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ORIGINAL ARTICLE

Dietary supplementation of Viligen™ to Nile tilapia improves growth and gut morphology

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Abstract

We evaluated the growth, gut histomorphometry, selected immune responses and resistance of Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae* infection when fed VILIGEN™, an additive blend containing sodium butyrate, dehydrated hydrolysed yeast and zinc proteinate. Increasing doses (0.00, 0.60, 1.20, 2.40 and 4.80 g kg^{-1}) were added to a basal diet and randomly assigned to six groups, each with 28 fish. The fish were fed to satiation twice daily for 60 days, infected *via* gavage with *S. agalactiae* at 1.0×10^9 CFU ml^{-1}, and mortality was recorded for 14 days. Dietary inclusion of VILIGEN™ promoted higher weight gain, gut-fold perimeter and the number of goblet cells. Through polynomial regression, the optimal inclusion level for these variables was estimated at 2.80, 3.30 and 3.50 g kg^{-1}, respectively. However, there was no clear effect of the additive on the immune responses tested before bacterial infection or in the survival after challenge. This is the first study on VILIGEN™ supplementation in fish diets. Higher growth and altered gut morphology were seen with the inclusion of VILIGEN™ in the range of 2.80 to 3.50 g kg^{-1} but the response to the bacterial challenge was not clearly affected.

KEYWORDS
bacterial challenge, feed additive, fish nutrition, growth, gut histology, immune response

1 | INTRODUCTION

The tilapia group is the second most produced fish worldwide, after the carps (FAO, 2018), and its production is expected to further increase to reach the global food demand (Tiengtam et al., 2017). Nile tilapia, *Oreochormis niloticus*, is the target species of this study; it presents highly favourable characteristics for aquaculture, such as fast growth in high densities, hardiness, easy reproduction and high market acceptance (Watanabe et al., 2002). However, the intensification of aquaculture can generate numerous stressors in fish, since it increases the stocking density, alters water quality, promotes excess organic matter and involves excessive fish handling (Barton, 2000). This may cause immunosuppression, decreased growth and an increased susceptibility to diseases caused by pathogens in fish (Tort, 2011). Within this context, diseases caused by bacteria are a major concern in aquaculture, as they have high proliferation rates, causing large losses in production systems (Pavanelli et al., 2008).

In tilapia farming, diseases caused by bacteria of the genus *Streptococcus* are responsible for huge economic losses and present the following clinical signs: appetite loss; displacement of the spine; exophtalmia; cornea opacity; and haemorrhaging of the gills, operculum and fins (Jiménez et al., 2007; Yanong & Floyd, 2002; Zamri-Saad et al., 2010; Zheng et al., 2018). The utilization of antibiotics against bacterial infections in aquaculture production is prohibited in several countries (Denev et al., 2009); besides leaving residues in fish meat, it also promotes the occurrence of resistant strains and reduces beneficial microbiota in the gastrointestinal tracts of fishes (Munir et al., 2016). Thus, the use of

TABLE 1 Formulation and composition of the experimental diets and feed additive composition

Ingredients	VILIGEN™ inclusion, g kg^{-1}				
	0.00	0.60	1.20	2.40	4.80
Ground corn[a]	432.90	432.30	431.70	430.50	428.10
Soybean meal[b]	366.00	366.00	366.00	366.00	366.00
Poultry by-product meal[c]	195.00	195.00	195.00	195.00	195.00
Vitamin–mineral premix[d]	5.00	5.00	5.00	5.00	5.00
Choline chloride[b,e]	1.00	1.00	1.00	1.00	1.00
Butylated hydroxytoluene	0.10	0.10	0.10	0.10	0.10
VILIGEN™[f]	0.00	0.60	1.20	2.40	4.80
Diet composition, g kg^{-1} dry matter					
Dry matter	907.00	914.60	845.60	904.30	897.90
Crude protein	365.20	365.90	365.60	368.90	367.80
Digestible protein[g]	340.00	340.00	340.00	340.00	340.00
Gross energy, kcal kg^{-1}	4600	4658	4621	4574	4630
Digestible energy[7], kcal kg^{-1}	3273	3273	3273	3273	3273
Ether extract	75.10	71.10	74.70	71.90	75.60
Mineral matter	57.40	58.30	56.80	56.30	56.20
VILIGEN™ composition, g kg^{-1} dry matter					
Crude protein	324.10				
Gross energy, kcal kg^{-1}	4633				
Ether extract	42.50				
Mineral matter	191.30				
Sodium butyrate	3.50				
Zinc	11.97				

[a]Supplied by Agroterra Agropecuária (Florianópolis, Brazil).; [b]Supplied by Nicoluzzi Rações Ltda (Penha, Brazil).; [c]Supplied by Kabsa S.A (Porto Alegre, Brazil).; [d]Supplied by Cargill (Toledo, Brazil), composition per kg product: folic acid 420 mg, pantothenic acid 8333 mg, BHT 25,000 mg, biotin 134 mg, cobalt sulphate 27 mg, copper sulphate 1833 mg, ferrous sulphate 8000 mg, calcium iodate 92 mg, manganese sulphate 3500 mg, niacin 8333 mg, selenite 100 mg, vitamin (vit.) A 1,666,670 IU, vit. B_1 2083 mg, vit. B_{12} 5000 μg, vit. B_2 4166 mg vit. B6 3166 mg, ascorbic acid equivalent 66.670 mg, vit. D_3 666.670 IU, vit. E 16,666 IU, vit. K_3 833 mg, zinc sulphate 23,330 mg, inositol 50,000 mg, and calcium propionate 250,000 mg.; [e]Choline source contained 600 g choline per kg product.; [f]Produced by Alltech Inc. (Nicholasville, USA) and imported by Alltech do Brazil Agroindustrial Ltda (Maringá Brazil).; [g]Estimated digestible values based on those proposed by Furuya et al. (2010).

alternative products that do not produce such negative side effects can promote the sustainability of the aquaculture industry (Doan et al., 2018).

A new trend in aquaculture is the use of feed additives, such as prebiotics, immunostimulants and organic salts, which can improve fish growth and health (Luckstadt, 2008; Tiengtam et al., 2017); they could be an environmentally viable alternative to antibiotics (Rashidian et al., 2019). Such additives, besides promoting growth, can also stimulate the growth of beneficial bacteria in the host gut, promoting positive immunomodulation that will minimize the action and proliferation of pathogens (Adorian et al., 2016; Gholampour et al., 2020; Ha et al., 2017; Rashidian et al., 2020; Ringo et al., 2010; Torrecillas et al., 2015; Vazirzadeh et al., 2020; Zhao et al., 2015).

VILIGEN™ (Alltech) is a new commercial feed additive, composed of zinc proteinate, dehydrated hydrolysed yeast and sodium butyrate; it was developed using nutrigenomic techniques to improve animal growth and gut health. Among the components of the additive, sodium butyrate is an organic acid that, besides being a bactericidal compound (Ng & Koh, 2017; Silva et al., 2013) and favouring the growth of lactic acid bacteria (Baruah et al., 2007; Luckstadt, 2008; Robles et al., 2013), acts by providing energy to the epithelial cells and, consequently, their proliferation. Hydrolysed yeast positively modulates the immune system through toll-like receptors that identify proteins and pathogen-associated molecular patterns, which triggers improved innate immune responses (Ha et al., 2017; Trichet, 2010). Zinc proteinate is an organic mineral that is more bioavailable than inorganic minerals; it has beneficial effects on growth performance and is important for cell proliferation in the immune system (Apines-Amar et al., 2004; Ibs & Rink, 2003; Lin et al., 2013; Rink & Haase, 2007). Therefore, using the three elements synergistically

could possibly result in faster growth and animal health responses than use of a single compound.

Thus, this study aimed to evaluate the influence of different inclusion levels of the feed additive VILIGEN™ in juvenile Nile tilapia diets on the growth performance, body composition, protein retention, gut histology, selected immune responses and survival after bacterial challenge with *Streptococcus agalactiae*.

2 | MATERIAL AND METHODS

2.1 | Experimental design, diets, feeding, fish and experimental procedures

The procedures adopted in the experiment were approved by the Animal Use Ethics Committee of the Federal University of Santa Catarina (CEUA, UFSC), under protocol number 2200250219.

A basal diet was formulated with practical ingredients to reach the nutritional requirements of Nile tilapia (Furuya et al., 2010; NRC, 2011). The additive was included in a basal diet at five levels: 0.00; 0.60; 1.20; 2.40; and 4.80 g kg^{-1}, replacing broken corn (Table 1).

The ingredients used in diet formulation had their approximate composition and energy content determined. Ingredients were individually ground in a hammer mill (1.0 mm screen mesh), manually sieved (0.5 mm), weighed and homogenized in a horizontal mixer. The moisture of the ingredients was adjusted to 210 g per kg using water. Diet extrusion was carried out in a single screw extruder, model MX-40 (Inbramaq). The extrusion parameters were as follows: temperature, 100°C; thread speed, 220 rpm; flow rate 1/5 of rated capacity; width to diameter ratio, 2.3:1; thread diameter, 92.5 mm; and cylinder length, 210 mm. After extrusion, diets were dried in a forced-air circulation oven at 55°C, and subsequently, they were packaged and stored in a dry environment at a constant temperature of 23°C.

Sex-reversed Nile tilapia juveniles from the GIFT strain were obtained at Piscicultura Pomerode (Pomerode, Santa Catarina, Brazil). Prior to the feeding trial, the fish were acclimated to laboratory conditions for one week in three 1000-L tanks that were connected to a recirculation system and equipped with biological and mechanical filtration, an air supply, and a heat exchanger. The temperature was set to 28°C, and the photoperiod was adjusted to 12 h.

After acclimation, groups of 28 fish were randomly stocked in experimental units (n = 30) in 200-L tanks. The fish remained under acclimation for another week and were fed the basal diet without the inclusion of the feed additive. Subsequently, the initial biometric parameters were determined (weight, 1.98 g ± 0.04; total length, 4.83 cm ± 0.04 [mean ± SD]). The experiment was conducted in a completely randomized design, using six replications for each tested level. Fish were fed twice daily (09:00 and 04:30 h) until apparent satiation for 60 days, and feed intake and mortality were recorded daily.

The inlet water flow was 40 ml s^{-1}, providing a water exchange rate of 36 times the total volume of each experimental unit per day. The temperature (28.33 ± 0.28°C), dissolved oxygen (6.35 ± 0.49 mg L^{-1}) and pH (6.74 ± 0.19) were measured daily, while the total ammonia (0.15 ± 0.11 mg L^{-1}), nitrite (0.03 ± 0.05 mg L^{-1}) and alkalinity (44.28 ± 10.61 $CaCO_3$ mg L^{-1}) were measured weekly. The water quality indicator values were within the range of known comfort levels for Nile tilapia (Popma & Lovshin, 1995).

2.2 | Growth and feed utilization parameters and sample collection

At the beginning and at the end of the experimental period, fish were anaesthetized with Eugenol® (Biodynamics Chemical and Pharmaceutical Ltda) at a dosage of 100 mg L^{-1} and subsequently weighed and individually measured with precision scales (0.001 g) and an ichthyometer, respectively. The initial body compositions of 50 fish, euthanized with Eugenol® (200 mg L^{-1}), were determined in triplicate.

The following variables were calculated from the fish biometric data and feed intake: weight gain (WG, g = final weight − initial weight), daily weight gain (DWG; g day^{-1} = weight gain/days), feed efficiency (FE; weight gain/feed intake), specific growth rate (SGR; % day^{-1} = [ln final weight − ln initial weight]/days) × 100], survival (SUR; % = [final fish number/initial fish number] × 100) and apparent net protein retention (ANPR;% = [(final weight ×final body protein concentration − initial weight × initial body protein concentration)/protein intake] × 100).

At the end of the feeding trial, three fish per experimental unit were anaesthetized with Eugenol® (100 mg L^{-1}) and blood samples were collected for further analysis of immune responses. Similarly, three fish per experimental unit were collected for the analyses of body composition and gut histology, after being euthanized with an overdose of Eugenol® (200 mg L^{-1}). Fish samples for body composition analysis were stored at −20°C until processing. For gut histology, samples were fixed in a 10% buffered formalin solution for 24 h and samples were stored in 70% alcohol until processed.

2.3 | Immune responses

Blood samples were taken from three fish for each experimental unit before and after the bacterial challenge to determine the selected innate humoral immune responses (lysozyme activity, agglutination titre and antimicrobial activity), as well as one specific humoral immune response (total immunoglobulin). The fish were anaesthetized with Eugenol® (100 mg L^{-1}), and blood was sampled by caudal vein puncture with ethylenediaminetetraacetic acid (EDTA 1%, 0.1 mg ml^{-1} of blood) anticoagulant insulin syringes (Hemstab®). Subsequently, a blood pool of three fish per tank was centrifuged at 1400 *g* for 15 min, and after centrifugation, blood plasma was collected and stored at −20°C until analysis.

The total plasma protein was determined with a commercial kit (Total Protein; Lab Test®, Lagoa Santa, Brazil) using bovine albumin

to determine the standard curve. The total immunoglobulin concentration was measured using the method described by Amar et al. (2000), in which 100 µl of plasma was mixed with 100 µl of 12% polyethylene glycol (PEG) solution (Sigma-Aldrich) and the mixture was incubated at 18°C for 2 h to precipitate the immunoglobulin molecules. The precipitate was removed by centrifugation (5000 *g* at 4°C for 10 min), and the supernatant was used to determine the total protein concentration. The immunoglobulin concentration was obtained from the following equation:

$$Total\ Ig = PTP - TPPEG$$

where Ig, immunoglobulin (mg ml^{-1}); PTP, plasma total protein; TPPEG, total protein in polyethylene glycol.

The plasmatic lysozyme concentration was determined using the methodology proposed by Sankaran and Gurnani (1972) with a few modifications: 90 µl plasma was transferred to a tube, and 600 µl of 0.5 mg ml^{-1} suspensions of lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich) was added. In sequence, the initial and final absorbance readings were taken at 492 nm. The absorbance reduction rate was converted to lysozyme (µg ml^{-1} mg^{-1}) concentration per milligram of protein, using a standard curve made with hen egg white lysozyme (Sigma-Aldrich).

The agglutination titre was determined in microplates, according to the methodology proposed by Silva et al. (2009). The plasma was diluted to 1:3 in phosphate-buffered saline (PBS) (Oxoid®) in the first well (150 µl PBS solution:50 µl plasma) and serially distributed at a ratio of 1:2 in the other wells until the 12th well. Subsequently, 50 µl of inactivated *S. agalactiae* was added to all wells. The microplate was incubated for 24 h at 35°C in a humidified chamber. Agglutination was confirmed by the formation of precipitate at the bottom of the well and was considered reciprocal to the last dilution that presented agglutination.

Plasma antimicrobial activity against *S. agalactiae* bacteria was evaluated in a 96-well flat-bottom microplate, according to Silva et al. (2009). The *S. agalactiae* inoculum was grown in Brain Heart Infusion (BHI) broth at 35°C for 24 h, prepared at a 0.5 McFarland standard and diluted 100,000 times in a poor broth (PB) medium. Subsequently, plasma dilution (1:2) in the PB medium was performed to the 12th well. For positive and blank controls, the saline solution was diluted in PB, as was done for the plasma. Finally, 20 µl of bacteria was added to each well of the diluted plasma sample and the positive control. The microplate containing *S. agalactiae* was incubated at 35°C for 24 h. Growth of the microorganisms was determined in a microplate reader at 550 nm. The antimicrobial titre was reciprocal to the last dilution with antibacterial activity.

2.4 | Diets and body composition

Composition of the experimental diets and fish's whole bodies were analysed according to the standards of the Association of Official Analytical Chemists (AOAC, 1999). The moisture was determined by drying at 105°C (method 950.01); ether extract was determined by Soxhlet (method 920.39C); crude protein was determined by the Kjeldahl method (945.01); and mineral matter was determined by incineration in a muffle furnace at 550°C (method 942.05).

Crude energy was determined in a bomb calorimeter (Model 6200; PARR), according to instructions from the manufacturer. Nitro-perchloric acid digestion was performed to determine the zinc composition in the experimental diets. Subsequently, its quantification was performed by flame atomic absorption spectrometry. The liquid chromatography method (HPLC), using a C18 column and sodium butyrate standard (Sigma-Aldrich), was performed to determine the sodium butyrate concentration of the experimental diets.

2.5 | Gut histology

Approximately 5-cm long transverse portions of the proximal, medial and distal parts of the gut were sampled to determine the gut-fold histomorphometry. Initially, the tissues were dehydrated in ascending concentrations of alcohol, clarified in xylol and embedded in paraffin. Cuts of 3- to 5-µm thickness (PAT-MR10 microtome) were stained with haematoxylin and eosin. After staining, the slides were mounted and analysed on an Axio Imager 2 interference phase-contrast microscope (Zeiss). We measured (1) number of folds; (2) fold height, corresponding to the distance from the apex of the fold to the end of the serosa; (3) fold width, measured perpendicularly to the height; (4) fold perimeter, corresponding to the sum of the fold contour; and (5) goblet cell number. All analyses were performed using the Zen Pro software (Zeiss).

2.6 | *Streptococcus agalactiae* challenge

After the dose-response trial, fish were challenged with the bacteria *S. agalactiae*. The pathogenic bacteria *S. agalactiae* (S13) was isolated from moribund tilapia during a mortality outbreak in Paraná State, Brazil. This strain was obtained from the Fish Bacteriology Laboratory (LABBEP) and identified by molecular identification (Facimoto et al., 2017). For infection, bacteria were cultured in a BHI broth culture medium (HiMedia, Mumbai, India) at 28°C for 24 h. Subsequently, the culture was centrifuged at 1500 *g* for 10 min, and the supernatant was discarded. Precipitated bacteria were resuspended in PBS solution (0.04 M sodium phosphate monobasic and 0.16 M sodium phosphate dibasic; pH 7.2) at a concentration of 1×10^9 CFU ml^{-1}.

Groups of 10 fish were randomly distributed in 24 experimental units. Fish from the same treatments used in the dose-response trial were utilized in the bacterial challenge. A pool of eight fish from all dietary treatments, totalling 40 fish, were used to create a negative control in the bacterial challenge. These fish

were inoculated only with PBS solution, without bacteria. The new arrangement for the bacterial challenge resulted in a total of six treatments with four replicates. Prior to the bacterial infection, fish were anaesthetized with Eugenol® (100 mg L^{-1}) and each fish received a 100-µl solution containing *S. agalactiae*, in a concentration of 1×10^9 CFU ml^{-1} inoculated into the oesophagus of the fish *via* gavage with a pipette. After bacterial infection, fish were observed for 14 days. Mortality and clinical signs of infection were recorded, and dead fish were immediately removed. After 14 days of infection, blood was sampled to analyse the immune responses previously mentioned. Surviving fish were anaesthetized with Eugenol® (100 mg L^{-1}) and subsequently euthanized through spinal cord section. Bacterial infection was confirmed by re-isolating bacteria from the liver, brain and kidney fragments of dead fish, as well as surviving infected fish. Samples were added to tubes containing a liquid BHI medium (HiMedia) for growth. Subsequently, the contents were streaked on tryptic soy agar medium (HiMedia) enriched with 5% defibrinated sheep blood and then incubated at 28°C for 24 h for the visualization and characterization of *S. agalactiae* colonies.

2.7 | Statistical analyses

Data were subjected to regression analysis to determine the best dietary inclusion level for VILIGEN™, depending on the variables evaluated. Data from the bacterial challenge were analysed by the Kaplan–Meier test. A significance level of 5.0% was adopted in all tests. All statistical procedures were performed using GraphPad Prism 6 software.

3 | RESULTS

3.1 | Growth performance

VILIGEN™ dietary supplementation resulted in a quadratic effect on final weight, weight gain, daily weight gain and specific growth rate. It was estimated that the dietary inclusion level of 2.80 g kg^{-1} VILIGEN™ promoted the highest response, in terms of daily weight gain (Figure 1), final weight, weight gain and specific growth rate (Table 2). The additive inclusion levels did not affect the feed efficiency or fish survival rate.

3.2 | Body composition and ANPR

The body protein composition and ANPR were influenced by dietary treatments in a quadratic fashion (Table 3). The lowest body protein content was estimated to be at 2.60 g kg^{-1} VILIGEN™ supplementation. However, the lowest protein retention rate was estimated at 2.70 g kg^{-1} of supplementation. There was no significant difference in body composition for moisture, lipid and mineral matter.

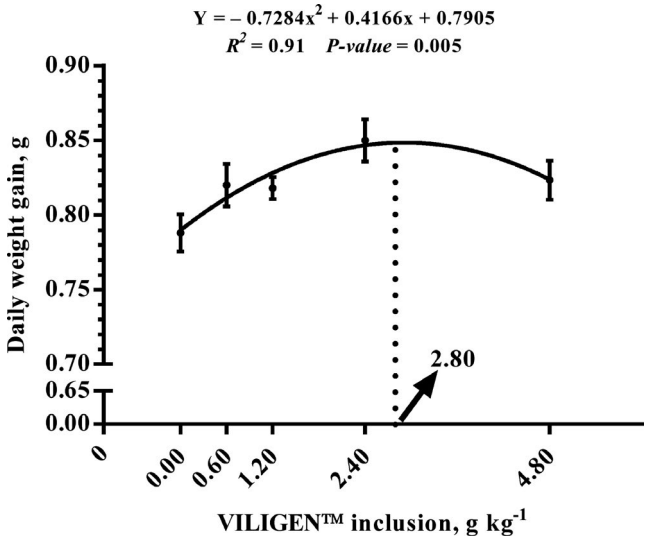

FIGURE 1 Daily weight gain of Nile tilapia juveniles fed diets containing increasing levels of VILIGEN™ for 60 days. Polynomial regression. The dashed line represents the highest response in daily weight gain according to the polynomial equation

3.3 | Gut histology

Goblet cell numbers and gut-fold perimeters from the proximal region were affected by VILIGEN™ supplementation; both variables resulted in a quadratic effect. The highest goblet cells number and the greatest perimeter were estimated to be at 3.30 (Figure 2a) and at 3.50 g kg^{-1} dietary inclusion (Figure 2b), respectively. In the proximal region, the gut-fold variables height, width and number were not affected by the dietary supplementation of VILIGEN™ (Table 4).

The distal region of the gut showed differences in the height (Figure 2c) and perimeter (Figure 2d) of the gut folds. These variables presented a linear and quadratic response to increasing dietary levels of VILIGEN™, respectively. The fold width and number, as well as the goblet cell number, were not influenced by the experimental diets.

3.4 | Immune responses

After the bacterial challenge, total immunoglobulin decreased in a linear fashion with the dietary supplementation of VILIGEN™ (Figure 3). However, supplementation did not affect the total protein, antimicrobial activity, agglutination titre, nor lysozyme activity prior to or after the bacterial challenge (Table 5).

TABLE 2 Growth and survival of Nile tilapia juveniles fed diets containing different levels of VILIGEN™ for 60 days

Variables	VILIGEN™ inclusion, g kg^{-1}					Pooled SEM	*p*-value regression
	0.00	0.6	1.20	2.40	4.80		
Final weight, g[a]	48.45	50.46	50.43	52.21	50.50	1.59	.005[b]
Weight gain, g	46.49	48.49	48.43	50.23	48.52	1.21	.005[c]
Feed efficiency	0.90	0.93	0.87	0.91	0.89	0.01	.710
Specific growth rate, %	5.44	5.49	5.47	5.52	5.49	0.04	.020[d]
Survival, %	88.57	92.86	95.14	92.26	93.45	4.87	.460

Note: Values are expressed as means of six replicates.

[a]Initial weight = 1.98 g.; [b]Polynomial regression: $y = -43.659x^2 + 24.95x + 48.594$; $R^2 = .91$.; [c]Polynomial regression: $y = -42.977x^2 + 24.578x + 46.639$; $R^2 = .91$.; [d]Polynomial regression: $y = -0.8073x^2 + 0.4758x + 5.447$; $R^2 = .73$.

TABLE 3 Initial and final body composition (expressed as wet weight), and apparent net protein retention (ANPR) in Nile tilapia juveniles fed diets containing increasing levels of VILIGEN™ for 60 days (n = 18)

Variables, g kg^{-1}	VILIGEN™ inclusion, g kg^{-1}						Pooled SEM	*p*-value regression
	Initial	0.00	0.60	1.20	2.40	4.80		
Moisture	766.5	690.6	688.1	694.7	699.5	688.6	0.74	.18
Protein	125.5	161.7	157.5	155.2	153.3	158.6	0.46	.01[a]
Lipids	70.8	119.0	124.5	122.6	115.2	124.8	0.74	.71
Mineral matter	32.1	28.5	29.3	28.1	27.2	28.3	0.18	.75
ANPR	–	40.44	40.32	37.03	38.03	39.44	1.78	.04[b]

Note: Values are expressed as means of six replicates.

[a]Polynomial regression: $y = 12.125x^2 - 6.4279x + 16.136$; $R^2 = .98$.; [b]Polynomial regression: $y = 38.403x^2 - 21x + 40.681$; $R^2 = .79$.

3.5 | Bacterial challenge

The first mortalities were verified three days after the bacterial infection (Figure 4) when fish started presenting the clinical signs of *S. agalactiae* infection, such as dermal ulcers, ascites, opercular haemorrhaging, erratic swimming, lethargy, corneal opacity, unilateral and bilateral exophthalmy, and changes in body colour. On days six, seven and eight, differences in accumulated survival were observed among fish fed different supplementation levels of VILIGEN™; fish fed 4.80 g kg^{-1} VILIGEN™ supplementation presented the highest survival rate, whereas fish fed 1.20 g kg^{-1} presented the lowest. However, from the 10th day to the end of the bacterial challenge, there were no significant differences in fish survival.

4 | DISCUSSION

This is the first study evaluating the inclusion of the feed additive VILIGEN™ in fish diets. The combined use of sodium butyrate, dehydrated hydrolysed yeast and zinc proteinate—the components of this feed additive—was effective in affecting growth of juvenile Nile tilapia. The highest daily weight gain was estimated to occur at a VILIGEN™ inclusion of 2.80 g kg^{-1}, representing a difference of 7.87% when compared to fish fed diets without the additive. In addition, regardless of the level of VILIGEN™ inclusion in the diet, it always resulted in higher growth when compared to fish fed the diet without the additive. There was slightly lower body protein composition and protein retention in fish fed 1.20 g kg^{-1} VILIGEN™; however, this difference was 4 percentage units when compared to the highest body protein content, shown by fish fed the diet without the additive.

Among the components of the additive VILIGEN™, butyrate is a short-chain fatty acid that is promptly absorbed in the intestines; it is metabolized even faster than a glucose molecule, thus providing energy to epithelial cells (Robles et al., 2013). In addition, the incorporation of butyrate in diets could avoid amino acid oxidation, increasing their availability and absorption (Robles et al., 2013). Our findings corroborate those found in Nile tilapia larvae fed diets supplemented with sodium butyrate (Jesus, Pereira, Owatari, Addam, et al., 2019), in which the highest growth performance and lowest feed conversion rate were reported on the diets containing sodium butyrate. The authors hypothesized that the growth results could be attributed to the increased energy supply to the gut epithelium, through sodium butyrate dietary supplementation. In that study, besides the higher growth results, fish fed sodium butyrate-supplemented diets also presented an increased nutrient absorption surface area in the gut (Jesus, Pereira, Owatari, Syracuse, et al., 2019). Indeed, we also found higher gut-fold perimeter of Nile tilapia when fed Viligen, which supports this hypothesis.

Additionally, VILIGEN™ contains a dehydrated hydrolysed yeast, which includes mannan. Mannan is a well-known immunomodulator that promotes the predominance of beneficial gut

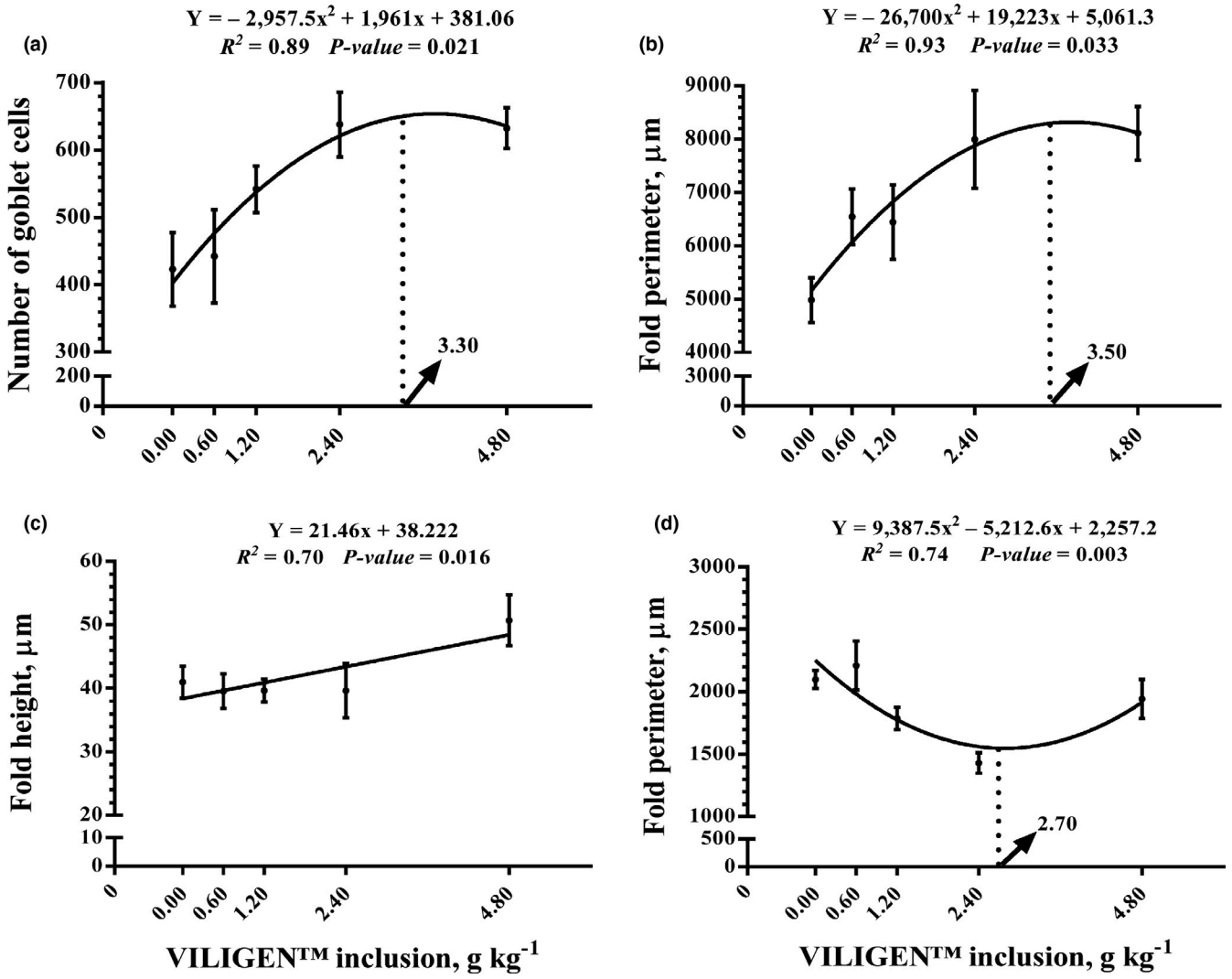

FIGURE 2 Gut morphometry of Nile tilapia juveniles fed diets containing increasing levels of VILIGEN™. (a) The number of goblet cells from the gut proximal region. Polynomial regression; (b) fold perimeter from the gut proximal region. Polynomial regression; (c) fold height from the gut distal region. Linear regression; and (d) fold perimeter from the gut distal region. Polynomial regression Dashed lines represent the highest or lowest values determined by the polynomial equation (n = 18, within each gut portion)

microflora (Ringo et al., 2010), leading to a better nutrient assimilation, and, consequently, fish growth (Vazquez et al., 2006; Vos et al., 2007). Thus, it is suggested that the synergistic use of sodium butyrate and dehydrated hydrolysed yeast favour the absorption and utilization of dietary nutrients, positively affecting fish growth. In support of this hypothesis, we registered an increase in the gut-fold perimeter in the proximal area, which represents the largest absorptive area of nutrients by gut microvilli. In Nile tilapia, the proximal region of the intestine is the site where the digestive enzymes present the highest activity, thus, representing the main site of nutrient absorption (Buddington et al., 1987). This may contribute to the improved performance of fish fed diets containing VILIGEN™.

Dietary supplementation with mannan-rich fractions (ACTIGEN®) affected positively the growth of health pigs (Edwards et al., 2014) and poultry (Mathis et al., 2012); however, such findings are conflicting in fish. In Nile tilapia, growth was not affected when ACTIGEN® was supplemented at low doses (0.40, 0.60 and 0.80 g kg^{-1}), regardless of the feeding period being 30 or 60 days and despite increasing both gut-fold height and gut-surface absorption area (Ha et al., 2017). Likewise, growth was not affected when ACTIGEN® was supplemented at higher doses (4.00 g kg^{-1}) in Nile tilapia diets (Cavalcante et al., 2020). Studies with silver catfish, *Rhamdia quelen* (Adorian et al., 2015), channel catfish, *Ictalurus punctatus* (Zhao et al., 2015), and greater amberjack, *Seriola dumerili* (Fernándes-Monteiro et al., 2019) also failed to demonstrate a positive response to growth when fed with ACTIGEN®. On the other hand, European sea bass, Dicentrarchus labrax, showed improved growth when fed ACTIGEN® at 1.6 g kg^{-1} for 30 or 60 days (Torrecillas et al., 2015) but they needed a longer feeding period of 90 days to show a positive growth effect when fed a mixture of the two generations of prebiotics ACTIGEN® and BIO-MOS® (Torrecillas et al., 2018). Therefore, differences in supplementation doses and feeding periods cannot be ruled out when explaining the conflicting growth results in fish.

TABLE 4 Gut morphometry, within the different gut portions, in Nile tilapia juveniles fed diets containing increasing levels of VILIGEN™ for 60 days (n = 18 within each gut portion)

Variables	VILIGEN™ inclusion, g kg^{-1}					Pooled SEM	p-value regression
	0.00	0.60	1.20	2.40	4.80		
Proximal							
Fold height, μm	86.14	78.87	85.05	96.00	84.39	11.91	.26
Fold width, μm	20.74	18.64	18.23	19.29	19.93	1.76	.09
Number of folds	38.67	40.33	40.00	39.20	42.33	4.25	.73
Medial							
Fold height, μm	49.85	40.48	47.77	42.59	45.21	6.59	.31
Fold width, μm	16.79	15.07	16.20	16.57	17.60	2.38	.55
Number of folds	32.33	32.66	30.00	30.00	31.66	2.46	.58
Goblet cell number	369.33	433.67	404.80	506.40	449.00	78.39	.08
Fold perimeter, μm	4138.00	3242.70	3274.50	3457.40	3608.10	566.10	.08
Distal							
Fold width, μm	17.65	15.69	16.69	16.56	18.05	2.99	.41
Number of folds	17.00	18.33	15.00	10.66	12.80	2.95	.12
Goblet cell number	14.60	37.66	20.66	14.00	31.40	39.34	.44

Note: Values are expressed as means of six replicates.

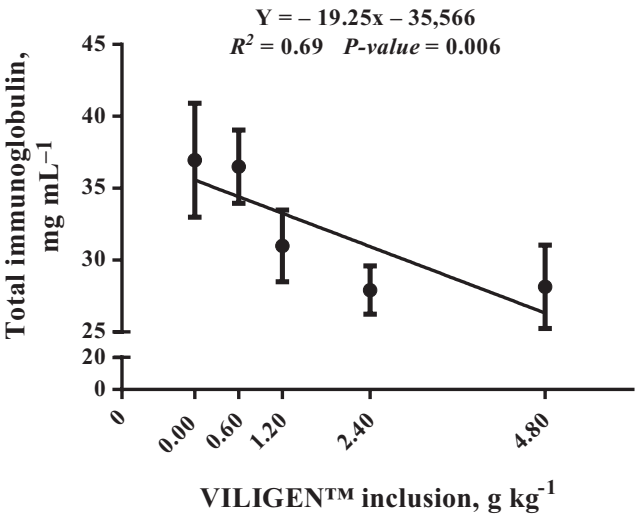

FIGURE 3 Total immunoglobulin in the blood plasma of Nile tilapia juveniles fed diets containing increasing levels of VILIGEN™, 14 days after the bacterial challenge (n = 18). Linear regression

The improved growth performance of fish fed VILIGEN™ in the present study, together with conflicting growth responses to ACTIGEN® in the previously mentioned studies in fish, is consistent with a potential advantage of combining sodium butyrate and zinc protein with dehydrated hydrolysed yeast, since the additive ACTIGEN® contains only mannan-rich fractions. The mannan-rich fraction in the hydrolysed yeast can probably positively modulate the bacteria in the intestine together with sodium butyrate, which is an energy supply substrate easily absorbed by the intestine; it is a condition that favours the absorption of nutrients, promoting the growth of fish. In addition, zinc proteinate is a protein-bound organic form of mineral that is more bioavailable than inorganic zinc sources and is a more stable molecule that does not interact with other nutrients (Ibs & Rink, 2003; Lin et al., 2013). Thus, this compound in organic form can be absorbed more thoroughly, favouring the growth of fish, and improving the immune and antioxidant systems of fish (Apines-Amar et al., 2004; Ibs & Rink, 2003).

The number of goblet cells is an important indicator of resistance to enteric disease, as these cells are responsible for the secretion of mucus, which covers the gut epithelium, forming the first-line lumen defence (Adorian et al., 2016; Lauriano et al., 2016). In this study, fish fed diets supplemented with VILIGEN™ had higher goblet cell numbers in the proximal intestine than those in the control group. Mucus provides chemical protection against toxins and antigens and has bactericidal properties that reduce the bacterial population in direct contact with the epithelial surface (Corfield et al., 2000; Gaudier et al., 2009; Hoebler et al., 2006). Thus, one could speculate that the gut mucosa of fish that received diets containing VILIGEN ™ had greater protection against infections. However, future studies evaluating mucins and mucus immunological properties would be necessary to validate this hypothesis.

When infection of *S. agalactiae* occurs by oral route, as in the present study, there is a systemic infection in the first hours after contact with the bacteria: brain, liver, spleen and kidney being the main affected organs (Iregui et al., 2016). Our survival results after bacterial infection were unclear but suggest that the highest number of goblet cells, and probable highest mucus production, was not enough to fully protect fish against bacterial infection.

Although without significant differences, fish fed the diet containing 0.60 g kg^{-1} VILIGEN™ presented the highest survival, 20% higher than in fish fed the control diet. One could speculate that this response may be related to the immunosuppression of the

TABLE 5 Immune responses of Nile tilapia juveniles fed diets containing increasing levels of VILIGEN™, prior and after the challenge with *Streptococcus agalactiae* (n = 18 prior challenge and n = 12 after challenge)

Variables	VILIGEN™ inclusion, g kg^{-1}					Pooled SEM	p-value regression
	0.00	0.60	1.20	2.40	4.80		
Prior to challenge							
Total protein, mg ml^{-1}	47.77	43.89	50.32	49.56	47.85	3.80	.18
Total immunoglobulin, mg ml^{-1}	31.00	31.10	31.63	32.15	31.57	3.42	.48
Antimicrobial activity, log 2	3.58	3.58	3.60	3.58	3.41	0.30	.32
Agglutination titre, log 2	6.42	5.58	5.42	5.42	5.58	0.92	.33
Lysozyme, µg mg^{-1}	1.59	4.78	1.33	1.05	2.18	1.45	.81
After challenge							
Total protein, mg ml^{-1}	50.98	51.11	44.18	40.74	42.85	6.59	.18
Antimicrobial activity, log 2	3.33	3.58	3.83	3.58	3.58	0.35	.48
Agglutination titre, log 2	3.58	3.58	3.60	3.58	3.41	1.24	.32
Lysozyme, µg mg^{-1}	1.83	0.54	2.24	1.89	2.38	1.65	.33

Note: Values are expressed as means of six replicates.

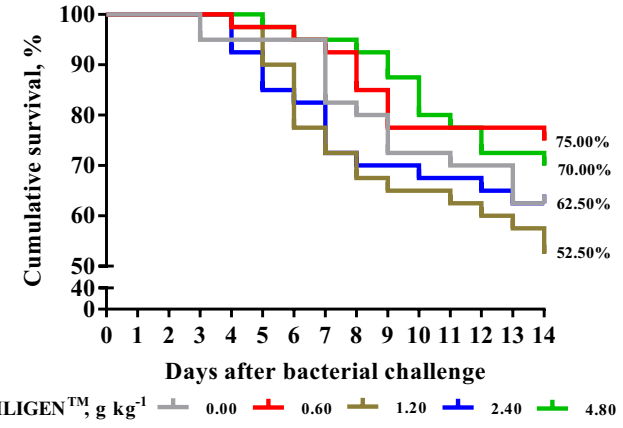

FIGURE 4 Cumulative survival of Nile tilapia juveniles after exposure to *Streptococcus agalactiae* at 10^9 CFU ml^{-1} (n = 40)

humoral immune system registered in fish fed the higher doses of VILIGEN ™. Indeed, administration of high doses of immunostimulants for long periods of time such as used in the present study (60 days) may not improve the immune system (Couso et al., 2003; Rodriquez et al., 2002; Welker et al., 2012) or even decrease resistance to pathogens (Whittington et al., 2005). However, in our study, fish fed the highest tested dose (4.80 g kg^{-1}) presented the second highest survival after the bacterial challenge, indicating that there is no obvious trend in survival that is consistent with the linear effect of the level of additive in immunoglobulins. A possible explanation for our findings where the lowest (0.60 g kg^{-1}) and highest (4.80 g kg^{-1}) tested doses promoted the best survival after the bacterial challenge may be linked to changes in the gut bacterial community. Indeed, Nile tilapia fed diets containing probiotic bacteria (*Psychrobacter namhaensis*, *Psychrobacter maritimus* or *Bacillus paralicheniformis*) showed an improvement in their immune system when compared to those fed diets without probiotics (Makled et al., 2017, 2019, 2020). The authors suggest that such improvement in the immune system could be linked to 1) a stimulation caused by the probiotic bacteria and 2) changes in the microbial gut community. Thus, further studies should be carried out in Nile tilapia to assess the ideal VILIGEN™ dose and duration of supplementation and how such variables affect disease resistance, immune responses, and gut microbial diversity.

In summary, this is the first study that has evaluated the inclusion of the feed additive VILIGEN™ in fish diets, and its use resulted in an increase of 7.87% on growth and beneficial changes in gut morphology of Nile tilapia juveniles when compared to control group. However, our findings on resistance to bacterial infection were not clear. It is noteworthy that inclusion levels above 0.60 g kg^{-1} when fed for a period of 60 days resulted in lower plasma total immunoglobulin, which could lead to immunosuppression. After bacterial challenge against *Streptococcus agalactiae*, fish fed the diet supplemented with 0.60 g kg^{-1} of VILIGEN™ had 20% higher survival than the control group without the additive. Further studies are needed to determine the optimal duration of the dietary supplementation with VILIGEN™ to optimize Nile tilapia immune responses and survival.

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CONFLICT OF INTEREST

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