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Ameliorative effects of trub-supplemented diet on the immune response and survival rate of *Oreochromis niloticus* exposed to *Streptococcus agalactiae*

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Abstract

Plant-based immunostimulants are being used more frequently as substitutes for antibiotics in aquaculture. The objective of this research was to assess if incorporating trub into the diet of Nile tilapia could enhance the fish's resistance to *Streptococcus agalactiae*. For this purpose, fish were fed with three experimental diets containing 0%, 1% or 2% trub for 50 days. The animals were then exposed to experimental infection, with the bacteria *S. agalactiae* being introduced through intragastric inoculation (gavage) at a concentration of 1×10^9 CFU·mL^{-1} in a volume of 100 µL per fish. The fish were observed for a period of 18 days. the findings showed that the number of erythrocytes increased (0.42 ± 0.041, 0.50 ± 0.06, and 0.58 ± 0.07) as the dietary inclusion of trub increased (0%, 1%, and 2%, respectively). The 1% treatment group showed higher haematocrit and mean corpuscular haemoglobin concentrations compared to the 0% control group. In the intestine, the 2% treatment group had increased number (32.47 ± 0.65) and width of villi (89.11 ± 4.82 µm) compared to the 0% control group (29.50 ± 1.14, and 81.78 ± 3.12 µm, respectively), while the 1% and 2% treatments showed higher area (32275.66 ± 130.61, and 30623.64 ± 276.85 µm^2, respectively) and perimeter (6252.54 ± 92.35, and 5239.20 ± 84.66 µm, respectively. A notable decrease in splenic steatosis was observed in the 1% and 2% treatment groups. Additionally, the 1% and 2% treatments enhanced the innate immunity of tilapia, with the lowest mortality rate (2.08%) observed in the 1% treatment group. These findings suggest that trub, a byproduct of the brewing industry, shows potential as a feed additive with immunostimulant properties for use in aquaculture.

Keywords Bioproducts, Brewing residue, Innate immunity, Plant-derived

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Introduction

Recently, the use of plant-derived immunostimulants has increased in strength as a sustainable alternative to the indiscriminate use of antibiotics in aquaculture [27, 39, 44]. This scenario presents opportunities for trub, an abundant residue from the brewing industry that is rich in proteins, carbohydrates, lipids, and natural bioactive compounds with immunostimulant, antimicrobial, antifungal, antioxidant and anti-inflammatory properties [12], making it potentially attractive for the aquaculture sector. Furthermore, research has revealed high concentrations of phenolic compounds (13.39 mg of gallic acid g^{-1} trub) [6] and (7.23 mg of gallic acid g^{-1} trub) [12], and high antioxidant activity (in vitro) in trub samples [22].

Despite all the scientific and technological knowledge about streptococcosis in aquaculture, the pathogen *Streptococcus agalactiae* remains one of the main causes of disease outbreaks and high mortality rates in Nile tilapia culture, generating significant economic losses for the sector [16]. Streptococcosis can arise when a combination of factors, which are found mainly in intensive cultivation, such as high fish stocking density, poor water quality, excess organic matter and nutritional deficiencies, simultaneously impacting the same fish populations [42].

Generally, the use of synthetic chemotherapeutics, such as antibiotics and antimicrobials, remains the primary source of disease control and treatment [27], negatively impacting the production sector, the environment, and possibly fish consumers [35]. Consequently, treatments involving chemotherapeutics have become a barrier to the international trade of aquacultured fish [36].

At present, international legislation regulates the application of antibiotics in aquaculture, establishing maximum residue limits in products of animal origin (e.g., in fin fish the maximum residue limit for oxytetracycline is 100 μg kg^{-1}) [11], and Brazil adopts the limits preestablished by the United States, Mercosur, the European Union and the Codex Alimentarius [32]. Given the concerning global situation surrounding the use of synthetic antibiotics, there has been a growing focus on research exploring alternative approaches to managing aquaculture diseases. One such approach involves incorporating immunostimulant natural products into diets to enhance disease control [10, 43]. In this case, trub, a byproduct of beer production, has the potential to enhance sustainability and promote economic circularity in aquaculture.

The use of natural bioactive compounds with immunostimulant properties in aquaculture for treating and preventing diseases is highly desirable. Trub, in addition to its appealing nutritional profile, has immunostimulant properties that make it a potentially effective health promoter and disease prevention agent in fish. The aim of this study was to assess the haematological, immunological, and histomorphometric parameters, as well as the survival rate of Nile tilapia (*Oreochromis niloticus*) when fed diets with varying levels of trub (0%, 1%, and 2%) for 50 days and subsequently exposed to the bacterium *S. agalactiae*.

Materials and methods

Experimental design

The brewing residue used in this study was supplied by the artisanal microbrewery Armada (São José, Santa Catarina). The sample was acquired from a single batch, which represented the brewing trub of an IPA-style beer–India Pale Ale–composed of the malts Pilsen, Pale Ale, Cara Ruby and Cara Gold and the hops Nugget, Cascade and Citra. The nutritional composition (moisture, crude protein, lipid, fibre, and ash contents) and phenolic extract content of the trub are described in supplementary file 1.

A total of 162 juvenile Nile tilapia from a commercial fish farm (Pomerode fish farming, Pomerode, Santa Catarina, Brazil) were divided into 18 circular 100 L experimental units and subjected to three treatments with six replicates: a control group with 0% trub, 1% trub, and 2% trub. The experimental units were connected to a recirculating aquaculture system with constant aeration, controlled temperature, physical and biological filtration and an ultraviolet (UV) sterilizer, according to Owatari et al. [29]. During the 50-day trial period, the average water temperature remained at 26.5 ± 1.5 °C, the pH was 6.8 ± 0.7, the total ammonia concentration was 1.0 ± 0.5 mg·L^{-1}, and the nitrite concentration was below 1.0 mg·L^{-1}.

After 50-day feeding trial, final weight was significantly higher among fish fed with 2% trub [Control = 47.58 ± 2.95^{b}; Trub 1% = 60.73 ± 6.85^{ab}; and Trub 2% = 62.77 ± 9.24^{a}]. Next, the fish underwent an experimental challenge where the bacterium *S. agalactiae* was introduced through intragastric inoculation (gavage) at a concentration of 1×10^{9} CFU·mL^{-1} in a volume of 100 μL per fish. This was done by pouring the inoculum into the gastric cavity using a micropipette connected to a urinary catheter [31]. The average weight of the animals at the beginning of the challenge was 55.29 ± 8.66 g. Throughout the experimental challenge, the fish were given their designated diets (0%, 1%, and 2%) until they appeared full, four times a day (9 am, 11 am, 2 pm, and 4 pm).

Experimental infection methodology

The *S. agalactiae* inoculum was incubated in test tubes with brain heart infusion (BHI) broth at 28 °C for 12 h. After this period, the inoculum was centrifuged for 30 min at 4,000 ×g. Then, the supernatant was discarded, and the precipitate was resuspended in 10 mL of

sterile saline solution containing 0.65% NaCl, resulting in a treatment of 1×10^9 CFU·mL^{-1}.

Before experimental challenge, the fish were fasted for a period of 24 h. After the fasting period, the animals were anaesthetized with Eugenol® solution (75 mg·L^{-1}) and exposed to the pathogen via intragastric inoculation (gavage) according to Owatari et al. [31]. For the anesthetic procedure, a 10 L container was filled with 8 L of water at 25 °C containing the clove oil solution. The fish were kept in the anesthetic solution until they showed signs of complete anesthesia, such as no response to stimuli and a significantly reduced opercular beat. Each fish received 100 µL of bacterial solution via gavage. Following the procedures, the fish were placed back into the experimental units and gradually regained motor activity and balance.

After inoculation, the fish were monitored for 18 days, and survival and feed consumption were recorded until mortality stabilized. After a period of 18 days, all remaining fish were removed from the experimental units, anaesthetized and subjected to haemato-immunological and histological analyses.

Haemato-immunological parameters

At the conclusion of the 18-day experimental challenge period, four animals from each experimental group were randomly chosen, and blood samples were obtained through caudal vein puncture using a 3-mL syringe filled with ethylenediaminetetraacetic acid (EDTA) solution (Hemstab®, Brazil) for hematological evaluations.

Immediately after blood collection, an aliquot was used to determine the plasma glucose concentration (Accu-Chek®, Roche Diagnóstica, Brazil), whereas another 5.0 µL blood aliquot was fixed in Dacie solution (1:200) (sodium citrate, Synth®, Brazil), 40% formalin (Dinamica®, Brazil) and toluidine blue (Vetec®, Brazil) for subsequent total erythrocyte counting in a Neubauer chamber according to Blaxhall and Daisley [3]. Another aliquot of blood was designated for determining haematocrit via the microhaematocrit method according to Goldenfarb et al. [13] and for determining total plasma protein via a portable refractometer (Biobrix® 301, São Paulo, Brazil).

Three microliter aliquots of blood were used for differential leukocyte counts, total thrombocyte counts, and total leukocyte counts via indirect methods according to Ishikawa et al. [14]. The slides with blood extensions were made in duplicate and stained with May-Grünwald-Giemsa-Wright according to the methodology proposed by Ranzani-Paiva et al. [33]. The haemoglobin concentration was determined via the cyanmethemoglobin method with a 16 µL aliquot of blood [33]. With respect to the haematological data, the mean corpuscular volume (MCV), mean corpuscular haemoglobin (HCM) and mean corpuscular haemoglobin concentration (MCHC) were determined.

The immunological analysis was carried out using blood samples from the same fish mentioned previously. The blood was collected and pooled, and the samples were incubated at 25 °C for 1 h before being centrifuged at 1,400 × g for 15 min to obtain plasma. The plasma was then stored at −20 °C for analysis of total plasma protein concentration (Biotécnica®, Varginha, Brazil), total immunoglobulin concentration according to Amar et al. [1], and agglutination titre according to Silva et al. [38]. To assess the serum antimicrobial activity, an inoculum of *S. agalactiae* was cultured in BHI broth medium (HiMedia, India) at 28 °C for 24 h, prepared at a concentration of 0.5 on the McFarland scale, and diluted 100,000 times in PB medium. Serial dilutions of the serum in PB medium (1% peptone, 0.5% NaCl, and 900 mL H_2O) were performed at a 1:2 ratio up to the 12 th well. Saline solution was used for the positive and white controls, diluted in PB in the same manner as the serum. Subsequently, 20 µL of the bacterial suspension was added to each well of the diluted serum sample and the positive control. The microplate containing *S. agalactiae* was then incubated at 28 °C for 24 h. The antimicrobial titre was determined using a microplate reader at 550 nm, and the minimum inhibitory concentration was defined as the last dilution of the serum where complete inhibition of microbial growth was observed.

Histological analysis

Following blood collection, the fish were euthanized by spinal cord sectioning, and tissue samples from the spleen, liver, and anterior and middle intestines were collected for histological examination. The samples were fixed in 10% buffered formalin, then washed, dehydrated in a series of increasing ethyl alcohol concentrations, clarified in xylol, and embedded in paraffin at 60 °C for sectioning into 4.0 µm slices using a microtome PAT- 54 MR10 (The Pathologist®, Brazil). These sections were subsequently stained with hematoxylin–eosin (H&E), mounted in Entellan® medium, and examined under an Axio Imager A.2 microscope with Zen Pro software (Zeiss®, Gottingen, Germany). Individual slides of liver, spleen, and intestine tissue were prepared for each fish. Histological alterations in the organs were graded based on the degree of intensity: 0 (no alteration), 1 (mild alteration, < 25% of tissue area), 2 (moderate alteration, 25–50% of tissue area), and 3 (severe alteration, > 50% of tissue area), following the method outlined by Schwaiger et al. [37] and adapted by Brum et al. [4]. To evaluate

intestinal integrity, measurements were taken for villi length, width, and perimeter, as well as the number of villi and goblet cells per villi.

Statistical analysis

Statistical analysis was conducted using Statistica 7.0 software (StatSoft, Inc., USA). Homoscedasticity was verified with Levene's test, and normality of the data was assessed using the Shapiro–Wilk test. Data that did not show homogeneity were $\log_{10}$ (x + 1) transformed. One-way analysis of variance (ANOVA) was performed on the transformed data, and Tukey's test was used to compare means. Immunological data were analyzed using ANOVA and compared using Tukey's test. Histological analysis was conducted using the nonparametric Kruskal–Wallis test followed by Dunn's test.

Results

Haemato-immunological parameters

At the end of the 18-day experimental challenge period, haemoglobin did not differ significantly ($p > 0.05$) between treatments; however, the other haematological parameters were significantly altered. The number of erythrocytes increased significantly ($p < 0.05$) as the amount of trub in the diet increased, reaching a maximum value of 5.80 ± 0.75 ($\times 10^6$ μL^{-1}).

Haematocrit (36.45 ± 1.36%) and MCHC (27.68 ± 0.29 $g{\cdot}dL^{-1}$) were significantly greater ($p < 0.05$) in fish from the 1% treatment compared to fish in the 0% control group (32.85 ± 2.68% and 21.60 ± 3.92 $g{\cdot}dL^{-1}$), whereas MCV was significantly lower ($p < 0.05$) in the fish from the 1% treatment (6.52 ± 0.51 fL) than in the fish from the 0% control group (7.59 ± 0.55 fL). The MCH was significantly lower ($p < 0.05$) in the fish in the 2% treatment (1.52 ± 0.22 $g{\cdot}dL^{-1}$) than in the fish in the 0% control group (2.14 ± 0.12 $g{\cdot}dL^{-1}$). The plasma glucose concentration was significantly greater in fish in the 0% control group (70.75 ± 5.88 $mg{\cdot}dL^{-1}$) than in fish in the 2% treatment (51.81 ± 5.51 $mg{\cdot}dL^{-1}$) (Table 1).

With respect to immunological parameters, no significant differences ($p > 0.05$) in antimicrobial activity or agglutination titre were detected between treatments. The total protein concentration was significantly greater ($p < 0.05$) in the fish in the 2% treatment (33.63 ± 3.88 $mg{\cdot}mL^{-1}$) than in the control group (26.87 ± 4.99 $mg{\cdot}mL^{-1}$). The total immunoglobulin concentration was significantly greater in the fish in the 2% treatment (20.82 ± 3.83 $mg{\cdot}mL^{-1}$) than in the fish in the 1% treatment (16.05 ± 1.88 $mg{\cdot}mL^{-1}$) (Table 1).

Histological analysis

At the end of the 18-day experimental challenge period, no significant differences ($p > 0.05$) were detected in the number of goblet cells or the villus height between treatments. A significant increase in the number of villi was observed as the amount of trub in the diet increased. The intestinal villi of the fish in the 2% treatment were greater than those of the fish in the 0% control group, whereas the villus width was significantly greater in the 2% treatment than in the 1% treatment and control groups. The areas and perimeters of the intestinal villi were significantly larger ($p < 0.05$) in the fish from the 1% and 2% treatments than in the fish from the 0% control group; however, the 1% and 2% treatments also differed significantly. Additionally, in

Table 1 Haemato-immunological parameters (mean ± standard deviation) of Nile tilapia (*Oreochromis niloticus*) fed diets with different levels of trub (0%, 1%, or 2%) for 50 days and exposed to *Streptococcus agalactiae*. Data were collected 18 days post-challenge. *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, and *MCHC* mean corpuscular hemoglobin concentration

	Treatments			
Parameters	**Trub 0%**	**Trub 1%**	**Trub 2%**	***p*-value**
Haemoglobin ($g\ dL^{-1}$)	7.48 ± 1.71	8.88 ± 1.29	8.97 ± 0.82	0.568
Haematocrit (%)	32.85 ± 2.68^{b}	36.45 ± 1.36^{a}	34.45 ± 1.83^{ab}	0.039
Erythrocytes ($\times 10^6$ μl^{-1})	0.42 ± 0.041^{c}	0.50 ± 0.06^{ab}	0.58 ± 0.07^{a}	0.019
MCV (fL)	7.59 ± 0.55^{a}	6.52 ± 0.51^{c}	6.53 ± 0.24^{bc}	0.009
MCH ($g\ dL^{-1}$)	2.14 ± 0.12^{a}	1.74 ± 0.35^{bc}	1.52 ± 0.22^{c}	0.014
MCHC ($g\ dL^{-1}$)	21.60 ± 3.92^{c}	27.68 ± 0.30^{a}	24.93 ± 1.11^{bc}	0.007
Glucose ($mg\ dL^{-1}$)	70.75 ± 5.88^{a}	61.25 ± 10.74^{ab}	51.81 ± 5.52^{b}	0.012
Total protein ($mg\ mL^{-1}$)	26.87 ± 4.99^{b}	29.88 ± 1.42^{ab}	33.63 ± 3.88^{a}	0.023
Total immunoglobulin ($mg\ mL^{-1}$)	17.99 ± 2.39^{ab}	16.05 ± 1.88^{b}	20.82 ± 3.83^{a}	0.039
Antimicrobial activity	2.73 ± 1.69	1.21 ± 1.90	2.74 ± 1.83	0.707
Agglutination titer	5.33 ± 2.66	5.67 ± 0.52	6.00 ± 0.00	0.852

*Different letters on the same line indicate statistical difference between treatments using the Tukey test (p 0.05)

the intestine, no severe lesions were identified, and no significant differences ($p > 0.05$) in eosinophilic infiltrate or vacuolation were detected between treatments (Fig. 1 and Table 2).

Cholestasis, congestion in the portal vein, dilation of the sinusoids, eosinophilic infiltrate, hypotrophy of the hepatocyte nucleus, loss of the hepatocyte nucleus and amyloidosis were not observed in the liver tissue.

Cordonal aspects were significantly preserved ($p < 0.05$) in the fish in the 1% and 2% treatment compared with the fish in the 0% control group, whereas the loss of pancreatic structure was significantly greater in the fish in the 2% treatment than in the fish in the control group. Ballooning hepatocytes, congestion in large vessels, and microsteatosis were significantly more common ($p < 0.05$), whereas sinusoidal congestion was significantly less common ($p < 0.05$) in fish from the control group. Displaced hepatocytes were significantly reduced ($p < 0.05$) in the fish in the 2% treatment compared with those in the fish in the 1% treatment. Hepatocyte hypertrophy was significantly greater ($p < 0.05$) in fish from the control group than in those from the 1% treatment. Pyknosis of the nuclei, karyolysis of the nuclei and karyorrhexis of the nuclei were significantly lower ($p < 0.05$) in the fish from the 1% treatment than in the fish from the 2% treatment. The other changes in liver tissue were not significantly different between treatments (Fig. 2 and Table 2).

No necrotic foci were observed in the splenic tissue. Splenic steatosis was significantly greater ($p < 0.05$) in the fish in the control group than in the 1% and 2% treatments. No significant differences were detected in the other parameters evaluated in the tilapia spleen (Table 2).

Experimental challenge

During the 18 days following exposure to *S. agalactiae*, tilapia developed characteristic clinical signs of streptococcosis, such as accelerated opercular movement, lethargy, eczema and petechiae on the body, exophthalmos, corneal opacity, erratic swimming, ascites, and death.

Up to 72 h postinfection, the fish continued to feed normally. However, on subsequent days, there was a reduction in food intake in all treatments. Until the 5th day postinfection, no deaths were observed between treatments. The highest incidence of mortality occurred on the 13 th day. The mortality rate was significantly lower ($p = 0.016$) among the fish in the 1% treatment (2.08%) than in the 2% treatment (19.23%) and control group (20.37%) (Fig. 3).

Discussion

The present study investigated the effects of trub, a residue from the brewing industry, on the health of Nile tilapia. Hematoimmunology is an important tool for monitoring the health, including nutritional and

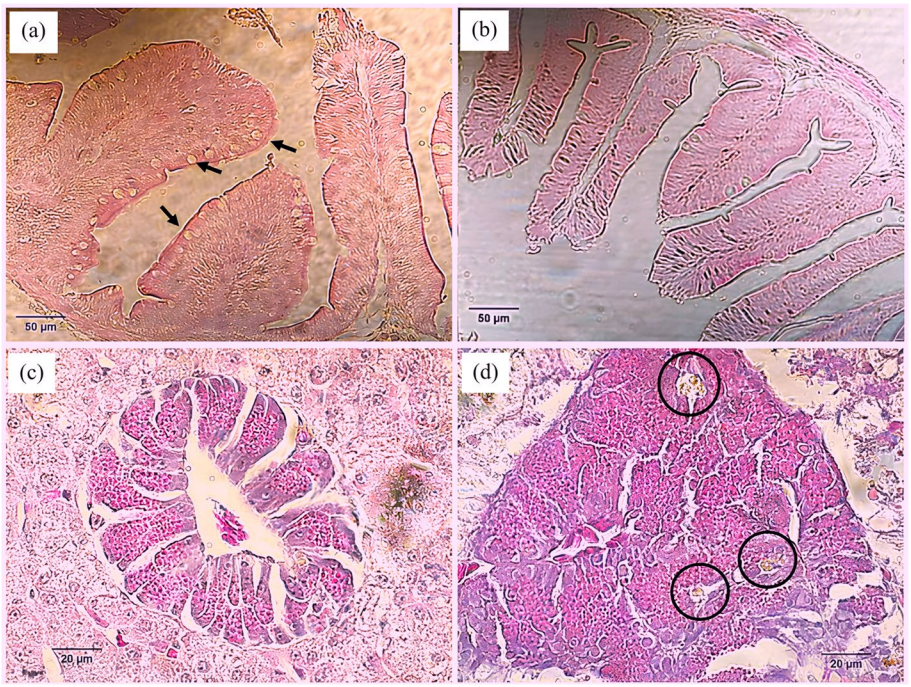

Fig. 1 Histological evaluation in intestinal and liver tissue of Nile tilapia (average weight 55 g) fed a diet supplemented with trub at concentrations of 0%, 1%, or 2%, and exposed to *Streptococcus agalactiae*. Note: (**a**) Goblet cells (black arrow) in the 2% treatment, and (**b**) control group. (**c**) Pancreatic structure in the 2% treatment, and (**d**) loss of pancreatic melanomacrophages (circles) in the control group. H&E staining

Table 2 Histological analyses of intestinal, hepatic and splenic tissue of juvenile Nile tilapia (*Oreochromis niloticus*) fed diet with different levels of trub (0%, 1%, or 2%) for 50 days and exposed to *Streptococcus agalactiae*. Data were collected 18 days post-challenge. For all organs, values were assigned for histological alteration, according to the degree of intensity; 0 (absence of alteration), 1 (mild 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). Values expressed as mean ± standard deviation

	Treatments			
Intestinal morphometry/alterations	Trub 0%	Trub 1%	Trub 2%	*p*-value
Average villi number	29.50 ± 1.14^{b}	32.11 ± 2.32^{ab}	32.47 ± 0.65^{a}	< 0.001
Average goblet cells number	471.73 ± 159.20	358.81 ± 72.69	350.43 ± 118.42	0.485
Villi Height (µm)	199.88 ± 32.52	199.95 ± 35.10	196.11 ± 27.15	0.986
Villi Width (µm)	81.78 ± 3.12^{b}	80.52 ± 2.47^{b}	89.11 ± 4.82^{a}	0.023
Villi area (μm^2)	$27{,}804.58 \pm 108.10^{c}$	$32{,}275.66 \pm 130.61^{a}$	$30{,}623.64 \pm 276.85^{b}$	< 0.001
Villi perimeter (µm)	3770.03 ± 109.68^{c}	6252.54 ± 92.35^{a}	5239.20 ± 84.66^{b}	< 0.001
Vacuolation	0.75 ± 0.50	0.33 ± 0.28	0.65 ± 0.14	0.485
Eosinophilic infiltrate	0.05 ± 0.11	0.12 ± 0.21	0.10 ± 0.22	0.098
Liver alterations	***Trub 0%***	***Trub 1%***	***Trub 2%***	p-value
Loss of cordonal aspect	1.00 ± 0.00^{c}	2.44 ± 0.10^{b}	2.17 ± 0.19^{a}	0.023
Uniformity of cells and nuclei	2.72 ± 0.30	2.79 ± 0.25	2.83 ± 0.16	0.181
Pancreas structure	2.05 ± 0.11^{b}	2.29 ± 0.35^{ab}	2.70 ± 0.30^{a}	0.022
Ballooning hepatocyte	1.00 ± 0.00^{a}	0.17 ± 0.33^{b}	0.17 ± 0.33^{b}	0.003
Congestion in large vessels	1.50 ± 0.00^{b}	2.25 ± 0.25^{a}	1.08 ± 0.17^{c}	0.024
Displaced hepatocytes nuclei	1.57 ± 0.18^{ab}	1.85 ± 0.17^{a}	1.35 ± 0.22^{c}	0.029
Sinusoidal congestion	0.33 ± 0.24^{b}	0.78 ± 0.25^{a}	1.00 ± 0.00^{a}	0.010
Hepatocyte hypertrophy	0.87 ± 0.25^{a}	0.44 ± 0.18^{c}	0.68 ± 0.21^{ab}	0.028
Macrosteatosis	1.00 ± 1.00	0.92 ± 0.87	0.60 ± 0.31	0.186
Microsteatosis	1.00 ± 1.00	0.92 ± 0.87	0.60 ± 0.31	0.987
Necrosis	2.62 ± 0.26	2.62 ± 0.70	2.52 ± 0.34	0.529
Pyknosis of the nuclei	1.92 ± 0.17^{ab}	2.06 ± 0.12^{a}	1.54 ± 0.36^{c}	0.005
Karyolysis of the nuclei	1.87 ± 0.18^{ab}	2.00 ± 0.00^{a}	1.62 ± 0.27^{c}	0.038
Karyorrhexis of the nuclei	1.87 ± 0.18^{ab}	2.00 ± 0.00^{a}	1.58 ± 0.30^{c}	0.017
Spleen alterations	***Trub 0%***	***Trub 1%***	***Trub 2%***	p-value
Vessels congestion	1.94 ± 0.47	1.65 ± 0.28	1.70 ± 0.27	0.367
Melanomacrophages centre	2.75 ± 0.35	2.54 ± 0.37	2.85 ± 0.22	0.873
Melanomacrophages	2.67 ± 0.47	2.25 ± 0.50	2.65 ± 0.55	0.345
Eosinophilic infiltrate	0.25 ± 0.25	0.12 ± 0.14	0.20 ± 0.21	0.726
Lymphocytic infiltrate	0.55 ± 0.41	1.00 ± 0.56	1.19 ± 0.37	0.763
Steatosis	0.93 ± 0.62^{a}	0.25 ± 0.32^{b}	0.20 ± 0.21^{b}	0.019
Hemosiderin	0.30 ± 0.41	0.46 ± 0.37	0.40 ± 0.42	0.021

*Different letters on the same line indicate statistical difference between treatments using the Tukey test (p 0.05)

infection responses, of aquaculture fish [9]. The increase in erythrocyte count, haematocrit levels and MCHC could be associated with the presence of trub phytochemicals, such as phenolic compounds and vitamins A and E, which are important in hematopoiesis, i.e., in the formation, development and maturation of blood elements [47]. Over the years, several studies have linked the immunostimulant potential of plant-derived compounds to their ability to improve haematological parameters [10, 21, 27].

An increase in circulating erythrocyte counts was detected in tilapia supplemented with plant-derived products. Sriyasak et al. [41] reported that crude extracts of common indigo (*Indigofera tinctoria* L.) significantly increased tilapia erythrocyte counts, whereas Mansour et al. [19] observed that the immunological response of Nile tilapia fed a diet supplemented with basil (*Ocimum basilicum*) extracts improved.

Similar findings to those of the current study were also reported in other species, including *Labeo rohita*, when

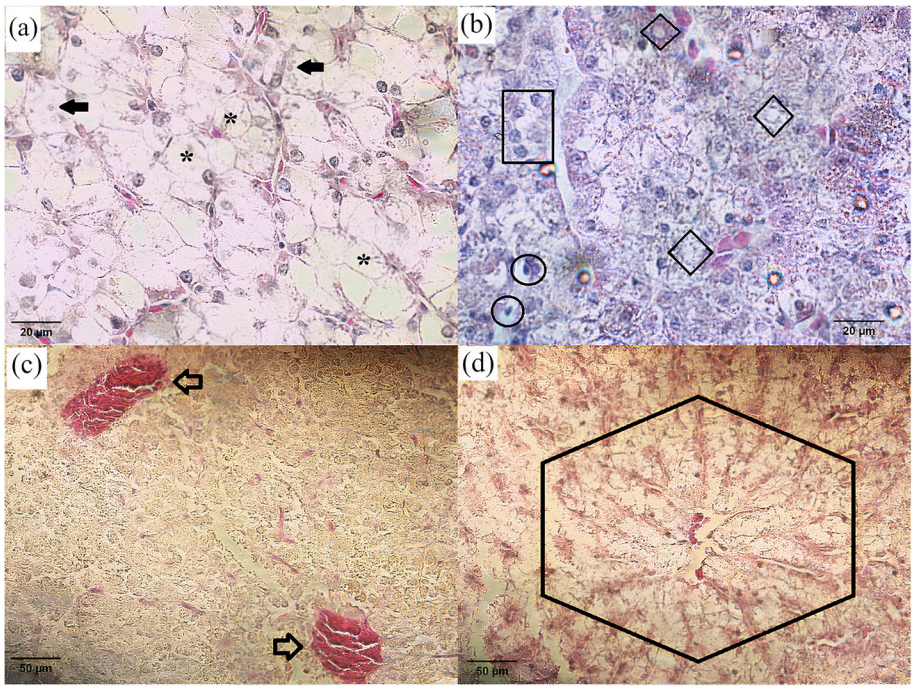

Fig. 2 Histological evaluation in liver tissue of Nile tilapia (average weight 55 g) fed a diet supplemented with trub at concentrations of 0%, 1%, or 2%, and exposed to *Streptococcus agalactiae*. Note: (**a**) Hepatocytes with pale cytoplasm (black arrow), and ballooning hepatocyte (asterisks). **b** pyknosis of the nuclei (circles), karyolysis of the nuclei (rectangle), and vacuolation (diamonds). **c** congestion of the great vessels (open arrows). **d** maintenance of the cordonal aspect. H&E staining

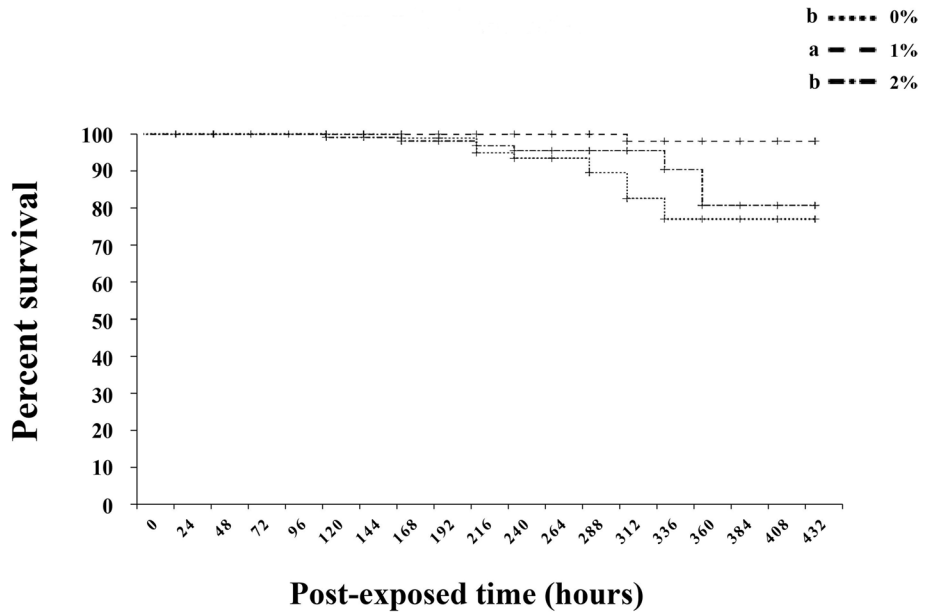

Fig. 3 Survival curves of the experimental challenge using the log-rank Mantel-Cox test. Note: The fish were infected with the *Streptococcus agalactiae* bacterial strain via intragastric [1×10^9 CFU] route and observed for up to 432 h post-infection. Mortalities were recorded during this period. Groups with different letters show significant variations

they were fed diets containing basil extract (*Ocimum sanctum*) at concentrations of 0.1%, 0.2%, 0.5%, and 1% [7] and Victoria Labeo (*Labeo victorianus*) supplemented with stinging nettle (*Urtica dioica*) at concentrations of 0%, 1%, 2% and 5% [28] and exposed to the bacterium *Aeromonas hydrophila*. As found in the phytochemical composition of trub, *O. sanctum* and *U. dioica* are composed of flavonoids, carotenoids and vitamins A and E, which are biocompounds that are related to immunological improvements in fish [18, 27].

In the current study, although there was no significant difference between treatments, the absolute number of haemoglobin count was high in the 1% and 2% treatment groups, suggesting a potentially improved response to the infection. Haemoglobin levels were also reduced in the blood of Nile tilapia after exposure to *S. agalactiae*; however, dietary supplementation with clove and ginger essential oils did not significantly affect haemoglobin, haematocrit, MCV or MCHC post-challenge [5]. Haemoglobin plays a crucial role in transporting oxygen to all organ tissues and allows fish to breathe properly to maintain metabolic functions [45]; thus, trub could act as a regulator of globin synthesis.

The lower plasma glucose levels observed in the blood of fish in the 2% treatment could indicate better physiological conditions provided by trub, considering that high plasma glucose levels are indicative of stress-modulating responses in fish [15]. Previous studies have reported a reduction in plasma glucose levels when plant-derived additives are applied as natural immunostimulants in fish diets [24, 28]. This reduction in glucose level could be related to the presence of phenolic compounds and vitamins (A and E) in trub, which probably act in the regulation of glucose metabolism and help to maintain this parameter in fish after 2% treatment [25].

Bioactive molecules like phenolic compounds, amino acids, and vitamins can enhance disease resistance by boosting the immune system and providing antioxidant and anti-inflammatory benefits [18, 21, 27], as observed in the present study. The results show that plant-based products like trub from brewing can be used as natural immunostimulants in the diet of Nile tilapia.

As observed in this study, the serum total protein and total immunoglobulin levels increased significantly in *L. rohita* fed diets containing *O. sanctum* for 42 days and exposed to *A. hydrophila* for 18 days, indicating that these effects were immunostimulatory and made the fish more resistant to bacterial infection [7]. On the other hand, immune response indicators such as the agglutination titre, antimicrobial activity, immunoglobulin concentration and total serum protein content of Nile tilapia were also not significantly altered after dietary supplementation with silymarin (*Silybum marianum*) or subsequent exposure to *S. agalactiae* [30]. Commonly, *S. agalactiae* causes severe damage to cells and tissues caused by toxins (e.g., proteases and hemolysins); however, the humoral immune response can be more evident in experimental challenges involving intraperitoneal injections [31].

Although the mechanisms of action of trub on the immune system have not been completely elucidated, it is known that plant-derived bioactive compounds are beneficial for fish immunity. The findings of this study indicate that the protective impact of trub is facilitated by the innate immune mechanisms of tilapia.

In terms of histology, the findings of this study suggest that dietary supplementation with trub provides greater energy for epithelial cells and favours the multiplication of enterocytes, thus resulting in an increase in the number of villi with greater widths, areas and perimeters. These improvements in intestinal morphology increase nutrient absorption capacity, improving the resistance of fish to infection. Nile tilapia fed diets without additives and exposed to *S. agalactiae* presented a reduction in the number and height of intestinal villi [31], whereas after dietary supplementation with plant-derived immunostimulants, an increase in the number of goblet cells and the height and width of intestinal villi was observed in tilapia [4]. This information supports the findings of the current study.

As previously reported, *S. agalactiae* causes severe damage to tissues and cells [31], which could be related to the changes in the structure of the tilapia liver and spleen observed in the present study. On the other hand, trub-enriched diets provided greater organ integrity during streptococcal infection.

For example, the reduction in splenic steatosis in tilapia from the 1% and 2% treatments could be related to the presence of phosphorus and phenolic compounds in the trub, considering that such phenolic compounds are capable of regulating glucose metabolism in the body, whereas phosphorus regulates the action of enzymes involved in gluconeogenesis, thus preventing the accumulation of fat in the liver tissue of these animals [2, 46]. Dietary supplementation with the essential oils 0.5% ginger and 0.5% basil also significantly reduced hepatic microsteatosis after exposure to *S. agalactiae* [4].

Some liver changes, such as ballooning appearance, hepatocyte hypertrophy, displacement of the hepatocyte nuclei, pyknosis of the nuclei, karyolysis of the nuclei and karyorrhexis, could be related to the excessive accumulation of fat in the organ [26] caused by the reduction in metabolic activity in the liver during the infection

process, causing injuries and morphological variability in hepatocytes and the nucleus [31, 34]. In this sense, the 2% treatment, which significantly reduced fat accumulation in liver tissue, had a possible hepatoprotective effect during bacterial infection.

Tilapia exposed to *S. agalactiae* exhibit necrosis, moderate microsteatosis and severe macrosteatosis, in addition to melanomacrophages, melanomacrophage centers and vascular congestion in the spleen [31]. However, in the present study, dietary supplementation with trub significantly reduced splenic steatosis in tilapia exposed to streptococci, which could be related to the presence of phosphorus and phenolic compounds in trub, which regulate the deposition of lipids in tissues [2, 46].

In the experimental challenge, the fish in the 1.0% treatment group showed a high survival rate, indicating a strong immune response to infection. Previous studies have reported low mortality rates (up to 4.76%) in juvenile Nile tilapia fed diets with ginger and basil essential oils and exposed to *S. agalactiae* for 10 days [5]. In contrast, mortality rates exceeding 20% were observed in Nile tilapia fed diets with *Mentha piperita* (0.075%, 0.125% and 0.25%) and exposed to *S. agalactiae* for seven days [40]. These differences in mortality rates could also be linked to the different factors that might affect the virulence of S. agalactiae in tilapia farming, such as strain serotype [8], nutritional status [23], and water temperature, which can rise in temperatures ranging from 27 °C to 32 °C [17, 20, 31]. In the present study, the low mortality rate in the 1% treatment group may be due to improvements in haemato-immunological parameters, possibly linked to the immunostimulatory effects of biomolecules present in trub.

Conclusion

Trub, a plentiful byproduct of the beer industry, is often overlooked despite its rich composition of proteins, carbohydrates, lipids, and various phenolic compounds. This makes it a valuable resource for research in different fields, including aquaculture. In this study, incorporating trub into the diet of Nile tilapia had a positive impact on their haemato-immunological and histomorphological parameters when the fish were challenged with the bacterium *S. agalactiae*. Notably, using 1% trub resulted in a low fish mortality rate of 2.08%. These findings suggest that trub has great potential as a natural immunostimulant in aquaculture, offering benefits and promising applications in the industry. Future studies should explore the potential benefits of incorporating different trub dietary inclusions for tilapia, as well as other aquacultured fish species.

Supplementary Information

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Supplementary Material 1.

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Animal welfare
All procedures in this research were approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Santa Catarina (UFSC) and are registered under protocol number 1879260819.

Authors' contributions
B.C.G.G. conceptualization, methodology, investigation, formal analysis, writing—original draft. M.S.O. Formal analysis, investigation, writing — original draft, writing—review and editing. G.d.M.G. methodology, investigation, formal analysis. S.A.P.D. methodology, investigation, formal analysis, writing — original draft. J.V.S.F. methodology, investigation, formal analysis. A.P.S. methodology, formal analysis. M.L.M. Conceptualization, methodology, investigation, writing—original draft. J.L.P.M. Conceptualization, methodology, investigation, writing—original draft. P.L.M.B. conceptualization, methodology, investigation, funding acquisition, project administration, writing—original draft (equal), writing— review and editing.

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Data availability
The original data measured in this study are available from the corresponding author and may be made available upon prior request.

Declarations

Competing interests
The authors declare no competing interests.

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References

1. Amar EC, Kiron V, Satoh S, Okamoto N, Watanabe T. Effects of dietary βcarotene on the immune response of rainbow trout *Oncorhynchus mykiss*. Fish Sci. 2000;66(6):1068–75. https://doi.org/10.1046/j.1444-2906.2000.00170.x.
2. Anhê FF, Desjardins Y, Pilon G, Dudonné S, Genovese MI, Lajolo FM, Marette A. Polyphenols and type 2 diabetes: a prospective review. PharmaNutrition. 2013;1(4):105–14. https://doi.org/10.1016/j.phanu.2013.07.004.

3. Blaxhall PC, Daisley KW. Routine haematological methods for use with fish blood. J Fish Biol. 1973;5(6):771–81. https://doi.org/10.1111/j.1095-8649.1973.tb04510.x.
4. Brum A, Cardoso L, Chagas EC, Chaves FCM, Mouriño JLP, Martins ML. Histological changes in Nile tilapia fed essential oils of clove basil and ginger after challenge with *Streptococcus agalactiae*. Aquaculture. 2018;490:98–107. https://doi.org/10.1016/j.aquaculture.2018.02.040.
5. Brum A, Pereira SA, Owatari MS, Chagas EC, Chaves FCM, Mouriño JLP, Martins ML. Effect of dietary essential oils of clove basil and ginger on Nile tilapia (*Oreochromis niloticus*) following challenge with *Streptococcus agalactiae*. Aquaculture. 2017;468:235–43. https://doi.org/10.1016/j.aquaculture.2016.10.020.
6. Costa FSF, Amparo TR, Seibert JB, Silveira BM, Silva RG, Pereira DI, Barbosa RGG, Santos ODH, Brandão GC, Teixeira LFM, Vieira PMA, Souza GHB. Reuse of hot trub as an active ingredient with antioxidant and antimicrobial potential. Waste Biomass Valor. 2021;12:2037–47. https://doi.org/10.1007/s12649-020-01163-6.
7. Das R, Raman RP, Saha H, Singh R. Effect of Ocimum sanctum Linn. (Tulsi) extract on the immunity and survival of Labeo rohita (Hamilton) infected with Aeromonas hydrophila. Aquac Res. 2015;46(5):1111–21. https://doi.org/10.1111/are.12264.
8. Delannoy CM, Samai H, Labrie L. *Streptococcus agalactiae* serotype IV in farmed tilapia. Aquaculture. 2021;544:737033. https://doi.org/10.1016/j.aquaculture.2021.737033.
9. Fazio F. Fish hematology analysis as an important tool of aquaculture: a review. Aquaculture. 2019;500:237–42. https://doi.org/10.1016/j.aquaculture.2018.10.030.
10. Ferreira MB, Santos PC, Santos GG, Anjos GBD, Silva GVGM, Souza ADP, Ferreira GB, Owatari MS, Mouriño JLP, Martins ML. *Azadirachta indica* as a sustainable nutritional alternative in Nile tilapia farming–A mini review. Probe Fish Sci Aquacult. 2024;6(1):2208. https://doi.org/10.18186/fsa.v6i1.2208.
11. Ferri G, Lauteri C, Vergara A. Antibiotic resistance in the finfish aquaculture industry: a review. Antibiotics. 2022;11(11):1574. https://doi.org/10.3390/antibiotics11111574.
12. Gandolpho BC, Almeida AR, Gandolpho GM, Freitas DZ, Gasparini OC, Machado MH, Barreto PL. Optimization of brewing waste's (trub) phenolic compounds extraction by ultrasound assisted using response surface methodology. Quim Nova. 2021;44:478–83. https://doi.org/10.21577/0100-4042.20170688.
13. Goldenfarb PB, Bowyer FP, Hall E, Brosious E. Reproducibility in the hematology laboratory: the microhematocrit determination. Am J Clin Pathol. 1971;56(1):35–9. https://doi.org/10.1093/ajcp/56.1.35.
14. Ishikawa NM, Ranzani-Paiva MJT, Lombardi JV. Total leukocyte counts methods in fish *Oreochromisniloticus*. Arch Vet Sci. 2008;13:54–63.
15. Jiang D, Wu Y, Huang D, Ren X, Wang Y. Effect of blood glucose level on acute stress response of grass carp *Ctenopharyngodon idella*. Fish Physiol Biochem. 2017;43:1433–42. https://doi.org/10.1007/s10695-017-0383-y.
16. Kayansamruaj P, Dinh-Hung N, Srisapoome P, Na-Nakorn U, Chatchaiphan S. Genomics-driven prophylactic measures to increase streptococcosis resistance in tilapia. J Fish Dis. 2023;46(6):597–610. https://doi.org/10.1111/jfd.13763.
17. Kayansamruaj P, Pirarat N, Hirono I, Rodkhum C. Increasing of temperature induces pathogenicity of *Streptococcus agalactiae* and the up-regulation of inflammatory related genes in infected Nile tilapia (*Oreochromis niloticus*). Vet Microbiol. 2014;172(1–2):265–71. https://doi.org/10.1016/j.vetmic.2014.04.013.
18. Kumar S, Choubey AK, Srivastava PK. The effects of dietary immunostimulants on the innate immune response of Indian major carp: a review. Fish Shellfish Immunol. 2022;123:36–49. https://doi.org/10.1016/j.fsi.2022.02.039.
19. Mansour AT, Diab AM, Khalil RH, Eldessouki EA, El-Sabbagh N, Elsamannoudy SI, Younis NA. Physiological and immunological responses of Nile tilapia fed dietary supplementation of sweet basil ethanolic and aqueous extracts. Front Mar Sci. 2023;9:1064455. https://doi.org/10.3389/fmars.2022.1064455.
20. Marcusso PF, Aguinaga JY, Claudiano GS, Eto SF, Fernandes DC, Mello H, Marinho Neto FA, Salvador R, Moraes JRE, Moraes FR. Influence of temperature on *Streptococcus agalactiae* infection in Nile tilapia. Braz J Vet Res Ani Sci. 2015;52(1):57–62. https://doi.org/10.11606/issn.1678-4456.v52i1p57-62.
21. Mariappan B, Kaliyamurthi V, Binesh A. Medicinal plants or plant derived compounds used in aquaculture. In: Recent Advances in Aquaculture Microbial Technology (eds. M. Jyothis, S.J. Midhun, E.K. Radhakrishnan and K. Ajay), pp. 153–207. Academic Press, Amsterdam, Netherlands. 2023. https://doi.org/10.1016/B978-0-323-90261-8.00003-1.
22. Mattioli S, Castellini C, Mancini S, Roscini V, Mancinelli AC, Cotozzolo E, Pauselli M, Dal Bosco A. Effect of trub and/or linseed dietary supplementation on *in vivo* oxidative status and some quality traits of rabbit meat. Meat Sci. 2020;163:108061. https://doi.org/10.1016/j.meatsci.2020.108061.
23. Maulu S, Hasimuna OJ, Mphande J, Munang'andu HM. Prevention and control of streptococcosis in tilapia culture: a systematic review. J Aquat Anim Health. 2021;33(3):162–77. https://doi.org/10.1002/aah.10132.
24. Mohamed RA, Yousef YM, El-Tras WF, Khalafallaa MM. Dietary essential oil extract from sweet orange (*Citrus sinensis*) and bitter lemon (*Citrus limon*) peels improved Nile tilapia performance and health status. Aquac Res. 2021;52(4):1463–79. https://doi.org/10.1111/are.15000.
25. Mohammadi G, Rafiee G, El Basuini MF, Abdel-Latif HM, Dawood MA. The growth performance, antioxidant capacity, immunological responses, and the resistance against *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*) fed *Pistacia vera* hulls derived polysaccharide. Fish Shellfish Immunol. 2020;106:36–43. https://doi.org/10.1016/j.fsi.2020.07.064.
26. Naiel MA, Negm SS, Ghazanfar S, Shukry M, Abdelnour SA. The risk assessment of high-fat diet in farmed fish and its mitigation approaches: a review. J Anim Physiol Anim Nutr. 2023;107(3):948–69. https://doi.org/10.1111/jpn.13759.
27. Ng JJY, Yusoff NAH, Elias NA, Norhan NAS, Harun NA, Abdullah F, Ishak AN, Hassan M. Phytotherapy use for disease control in aquaculture: a review of the last 5 years. Aquacult Int. 2024;32(3):2687–712. https://doi.org/10.1007/s10499-023-01292-4.
28. Ngugi CC, Oyoo-Okoth E, Mugo-Bundi J, Orina PS, Chemoiwa EJ, Aloo PA. Effects of dietary administration of stinging nettle (*Urtica dioica*) on the growth performance, biochemical, hematological and immunological parameters in juvenile and adult Victoria Labeo (*Labeo victorianus*) challenged with *Aeromonas hydrophila*. Fish Shellfish Immunol. 2015;44(2):533–41. https://doi.org/10.1016/j.fsi.2015.03.025.
29. Owatari MS, Jesus GFA, Melo Filho MES, Lapa KR, Martins ML, Mouriño JLP. Synthetic fibre as biological support in freshwater recirculating aquaculture systems (RAS). Aquacult Eng. 2018;82:56–62. https://doi.org/10.1016/j.aquaeng.2018.06.001.
30. Owatari MS, Jesus GFA, Brum A, Pereira SA, Lehmann NB, Pereira UP, Martins ML, Mouriño JLP. Sylimarin as hepatic protector and immunomodulator in Nile tilapia during *Streptococcus agalactiae* infection. Fish Shellfish Immunol. 2018;82:565–72. https://doi.org/10.1016/j.fsi.2018.08.061.
31. Owatari MS, Cardoso L, Pereira SA, Pereira UP, Tachibana L, Martins ML, Mouriño JLP. Laboratory-controlled challenges of streptococcosis in Nile tilapia using the oral route (infected-feed) for infection. Fish Shellfish Immunol. 2022;120:295–303. https://doi.org/10.1016/j.fsi.2021.11.039.
32. Pacheco-Silva É, Souza JRD, Caldas ED. Veterinary drug residues in milk and eggs. Quim Nova. 2014;37:111–22. https://doi.org/10.1590/S0100-40422014000100020.
33. Ranzani-Paiva MJT, Pádua SB, Tavares-Dias M, Egami MI. Methods for Hematological Analysis in Fish. EdUEM, Maringá, Brazil. 2013. 135 pp. (in Portuguese).
34. Rašković B, Stanković M, Marković Z, Poleksić V. Histological methods in the assessment of different feed effects on liver and intestine of fish. J Agric Sci. 2011;56(1):87–100.
35. Rigos G, Kogiannou D. Antimicrobial drugs in aquaculture: use and abuse. In: Present Knowledge in Food Safety (eds. M.E. Knowles, L.E. Anelich, A.R. Boobis and B. Popping), 2023;pp. 142–161. Academic Press, Amsterdam, Netherlands. https://doi.org/10.1016/B978-0-12-819470-6.00027-5.
36. Santos L, Ramos F. Analytical strategies for the detection and quantification of antibiotic residues in aquaculture fishes: a review. Trends Food Sci Technol. 2016;52:16–30. https://doi.org/10.1016/j.tifs.2016.03.015.
37. Schwaiger J, Wanke R, Adam S, Pawert M, Honnen W, Triebskorn R. The use of histopathological indicators to evaluate contaminant-related stress in fish. J Aquat Ecosyst Stress Recovery. 1997;6:75–86. https://doi.org/10.1023/A:1008212000208.
38. Silva BC, Martins ML, Jatobá A, Buglione Neto CC, Vieira FN, Pereira GV, Jerônimo GT, Seiffert WQ, Mouriño JLP. Hematological and

immunological responses of Nile tilapia after polyvalent vaccine administration by different routes. Pesq Vet Bras. 2009;29:874–80. https://doi.org/10.1590/S0100-736X2009001100002.
39. Silva CSD, Brasil EM, Cipriano FD, Magnotti C, Rocha VMD, Furtado WE, Chagas EC, Chaves FCM, Owatari MS, Martins ML, Cerqueira VR. Antiparasitic efficacy of essential oils for *Neobenedenia melleni* infecting farmed Lebranche mullet (*Mugil liza*). Int Aquat Res. 2024;16(2):187–94. https://doi.org/10.22034/iar.2024.2008628.1671.
40. Silva LTS, Pereira UP, Oliveira HM, Brasil EM, Pereira SA, Chagas EC, Jesus GFA, Cardoso L, Mouriño JLP, Martins ML. Hemato-immunological and zootechnical parameters of Nile tilapia fed essential oil of *Mentha piperita* after challenge with *Streptococcus agalactiae*. Aquaculture. 2019;506:205–11. https://doi.org/10.1016/j.aquaculture.2019.03.035.
41. Sriyasak P, Senasri N, Chumnanka N, Tongkasee P, Phlaetita W, Pimolrat P, Suwanpakdee S. Effectiveness of crude extracts from common indigo (Indigofera tinctoria L.) in inhibiting Streptococcus agalactiae in Nile tilapia. Int Aquat Res. 2023;15(3):219–33. https://doi.org/10.22034/iar.2023.1986215.1443.
42. Van Doan H, Soltani M, Leitão A, Shafiei S, Asadi S, Lymbery AJ, Ringø E. Streptococcosis a re-emerging disease in aquaculture: significance and phytotherapy. Animals. 2022;12(18):2443. https://doi.org/10.3390/ani12182443.
43. Vijayaram S, Sun YZ, Zuorro A, Ghafarifarsani H, Van Doan H, Hoseinifar SH. Bioactive immunostimulants as health-promoting feed additives in aquaculture: a review. Fish Shellfish Immunol. 2022;130:294–308. https://doi.org/10.1016/j.fsi.2022.09.011.
44. Vijayaram S, Ringø E, Zuorro A, Van Doan H, Sun Y. Beneficial roles of nutrients as immunostimulants in aquaculture: a review. Aquac Fish. 2023;9(5):707–20. https://doi.org/10.1016/j.aaf.2023.02.001.
45. Xu Z, Cao J, Qin X, Qiu W, Mei J, Xie J. Toxic effects on bioaccumulation, hematological parameters, oxidative stress, immune responses and tissue structure in fish exposed to ammonia nitrogen: a review. Animals. 2021;11(11):3304. https://doi.org/10.3390/ani11113304.
46. Yang SD, Lin TS, Liu FG, Liou CH. Influence of dietary phosphorus levels on growth, metabolic response and body composition of juvenile silver perch (*Bidyanus bidyanus*). Aquaculture. 2006;253(1–4):592–601. https://doi.org/10.1016/j.aquaculture.2005.09.002.
47. Zare M, Esmaeili N, Paolacci S, Stejskal V. Nettle (*Urtica dioica*) additive as a growth promoter and immune stimulator in fish. Aquac Nutr. 2023;2023(1):8261473. https://doi.org/10.1155/2023/8261473.

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