophages via IgE-dependent mechanisms that increase the levels of this cytokine (Ohkawara et al., 1992). A diet with the addition of probiotics increases the expression of pro-inflammatory (cytokines), including IL-1 and TNF-α in fish, increasing the nonspecific immune response (Low et al., 2003).

HSPs (heat shock proteins), in particular members of the HSP 70 kDa family (HSP-70), are expressed in cells under normal conditions and function as chaperone proteins that ensure the proper conformation of newly synthesized proteins (Tine et al., 2010).

However, when the animal is under stress, HSP-70 is produced by the body protecting it against cell damage. Under stress conditions, HSPs act in a way to prevent the denaturation or aggregation of cytoplasmic proteins (Fink, 1999; Place and Hofmann, 2001; Dahlhoff, 2004; Hofmann, 2005). Heat shock response (HSR), measured by the activity or expression of HSPs, contributes to the determination of the tolerance limits of organisms (Tomanek, 2008). Therefore, studies have addressed HSP-70 as a biomarker of stress in fish (Gutierre, 2011).

Thus, studies of HSP-70 gene expression in Nile tilapia are important because of stress-induced changes in animal performance. In addition, changes in dietary pattern may cause changes in the gene expression of the somatotrophic axis, IGF-I, GHR and GH, and also affect performance (Gutierre, 2011). The objective of this study was to evaluate the effect of the probiotic *Bacillus subtilis* plus *Lactobacillus plantarum* in the diet of Nile tilapia, in the sex reversal phase, on the zootechnical performance parameters survival, microbial intestinal colonization, and gene expression.

MATERIAL AND METHODS

The study was carried out in CPA (Aquaculture Research Center) at the Fisheries Institute of São Paulo, using post-larvae of Nile tilapia (5 days after hatching) during the sex reversal phase. A commercial probiotic composed of *L. plantarum* ($1.51x10^6$ CFU g^{-1}) and *B. subtilis* ($1.34x10^7$ CFU g^{-1}), the counting of bacteria was done on tryptic soy agar (TSA) and modified *Lactobacillus* agar (MLA) plates) of ration used in the experiment of the post-larvae. 60 mg kg^{-1} of the 17-α-methyltestosterone was dissolved in 0.5 liter of 98% ethanol and added to commercial feed. The experimental design was completely randomized, with four treatments and five replicates. A total of 900 tilapia fry kept in aquariums with 15 L, previously acclimatized for 7 days, were used. The treatments were: 1) probiotic in feed; 2) probiotic in water; 3) probiotic in feed and water; and 4) control. The probiotic was added to the feed at a proportion of 0.02 g kg^{-1} of the feed, and in the aquarium water, the probiotic was administered daily at 0.625 g, every 2 days, after cleaning by siphoning (Zhou et al., 2010), depending on experimental treatment.

The feed utilized containing 48% crude protein, 8% ethereal extract, 6% crude fiber and 10% ash and was supplied five times daily *ad libitum*. The fish were fed experimental diets at 8:00, 10:00, 12:00, 14:00 and 16:00 for 28 days.

During the experiment, water quality in the aquarium was determined by monitoring dissolved oxygen levels with a digital oximeter and using an Alcon® kit for total ammonia, pH and temperature (digital thermometer). Siphoning was performed from the bottom of the aquariums every 2 days, removing 25% of the aquarium volume, and the water temperature was maintained at 25ºC. The photoperiod was controlled at 10:14 h (light:dark). After 28 days of treatment, the samples were collected to perform the analyses to evaluate zootechnical performance, namely survival, microbiology and TNF-α and HSP-70 gene expression.

Zootechnical performance

Zootechnical performance parameters were evaluated by biometry at the beginning and end of the experiment, and 25 animals were used per treatment, obtaining the total length and weight of the fish. The evaluated parameters were: weight gain (WG), survival (S%), condition factor (CF) and specific growth rate (SGR) (Carneiro et al., 1999).

Microbiology

Two fish from each aquarium were anesthetized with eugenol and killed by spinal dissection. The intestine was removed and macerated with a glass rod, and serial dilutions of 10^{-1}, 10^{-2}, 10^{-3},

10^{-4} and 10^{-5} were prepared. Each dilution was seeded with a Drigalsky spatula in Petri dishes containing approximately 20 mL of TSA or MLA, in duplicate (Irianto and Austin, 2002). Plates were incubated in an oven at 30°C for 36 h. The colony forming units (CFU) characteristic of *B. subtilis* and *Lactobacillus* spp. were counted.

RT-qPCR analyses

RT-qPCR analyses were performed at the Microbiology Laboratory of the University of Málaga (UMA), Spain. We selected two genes related to the immune system (TNF-α) and to stress (HSP-70). The sequences of the primers for intestinal analysis by PCR are shown in Table 1.

Two fish from each experimental group were used to determine qPCR expression. The samples were homogenized and total RNA was isolated using the TRIsure extraction kit (BIOLINE), and the RNA isolation procedure was performed according to the manufacturer's protocol. Possible traces of fish genomic DNA were removed by treating the samples with the RNase-free DNAse kit (Thermo Scientific) for 30 min. The quality of the RNA sample was checked on agarose gel and stained with ethidium bromide. Total RNA (1 µg) from each sample was reverse transcribed for the conversion of RNA into DNA using the iScript_cDNA Synthesis Kit (Bio-Rad).

Table 1. Primer sequences of TNF-α and HSP-70 genes used for evaluation of gene expression in intestinal tissue of Nile tilapia post-larvae exposed to probiotic composed of *Lactobacillus plantarum* and *Bacillus subtilis* added to feed and/or water.

Gene	Primer sequence	GenBank accession No.
TNF-α	F: CTTCCCATAGACTCTGAGTAGCG R: GAGGCCAACAAAATCATCATCCC	JF957367
HSP-70	F: TGCCTTTGTCCAGACCGTAG R: GTGTCCAACGCTGTCATCAC	JF957370

F = *forward*; R = *reverse*

mRNA was isolated from total RNA using a primer with 15 thymine units (poly-T) that specifically binds to the tail of eukaryotic mRNA molecules. The absence of genomic DNA contamination was confirmed by PCR amplification of RNA samples with no cDNA synthesis. Real-time PCR analysis was performed using an iCycler (Bio-Rad). Reactions were performed in a total volume of 25 µL containing cDNA generated from 20 ng of original RNA template, 300 nM each of the forward and reverse primers and 25 µL of iQ SYBR Green Supermix (Bio-Rad). The amplification protocol used started with initial denaturation and activation of enzymes for 7 min at 95°C, followed by 40 cycles of 95°C for 15 s and 65°C for 30 s. Each assay was performed in triplicate. Data were analyzed for relative quantification relating the PCR signal from the target transcript in a treatment group to another sample, such as an untreated control. The 2-ΔΔCt method is a convenient way to analyze relative changes in gene expression (Livak and Schmittgent, 2001) and 18S gene was included as an internal reference for normalization of gene expression data.

Statistical analyses

After tests for normality and homoscedasticity, the data were submitted to analysis of variance (ANOVA), followed by the Tukey test ($\alpha<0.05$) with the help of statistics software SAS 9.1.

RESULTS

The results of the water quality parameters of the aquariums where Nile tilapia were fed a diet containing probiotic composed of *L. plantarum* ($1.0x10^6$ CFU g^{-1}) and *B. subtilis* ($1.0x10^7$ CFU g^{-1}) added to feed and/or water, are shown in Table 2. There was no significant difference between treatments ($P>0.05$).

The results of zootechnical performance did not exhibit statistically significant differences between the treatments regarding the parameters evaluated ($P>0.05$) (Table 3).

Microbiological analyses not showed differences ($P<0.05$) in CFU recovered from the fish intestine, total counts in TSA of *Bacillus* spp. and in MLA of *Lactobacillus*, between treatment (Table 4).

Expression of genes related to immune system (TNF-α) and stress response (HSP-70) was not significantly different between the treatments ($P>0.05$) (Figure 1).

Table 2. Aquarium water quality parameters for Nile tilapia supplemented with probiotic composed of *Lactobacillus plantarum* ($1.0x10^6$ CFU g^{-1}) and *Bacillus subtilis* ($1.0x10^7$ CFU g^{-1}) added to feed and/or water.

Treatment	Water quality parameters			
	pH	Dissolved oxygen (mg L^{-1})	Total ammonia (mg L^{-1})	Temperature
Probiotic in feed	7.0±0.59	5.98±0.59	0.25±0.22	27.0 ºC±0.24
Probiotic in feed + H_2O	7.0±0.85	6.2±0.85	0.22±0.52	27.0 ºC±0.19
Probiotic in H_2O	7.0±0.88	5.94±0.88	0.21±0.20	27.0 ºC±0.12
Control	7.0±0.57	5.95±0.57	0.24±0.17	27.0 ºC±0.14

Table 3. Growth performance of post-larvae fed for 28 days with probiotic composed of *Lactobacillus plantarum* ($1.0x10^6$ CFU g^{-1}) and *Bacillus subtilis* ($1.0x10^7$ CFU g^{-1}) added to feed and/or water.

Treatment	Total length (mm)	Final weight (mg)	Survival (%)	SGR (%)
Probiotic in feed	19.38±1.07	1.188±1.80	73.33±9.22	2.82±0.24
Probiotic in feed + H_2O	18.71±0.85	1.222±0.82	76.00±5.52	2.76±0.19
Probiotic in H_2O	19.88±0.88	1.310±1.39	72.00±10.00	2.86±0.08
Control	19.96±0.57	1.392±1.38	68.44±10.17	2.96±0.14

Table 4. Colony forming units (CFU) recovered from tryptic soy agar (TSA) and modified *Lactobacillus* agar (MLA) Himidia) agar per gram of Nile tilapia gut fed for 28 days with the probiotic composed of *Lactobacillus plantarum* and *Bacillus subtilis* added to feed and/or water.

	Mean $\log_{10}$ g^{-1} of intestine		
Treatment	Total count in TSA	*Bacillus* spp.	*Lactobacillus* spp.
Probiotic in feed	6.20_a±1.09	5.86_b±1.01	6.20_c±0.74
Probiotic in feed + H_2O	7.15_b±0.54	6.80_{ab}±0.57	6.64_{bc}±0.53
Probiotic in H_2O	7.85_b±0.45	7.72_a±0.51	7.16_{ab}±0.56
Control	7.72_b±0.61	6.41_b±1.21	7.60_a ±0.85

a, b, c Different letters in column indicate statistically significant difference according to Tukey test ($P<0.05$).

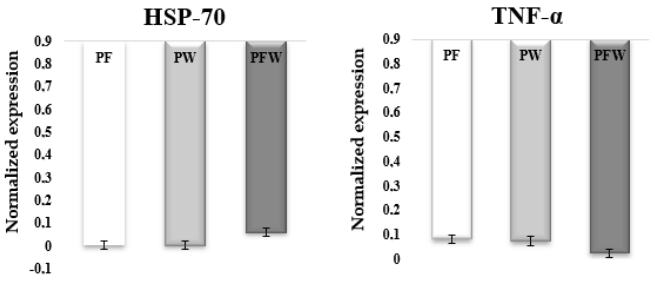

Figure 1. Gene expression of HSP-70 and TNF-α in intestine of post-larvae of Nile tilapia (*Oreochromis niloticus*) the probiotic *Bacillus subtilis* and *Lactobacillus plantarum*. Probiotic in feed (PF), probiotic in water (PW), and probiotic in feed + water (PFW).

DISCUSSION

The inclusion of probiotic in feed and water had no effect on weight gain and SGR. This result was also observed by Fagundes et al. (2016), in which Nile tilapia fed diets containing *L. plantarum* at concentrations of 10^4, 10^6 and 10^8 CFU.g^{-1} did not show significant differences in relation to the performance of animals. Tachibana et al. (2012) observed that the inclusion of probiotic containing *B. subtilis* in the feed did not cause significant differences in the performance of Nile tilapia fry. Some probiotics require a stressor to demonstrate their beneficial effects, such as an increase in digestibility, survival and zootechnical performance, and especially improvement in immune response against pathogens.

In this work, no significant differences in fish survival between treatments were observed, but Addo et al. (2017) observed a significant effect of the addition of probiotic *B. subtilis* on feeding (for 56 days) on the survival of Nile tilapia after challenge of oral infection with *Aeromonas hydrophila*. The effect of probiotics on the host is also related to feeding time and the type of stress caused.

Essa et al. (2010) observed a significant effect of added probiotics (*L. plantarum*, *B. subtilis* and *Saccharomyces cerevisiae*) on the growth performance of Nile tilapia juveniles. The bacteria used in this experiment did not promote improvement in the growth performance of the fish. According to Telli et al. (2014), the performance of Nile tilapia fed the diet with added probiotic bacterium, *B. subtilis*, was not superior to that of individuals that received the control diet, explained by the low level of CFU per gram of intestinal tissue. In the intestinal tract of the Nile tilapia fry, a lower number of CFU g^{-1} was observed in the total TSA count in the treatment with probiotic in the feed compared to the other treatments, which did not show statistical differences between them. However, the same trend was not observed in relation to the *Bacillus* spp. count, in which probiotic treatments in the feed and control displayed lower results than the probiotic treatment in water. The effect of probiotics on growth performance in tilapia and other fish may be related to different interactions between probiotics, intestinal microbiota and host (Addo et al., 2017).

Lactobacillus spp. were present at higher CFU count in control, followed by probiotic in water, probiotic feed + water and probiotic in feed. The increase in the amount of lactic acid bacteria was expected in fish fed the diet with added probiotic, in relation to the control (Jatobá and Mouriño, 2015). Makridis et al. (2000) obtained similar values in the CFU count among turtle specimens (*Scophthalmus maximus*) fed rotifers containing strains of bacteria 4:44 and PB52 and those that were subjected to water immersion with these strains.

The gut-colonizing microorganisms, contained in the probiotics, must adapt to the specificity of the intestinal environment, and resist the actions of digestive enzymes, host immune system, anaerobiosis and pH variations. The success of colonization also involves competition with other bacteria for binding sites, nutrients and resistance to toxins produced by other bacteria (Makridis et al., 2000).

Among the HSPs, HSP-70 are widely studied for their characterization and induction in response to environmental stress in various species (Elewaut et al., 1999), including Atlantic salmon, *Salmo salar* L. (Seppola et al., 2008) and Nile tilapia, *Oreochromis niloticus* (He et al., 2013). TNF-α is an important cytokine, which induces apoptosis and increases neutrophil migration and the respiratory activity of macrophages. Many functions of the TNF gene have been reported in vertebrates, mammals and fish (Tort et al., 2003). In addition, TNF-α is widely accepted as a pro-inflammatory factor (He et al., 2013), since it shows an acute response to stress, these factors being induced by *L. plantarum* JCM 1149 and *Aeromonas hydrophila* NJ-1; although the extent of changes is different, it was found that the intestine presents an acute response to these bacteria according to the expression profile of these cytokines (Ren et al., 2013).

The addition of probiotic to water or feed did not increase TNF-α or HSP-70 gene expression. The results observed in this experiment differed from those reported by some authors. Selim and Reda (2015) observed a significant effect of probiotics (*B. amyloliquefaciens*) on TNF-α and IL-1 gene expression in Nile tilapia. According to Telli et al. (2014), the possible divergences found in this experiment might have occurred because of the amount of probiotic, which was not effective in reducing the stress caused by the breeding system used. He et al. (2013) observed significant increases ($P<0.05$) in the expression of IL-1β, TNF-α and HSP-70 genes in Nile tilapia with diets supplemented with *B. subtilis*. The probiotic in this experiment was not sufficient to produce a significant increase in the expression of the TNF-α and HSP-70 genes of the animals that received the probiotic in compared to those that received control diet. He et al. (2013) reported that TNF-α and HSP-70 gene expression showed significant differences in Nile tilapia fed *B. subtilis* after 56 days of feeding. Likely, 28 days of supplementation with probiotics, *B. subtilis* and *L. plantarum* was not sufficient to modulate TNF-α and HSP-70 gene expression in post-larvae of Nile tilapia.

CONCLUSION

The probiotic *B. subtilis* plus *L. plantarum*, included in the diet of Nile tilapia fry during the sex reversal phase did not produce significant differences in zootechnical performance parameters of growth, survival, and TNF-α and HSP-70 gene expression, but modifiedStheSintestinalSmicrobiotaSofStheSfish.

ACKNOWLEDGEMENTS

We thank FAPESP for granting the Scientific Initiation Fellowship, process No. 2015/07274-4 and BEPE – Bolsa Estágio Pesquisa no Exterior, No. 2016/22355-3. We are grateful to Professor Dr. Miguel Angel Moriñigo and Dr. Silvana Tapia-Paniagua, Department of Microbiology, University of Málaga, Spain. Dr. Mariene Miyoko Natori reviewed the paper, and Dr. A. Leyva provided English translation and editing of the manuscript.

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