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### **Short Communication**

# Use of probiotics *Bacillus cereus* var. *toyoi* and *Bacillus subtilis* C-3102 in the diet of juvenile Nile tilapia cultured in cages

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**ABSTRACT.** This study aimed to evaluate gut colonization by probiotic bacteria, and their influence on the bacterial microflora, hematological profile, growth rate and proximate composition, and water quality parameters in the cultivation of Nile tilapia juveniles of the Genetically Improved Farmed Tilapia (GIFT) strain. 1800 fry were randomly distributed into four experimental groups ( $T_1$  = basal diet (BD) without probiotic addition;  $T_2$  = BD + 0.5% *Bacillus cereus* var. *toyoi* (BC);  $T_3$  = BD + 0.5% *B. subtilis* C-3102 (BS);  $T_4$  = BD + 0.25% BC + 0.25% BS) with five replicates. Cages (0.175 m³ capacity) containing 90 tilapia fry (0.34 ± 0.06 g) were used as culture units and individually installed in 8.4 m³ ponds. Except for hemoglobin, hematocrit, glucose, and neutrophil levels, no differences in hematological profile were observed among the groups after 127 days of culture (P > 0.05). The probiotic bacteria either added individually or in combination, successfully colonized the intestines of the fish, without negatively influencing the intestinal bacterial microflora, growth rate, proximate composition, or water quality parameters (P > 0.05). Thus, dietary probiotic supplementation of juvenile Nile tilapia alters the hematological profile, especially hemoglobin, hematocrit, glucose, and neutrophil levels.

Keywords: hematological profile, tilapia, intestinal microflora, probiotics, growth, aquaculture.

# Uso de los probióticos *Bacillus cereus* var. *toyoi* y *Bacillus subtilis* C-3102 en la dieta de juveniles de tilapia del Nilo cultivada en jaulas

**RESUMEN.** Este estudio tuvo como objetivo evaluar la colonización por bacterias probióticas del intestino y su influencia en la microflora bacteriana, perfil hematológico, tasa de crecimiento, composición proximal, y los parámetros de calidad del agua en el cultivo de juveniles de tilapia del Nilo de la cepa Genetically Improved Farmed Tilapia (GIFT). 1.800 alevines fueron distribuidos aleatoriamente en cuatro grupos experimentales ( $T_1$  = dieta basal (BD) sin adición de probióticos;  $T_2$  = BD + 0,5% *B. cereus* var. *toyoi* (BC);  $T_3$  = BD + 0,5% *B. subtilis* C-3102 (BS);  $T_4$  = BD + 0,25% BC + 0,25% BS) con cinco repeticiones. Se utilizaron jaulas (0,175 m³ de capacidad) con 90 alevines de tilapia (0,34 ± 0,06 g) como unidades de cultivo e instaladas de forma individual en estanques de 8,4 m³. No se observaron diferencias en el perfil hematológico entre los grupos después de 127 días de cultivo (P > 0,05), excepto para la hemoglobina, hematocrito, glucosa y niveles de neutrófilos. Las bacterias probióticas, suministradas individualmente o en combinación, colonizaron los intestinos de los peces, sin influir negativamente en la microflora intestinal bacteriana, tasa de crecimiento, tasa de composición corporal o los parámetros de calidad del agua (P > 0,05). Por lo tanto, la suplementación con probióticos en la dieta de juveniles de tilapia del Nilo altera el perfil hematológico, especialmente hemoglobina, hematocrito, glucosa y niveles de neutrófilos.

Palabras clave: perfil hematológico, tilapia, microflora intestinal, probióticos, crecimiento, acuicultura.

Aquaculture is of global economic importance. To improve productivity, performance, and fish health researchers have begun to investigate the use of alternatives to antibiotics, such as probiotics, prebiotics, symbiotic organisms, organic acids, and herbal remedies (Nayak, 2010; Cruz *et al.*, 2012; Mohapatra *et al.*, 2013).

Probiotics are used worldwide in ecofriendly and sustainable aquaculture practices. Probiotics are living microorganisms that are used as dietary additives; they live in the gastrointestinal tract of the host, adhering to the epithelial wall and proliferating in the intestine. Probiotics may act in the reduction and prevention of infection by pathogens by improving the intestinal bacterial flora, reducing the load of harmful bacteria by producing inhibitory substances, and assisting in digestion by producing digestive enzymes and further stimulating the immune system. If they will be used as dietary supplements, they should be able to survive for long periods of storage (Cutting, 2011; Cruz *et al.*, 2012; Mohapatra *et al.*, 2013).

The use of microorganisms of the genus *Bacillus* in aquaculture is a nutritional management practice that is expanding rapidly in regions with intensive fish farming, mainly in Asia (Kesarcodi-Watson *et al.*, 2008). *Bacillus cereus* var. *toyoi* and *B. subtilis* C-3102 are products approved by the European Food Safety Authority (EFSA). They are composed of lyophilized spores added to animal feed that contribute to the growth, feed efficiency, and modulation of the gastrointestinal flora. These strains are susceptible to antibiotics and have no toxigenic potential. These probiotics are commercially used for cattle, poultry, rabbits, and swine (Silley, 2006), and recently in fish (Albuquerque *et al.*, 2013; Mohapatra *et al.*, 2013; Nakandakare *et al.*, 2013; Wild *et al.*, 2014).

Evaluation of hematological profiles has become a useful tool for revealing possible homeostatic changes in fish, allowing inferences about their health when exposed to adverse situations. Several authors have studied the influence of dietary supplements on hematological parameters in Nile tilapia (Ghiraldelli *et al.*, 2006; Nakandakare *et al.*, 2013), although further development of this technique is required.

This study aimed to evaluate gut colonization by probiotics, and their influence on the bacterial microflora, hematological profiles, growth rate and proximate composition, and water quality parameters during growth of juvenile Nile tilapia of the Genetically Improved Farmed Tilapia (GIFT) strain cultured in cages with commercial fish feed supplemented with *B. cereus* var. *toyoi* and *B. subtilis* C-3102.

The experiment was performed at the Instituto de Pesquisa em Aquicultura Ambiental (InPAA) in Toledo, Paraná, Brazil, from November 2009 to April 2010, over 127 days. 1800 Nile tilapia fry (*Oreochromis niloticus*) of the GIFT strain, sexually reverted, with an initial average weight of  $0.34 \pm 0.06$  g, and an initial average size of  $2.89 \pm 0.26$  cm, were purchased from a commercial fish farming operation.

The fingerlings were distributed in a completely randomized design comprising 20 cages of 0.175 m<sup>3</sup> capacity  $(0.5\times0.5\times0.7 \text{ m})$ . The cages (4.0 mm mesh)were individually installed in 8.4 m<sup>3</sup> ponds. Each cage containing 90 fish was considered an experimental unit. The water temperature (°C) and dissolved oxygen (mg L<sup>-1</sup>) were monitored with a HI 9828 oxymeter. The hydrogen potential was measured with a HI 8424-pH meter, and conductivity (µS cm<sup>-1</sup>) was recorded with a HI 9835 conductivity meter (Hanna<sup>®</sup> Instruments, São Paulo, Brazil). The water transparency (cm) was measured with a Secchi disk (Alfakit Ltda. Florianópolis, Santa Catarina, Brazil). The rainfall (mm) was monitored using a Vantage Pro2<sup>TM</sup> (Agrosystem, Ribeirão Preto, São Paulo, Brazil) installed in the Meteorological Station of Catholic University of Parana, PUCPR/ Toledo, Paraná, Brazil. Data were collected weekly.

The experimental diet consisted of the commercial feed (Algomix® Agroindustria Ltda., Toledo, Paraná, Brasil) indicated for each phase of Nile tilapia culture supplemented with 0.5% lyophilized probiotics dissolved in 2% vegetable oil. Supplements of B. subtilis C-3102 (Comércio e Indústria Uniquimica Ltda.) and B. cereus var. toyoi (Sumitomo Chemical do Brazil Ltda.) were used, each containing 500 million spores per gram, proportionally; these were tested individually and combined. The control group that was not fed probiotics  $(T_1)$  was fed the basal diet supplemented with vegetable oil in the same proportion used in all experimental groups. The basal diet was supplemented with 0.5% Bacillus cereus var. tovoi  $(T_2)$ , 0.5% Bacillus subtilis C-3102 ( $T_3$ ), or 0.25% B. cereus var. toyoi + 0.25% B. subtilis C-3102 ( $T_4$ ).

Biometry was performed monthly, and all individuals in each cage were collected to determine the total biomass, weight, and total amount of fish. The amount of feed to be provided was adjusted accordingly. For biometry, three fish from each cage were randomly collected for extraction of intestines and 10 mL of water, in order to quantify the total number of mesophilic bacteria, total coliforms, and colonization by probiotics per gram of intestine and per milliliter of water. Microbiological analyses were performed in the Laboratory of Microbiology at the Catholic University of Parana, PUCPR/Toledo, using the official analytical methods for microbiological analysis for control of animal

products and water, proposed by the Ministry of Agriculture, Livestock, and Supply (Brasil, 2003).

The zootechnical performance was evaluated monthly using the biometry data. Thus, the growth performance was determined at the end of the experiment. Thirty-five fish per treatment were fasted, anesthetized, slaughtered, and dissected to determine the viscerosomatic and hepatosomatic body composition indices and visceral fat (Marengoni *et al.*, 2010; Albuquerque *et al.*, 2013). The chemical composition of the carcasses, including crude protein, dry matter, mineral matter, and ether extract was evaluated according to AOAC (2006).

After 127 days rearing, five fish were randomly collected, from each experimental unit; and anesthetized (75 mg eugenol L<sup>-1</sup>) prior to blood sampling by caudal vein puncture with sterilized syringes (2 mL) and needles containing EDTA at 10%, as an anticoagulant agent. The blood was transferred to the Allabor laboratory (Allabor Laboratório de Alimentos Ltda., Toledo, PR) to determine the white blood cell (WBC) count and leukocyte differential count (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) using blood smears stained with May-Grunwald-Giemsa stain (Vallada, 1999). The erythrocyte series corresponding to red blood cells, hemoglobin and hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), variation in red cell volume (RDW), and platelet counts were assessed by an automatic counter according to the standard procedure (Campbell & Ellia, 2007).

Determination of glucose in fish blood was performed immediately after sampling, by applying a single drop of blood to an automatic glucose meter (model Advantage ACCU-CHEK® Softclix, with 200 Soft Clix lancets, F. Hoffmann-La Roche Ltd.).

The normality of experimental errors and homogeneity of variance for each trait were previously evaluated using the Shapiro-Wilk and Levene tests, respectively. The mean values were subjected to MANOVA or ANOVA and Tukey's test ( $\alpha = 0.05$ ) when significant differences were detected (StatSoft, 2005). Pearson correlation coefficient between results of counts on total mesophilic bacteria of samples from intestine and water were estimated.

Addition of probiotic *B. cereus* var. *toyoi* and *B. subtilis* C-3102 to the diet of Nile tilapia fingerlings resulted in colonization of the fishes' intestines and the aquatic culture environment without causing disease or mortality. The total number of mesophilic aerobic bacteria, as well as the number of coliforms, did not differ among the experimental groups, in either intestinal or water samples (P > 0.05). Mean CFU

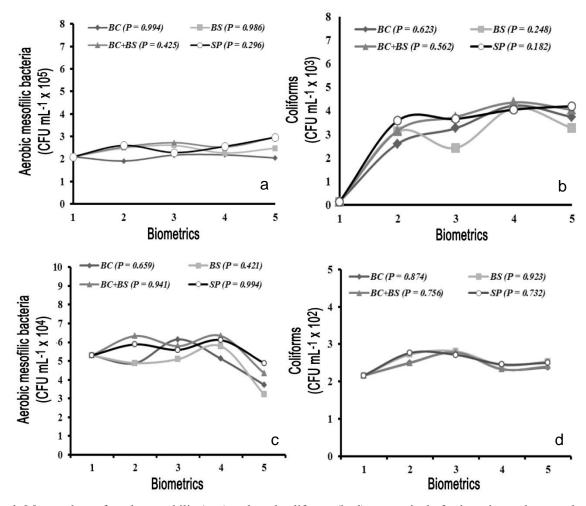
values for samples from tilapia intestines ranged from  $2.08 \times 10^5$  to  $3.85 \times 10^5$  for mesophilic and  $2.61 \times 10^3$  to  $3.85 \times 10^3$  for coliform bacteria, respectively. In the biometric analysis, no significant differences were observed among the groups for these parameters (P > 0.05). Values for total mesophilic bacteria were stable in all biometric analyses with no significant variation, regardless of the daily supply of probiotics or rainfall (P > 0.05) (Fig. 1). Throughout the experimental period, the counts for total mesophilic bacteria in samples from intestines and water showed no Pearson correlation (r = 0.003). No significant differences in the mean values for water quality parameters, assessed biweekly, were observed (P > 0.05).

After 127 days, the hemoglobin density was significantly higher in fish fed diets containing B. cereus than in those without probiotic addition (P = 0.033). The fish fed diets supplemented with B. subtilis alone and with B. cereus and B. subtilis combined differed significantly from those without probiotic supplementation in hematocrit concentration (P = 0.027). No significant difference was observed in values of RBC, WBC, MCH, MCHC, RDW, platelets, or MPV among the experimental groups (P > 0.05). The glucose concentration was higher in tilapia fed probiotic-free diets than in those receiving probiotics (P = 0.002) (Table 1).

Measurement of immune cells present in the blood showed a predominance of lymphocytes, followed by neutrophils, monocytes, and eosinophils. The neutrophil value was higher in the fish group fed a diet containing B. subtilis than in the group lacking probiotics (P = 0.006). The mean values for lymphocytes, eosinophils, and monocytes did not show significant differences among the experimental groups (P > 0.05) (Table 1).

The addition of probiotic *B. cereus* var. *toyoi* and/or *B. subtilis* C-3102 did not influence growth performance, body composition indices, or proximate composition of carcasses of juvenile Nile tilapia (P > 0.05). Similarly, Albuquerque *et al.* (2013) did not observe significant differences in hepatosomatic or viscerosomatic indices or visceral fat, but they reported lower values overall than those presented in this study, due to differences in the size of the fish. Visceral fat deposition was 2.1% to 1.7%, respectively, for fish fed diets containing *B. subtilis* C-3102 and those without probiotic supplementation, which is within expected levels for good performance of Nile tilapia, despite the addition of vegetable oil at 2% kg<sup>-1</sup> for the addition of probiotics (Table 2).

B. cereus and/or B. subtilis colonized the intestines and the water in which the Nile tilapia were grown. Regardless of its source, the microorganism must be



**Figure 1.** Mean values of total mesophilic (a, c) and total coliforms (b, d), respectively for intestine and water, during the cultivation of Nile tilapia fed on dietary probiotics into four experimental groups (0.5% *B. cereus* var. *toyoi* - BC, 0.5% *B. subtilis* C-3102 - BS, 0.25% *B. cereus* and 0.25 *B. subtilis* - BC + BS and without probiotic addition - SP).

able to establish, colonize, and multiply in the host gut without causing adverse effects, as observed in the present study (Nayak, 2010). Günther & Jimenez-Montealegre (2004), using *B. subtilis* as a probiotic for Nile tilapia, also found that the influence on the mesophilic bacterial community was relatively low; however, in the intestinal environment as well as in the water, this behavior could be offset by other positive effects such as production of bacteriocins to control pathogenic bacteria.

The environmental parameters used are considered suitable for cultivation of this species, and only the mean values for daily maximum temperature, which ranged from 23.4°C to 26.6°C, were somewhat below the ideal temperature indicated for growth of Nile tilapia (El-Sayed, 2006). Thus, the hematological profile values (Table 1) suggest that the addition of probiotics may have aided in tolerance of the stress caused by low temperatures during the rearing of tilapia

juveniles in cages. Ghiraldelli *et al.* (2006) reported that the hematocrit could indicate the level of stress to which the fish are subjected. In this study, the values for hemoglobin and hematocrit concentration were significantly higher in fish fed diets containing *B. cereus* and/or *B. subtilis* compared to those without probiotics (Table 1). This suggests that juvenile tilapia fed dietary prebiotics were not stressed, as shown by the absence of mortality and the high levels of blood cell indices, indicating that fish were healthy, thus corroborating the studies by Nakandakare *et al.* (2013).

Concordant with the results reported here, Marengoni *et al.* (2010), Ridha & Azad (2012), and Albuquerque *et al.* (2013) found that addition of probiotics to the diet produced no differences in the performance of tilapia fingerlings. The authors reported the low temperature at which the fish were cultivated may have impaired nutritional efficiency, and suggested

**Table 1.** Hematological parameters (means  $\pm$  SD) of juvenile Nile tilapia fed on dietary probiotics into four experimental groups  $T_I$  = basal diet (BD) without probiotic,  $T_2$  = BD + 0.5 % *B. cereus* (BC),  $T_3$  = BD + 0.5% *B. subtilis* (BS);  $T_4$  = BD + 0.25 % BC + 0.25 % BS. <sup>1</sup>RBC: red blood count, <sup>2</sup>MCV: mean corpuscular volume, <sup>3</sup>MCH: mean corpuscular hemoglobin, <sup>4</sup>MCHC: mean corpuscular hemoglobin concentration, <sup>5</sup>RDW: red cell distribution width, <sup>6</sup>WBC: white blood count, <sup>7</sup>MPV: mean platelet volume. *P*-values for F test significance probability. CV: coefficient of variation. Different superscripts to values at the same line denote differences by Tukey test ( $\alpha$  = 0.05).

Danamatan	Probiotic diet group					CV
Parameter	$T_{I}$	$T_2$	$T_3$	$T_4$	value	(%)
RBC $(10^6  \mu L^{-1})^1$	$1.60 \pm 0.62$	$2.14 \pm 0.42$	$2.24 \pm 0.29$	$2.04 \pm 0.13$	0.055	17.18
Hemoglobina (g dL <sup>-1</sup> )	$8.46 \pm 0.90^{b}$	$10.34 \pm 1.14^{a}$	$9.60 \pm 0.98^{ab}$	$9.14 \pm 0.90^{ab}$	0.033	9.32
Hematocrit (%)	$26.75 \pm 10.19^{b}$	$36.40 \pm 8.29^{ab}$	$38.10 \pm 3.97^{a}$	$37.84 \pm 3.93^{a}$	0.026	16.48
$MCV (fL)^2$	$167.01 \pm 10.60$	$171.46 \pm 11.78$	$169.62 \pm 6.97$	$185.82 \pm 19.62$	0.143	7.35
$MCH (pg)^3$	$66.28 \pm 34.00$	$49.70 \pm 10.60$	$42.96 \pm 5.06$	$44.92 \pm 4.91$	0.154	32.13
MCHC (g dL <sup>-1</sup> ) <sup>4</sup>	$39.51 \pm 19.82$	$29.40 \pm 8.47$	$25.26 \pm 2.22$	$24.60 \pm 5.29$	0.106	32.57
RDW (%) <sup>5</sup>	$18.18 \pm 4.64$	$16.68 \pm 1.91$	$12.98 \pm 3.36$	$11.84 \pm 5.17$	0.110	28.50
Glucose (mg dL <sup>-1</sup> )	$199.25 \pm 28.92^{a}$	$104.35 \pm 72.24^{b}$	$69.6 \pm 21.87^{b}$	$90.6 \pm 30.90^{b}$	0.002	39.47
WBC $(10^3  \mu L^{-1})^6$	$13.31 \pm 3.60$	$22.43 \pm 1.71$	$16.60 \pm 1.85$	$14.53 \pm 3.02$	0.226	51.08
Lymphocyte $(10^3  \mu L^{-1})$	$11.5 \pm 3.00$	$14.5 \pm 6.70$	$13.2 \pm 1.50$	$12.6 \pm 3.10$	0.245	17.76
Monocyte $(10^3 \mu L^{-1})$	$0.36 \pm 0.23$	$0.58 \pm 0.27$	$0.55 \pm 0.68$	$0.34 \pm 0.11$	0.105	38.86
Neutrophil (μL <sup>-1</sup> )	$1.28 \pm 0.49^{b}$	$1.55 \pm 0.79^{ab}$	$2.29 \pm 0.52^{a}$	$1.38 \pm 0.18^{b}$	0.006	25.43
Eosinophil (μL <sup>-1</sup> )	$0.22 \pm 0.17$	$0.27 \pm 0.16$	$0.30 \pm 0.23$	$0.34 \pm 0.49$	0.553	46,53
Platelet (10 <sup>3</sup> µL <sup>-1</sup> )	$11.5 \pm 3.60$	$13.6 \pm 3.5$	$12.2 \pm 4.2$	$12.2 \pm 3.82$	0.856	31.45
$MPV (fL)^7$	$11.56 \pm 6.51$	$11.03 \pm 6.05$	$10.86 \pm 5.99$	$11.00 \pm 6.15$	0.952	18.43

**Table 2.** Growth rates and proximate composition (means  $\pm$  standard deviation) of the Nile tilapia juveniles subject to fed on dietary probiotics into four experimental groups ( $T_1$  = basal diet - BD without probiotic,  $T_2$  = BD + 0.5 % B. cereus - BC,  $T_3$  = BD + 0.5 % B. subtilis - BS;  $T_4$  = BD + 0.25 % BS).

Parameter	Probiotic diet group				
T drameter	$T_1$	$T_2$	$T_3$	$T_4$	P
Final body weight (g fish <sup>-1</sup> )	$89.52 \pm 16.87$	$88.95 \pm 16.59$	$100.52 \pm 8.14$	$87.63 \pm 11.39$	0.44
Final biomass (kg)	$6.5 \pm 1.85$	$6.28 \pm 1.03$	$7.69 \pm 0.71$	$6.54 \pm 1.17$	0.19
Feed intake (kg)	$10.43 \pm 1.97$	$11.94 \pm 0.92$	$12.83 \pm 1.71$	$10.56 \pm 0.00$	0.23
Feed conversion ratio (kg kg <sup>-1</sup> )	$1.96 \pm 0.94$	$1.95 \pm 0.31$	$1.69 \pm 0.29$	$1.71 \pm 0.71$	0.84
Survival (%)	$80 \pm 18.00$	$85 \pm 9.00$	$90 \pm 2.00$	$88 \pm 7.00$	0.43
Daily weight gain (g fish-1)	$0.70 \pm 0.13$	$0.70 \pm 0.13$	$0.79 \pm 0.06$	$0.69 \pm 0.09$	0.44
Specific growth rate (% day <sup>-1</sup> )	$4.15 \pm 0.15$	$4.14 \pm 0.15$	$4.24 \pm 0.06$	$4.14 \pm 0.10$	0.47
Viscerosomatic index (%)	$10.31 \pm 1.00$	$10.4 \pm 1.35$	$10.9 \pm 3.03$	$9.71 \pm 0.62$	0.77
Hepatosomatic index (%)	$2.24 \pm 0.47$	$2.50 \pm 0.39$	$3.00 \pm 0.45$	$2.27 \pm 0.33$	0.26
Visceral fat (%)	$1.67 \pm 0.46$	$2.02 \pm 0.29$	$2.10 \pm 0.44$	$1.72 \pm 0.39$	0.56
Dry matter (%)	$23.08 \pm 1.42$	$23.80 \pm 0.83$	$23.60 \pm 1.14$	$23.6 \pm 1.51$	0.83
Crude protein (%)	$13.48 \pm 1.38$	$13.78 \pm 0.90$	$13.65 \pm 1.38$	$13.08 \pm 0.58$	0.78
Ether extract (%)	$4.49 \pm 0.67$	$4.78 \pm 0.84$	$4.33 \pm 1.00$	$4.13 \pm 0.95$	0.69
Mineral matter (%)	$3.10 \pm 1.11$	$3.08 \pm 0.80$	$2.20 \pm 0.64$	$2.96 \pm 1.28$	0.44

that food additives must be provided for a long period to produce beneficial effects. In contrast to our observations, Nakandakare *et al.* (2013) observed differences in the growth performance of Nile tilapia juveniles fed 4 g kg<sup>-1</sup> of *B. subtilis* and *B. toyoi* over 63 days.

The addition of dietary *Bacillus* may have improved the nutrient absorption efficiency, which may have favored survival and nutrient retention efficiency in tilapia juveniles. According to Wild *et al.* (2014), the addition of probiotics, although it increases the cost of production, can contribute to the environmental sustai-

nability of systems for production of Nile tilapia, because it results in lower nutrient input into the ponds.

The probiotics *B. cereus* var. *toyoi* and *B. subtilis* C-3102 added individually or in combination at 0.5% in tilapia feed, colonized fish intestines without negatively affecting the intestinal bacterial microflora, or growth rates and proximate composition, or water quality parameters in the cultivation of Nile tilapia juveniles of the GIFT strain. Addition of dietary probiotics to juvenile tilapia influenced the hematological profile, especially the hemoglobin, hematocrit, glucose, and neutrophil levels, without strong variation, indicating that hematopoietic activity was not suppressed in either group.

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