GROWTH PERFORMANCE, HEMATOLOGICAL AND HISTOLOGICAL PARAMETERS OF NILE TILAPIA LARVAE FED DIETS SUPPLEMENTED WITH B-GLUCANES AND NUCLEOTIDES

DESEMPENHO DE CRESCIMENTO, PARÂMETROS HEMATOLÓGICOS E HISTOLÓGICOS DE LARVAS DE TILÁPIA-DO-NILO ALIMENTADAS COM DIETAS SUPLEMENTADAS COM B-GLUCANAS E NUCLEOTÍDEOS

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ABSTRACT

Food additives such as nucleotides and β-glucans are used to enhance the performance and immunity of aquatic organisms. This study aimed to evaluate the addition of these substances in the diet of Nile tilapia larvae, assessing growth, survival, hematological parameters, histological changes, and stress resistance. The experiment lasted 30 days, with 800 larvae distributed across 16 aquariums and divided into four treatments: control (no supplementation), β-glucan (0.75 g kg^{-1}), nucleotide (2 g kg^{-1}), and β-glucan + nucleotide (0.75 + 2 g kg^{-1}). The larvae fed diets supplemented with β-glucan showed higher final weight, daily weight gain, total length, and specific growth rate, and better performance in the hypoxia test. Larvae fed the combination of β-glucan + nucleotides also differed from the control for the same parameters, except for length. After stress challenges with salinity and hypoxia, larvae in the control group showed lower survival, demonstrating that the supplementation of β-glucan and nucleotides improves stress resistance and animal well-being. Intestinal villus thickness was reduced in the nucleotide-supplemented diet. In conclusion, the supplementation of β-glucan and nucleotides has positive effects on the survival and performance of Nile tilapia larvae, particularly under stress conditions.

KEYWORDS: Food additives, hypoxia, salinity.

RESUMO

Aditivos alimentares como nucleotídeos e β-glucanas são utilizados para melhorar o desempenho e a imunidade de organismos aquáticos. Este estudo teve como objetivo avaliar a adição dessas substâncias na dieta de larvas de tilápia do Nilo,

analisando crescimento, sobrevivência, parâmetros hematoimunológicos, alterações histológicas e resistência ao estresse. O experimento durou 30 dias, com 800 larvas distribuídas em 16 aquários, divididas em quatro tratamentos: controle (sem suplementação), β-glucana (0,75 g kg^{-1}), nucleotídeos (2 g kg^{-1}) e β-glucana + nucleotídeos (0,75 + 2 g kg^{-1}). As larvas alimentadas com dietas suplementadas com β-glucana apresentaram maior peso final, ganho de peso diário, comprimento total e taxa de crescimento específico, além de melhor desempenho no teste de hipóxia. As larvas alimentadas com a combinação de β-glucana + nucleotídeos também diferiram das do controle para os mesmos parâmetros, exceto para o comprimento. Após desafios de estresse por salinidade e hipóxia, as larvas do grupo controle mostraram menor sobrevivência, evidenciando que a suplementação com β-glucana e nucleotídeos melhora a resistência ao estresse e o bem-estar dos animais. A espessura das vilosidades intestinais foi reduzida na dieta com nucleotídeos. Conclui-se que a suplementação com β-glucana e nucleotídeos tem efeitos positivos na sobrevivência e no desempenho das larvas de tilápia do Nilo, especialmente em condições de estresse.
PALAVRAS-CHAVE: Aditivos, hipóxia, salinidade.

INTRODUCTION

Oreochromis niloticus Linnaeus, 1758 (Nile tilapia) is one of the most cultivated tropical freshwater species, and its cultivation is expanding at an extremely high rate. Out of the 49,120,000 tons of production of aquaculture species, 4.41 thousand tons originate from tilapia culture, representing 9% of the total production in 2020[1]. In Brazil, tilapia production was 63.93% (550,060 tons) of all Brazilian fish cultivation in 2022, an increase of 3% over the previous year, maintaining the country as the world's 4th largest producer[2].

Larviculture plays a vital role for achieving both the quantity and quality of fish necessary for maximizing productivity. Proper nutrition is a fundamental requirement influencing productivity and is essential for successful cultivation. This aspect is explored in the context of large-scale production to enhance the survival rate and growth of individuals, facilitated using commercial feed from the early stages of fish life[3]. Additionally, it is crucial to provide highly nutritious and easily assimilable foods, along with a specific feeding frequency[4]. As a strategy, additives containing immunostimulants have been incorporated into diets[5].

Due to restriction on the use of antibiotics and some chemotherapy drugs as growth promoters, immunostimulants have become prominent[6]. Among the

additives with immunostimulant potential in fish nutrition, β-glucans, and nucleotides have been studied. β-glucans are polysaccharides that are structural constituents of the cell wall of yeasts, fungi, and some cereals, with various beneficial effects such as antitumor, anti-inflammatory, antimutagenic, hypocholesterolemic, and hypoglycemic properties[7-8], observed that the survival rate in Nile tilapia was 40% and 45% higher when supplemented with 0.1g kg^{-1} of β-glucans, compared to animals without supplementation after sanitary challenge with an intraperitoneal injection of *S. agalactiae*. Other studies also reported that β-glucans increased the productive performance of Nile tilapia[9-11].

Nucleotides are biochemical compounds of low molecular weight that perform many physiological and biochemical functions essential to the cell, such as construction of monomeric nucleic acid units, modulation of energy metabolism, involvement in biosynthetic pathways, regulation of biological processes, and serving as coenzyme components[12]. The addition of β-glucans and nucleotides to the diet has been shown to promote greater growth of golden (*Megalobrama amblycephala*)[13], gibel carp (*Carassius auratus*)[14], Jian carp (*Cuprinus carpio* var. Jian)[15], and tilapia[16,17].

Although some studies indicate the benefits of β-glucan and nucleotides in the fish diet, data on the benefits of these additives, either individually or in combination, during the larviculture phase are scarce. Therefore, the use of β-glucan may promote greater weight gain, while nucleotides may result in a lower mortality rate, especially in places with induction of environmental stress. Then, combining both additives may lead to improved survival and performance rates.

Thus, we aimed to evaluate the dietary addition of nucleotides and purified β-1.3/1.6 glucans, individually or in combination, on performance, histological and blood parameters, and resistance to the challenge with salinity and hypoxia of the larval phase and Nile tilapia.

MATERIAL AND METHODS

The experiment was carried out at the Laboratory of Aquaculture and Aquatic Ecology of the Federal University of Vales do Jequitinhonha e Mucuri (JK Campus), in Diamantina, MG, for 30 days. All procedures with animal use were previously approved by the Ethics Committee on Animal Use (ECAU) of UFVJM (process 006/2020 ECAU, UFVJM, Brazil).

Were evenly distributed 800 Nile tilapia larvae, with an initial weight of 0.0156 ± 0.0063mg and length of 1.004 ± 0.065cm, among 16 aquariums (40 L useful volume), at the density of 1.0 fish L^{-1} (40 fish aquarium-1). They had constant aeration and controlled photoperiod (12 h of light and 12 h of darkness), conditions in which the animals were previously acclimatized for seven days.

The larvae were distributed in a completely random design into four dietary groups: commercial feed without supplementation (CF); commercial feed supplemented with β-glucan at 0.75 g kg^{-1} (CFB); commercial feed supplemented with nucleotide at 2 g kg^{-1} (CFN); and commercial feed supplemented with β-glucan (0.75 g kg^{-1}) and nucleotide (2 g kg^{-1}) (CFBN). Each treatment consisted of four aquariums, serving as the sampling units.

EXPERIMENTAL FOOD AND DIET

The fish were fed five times a day (at 8 am, 10 am, 12 am, 2 pm, and 4 pm) at 20% of the biomass of the aquarium. The amount of feed was adjusted according to [18]. A commercial mashed feed (SUPRA®) was used with 50% crude protein, 12% humidity, 9% ether extract, 3% crude fiber, and 14% mineral matter. The additives were incorporated into each 1 kg of commercial feed according to the treatment (Table 1). The additives used in the experiment were produced and transferred by the Biorigin® company. The nucleotide source was an extract of the yeast Saccharomyces cerevisiae with high concentration and availability of nucleotides and proteins and purified β-1.3/1.6 glucans, from a selected strain of the yeast *Saccharomyces cerevisiae*. To incorporate the immunostimulants into the

respective diets, the products were initially diluted in 100 ml of absolute alcohol and sprayed onto the feed while manual mixing was performed. Following, the adding the additives, the diets were dried in greenhouses at 40°C for 24 h.

Table 1. Composition of experimental diets (g MS).

Items	**Diets**			
	CF	**CFB**	**CFN**	**CFBN**
Commercial Feed (g)[1]	1000	1000	1000	1000
Purified β-glucans (g)[2]	0.00	0.75	0.00	0.75
Nucleotides (g)[3]	0.00	0.00	2.00	2.00
	Composition (%)			
Dry matter	80.47	81.55	78.86	73.47
Organic matter	19.53	18.45	21.14	26.53
Mineral matter	11.90	11.69	13.62	12.69
Crude protein	43.85	47.08	44.57	48.53
Ether extract	4.00	6.45	10.70	5.02
Crude fiber	4.02	6.44	6.77	2.62
Non-fibrous carbohydrate	30.5	29.1	31.9	30.6

CF - Commercial feed without supplementation; CFB - commercial feed supplemented with β-glucan; CFN - commercial feed supplemented with nucleotide; CFBN - commercial feed supplemented with β-glucan and nucleotide.

[1] Commercial Ration - Manufacturer's Packaging Data

[2] Purified β-1.3/1.6 glucans produced from a strain of *Saccharomyces cervisae* yeast.

[3] Produced by yeast extract with high concentration and availability of nucleotides and proteins

WATER QUALITY

The aquariums were cleaned twice a week with a renewal of 20% of the water volume to remove excess organic matter. During the experimental period, the physical parameters of water, temperature, and dissolved oxygen were measured daily (EcoSense®DO200A). The parameters of electrical conductivity (Conductivity© METTLER TOLEDO), light intensity (Digital Lux Meter ©ICEL LD-510), pH (pHmetro ©AKASO AK 151), turbidity, total suspended solids, and salinity (Horiba W-22XDD) were measured weekly. The analyses of ammonia, nitrite, nitrate, alkalinity, and hardness were performed weekly[19].

GROWTH PERFORMANCE

On the 13th day, all larvae were anesthetized with eugenol solution (75 mg · L^{-1})[20]. The larvae of each aquarium were counted to estimate the survival rate (survival rate = (number of fish alive at the end of the period . total fish alive at the beginning of the period^{-1}) × 100). Then, the larvae were weighed with a precision analytical balance (0.01 g), and the standard and total lengths were measured with a digital pachymeter (0.01 mm).

These data were used to estimate biomass (sum of the weight of aquarium fish), Fulton's condition factor (K = 100 × (weight × length^{-3}), feed conversion ((FC = feed weight offered . biomass gain^{-1}), weight gain (WG = final weight − initial weight), daily weight gain (DWG = weight gain . days of experiment^{-1}), and specific growth rate (SGR = 100 (lnWtf − lnWti) . Δt^{-1}, considering Δt the duration, in days, between the samples, Wi the initial weight and Wf the final weight).

BLOOD PARAMETERS

Three fish from each box were euthanized by medullary section and caudal section for blood collection. The blood was used to prepare stained blood smears with the Rosenfeld May-Grunwald-Giemsa technique (1974), for counting red blood cells and leukocyte, and leukocyte differential (lymphocytes, neutrophil eosinophils, basophils, and monocytes). The count was performed with an optical microscope (Nikon®), with an increase of 1000 times. Moreover, the differential count in "zigzag" was performed throughout the blood smear, and the percentage of each cell was subsequently estimated.

STRESS CHALLENGE

After 24 hours since the last biometrics, 16 fish from each aquarium were separated into two groups, eight larvae each, to be tested by two acute stresses: salinity stress and air exposure stress. The first test was performed with 40 gL^{-1} of NaCl, a concentration established by a previous experiment[21]. Different aquariums received 1 L of saline water (40 g L^{-1}), where eight larvae (8 fish L^{-1}) were placed.

The larvae were kept in brackish water until they expressed disoriented behaviors or inactivity, the time was measured for subsequent comparison. After exposure to high salinity, the larvae returned to the experimental units containing fresh water with constant ventilation for 24 hours to determine survival after stress[22].

To perform the second challenge, Tilapia larvae were allocated on a countertop and kept on a damp cloth to avoid possible lesions. The fish were exposed to air for 15 minutes, a time determined by the previous experiment. After this period, the animals were relocated in the aquariums of their respective treatments, with constant ventilation. The survival rate after exposure to air was determined after 24 hours[23.]

MORPHOLOGICAL ANALYSIS

Three fish per treatment were euthanized by eugenol overdose (300 $mg.L^{-1}$). The liver and viscera were weighed to determine the hepatosomatic index (HSI) and the viscerosomatic index (VSI). Transverse sections were collected from the medial region of the intestine, then dissected and washed with paraformaldehyde (10%) before they were fixed in paraformaldehyde (10%) for 24 h at room temperature. After this period, the fragments of the intestine were washed in running water for 12 h and subsequently immersed in 70% ethanol until the processing. The samples were dehydrated in graded ethanol series, cleaned in xylene, and enused in paraplast. The intestine was sectioned 5 μm thick and stained with hematoxylin and eosin (H&E) for histological analysis. Two segments from each section of the intestine were analyzed, focusing on morphological variables such as villi quantification, villi height, and villi thickness[24-26].

Quantitative and morphometric data of intestinal villi were obtained in the anatomy laboratory of Universidade Federal de Alfenas, Campus Alfenas, MG, via an adaptation of the methodology used by[27-29]. For this study, villi were identified and quantified in a 20× or 40× objective; after quantification three of these structures were randomly selected for measurement of the length and thickness. The length was considered from the apex to the base, with the limit in the area adjacent

to the crypt zone, and the thickness was obtained from the top of an enterocyte to the lamina propria of the villi. This measurement was performed under a light microscope with an increase of 20× or 40×. Both quantitative analysis and morphometry used tools of the AxioVision Rel. software 4.8.2 and AxioVision 4 Module Interactive Measurement of the brand Carl Zeiss.

STATISTICAL ANALYSIS

To compare the efficiency of diets on larvae performance, the data were submitted to normality and homogeneity tests. Followed to One way ANOVA and Tukey's test, at 5% probability, using the statistical software R® (Version 1.3.1093, 2009–2020). The results obtained were expressed as mean ± standard error of the mean. To interpret histology data were also performed normality and homogeneity tests, and One-Way Analysis of Variance (ANOVA), followed by Tukey's test, using GraphPadPrism 8 software (GraphPad Software, La Jolla, CA, USA) and adopting the significance level of 5% for all analyses.

RESULTS

WATER QUALITY

The water quality parameters are presented in Table2. We observed no statistical differences ($p > 0.05$) between treatments for parameters dissolved oxygen, temperature, ammonia, nitrite, nitrate, phosphate, alkalinity, hardness, and pH. The values observed for nitrite and electrical conductivity were higher ($p < 0.05$) in the CF treatment (0.013 $mg.L^{-1}$ and 0.168 $mS.cm^{-1}$) compared to the CFBN treatment (0.007 $mg.L^{-1}$ and 0.163 $mS.cm^{-1}$); however, both showed no difference when compared with the values observed in the aquariums where the larvae received CFB (0.009 $mg.L^{-1}$ and 0.165 $mS.cm^{-1}$) and CFN (0.008 $mg.L^{-1}$ and 0.164 $mS.cm^{-1}$), and were within the recommended for this species.

Table 2. Mean values and standard error of the physical-chemical parameters of water in the cultivation of Nile tilapia larvae fed commercial feed with or without supplementation.

Parameters	Treatments				SE	P-value	Reference value
	CF	CFB	CFN	CFBN			
Dissolved oxygen ($mg.L^{-1}$)	6.0	5.8	5.9	6.0	0.036	0.38	>3.5[1]
Temperature (°C)	28.1	28.3	28.2	28.3	0.054	0.61	27–32[1]
Ammonia ($mg.L^{-1}$)	0.14	0.13	0.13	0.14	0.001	0.59	0.20[1]
Nitrite ($mg.L^{-1}$)*	0.013[a]	0.009[ab]	0.008[ab]	0.007[b]	0.001	<0.05	≤1[1]
Nitrate ($mg.L^{-1}$)	0.014	0.014	0.015	0.015	0.0008	0.84	≤10[2]
Phosphate ($mg.L^{-1}$)	0.55	0.51	0.55	0.64	0.019	0.09	<100[3]
Alkalinity ($mgCaCo_3.L^{-1}$)	33.26	40.20	33.44	38.72	3.293	0.36	>20[3]
Hardness ($mgCaCo_3.L^{-1}$)	54.54	56.24	48.32	54.96	9.106	0.83	>20[5]
pH	7.23	6.94	6.94	6.76	0.184	0.89	6.5–8[1]
Conductivity ($\mu S.cm^{-1}$)*	1.7[a]	1.6[ab]	1.6[ab]	1.6[b]	0.004	<0.01	1.2–5[5]
TSS ($g.L^{-1}$)	0.06	0.05	0.05	0.06	0.002	0.39	0.06[2]
Luximeter (lux)	211.4	210.4	247.6	220.6	6.717	0.10	180–500[6]
Turbidity (NTU)	108.1	101	91.4	103	5.387	0.41	≤100[2]

CF - Commercial feed without supplementation; CFB - commercial feed supplemented with β-glucan; CFN - commercial feed supplemented with nucleotide; CFBN - commercial feed supplemented with β-glucan and nucleotide [18, 30-33].

*means with different lowercase letter in the same line indicate significant treatment effect (Tukey $p < 0.05$)

TSS: total suspended solids; SE: standard error

GROWTH PERFORMANCE

The growth performance of Nile tilapia larvae fed with food enriched with two supplements is presented in the Table 3. The initial weight of the larvae, final biomass, feed conversion, and Fulton's condition factor (K) were the same among treatments ($p>0.05$). Tilapia larvae fed with CFB and CFBN presented higher final weight (0.80 and 0.78 g), daily weight gain (0.026 and 0.025g), total length (3.46 and 3.56 cm), and SGR (2.6 and 2.5%) compared to larvae without supplementation in the control treatment (0.52 g; 0.016g; 3.04 cm and 1.7%, respectively). Regarding these variables, animals consuming supplemented feed with nucleotide ($p > 0.05$) had the same results as those from the other treatments.

Table 3. Growth performance (means) in the cultivation of Nile tilapia larvae fed commercial feed with or without supplementation.

Parameters	Diets				SE	P-value
	CF	CFB	CFN	CFBN		
Initial Weight (g)	0.014	0.013	0.014	0.014	0.0009	0.78
Final Weight (g)*	0.52[b]	0.80[a]	0.65[ab]	0.78[a]	0.035	<0.01
Daily Weight Gain (g)*	0.017[b]	0.026[a]	0.021[b]	0.025[a]	0.001	<0.01
Total Length (cm)*	3.05[b]	3.46[a]	3.29[ab]	3.46[a]	0.063	<0.01
Standard Length (cm)*	1.78[b]	2.20[a]	2.07[ab]	2.05[ab]	0.054	0.02
Consumption (g)	112.03	113.53	96.16	111.76	0.021	<0.01
Biomass (g)	13.73	20.11	16.97	18.11	1.003	0.14
Apparent Feed Conversion (g)	0.91	1.29	1.13	1.26	0.063	0.11
Fulton (K)	1.81	1.93	1.83	1.74	0.040	0.45
Specific Growth Rate (%)*	1.69[b]	2.61[a]	2.11[ab]	2.55[a]	0.117	<0.01

CF - Commercial feed without supplementation; CFB - commercial feed supplemented with β-glucan; CFN - commercial feed supplemented with nucleotide; CFBN - commercial feed supplemented with β-glucan and nucleotide.
*Means with different lowercase letter in the same line indicate significant treatment effect (Tukey $p < 0.05$).
SE: standard error

BLOOD PARAMETERS

The hematological parameters of Nile tilapia larvae fed with food enriched with two supplements are presented in Table 4. The additives in commercial feeds had no effect ($p > 0.01$) on the hematological parameters of Nile tilapia larvae and all were within the recommended range for the species.

Table 4. Means, standard error, and p-value of hematological variables in the cultivation of Nile tilapia larvae fed commercial feed with or without supplementation.

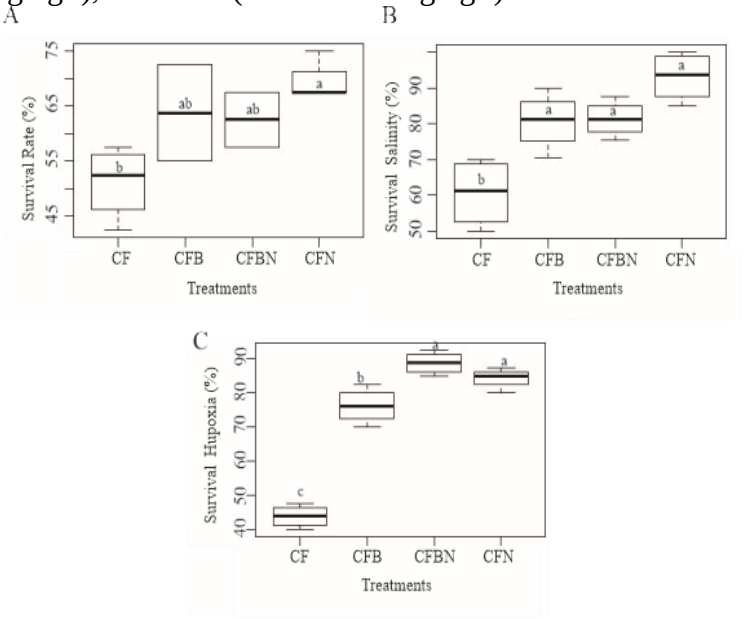
Variables (%)	Diets				SE	P-value	Reference Values[1]
	CF	CFB	CFN	CFBN			
Red Blood Cells	41.47	44.48	41.99	41.42	0.682	0.3634	21-44
Lymphocytes	32.26	29.72	32.92	32.73	0.524	0.0950	16-69
Eosinophils	0	0	0	0	0	-	0
Monocytes	1.36	1.43	1.65	1.54	0.049	0.1621	1-12
Neutrophils	23.44	23.34	23.76	24.08	0.487	0.9596	25-82
Basophils	1.27	1.42	1.40	1.95	0.202	0.6871	0-2

CF - Commercial feed without supplementation; CFB - commercial feed supplemented with β-glucan; CFN - commercial feed supplemented with nucleotide; CFBN - commercial feed supplemented with β-glucan and nucleotide [34.]

The survival rates, after the feeding period (30 days) and resistance tests, showed a significant difference (Figure 1). The mean survival rate after the feeding

period, in which all water variables were monitored to properly develop larvae, was 61.5%. We also observed a lower survival rate of larvae fed with CF (51%) compared to those that consumed CFN (69%). Survival rates of larvae consuming CFB (63%) and CFBN (62%) did not differ significantly from other treatments (Figure 1A). After stress induction, the results of supplementation with additives in the diet improved ($p < 0.05$) the survival rates of larvae compared to treatment without supplementation. In the salinity test, non-supplemented larvae presented a lower survival rate (60%) than larvae consuming CFN (93%), CFB (80%), and CFBN (81%), which had the same survival rate among each other (Figure 1B). After the hypoxia test, we observed the best results in the treatments with nucleotides, CFN (84%), and CFBN (88%), followed by treatment with β-glucan, CFB (76%). Moreover, we observed the lowest survival rates in larvae without supplementation, CF (43%) (Figure 1C).

Figure 1. Survival rate at the end of 30 days and on the sanitary challenges of salinity (40 g L^{-1}) and hypoxia (15 min of air exposure) in the larval phase of Nile tilapia fed with commercial feed without supplementation (control) or supplemented with CFB (β-glucan 0. 75g kg^{-1}), CFBN (β-glucan 0.75g kg^{-1}+ Nucleotide 2g kg^{-1}), and CFN (Nucleotides 2g kg^{-1}).



MORPHOLOGICAL ANALYSIS

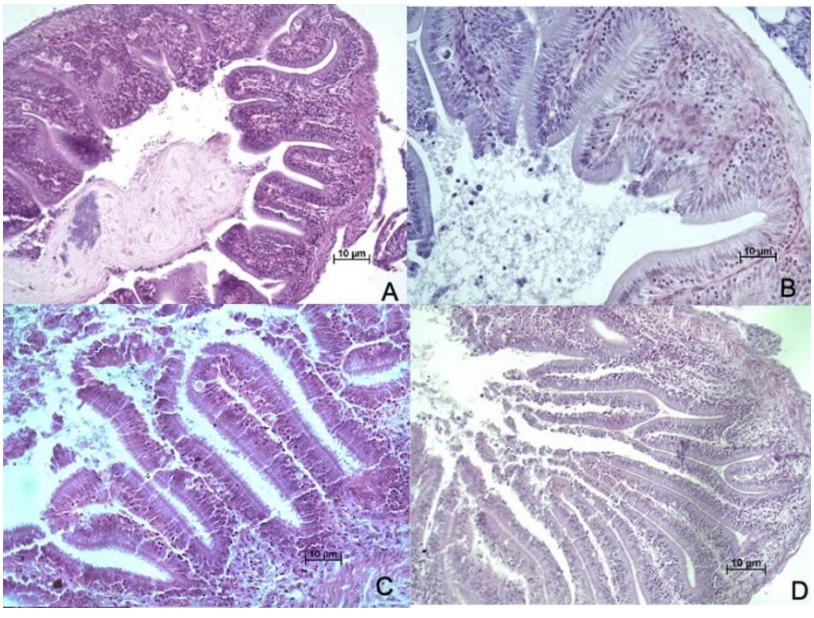
For all treatments, the villi quantification was the same (Table 5) (Figure 2). The villi height was higher ($p > 0.05$) in treatments without supplementation and CFB (commercial feed supplemented with β-glucan) than the villi of the larvae of the CFN treatment.

Table 5. Histological analysis in the cultivation of Nile tilapia larvae fed commercial feed with or without supplementation.

	Variable		
Treatment	Villi Quantification[1]	Villi Height[2]	Villi Thickness[2]
CF*	3.47	30.51[a]	5.87[a]
CFB*	3.14	25.32[ab]	6.15[a]
CFN*	4.24	18.92[c]	4.35[b]
CFBN*	3.71	20.97[bc]	5.26[ab]

CF - Commercial feed without supplementation; CFB - commercial feed supplemented with β-glucan; CFN - commercial feed supplemented with nucleotide; CFBN - commercial feed supplemented with β-glucan and nucleotide.
*Means with different lowercase letter in the same line indicate significant effect of treatment (Kruskal-Walis $P < 0.05$).

Figure 2. Intestinal villi of tilapia in the larval phase submitted to control treatments (A), supplementation with β-glucan (0. 75g.kg^{-1}) (B), Nucleotides (2g kg^{-1}) (C), and β-glucan + Nucleotide (β-glucan 0.75g kg^{-1}+ Nucleotide 2g kg^{-1}) (D), for 30 days.



DISCUSSION

When supplementing the diet of aquatic organisms, there is a concern that the increased input of nutrients may lead to deterioration in the quality of the fish farming water. However, while the treatment of larvae fed with commercial feed without supplementation showed higher concentrations of nitrite and electrical conductivity, the observed values were not alarming. They did not exceed the maximum recommended levels for the species: 1 mg L^{-1} of NO_2 and up to 1000 μS cm^{-1}. Therefore, we corroborate that all the observed values for the physical-chemical parameters of the water were suitable for Nile tilapia cultivation, and as indicated in an important reference, these results reflect adequate environmental management[36]. Therefore, we can assume that the cultivation environment did not provide differences in the growth performance of the animals, which is typically a factor that influences their performance. In this case, however, the environment did not interfere with the differences observed between the treatments. All the results found can be attributed to the treatments tested. Larvae supplemented with CFB and CFBN showed the best results for performance variables, including final weight, daily weight gain, total length, standard length, and specific growth rate. The hypothesis for these observations is that β-glucan stimulated intestinal digestive activity, allowing better absorption of nutrients and, consequently, better performance of tilapia larvae. Thus, our results corroborate the literature[37-41], which indicates that supplementation with β-glucan benefits the productive performance of fish, especially regarding the increase in growth rate. However, the response may vary depending on the species, method of administration, amount of supplement incorporated in the diet, duration of administration, and environmental temperature[12,37,40-43].

The results of this study allow us to affirm that 30 days of supplementation were sufficient for the beneficial effects to appear. Supplementation with additives for thirty days positively affected tilapia larvae, as evidenced by the greater growth of larvae fed diets supplemented with beta-glucan and with both beta-glucan and

nucleotides, as well as the increased survival of larvae subjected to stress tests, particularly those fed diets supplemented with nucleotides.

The parameters of final biomass, apparent feed conversion, and Fulton's factor were the same between treatments, which may be related to the short supplementation time (30 days). According to (Chagas et al., 2013)[44], the time of administration to achieve better efficacy is still under discussion. In a study with Nile tilapia and "sea bass" (*Dicentrarchus labrax*) fed diets supplemented with β-glucan, and another with bijupirá juveniles (*Rachycentron canadum*) supplemented with 780 g kg^{-1} of nucleotides, no effect of the additives on these variables was observed[42,44-45]. In our study, as well in several others, including prebiotic did not lead to increase intestine development[24,47-49]**,** however, these authors did not observe reduced development compared to the control diet.

Although in most studies, nucleotides have beneficial effects on the intestinal morphology of fish[50-51], this was not observed in the present study with tilapia fed nucleotides. However, this additive increased the fish's resistance to stress and provided intermediate growth performance, positioned between the control, which showed the worst growth, and the other treatments. A suitable approach to assess the effects of prebiotics on fish health involves subjecting the animals to a form of stress. Saline challenges are a stressful factor used to assess osmotic capacity as an indicator of the general rusticity of larvae[52]. However, over a short period, due to ontogenetic changes associated with adaptive strategies, larvae increase their osmoregulatory capacity[52]. In the present study, supplemented larvae exhibited a higher survival rate during the saline stress test compared to the non-supplemented ones. Therefore, we hypothesize that the use of immunostimulants triggered the non-specific defense mechanisms in fish subjected to immunosuppression due to the acute stress of the test, resulting in a higher survival rate.

A study on β-glucan and nucleotides in tambaqui (*Colossoma macropomum*) challenged with *Aeromonas hydrophila* reported that these additives stimulate non-specific defense mechanisms, enhancing protection and reducing

diseases and mortality[44]. The early developmental phase represents a critical stage due to higher mortality rates and increased susceptibility to diseases and stress in fish. Thus, hematological parameters serve as significant indicators for monitoring health, nutritional status, and environmental conditions that affect fish, as these parameters can reflect immune response[53,54].

One of the benefits of immunostimulants is their ability to increase the activity of macrophages, enhance phagocytosis by neutrophils and monocytes, and promote higher production of lymphocytes, immunoglobulins, and lysozyme[55]. This ultimately leads to greater resistance of fish to opportunistic microorganisms[56]. The increase in leukocyte following β-glucan administration in the diet may indicate stimulation of non-specific immunity[38] and possibly result in the improvement of the defense system, which can increase resistance to environmental stress or pathogens.

This is supported by the results of a study that showed greater survival of pompano fish when exposed to salinity stress[57]. Lymphocytes play a crucial role in initiating and executing the adaptive or specific immune response by producing and releasing antibodies from T and B lymphocytes[58,59]. Stressed fish may exhibit elevated levels stress hormones, which can influence immune functions such as reducing lymphocyte counts and suppressing immunoglobulin synthesis[60]. However, supplementation with β-glucan or nucleotides did not interfere with the concentrations of these cells, which remained within the reference values 16-69%[34].

Basophils also did not differ between the treatments as expected, since they are typically present in very low concentration in fish blood. They may appear in higher concentration, along eosinophils, when fish experience an allergic reaction or are infected by parasites[61]. Therefore, the absence is justified, since fish were not infected or subjected to an allergic process, which is corroborated by the non-detection of circulating eosinophils-cells that are distributed in the connective tissues, mainly in the digestive tract and gills present in the bloodstream in allergic situations or when an acute inflammatory response is necessary[62].

The low levels of basophils may hinder the elucidation of immunostimulant action. β-glucan was administered to tilapia to evaluate stress indicators, and a decrease in basophil levels was found[63]. The immunostimulants did not affect the concentration of monocytes and neutrophils, as the proposed challenges were not sufficient to alter the metabolism of the fish. This corroborates findings from studies that reported the same monocyte values after the addition of prebiotics and probiotics to the diet of Nile tilapia and matrinxã (*Brycon amazonicus*)[64,65]. Monocytes and other leukocytes collaborate in the phagocytosis and elimination of dead or injured tissues, foreign materials, cellular debris, and the destruction of cancer cells, as well as contributing to the regulation of the body's immunity[59].

Neutrophils play a crucial role in the acute inflammatory defense and serve as the organism's first line of defense against bacterial agents by performing phagocytosis. They can also combat fungi, yeasts, algae, parasites, and viruses[59]. Thus, in bacterial infections, neutrophils can be found in greater amounts in the blood[66,67]. Interestingly, tambaquis (*C. macropomum*) fed with β-glucan had a decrease in neutrophil count, whereas with nucleotides neutrophil levels remained elevated[44]. Nile tilapia supplemented with 500 mg kg^{-1} of vitamins C and E also showed a reduction in the total number of circulating neutrophils in the blood after receiving intraperitoneal injections with lipopolysaccharides (LPS) from *Escherichia coli*[68]. Another environmental stress that the animals were subjected to evaluate their ability to improve health was resistance to hypoxia. This challenge can promote morphological, physiological, and behavioral changes at various stages of fish life[69]. The best results observed in nucleotide-supplemented larvae suggest that, in stressful situations, the use of nucleotides benefits the immune system of fish by inhibiting cortisol release[12], increasing disease resistance[70].

To comprehend the mechanism of action of immunostimulants and their impact on the body's defense, it is crucial to understand the efficiency of their absorption in the digestive tract. Larvae, being in the initial development phase, have a limited capacity to assimilate food. Despite tilapia larvae starting to consume feed as soon as the mouth opens, the digestive system is still forming[71]. While we

observed an improvement in the performance of supplemented larvae, their intestine still has a low absorption rate, which may explain the lack of interference of the β-glucan and nucleotides on blood parameters. Moreover, the surface area for microbiota fixation in the digestive tract of larvae is relatively smaller compared to animals of the same species in later development phases. The simple digestive system of the larvae of the present study affects digestion and absorption of nutrients.

Therefore, the establishment of immunostimulation methods that improve productive performance, stress resistance, and fish health conditions, contributing to prevent diseases in cultivation and to reduce mortality from stress, are crucial to develop the larvae activity[44].

CONCLUSIONS

Supplementation with immunostimulants, including β-glucan (0.75 g kg^{-1}) and nucleotides (2 g kg^{-1}), positively influenced the growth of Nile tilapia larvae, while maintaining hematological parameters within the reference values for the species. However, supplementation with nucleotides alone did not promote increased growth. In stressful environments, the use of nucleotides and β-glucan, either separately or in combination, effectively reduced larval mortality. The association of both additives did not result in cumulative effects that would justify recommending their combined use to improve growth and immunity in larviculture. Since the marketing of fingerlings is based on the number of individuals, we recommend the addition of nucleotides in larviculture, as they increase survival rates without negatively affecting fish growth.

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