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Nucleotides, β-glucans, ascorbic acid, α-tocoferol, and different concentrations of a vitamin–mineral premix promote growth of Nile tilapia juveniles

[*Nucleotídeos, β-glucanos, ácido ascórbico, α-tocoferol e diferentes concentrações de uma pré-mistura de vitaminas e minerais promovem o crescimento de juvenis de tilápia do Nilo*]

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ABSTRACT

Feed additives, such as β-glucans, nucleotides, ascorbic acid (vitamin C), and α-tocopherol (vitamin E) can improve fish immunity and contribute to enhanced zootechnical performance of the fish. This study aimed to evaluate the effect of different concentrations of vitamin- mineral premix with or without the inclusion of an immunostimulant boost (β-glucans, nucleotides and vitamins C and E) on the zootechnical performance, hemato-immunological parameters, histological changes, and survival of Nile tilapia juveniles. To this end, isocaloric and isoproteic diets were prepared with three different concentrations of vitamin–mineral premix (1.0, 1.5, and 2.0 kg ton-1); additionally, the effects of 4% immunostimulant boost in the diet were examined considering six diets (without the immunostimulant boost: 0.1%, 0.15%, and 0.2%, and with the immunostimulant boost: 0.1% I, 0.15% I, and 0.2% I). We used 24 experimental units, each including 15 fish. Nile tilapia juveniles (1.88 g±0.25) were fed for 50 d with supplemented diets. Further, zootechnical indexes; histological changes in the liver, spleen, and intestine; intestinal morphology; and hemato-immunological parameters were evaluated. The fish that received supplementation showed higher zootechnical values, compared to those that did not receive the supplementation of immunostimulant reinforcement. Weight gain (30.45g±3.06), daily weight gain (0.60g±0.06), final weight (32.41g±3.15) and feed conversion (0.95±0.06) were higher in tilapia fed with an inclusion concentration of 0.2%. Hematological parameters were not affected by either the pre-mix concentrations or the booster of the immunostimulant. There was an increase in the number of intestinal folds, length of the fold, total area and number of goblet cells in the group supplemented with immunostimulant reinforcement. Supplementation with the immunostimulant promoted growth and improved intestinal morphology and immunological parameters of Nile tilapia juveniles.

Keywords: fish farming, immunostimulant, feed additives, vitamins, minerals

RESUMO

Os aditivos alimentares, tais como β-glucanos, nucleotídeos, ácido ascórbico (vitamina C) e α-tocoferol (vitamina E), podem melhorar a imunidade dos peixes e contribuir para um melhor desempenho zootécnico. Este estudo teve como objetivo avaliar o efeito de diferentes concentrações de premix vitamínico mineral com ou sem a inclusão de um imunoestimulante (β-glucanos, nucleotídeos e vitaminas C e E) no desempenho zootécnico, nos parâmetros hematoimunológicos, nas alterações histológicas e na sobrevivência dos juvenis de tilápia-do-Nilo. Para esse fim, foram preparadas dietas isocalóricas e isoproteicas com três concentrações diferentes de premix vitamínico e mineral (1,0, 1,5, e 2,0kg ton-1); além disso, foram examinados os efeitos da inclusão de 4% de imunoestimulante na dieta, considerando-se seis dietas (sem o imunoestimulante: 0,1%, 0,15% e 0,2%, e com a inclusão de imunoestimulante:

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0,1% I, 0,15% I e 0,2% I). Foram utilizadas 24 unidades experimentais, cada uma com 15 peixes. Os juvenis de tilápia-do-Nilo (1,88g±0,25) foram alimentados, durante 50 dias, com as dietas suplementadas. Além disso, foram avaliados índices zootécnicos; alterações histológicas no fígado, baço e intestino; morfologia intestinal; e parâmetros hematoimunológicos. Os peixes que receberam suplementação apresentaram valores zootécnicos mais elevados, em comparação aos que não receberam a suplementação de reforço imunoestimulante. O ganho de peso (30,45g±3,06), o ganho de peso diário (0,60g ± 0,06), o peso final (32,41g±3,15) e a conversão alimentar (0,95±0,06) foram maiores nas tilápias alimentadas com uma concentração de inclusão de 0,2%. Os parâmetros hematológicos não foram afetados nem pelas concentrações de premix nem pelo reforço do imunoestimulante. Foi observado o aumento no número de pregas intestinais, no comprimento da prega, na área total e no número de células caliciformes no grupo suplementado com o boost imunoestimulante. A suplementação com o imunoestimulante promoveu o crescimento e melhorou a morfologia intestinal e os parâmetros imunológicos de juvenis de tilápia-do-Nilo.

Palavras-chave: piscicultura, imunoestimulante, aditivos alimentares, vitaminas, minerais

INTRODUCTION

Aquaculture feeds are formulated with various components, which when provided to an animal, supply essential nutrients to perform normal physiological functions, including maintenance of a strong natural immune system, growth, and reproduction (Encarnação, 2015).

Functional aquafeeds are an emerging new standard to develop diets for fish and crustaceans (Encarnação, 2015). In this context, feed additives can improve the health and zootechnical performance of fish (Carbone and Faggio, 2016). Feed is transformed into biomass gain in the animal's digestive system (Encarnação, 2015).

Components such as β-glucans, nucleotides, ascorbic acid (vitamin C), and α-tocopherol (vitamin E), are commonly added in functional feeds. These additives can improve the immunity and zootechnical performance of fish (Trichet *et al*., 2015; Ibrahem *et al*., 2010; Ringø *et al*., 2011; Gao *et al*., 2014; Aramli *et al*., 2015; Izquierdo; Betancor, 2015 Dawood *et al.*, 2017; Jiang *et al*., 2019).

The abovementioned feed additives contribute positively to environmental and economic sustainability that is essential in increasing aquaculture production globally, especially in intensive production systems, because the health and functionality status are directly correlated with the economic output of aquaculture farms (Encarnação, 2015).

β-glucans has high application potential in the aquaculture industry. It is a commonly used immunostimulant, as it influences the innate immune system of organisms, consequently, promoting the recognition of pathogen-associated molecular patterns (PAMPs) (Dawood *et al*., 2017; Meena *et al*., 2013). Moreover, β-glucans activate macrophages in fish, thereby increasing their ability to fight pathogens (Ringø and Song, 2016). Cornet *et al*. (2021), found that β-glucan supplemented diets did not affect growth performance, mortality, splenic index, or leukocyte respiratory burst activity in rainbow trout, however, β-glucan triggered different immune effectors, depending on the doses or length of exposure compared to others and/or the negative control.

Gil (2002) reported that nucleotides, when added in rodent diet, showed modulatory effects, such as maturation, activation, and proliferation of lymphocytes; phagocytosis of macrophages; immunoglobulin responses; intestinal microbiota; and gene expression of specific cytokines. Moreover, administration of nucleotides intravenously or through diet modifies the immune response and recovery of organs that have suffered from metabolic or inflammatory damages (Ringø *et al*., 2011).

Further, vitamins C and E are important antioxidant additives used in the food industry, as they reduce oxidative stress in animals, and their combined use increases their antioxidant potential (Gao *et al*., 2014). In most aquatic species, especially fish, vitamin C is not biosynthesized, due to the absence of the last enzyme, L-gluconolactone oxidase, of the

biosynthetic route (Abo-Al-Ela *et al*., 2017). As vitamin C is an essential micronutrient, it should be included in the diet (Trichet *et al*., 2015).

Among the various functions of vitamin E that influence the immune system, its antioxidant activity is the most widely documented function. Adequate amounts of this vitamin in diets can improve fish health and promote the resistance of fish to stress and infectious diseases (Izquierdo and Betancor, 2015).

Although the use of feed additives has been extensively studied, the mechanisms by which these additives alter fish metabolism and immune capacity are still not well-understood (Elkatatny *et al*., 2020). Moreover, although many researchers have reported that the individual use of β-glucans, nucleotides, and vitamins C and E, can improve the immune system and zootechnical performance of Nile tilapia, knowledge on their combined use is scarce.

The aim of this study was to evaluate the effect of different inclusion concentrations of a vitamin-mineral premix, with or without the inclusion of immunostimulants (β-glucans, nucleotides and vitamins C and E) on the zootechnical performance, hemato-immunological parameters, and histological changes of Nile tilapia juveniles. (*Oreochromis niloticus*).

MATERIALS AND METHODS

Specific diets were formulated to meet the nutritional requirements of Nile tilapia, according to the Nutrient Requirements of Fish and Shrimp (Nutrient…, 2011) (Table 1). Six isocaloric and isoproteic diets with three different concentrations of Premix Rovimix DSM® (1.0, 1.5, and 2.0kg ton^{-1}) were formulated, and the effects of inclusion or exclusion of the immunostimulant boost, Rovimax Boost DSM® (4.0kg ton^{-1}), in the feeds was assessed in six diets. (Table 1). The recommended amount for the inclusion of Premix Rovimix DSM® was 1.5–2.0kg t^{-1}.

The diets were produced by extrusion in 2-mm pellets. A horizontal mixer (Inbramaq, Riberão Preto, Brazil) was used to mix the dry components and extrusion was performed in a single thread extruder (MX40, Inbramaq, Riberão Preto, Brazil). Extrusion conditions were previously tested and adjusted to a temperature of 85 °C in the cannon head and 24% humidity, using deionized water. After extrusion, the feed was dried in an oven at 50 °C for 4 h, followed by packaging and storage at −20 °C until further use.

Table 1. Centesimal formulation and composition (in dry matter) of the experimental diets used

Ingredient, g kg^{-1}	0,1%	0,1% I	0,15%	0,15% I	0,2%	0,2% I
Soybean meal	500.0	470.0	500.0	470.0	510.0	470.0
Poultry meal	100.0	130.0	100.0	130.0	100.0	130.0
Broken rice	208.0	250.0	218.5	249.5	219.0	249.0
Corn	182.0	100.0	171.0	100.0	160.0	100.0
Soybean oil	7.0	7.0	7.0	7.0	7.0	7.0
Choline chloride	2.0	2.0	2.0	2.0	2.0	2.0
Premix[1]	1.0	1.0	1.5	1.5	2.0	2.0
Immunostimulant [2]	0.0	40.0	0.0	40.0	0.0	40.0
Centesimal composition (g kg^{-1})						
Dry matter	873.8	878.0	871.6	890.0	889.9	897.5
Crude protein[3]	353.6	349.9	350.8	358.2	343.4	359.9
Ether extract[3]	54.2	543.	55.9	52.0	54.5	53.5
Mineral matter[3]	58.4	73.6	54.3	78.6	64.3	61.9

[1]Composition Premix DSM Rovimix®: Vitamin A 5.333.000 IU kg^{-1}; Vitamin D3 1.000.000 IU kg^{-1}; Vitamin E 66,7 g kg^{-1}; Vitamin K3 3,33 g kg^{-1}; Vitamin B1 6,67 g kg^{-1}; Vitamin B2 10 g kg^{-1}; Vitamin B6 10 g kg^{-1}; Vitamin B12 0,013 g kg^{-1}; Niancine 53,33 g kg^{-1}; Pantothenic acid 26,67 g kg^{-1}; Biotin 0,333 g kg^{-1}; Folic acid 2,67 g kg^{-1}; Vitamin C 100 g kg^{-1}; Copper 3,33 g kg^{-1}; Iron 20 g kg^{-1}; Manganese 16,67 g kg^{-1}; Iodine 0,67 g kg^{-1}; Cobalt 0,033 g kg^{-1}; Zinc 26,6 g kg^{-1}; Selenium 0,167 g kg^{-1}. [2] Composition of immunostimulant Rovimax *Boost* DSM®: Vitamin C 10 g t^{-1}; Vitamin E 200000 UI t^{-1}; β -glucans 200 µg t^{-1}; Nucleotides 150 g t^{-1}. [3] Centesimal compositions in dry matter.

The centesimal composition of the diets was performed at the UFSC (Federal University of Santa Catarina) Nutrition Laboratory (LabNutri/UFSC), following the standard procedures given by the Association of Official Analytical Chemists (Cunniff, 1997), including humidity levels (samples were dried at 105 °C to a constant weight, method 950.01), crude protein (Kjeldahl, method 945.01), ether extract (Soxhlet, method 920.39C) and mineral matter (muffle incineration, method 942.05) (Table 1).

The experimental fish were Nile tilapia (*Oreochromis niloticus*) juveniles, male, sexually reversed from the GIFT lineage, acquired from the Santa Catarina Agricultural Research and Rural Extension Company (EPAGRI). Fish management followed protocol nº 8409161118, approved by the Animal Use Ethics Committee of the Federal University of Santa Catarina (CEUA, UFSC). For trial acclimatization, fish were stored in five 100 L tanks, connected to a recirculating aquaculture system (RAS), for 21 days. Subsequently, fish were redistributed to experimental units (100L tanks). During this period, fish were fed with a control diet (0.1%). A total of 360 fish were randomly stored in groups of 15 fish (initial weight 1.88g±0.25g, mean ± standard deviation), per experimental unit, in six dietary treatments and with four repetitions each, totaling 24 experimental units. The feed offered was weighted daily and feed consumption recorded individually for each tank. The fish were fed to apparent satiety four times a day for 50 days.

All the tanks were connected to a RAS, with a continuous flow of 0,55L min^{-1}. The system was equipped with mechanical and biological filters, and ultraviolet disinfection. The temperature of water and dissolved oxygen were monitored daily, while other water quality parameters were monitored weekly. The measurements were performed with digital oximeter (temperature and oxygen), multiparametric sensor (pH) and colorimetric kit (total ammonia, nitrite, and alkalinity). Water quality parameters remain at nile tilapia ideal range (El-Sherif and El-Feky, 2009; Tran-Duy *et al*., 2008), with (mean ± standard deviation): temperature 27,67 °C±0,35, dissolved oxygen 8,45mg L^{-1}±0,74, pH 7,87±0,12, total ammonia 0,14mg L^{-1}±0,09, nitrite 0,01 mg L^{-1}±0,00 and alkalinity 40 mg $CaCO_3$ L^{-1}.

The initial weight of the fish was recorded during the random distribution in the tanks and the final weight data obtained after 50 days of supplementation with the different diets. The growth performance parameters were calculated according to the following formula:

Weight gain (g) = final weight (g) – initial weight (g)
Daily weight gain (g) = final weight (g) – initial weight (g)/ duration of the feeding
Conversion Ratio = quantity of feed offered/weight gain
Survival (%) = (final number of fish/initial number of fish) × 100

At the end of the feeding experiment, three animals per experimental unit were anesthetized with Eugenol Vetec® (75mg L^{-1}) and the blood was collected by puncture of the caudal vein with a syringe containing ethylenediaminetetraacetic acid solution (EDTA) for different hematological analyses.

An aliquot of blood was used for the determination of hematocrit by the microhematocrit method (Goldenfarb *et al*., 1971). Another aliquot of blood was destined for total erythrocyte count in the Neubauer chamber, after dilution (1:200) in modified Dacie fluid (Blaxhall and Daisley, 2018). Hemoglobin concentration was determined by the cyanmethemoglobin method and hematimetric equations were applied to determine mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) (Ranzani-Paiva *et al*., 2013).

After collecting the blood samples, a pool was established using three samples from each experimental unit was rested for 1 h. Subsequently, the pool was centrifuged at 1,400 g for 15 min at 4 °C to obtain blood plasma, which was stored at −20 °C for further immunological analysis.

The total serum protein concentration was measured according to the Total Protein kit (Biotécnica, Varginha, MG, Brazil) and the total immunoglobulin concentration (mg mL^{-1}) was measured according to the method described by Amar *et al*. (2000).

The plasma agglutinating activity was performed in a microplate containing 96 U-bottom wells. The plasma was diluted with phosphate buffered saline (PBS) at 1:1 ratio (50 µL PBS: 50 µL plasma solution) in the first well, after which the plasma was diluted serially in the other wells at a factor ratio of 1:2. Later, 50µL of *Streptococcus agalactiae* S13 serotype Ib (Facimoto *et al*., 2017) inactivated at a concentration of 1×10^8 colony forming unit (CFU) mL^{-1} was added to all wells, after which the microplate was incubated at 25°C for 18 h in a humid chamber. The bonding title is considered as the reciprocal of the last dilution for which agglutination is observed, that is, when aggregates can be observed at the well bottom with the naked eye (Silva *et al*., 2009).

Further, minimum inhibitory concentration (MIC) title was determined in a 96-well flat bottom microplate, in which 150 µL of brain heart infusion broth (BHI) was added in the first well, while 100 µL was added in the other wells. Subsequently, 50 µL of plasma was added to the first well and a serial dilution by a factor of two was followed until the last well. Later, 20 µL the *Streptococcus agalactiae* bacterial suspension (1×10^8 CFU mL^{-1}) diluted in BHI was added. The microplate was incubated at 28 °C for 24 h. The lowest inhibitory concentration was determined as the last plasma dilution wherein bacterial growth was completely inhibited (Silva *et al*., 2009).

After 50 d of feeding, three fish per experimental unit (12 per treatment) were anesthetized in eugenol solution (75 mg L^{-1}) and euthanized per section of the medulla histological samples. Fragments of the liver, spleen, and median intestine were fixed in 10% buffered formalin. Subsequently, the tissues were dehydrated using a series of ethanol, clarified in xylol, and finally soaked in paraffin at 60 °C. Fragments of 3–5 µm thickness (PAT-MR10 microtome) were stained with Harris hematoxylin eosin. After staining, the slides were mounted on Entellan® medium and analyzed under a phase interference contrast microscope Axio Imager A.2 (DIC) (Zeiss, Gottingen, Germany).

During the morphological analysis of the median intestine, the number of intestinal folds, length (µm) and width (µm) of the intestinal folds, number of goblet cells, total area (μm^2), and total perimeter (µm) were measured in each histological section. The length, width, perimeter, and area of villi were measured using Zen Pro software (Zeiss, Oberkochen, Germany).

For the histological analysis of all organs, the following values were assigned to the histological alterations according to the degree of alteration intensity: 0 (no alteration), 1 (slight alteration, corresponding to < 25% of the tissue area), 2 (moderate alteration, 25%–50% of the tissue area), and 3 (severe alteration, > 50% of the tissue area), following the method described by Schwaiger *et al*. (1997) with minor modifications, proposed by Brum *et al*. (2018).

Furthermore, alterations in the following organs/cells were considered in the liver: cordial appearance of the hepatocytes; uniform size of cells and nuclei; pancreas with intact acids and zymogen granules; balloon-like appearance in the hepatocytes; pancreas and sinusoid congestion; eosinophilic and lymphocytic infiltrates; hepatocyte hypertrophy; loose melanomacrophages; and macroesteatosis, microesteatosis, and necrosis. In the spleen, alterations in the integrity of the white and red pulps and centers of melanomacrophages, loose melanomacrophages, and necrosis were evaluated, while in the median intestine, histological alterations were evaluated as eosinophilic infiltrate and loose melanomacrophages.

All data were submitted to the Shapiro-Wilk test to evaluate normality and Levene test to verify homoscedasticity of variance. Later, the data were submitted to factor variance analysis for all analyses (Two-way ANOVA). Tukey test was used to separate means when necessary. analyses were performed using Statistica 10.0 software (Statsoft Inc., Tulsa, USA). Data transformations were applied when necessary. For all tests, the significance level of 5% was considered.

RESULTS

Final weight, weight gain, daily weight gain, and food conversion were affected by the premix concentrations, with the treatments with the highest inclusion concentration (0.2%) showing better results compared to the treatments with 0.1% inclusion level (Table 2).

Table 2. Zootechnical parameters of tilapia juvenile fed for 50 days with diets supplemented with different levels of premix (1.0; 1.5; 2.0 g kg-1) and supplemented or not with immunostimulant boost (40g kg-1). Values are presented as mean ± standard deviation

	0.1%	0.1% I	0.15%	0.15% I	0.2%	0.2% I	*P* Premix	*P* Immune	*P* Interaction
Final weight, g	16.36$^{b.y}$ ±0.34	28.28$^{a.y}$ ±3.70	18.46$^{b.xy}$ ±1.28	28.59$^{a.\ xy}$ ±2.29	19.37$^{b.x}$ ±0.27	32.41$^{a.\ x}$ ±3.15	0.0457	< 0.0001	0.3947
Weight gain, g	14.51$^{b.y}$ ±0.29	26.29$^{a.y}$ ±3.54	16.79$^{b.xy}$ ±0.99	26.63$^{a.\ xy}$ ±2.25	17.32$^{b.x}$ ±0.27	30.45$^{a.\ x}$ ±3.06	0.0507	< 0.0001	0.4571
Daily weight gain, g	0.29$^{b.y}$ ±00.00	0.52$^{a.y}$ ±0.07	0.33$^{b.xy}$ ±0.01	0.53$^{a.\ xy}$ ±0.04	0.34$^{b.x}$ ±0.00	0.60$^{\ a.\ x}$ ±0.06	0.0506	< 0.0001	0.4598
Food conversion	1.32$^{b.y}$ ±0.00	1.04$^{a.y}$ ±0.04	1.22$^{b.xy}$ ±0.06	0.96$^{a.\ xy}$ ±0.06	1.25$^{b.x}$ ±0.03	0.95$^{\ a.\ x}$ ±0.06	0.0189	< 0.0001	0.7682
Survival, %	57.77 ±38.61	100.00 ±0.00	76.66 ±26.03	85.00 ± 15.18	62.22 ±39.37	75.55 ±34.57	0.7852	0.1640	0.5925

a, b: Indicates statistical difference between supplementation with the immunostimulant boost by the Tukey test with a 5% significance level. x, y: Indicates statistical difference between supplementation with the levels of premix by the Tukey test with a 5% significance level. The letters "a" and "x" represent the best results for each zootechnical parameter (not the highest absolute result).

Moreover, immunostimulant boost affected the final weight, weight gain, and feed conversion. Particularly, fish that received supplementation (0.1% I, 0.15% I, and 0.2% I) showed higher values compared to those that did not receive the immunostimulant boost supplementation (0.1%, 0.15%, and 0.2%) (Table 2). No statistical difference was found between the survival values. The variation in survival among treatments is due to the territorial behavior of Nile tilapia.

Hematological parameters were affected neither by the premix concentrations nor by the immunostimulant boost; moreover, these factors did not interact at the end of the 50-d supplementation period (Table 3).

Table 3. Hematological parameters of Nile tilapia juveniles fed for 50 days with diets supplemented with different levels of premix (1.0; 1.5; 2.0g kg^{-1}) and supplemented or not with immunostimulant boost (40g kg^{-1}). Values are presented as mean ± standard deviation

	0.1%	0.1%I	0.15%	0.15%I	0.2%	0.2%I	*P* Premix	*P* Imuno	*P* Interaction
After 50 days of supplementation									
Eritrocit (cel mL^{-1})10^6	1.38 ±0.17	1.41 ±0.22	1.60 ±0.15	1.63 ±0.75	1.48 ±0.13	1.47 ±0.24	0.0534	0.8190	0.9708
Hematocrit (%)	26.62 ±4.21	29.31 ±1.34	28.31 ±1.39	30.54 ±2.91	28.81 ±0.66	30.22 ±1.64	0.6297	0.1650	0.9375
Hemoglobin (g dL^{-1})	15.67 ±5.41	12.03 ±2.40	14.14 ±6.36	16.28 ±7.85	11.00 ±4.22	12.81 ±5.89	0.9654	0.5103	0.5264
MCV (10^4 fL)	1.8 ±0.23	2.22 ±0.52	1.79 ±0.20	1.90 ±0.23	1.95 ±0.41	1.96 ±014	0.1862	0.585	0.4367
MCH (10^5 pg)	1.07 ±0.38	0.89 ±0.25	0.87 ±3.9	1.05 ±0.38	0.84 ±0.20	0.82 ±0.30	0.9540	0.6012	0.5839
MCHC (g dl^{-1})	60.51 ±25.47	41.41 ±9.76	45.16 ±12.51	56.81 ±24.82	43.14 ±14.15	42.15 ±18.03	0.7145	0.5892	0.2711

MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration.

At the end of the 50-d supplementation period, treatments of diets containing the immunostimulant boost (0.1% I, 0.15% I, and 0.2% I) showed higher concentrations of total protein and total plasma immunoglobulin (Table 4). However, the premix concentrations did not interact with the immunostimulant boost at the end of the 50-d period.

After the 50-d period of supplementation with the immunostimulant boost (0.1% I, 0.15% I, and 0.2% I), the number and length of intestinal

folds, number of goblet cells, and total area increased (Table 5).

In the liver tissues, the number of loose melanomacrophages was high in treatments lacking supplementation with the immunostimulant boost (0.1%, 0.15%, and 0.2%), while it was low in the treatments containing the immunostimulant boost (0.1% I, 0.15% I, and 0.2% I) (Table 6). However, no significant statistical differences were observed in the spleen and intestine among the different treatments (Table 5 and Table 7).

Table 4. Immunological parameters of Nile tilapia juveniles fed for 50 days with diets supplemented with different levels of premix (1.0; 1.5; 2.0g kg-1) and supplemented or not with immunostimulant boost (40g kg^{-1}). Values are presented as mean ± standard deviation

	0.1%	0.1%I	0.15%	0.15%I	0.2%	0.2%I	*P* Premix	*P* Imuno	*P* Interaction
After 50 days of supplementation									
Total protein (mg mL^{-1})	38.89^{b} ±4.7	45.31^{a} ±9.69	40.25^{b} ±2.5	37.71^{a} ±3.75	35.71^{b} ±1.48	46.01^{a} ±2.6	0.5203	0.0489	0.0898
Total Ig (mg mL^{-1})	17.44^{b} ±3.6	25.42^{a} ±10.05	16.09^{b} ±3.60	17.80^{a} ±2.63	14.08^{b} ±1.30	23.24^{a} ±2.9	0.2172	0.0069	0.2957
Agglutinating (Log^{2})	6.08 ±2.12	5.58 ±0.82	4.25 ±0.58	5.25 ±0.58	5.08 ±0.71	5.08 ±0.58	0.3731	0.1531	0.7069
MIC (Log^{2})	4.08 ±0.58	3.58 ±0.82	3.58 ±0.00	3.58 ±0.00	3.83 ±0.50	3.08 ±0.58	0.0624	0.3526	0.3526

a, b: Indicates statistical difference between supplementation with the immunostimulant boost by the Tukey test with a 5% significance level. MIC: minimum inhibitory concentration.

Table 5. Intestinal morphology and intensity of histological changes in the intestine of Nile tilapia juveniles fed with a diet supplemented with different levels of premix (1.0; 1.5; 2.0 g kg^{-1}) and supplemented or not with immunostimulant boost (40 g kg^{-1}) for 50 days. Values are presented as mean ± standard deviation

	0.1%	0.1%I	0.15%	0.15%I	0.2%	0.2%I	*P* Premix	*P* Imuno	*P* Interaction
Intestinal morphology									
NF	36.16 ±2.25^{b}	42.66 ±4.61^{a}	36.41 ±7.32^{b}	43.00 ±1.15^{a}	33.66 ±3.28^{b}	44.16 ±14.53^{a}	0.9753	0.0288	0.8454
L	24.32 ±8.21^{b}	34.62 ±1.41^{a}	28.27 ±12.93^{b}	37.37 ±3.58^{a}	26.87 ±7.34^{b}	30.12 ±10.13^{a}	0.9229	0.0106	0.3719
W	11.18 ±3.51	15.54 ±1.20	13.45 ±6.23	15.94 ±3.11	11.54 ±4.36	10.62 ±2.76	0.2037	0.1876	0.4344
NGC	47.1 ±19.13^{b}	51.8 ±7.6^{a}	33.7 ±7.6^{b}	45.1 ±12.0^{a}	35.7 ±9.0^{b}	56.2 ±10.5^{a}	0.4992	0.0164	0.4451
TA	17.20 ±8.72^{b}	38.10 ±7.25^{a}	31.19 ±15.91^{b}	38.76 ±9.95^{a}	17.71 ±9.84^{b}	26.3 ±19.69^{a}	0.1495	0.0331	0.6628
TP	3.36 ±1.24	5.14 ±0.84	4.35 ±1.16	5.02 ±0.57	3.15 ±1.06	4.19 ±2.32	0.3956	0.0752	0.8218
Histological alterations									
EI	1.08 ±0.31	0.95 ±0.55	1.00 ±0.00	1.04 ±1.67	1.00 ±0.00	1.66 ±0.47	0.2145	0.2421	0.1346
LM	0.00 ±0.00	0.08 ±0.16	0.08 ±0.16	0.00 ±0.00	0.08 ±0.16	0.25 ±0.46	0.4866	0.5709	0.5682

a, b: Indicates statistical difference between supplementation with the immunostimulant boost by the Tukey test with a 5% significance level. NF: Number of intestinal folds; L: length (µm); W: width (µm); NGC: number of goblet cells (x10); TA: Total area (x10^{3} µm^{2}); TP: Total perimeter (x10^{3} µm); EI: Eosinophilic infiltrate; LM: loose melanomacrophages.

Table 6. Intensity of histological changes in liver tissue of Nile tilapia juveniles fed with a diet supplemented with different levels of premix (1.0; 1.5; 2.0g kg^{-1}) and supplemented or not with immunostimulant boost (40g kg^{-1}) for 50 days. Values are presented as mean ± standard deviation

	0.1%	0.1%I	0.15%	0.15%I	0.2%	0.2%I	*P* Premix	*P* Imuno	*P* Interaction
Cordial appearance	1.91 ±0.56	1.33 ±0.72	1.41 ±0.41	1.83 ±0.63	1.33 ±0.81	1.62 ±1.23	0.9107	0.8969	0.3928
Balloon appearance in the hepatocytes	1.91 ±0.68	2.08 ±0.31	2.16 ±0.43	1.83 ±0.19	1.66 ±0.27	1.66 ±0.47	0.2241	0.7535	0.504
Congestion of the pancreas	1.25 ±0.41	1.58 ±0.56	1.58 ±0.16	1.50 ±0.19	1.41 ±0.31	1.66 ±0.47	0.7581	0.3029	0.5308
Congestion of the sinusoids	1.08 ±0.31	1.41 ±0.16	1.00 ±0.27	1.33 ±0.60	1.25 ±0.56	1.08 ±0.56	0.9135	0.3777	0.457
Eosinophilic infiltrate	1.58 ±0.56	1.83 ±0.57	1.5 ±0.33	1.75 ±0.16	1.75 ±0.73	1.95 ±0.94	0.7528	0.3562	0.9969
Lymphocytic infiltrate	0.5 ±0.63	0.41 ±0.5	0.50 ±0.57	0.16 ±0.19	0.25 ±0.31	0.5 ±0.43	0.8636	0.7746	0.4726
Hypertrophy of the hepatocytes	2.33 ±0.27	2.25 ±0.56	2.16 ±0.43	1.62 ±0.75	1.83 ±0.43	2.00 ±0.72	0.3001	0.5089	0.4491
Loose melanomacrophages	0.08 ±0.16[a]	0.00 ±00[b]	0.25 ±0.31[a]	0.00 ±0.00[b]	0.50 ±0.57[a]	0.00 ±0.00[b]	0.3419	0.0248	0.3419
Macroesteatosis	1.83 ±1.13	2.16 ±1.03	2.41 ±0.56	1.75 ±0.68	1.83 ±0.57	2.33 ±0.60	0.9717	0.8673	0.3145
Microesteatosis	0.16±0.33	0.50±1.00	0.33±0.38	0.25±0.50	0.33±0.66	0.25±0.31	0.9866	0.8188	0.7177
Necrosis	2.5 ±0.19	2.5 ±0.57	2.25 ±0.73	1.66 ±0.47	2.75 ±0.31	2.08 ±0.68	0.1207	0.0722	0.4148

a, b: Indicates statistical difference between supplementation with the immunostimulant boost by the Tukey test with a 5% significance level.

Table 7. Intensity of histological changes in the spleen tissue of Nile tilapia juveniles fed with a diet supplemented with different levels of premix (1.0; 1.5; 2.0 g kg^{-1}) and supplemented or not with immunostimulant boost (40 g kg^{-1}) for 50 days. Values are presented as mean ± standard deviation

	0.1%	0.1%I	0.15%	0.15%I	0.2%	0.2%I	*P* Premix	*P* Imuno	*P* Interaction
Integrity of the white pulp	3.00 ±0.00	3.00 ±0.00	2.87 ±0.25	2.75 ±0.28	3.00 ±0.00	2.87 ±0.25	0.1515	0.2878	0.7445
Integrity of the red pulp	3.00 ±00	3.00 ±0.00	2.87 ±0.25	2.75 ±0.28	3.00 ±0.00	2.87 ±0.25	0.1515	0.2878	0.7445
Centers of melanomacrophages	1.12 ±0.25	1.00 ±0.00	1.37 ±0.75	1.00 ±0.00	1.12 ±0.25	1.25 ±0.50	0.7689	0.4486	0.4647
Loose melanomacrophages	1.00 ±00	1.00 ±00	0.87 ±0.25	1.00 ±0.00	1.12 ±0.25	1.12 ±0.25	0.1256	0.5709	0.7209
Necrosis	0.25 ±0.50	0.37 ±0.47	0.87 ±0.62	0.87 ±0.62	0.75 ±0.28	0.87 ±0.62	0.1013	0.7099	0.9650

DISCUSSION

The premix doses influenced the zootechnical parameters of the Nile tilapia. For example, supplementation with 0.2% premix improved the final weight, weight gain, daily weight gain, and feed conversion compared with supplementation with 0.1% premix. The improvement in the zootechnical parameters could be related to the micromineral composition of the dietary components, as the estimated concentrations of these nutrients were higher than the nutritional requirements of the species. The Fe, Zn, Mn, and Cu concentrations in the diet supplemented with the lowest micromineral concentration (0.1% premix) were approximately 130, 65, 36, and 14mg kg^{-1}, respectively, while the actual mineral requirements are 85 mg kg^{-1} (Shiau and Su, 2003), 30mg kg^{-1} (Eid and Ghonim, 1994), 7mg kg^{-1} (Lin *et al*., 2008), and 5mg kg^{-1} (Ferrari *et al*., 2004), respectively.

Further, the best zootechnical performance can be correlated with the bioavailability of dietary minerals. The bioavailability of a nutrient indicates the proportion of a nutrient that has been effectively used by an organism to perform metabolic functions (Watanabe *et al*., 1997).

Additionally, several factors including inclusion level, nutrient form, particle size and its digestibility, synergistic or antagonistic interaction with biomolecules, physiological and pathological conditions of fish, mineral concentration in water, and target species, can influence the bioavailability of dietary minerals (Webster and Lim, 2015). Thus, better availability of minerals for Nile tilapia through the diet containing 0.2% premix could be attributed to these factors, which in turn promoted better zootechnical indexes.

Mineral bioavailability can also be affected by components, such as soy, corn, and rice, of vegetable origin, as they exhibit high concentrations of anti-nutritional factors, especially phytate, which can interact strongly with metallic ions (Reddy and Sathe, 2002). In this study, approximately 89% of the dietary formulation comprised components of vegetable origin. However, this did not affect the availability of dietary minerals, as the micromineral contribution from the premix was approximately 14%, 19%, and 24% for diets with 0.1%, 0.15%, and 0.2% supplementation, respectively.

Thus, the majority of the total metallic ion concentrations in the diets originated from other ingredients, mainly those of vegetable origin. However, most bioavailable micromineral sources originate from the premix, even if the premix was added in small amounts. Therefore, mineral supplementation possibly influenced the zootechnical parameters found among treatments with different concentrations of premix supplementation.

The isolated effects of each immunostimulant (β-glucans, nucleotides, vitamins C and E) evaluated in the present study have already been studied previously (Ringø *et al*., 2011). However, knowledge on the synergism among these compounds is limited. We observed that the final weight, weight gain, daily weight gain, and food conversion improved in the group with 0.4% immunostimulant boost compared to those in the group without supplementation. This was consistent with previous studies, which reported that the zootechnical parameters of Nile tilapia improved with the use of β-glucans (Aramli *et al*., 2015) nucleotides (Ringø *et al*., 2011), vitamins C (Ibrahem *et al*., 2010), and vitamin E (Jiang *et al*., 2019). In this study, immunostimulants showed positive synergism on the zootechnical parameters.

However, the combined use of these immunostimulants did not negatively influence the health of juvenile tilapia. A small proportion of mortalities could be attributed to the aggressive behavior (dominance) of some fish in the tank (Situmorang *et al*., 2016).

Overall, ß-glucans (Amphan *et al*., 2019; Barros *et al*., 2014), nucleotides (Machado *et al*., 2018), vitamin C (Barros *et al*., 2014; Ibrahem *et al*., 2010; Trichet *et al*., 2015), and vitamin E (Izquierdo and Betancor, 2015; Jiang *et al*., 2019; Saber *et al*., 2019) considerably influence the immune system, improve the health, and promote resistance of fish to stress and potentially, to infectious diseases.

Intestinal morphology reflects the association between the health status of fish with the capacity of nutrient assimilation and immune functions (Nicholson *et al*., 2012). An increase in the intestinal fold surface area indicates an improvement in intestinal health and increased nutrient absorption capacity (Mohamed *et al*., 2014). These factors could have possibly contributed to better zootechnical indices in the fish supplemented with the immunostimulant boost, because an increase in the fold number, fold length, total area, and number of goblet cells was observed in the group supplemented with the immunostimulant boost.

The mucus in the epidermal layer of fish has numerous functions; for example, it acts as an integral component of the innate immune mechanism, acts as a mechanical barrier that hinders the entry of pathogenic bacteria, and contains several antimicrobial components, such as lysozymes, immunoglobulins, complement system proteins, and lectins, of the innate immune response (Subramanian *et al*., 2007). These functions could fix beneficial bacterial microbiota to the immune system for better consumption of the nutrients, which in turn may have improved the zootechnical rates in the groups supplemented with the immunostimulant boost.

Increases in the total proteins and immunoglobulins in fish plasma are associated

with strong innate and acquired response mechanisms (Wiegertjes *et al*., 1996). Innate responses are developed after the invasion of pathogenic organisms and include a boost network of molecules and cells designed to kill or inactivate the pathogen (Buonocore and Scapigliati, 2009). Particularly, immunoglobulins play a significant role in the immune protection mechanisms of fish as a first line of defense (Misra *et al*., 2006).

In this study, total plasma protein and immunoglobulin concentrations increased with supplementation with the immunostimulant boost after the 50-d supplementation period, thus, suggesting that fish fed with immunostimulants were more immunologically strong and could fight pathogenic attacks more rapidly.

Hematopoietic tissues contain variable amounts of melanomacrophages or macrophage aggregates, particularly in the spleen and kidneys (Meseguer and Esteban, 1994). The premix inclusion levels with or without the supplementation of the immunostimulant boost did not impair the tilapia functions and were not toxic to the animals, as majority of the analyzed parameters did not show any significant difference.

Although loose melanomacrophages were observed in the liver tissue in the treatments without the immunostimulant boost, they were not observed in the splenic tissue. According to some studies, hematopoietic tissues include variable amounts of melanomacrophages or aggregates of macrophages, particularly in the spleen and kidney (Meseguer and Esteban, 1994) but this does not hinder their functional traits.

CONCLUSION

The best premix concentration for optimal zootechnical indexes was 0.2% with immunostimulant, when compared to diets with premix but without immunostimulant. Furthermore, there was a positive relationship between the use of the immunostimulant and the effect of the premix, as it was possible to observe an improvement as the doses increased. Supplementation with the immunostimulant boost promoted maximum growth and improved intestinal morphology and immunological parameters in Nile tilapia juveniles.

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