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Effects of *Citrus limon* extract on growth performance and immunity in striped catfish (*Pangasius hypophthalmus*)

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Abstract

This study aimed to investigate the chemical composition and effects of dietary supplementation with *Citrus limon* extract (CLE) (Nor-Spice AB®) on growth, hematological, and innate immunity parameters of striped catfish juveniles *(Pangasius hypophthalmus*). Chemical composition was detected using nuclear magnetic resonance and mass spectrometry. Six diets with different levels of CLE —0.0 (control), 0.2, 0.4, 0.8, 1.6, and 3.2 g kg diet^{-1} — were evaluated for 90 days, followed by 8 days of bacterial infection by *Aeromonas hydrophila*. CLE presented phenolic compounds (mainly flavonoids) and polysaccharides as major constituents. Fish supplemented with 0.4 g CLE kg diet^{-1} showed final weight, weight gain, final biomass, specific growth rate, and feed conversion ratio higher than fish of the control group, or those supplemented with diets between 0.8 and 3.2 g CLE kg^{-1}. Fish supplemented with 3.2 g CLE kg diet^{-1} showed plasma albumin levels, respiratory burst total thrombocytes, total leukocytes, lymphocytes, and monocytes significantly higher than fish supplemented with other diets. After bacterial infection, fish fed CLE maintained biochemical, hematological, and immunological parameters similar to the control group, except for plasma total proteins and neutrophils levels that decreased as the concentration of CLE was increased in the diet. In conclusion, the addition of 0.4 g CLE kg diet^{-1} is recommended for improving the growth and immune resistance of striped catfish in intensive culture.

Keywords *Aeromonas hydrophila* · Feed conversion ratio · Immune resistance · Phenolic · Polysaccharides

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Introduction

Striped catfish *(Pangasius hypophthalmus*, Sauvage, 1878) are one of the most extensively farmed fish globally (Naylor et al. 2021). It is a profitable farmed fish because of its high demand in local and international markets and its high nutritional value at a low consumer cost (Hoque et al. 2021). This species presents favorable characteristics for cultivation, such as tolerance of low dissolved oxygen (Lefevre et al. 2011), omnivorous feeding habits, and fast growth (Patel et al. 2022). However, when this species is cultured under high stocking density in aquaculture, it is highly vulnerable to infectious diseases caused by pathogenic bacteria such as *Aeromonas hydrophila* (Hayatgheib et al. 2020), resulting in great economic losses for fish farmers. *Aeromonas* sp. infection can decrease the functions of the immune system (Souza et al. 2020), and motile *Aeromonas* septicemia, also known as red spot disease or hemorrhage disease, leads to a mortality of up to 80% in striped catfish (Dung et al. 2008). Although antibiotics can be used for growth promotion and to treat *A. hydrophila* infections (Dawood et al. 2018), they increase the risk of selection of resistant pathogens (Souza et al. 2018), contamination of the environment (e.g., recirculating aquaculture system), and the death of bacteria beneficial to aquaculture (Felix e Silva et al. 2022). This reduces consumer acceptance of striped catfish aquaculture and discredits their nutritional benefits (Hasan et al. 2021). Therefore, it is necessary to use dietary supplements that promote greater performance and strengthen the health of fish, with a consequent contribution to the food safety of consumers.

Plant extracts have been highlighted among the supplements to be used as additives in fish diets because they are a safe food derived from eco-friendly farming practices (Morante et al. 2021; Souza et al. 2021) and a natural alternative to synthetic drugs (Yilmaz 2019). In addition, some plants have potential bioactive compounds such as polysaccharides (carbohydrates), tannins, flavonoids, and organic acids (Pan et al. 2011) that are closely linked to factors related to growth and metabolism in fish (Zhu 2020). The beneficial effects of plant-derived products on the development, survival, and health of striped catfish have been reported from previous studies (Güroy et al. 2014; Labh et al. 2017; Nhu et al. 2019; 2020; Hasan et al. 2021; Maiti et al. 2021; Patel et al. 2022). The mode of action of these herbs is usually the enhancement of the immune response through the elevation of immune parameters and control of infectious diseases by mitigating many side effects involving the synthesis of antimicrobials (Punitha et al. 2008).

Citrus genus plants are grown in over 140 tropical and subtropical countries, producing more than 140 million tons per hectare (FAO 2021). Lemon (*Citrus limon*) extract (hereinafter CLE) is rich in polysaccharides (pectin), flavonoids, essential oils, vitamins, and minerals (González-Molina et al. 2010). *Citrus* pectin has already demonstrated prebiotic potential (Ho et al. 2017) and growth promotion in fish (Hosseini et al. 2020), as well as antibacterial activity against *Lactobacillus paracasei* and *Bifidobacterium bifidum* (Zhang et al. 2017). Flavonoids play a role in growth promotion (Samavat et al. 2019; Morante et al. 2021; Souza et al. 2021) and antioxidant action in fish (Nazeri et al. 2017). The antibacterial activities of *Citrus* are also associated with flavonoid contents (Negi and Jayaprakasha 2001; Samavat et al. 2019). Polysaccharides have prebiotic properties, increasing nutrient digestibility, absorption, and assimilation capacity in fish through improved gastrointestinal morphology or digestive systems (Heidarieh et al. 2013; Gabriel and González-Redondo 2021). In general, *Citrus* are biologically active in improving fish health through antimicrobial, antioxidant, physiological, metabolic,

immunological, and intestinal benefits (Baba et al. 2016; Beltrán et al. 2017; Ngugi et al. 2017; Rahman et al. 2019; Samavat et al. 2019; Kesbiç et al. 2020; Harikrishnan et al. 2020; Chekani et al. 2021).

Although lemons have several bioactive compounds, there is no study on the effect of dietary CLE on striped catfish. Hence, in this study, different concentrations of CLE were incorporated into the diet fed to striped catfish juveniles to investigate its effects on growth performance, metabolism, hematology, immunology, and resistance to *A. hydrophila* infection.

Materials and methods

Location, fish, and experimental conditions

Striped catfish juveniles were supplied by a commercial fish farm in Juazeiro do Norte, CE, Brazil. The fish were transported to the aquaculture laboratory at Universidade Federal do Vale do São Francisco (UNIVASF). During acclimatization, the fish were fed extruded commercial feed containing 40% crude protein and 3000 kcal kg^{-1} of digestible energy (GUABI, P-40, 2 mm) six times a day (07:00, 09:00, 11:00, 13:00, 15:00, and 17:00 h) at a rate of 20% of the initial biomass. The experiment lasted 90 days, followed by 8 days of bacterial infection by *A. hydrophila*.

After two weeks of acclimatization, 234 fish (1.74 ± 0.05 g) were distributed in a completely randomized experimental design with six treatments and three replicates in 1000-L fiberglass tanks ($n = 13$ fish per tank) in a recirculating aquaculture system, with constant aeration, physical and biological filters, water heated to 28.0 °C (Full Gauge, model Tic-17rgti, Canoas, Brazil), and a natural photoperiod (about 12 L:12 D).

The water quality parameters remained stable during the experiment. The water quality parameters for dissolved oxygen (5.90 ± 0.40 mg O_2 L^{-1}) and water temperature (28.0 ± 0.30 °C) were monitored with the aid of an oximeter (Pol-60, Politerm®, São Paulo, Brazil), and pH (6.80 ± 0.30) was monitored using a pH meter (HI 98,130, Hanna®, Barueri, Brazil) every day. Alkalinity (50.00 ± 0.01 mg CaCO3 L^{-1}), non-ionized ammonia (0.22 ± 0.08 mg NH3 L^{-1}), and nitrite (0.05 ± 0.01 mg N-NO_2 L^{-1}) were monitored by kit (Alfatecnoquímica, Florianópolis, Brazil) twice a week. The tanks were cleaned daily with a siphon to remove excess feces and feed residues.

Composition of Citrus limon extract

The chemical characterization of CLE was performed by nuclear magnetic resonance (NMR) analysis. For this, 1D and 2D NMR data were acquired at 298 K in DMSO-d_6 on a Bruker AVANCE III 400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 9.4 T, observing ^{1}H and ^{13}C at 400 and 100 MHz, respectively. The NMR spectrometer was equipped with a 5-mm direct detection probe with a *z*-gradient. One-bond (^{1}H-^{13}C HSQC) and long-range (^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HMBC) NMR correlation experiments were optimized for average coupling constants $^{1}J_{(C,H)}$ and $^{LR}J_{(C,H)}$ of 140 and 8 Hz, respectively. All ^{1}H and ^{13}C NMR chemical shifts (δ) were given in ppm related to the TMS signal at 0.00 as an internal reference and the coupling constants (*J*) in Hz.

A stock solution (1 mg mL^{-1}) of CLE was prepared in methanol. A 5-µL aliquot of the stock solution was further diluted to 5 µg mL^{-1}, and the resulting solution was analyzed by direct infusion in the mass spectrometer (Bruker Daltonics, Germany). All mass spectra were recorded in continuous monitoring mode using TSQ Quantum Access equipment with an Atmospheric pressure chemical ionization (APCI) source and operating in positive and negative acquisition mode for mass spectrometry (MS) and MS/MS analyses. The spectra were obtained from the average of at least ten acquired spectra. The samples were infused into the APCI source through the equipment's syringe pump (10 µL min^{-1}). The MS/MS spectra were obtained from tap-plying energy from 25 to 35 eV.

Performance assay and diet preparation

The experimental diets were supplemented with CLE (Nor-Spice AB®, Nor-Feed, Beaucozé, France; organic production certificate CE n°834/2007 & CE n°889/2008 by FR-BIO

Table 1 Composition of experimental diets supplied to striped catfish (*Pangasius hypophthalmus*)

Ingredients	%
Soybean meal	50.00
Fish meal	19.73
Meat and bone meal	6.56
Corn bran	15.62
Premix[1]	5.00
Soya oil	2.97
Salt	0.50
Vitamin C	0.05
Butylated hydroxytoluene	0.001
Antifungal[2]	0.001
Nor-Spice AB®[3]	-
Total	
Analyzed proximate composition	
Crude protein (g kg^{-1})	43.43
Crude lipid (g kg^{-1})	4.91
Digestible energy (kcal)	3,800
Lysine (%)	2.50
Methionine (%)	0.64
Phosphorus (%)	1.14
Calcium (%)	1.70

[1]Mineral mixture e vitamins for fish (Neovia nutrition and animal health LTDA, São Lourenço da Mata, PE, Brazil) — chemical composition analyzed per kg of the product: vit. A, 1.200.000 UI; vit. B1, 4.800 mg; vit. B12, 4.8 mg; vit. B2, 4.800 mg; vit. B6, 4.800 mg; vit. C, 48 g; vit. D3, 200.000 UI; vit. E, 1.200 mg; vit. K3, 2.400 mg; folic acid, 1.200 mg; biotin, 48 mg; D-calcium pantothenate, 12.000 mg; choline chloride, 108 g; niacin, 24.000 mg; Se, 100 mg; I, 100 mg; Co, 10 mg; Cu, 3.000 mg; Fe, 50.000 mg; Mn, 20.000 mg; Zn, 30.000 mg; 1.000 g; antioxidant, 25 g. [2]Calcium propionate. [3]Composition supplied by the Nor-Spice AB® factory: protein 2.5%, fat 1%, fiber 1.2%, and ash 37%

10). Six isoproteic and isocaloric diets (0.0 (control), 0.2, 0.4, 0.8, 1.6, and 3.2 g CLE kg diet^{-1} g CLE kg diet^{-1}) were formulated (Table 1).

CLE was added along with the other ingredients. The ingredients were ground with a hammer grinder in 1 mm sieves, mixed, and processed to produce the experimental diets. Diet extrusion was performed by wetting the mixture with water (30%) in a commercial extruder (Inbramaq, São Paulo, Brazil) using a 5-mm die plate. Subsequently, the pellets were extruded at 90 °C by 2 s and dehydrated in a forced-air recycling system oven for 24 h at 55 °C. The pellets were refrigerated (− 20 °C) in glass containers with hermetically sealed caps until use.

Growth performance variables

The fish fasted for 24 h before growth performance analysis and sample collection. The weight (g) of striped catfish from all experimental units was measured to calculate growth performance (day 90), except for hepatosomatic index and viscerosomatic index, where three fish per treatment were randomly sampled, removed from the tanks, and anesthetized with benzocaine (30 mg L^{-1}) for tissue collection. The production variables were:

- Weight gain (WG, g) = final body weight (g) − initial body weight (g);
- Specific growth rate (SGR, % per day^{-1}) = 100 × (Ln final weight (g) − Ln initial weight (g))/time (days);
- Feed conversion ratio (FCR) = feed intake (g) / weight gain (g);
- Hepatosomatic index (HSI, %) = 100 x (liver weight (g)/body weight (g);
- Viscerosomatic index (VSI, %) = 100 x (viscera weight (g)/fish weight (g));
- Survival (%) = (final fish number / initial fish number) × 100.

Sample collection

At the end of the experimental period (90 days), the same three fish per treatment used for HSI and VSI analyses were used for blood collection, and 2 mL of blood was collected from each fish via a venocaudal puncture using a sterile syringe containing ethylenediaminetetraacetic acid (EDTA). Two blood aliquots were collected. The first aliquot (1 mL) was used for hematological analyses. The second aliquot (1 mL) was centrifuged at 3000 × g at 4 °C for 10 min (UniCen M, Helolab) to separate the plasma, and the samples were stored at − 20 °C for biochemical determinations.

Hematological and plasma analyses

The blood smears were made immediately after blood collection for the erythrocyte analysis. The erythrocytes were counted in a Neubauer counting chamber (1 × 10^6 μL^{-1}) using a microscope at 400 × magnification. The hemoglobin concentration was determined by the cyanmethemoglobin method. Hematocrit was determined in duplicate using heparinized capillary tubes, filled to two-thirds of its total volume, and centrifuged at 12,000 × g for 5 min. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to the following formulae:

$$\text{MCV (fL)} = (\text{hematocrit} * 10)/\left(\text{N° erythrocytes} * 106\ \mu\text{L}^{-1}\right)$$
$$\text{MCH (pg)} = (\text{hematocrit} * 10)/\text{N° erythrocytes};$$
$$\text{MCHC (g dL} - 1) = (\text{hemoglobin rate} * 100)/\text{hematocrit}.$$

Blood smears were prepared and panchromatically stained with May Grünwald-Giemsa-Wright for differential leukocyte counts. Two hundred cells of bloodstaining were counted to perform the differential count of leucocytes, defining the percentage of each component of interest (Tavares-Dias et al. 2007). These blood extensions were also used to determine the number of total leukocytes and thrombocytes. Total leucocytes and thrombocyte count were performed considering the total erythrocyte amount as obtained by the Neubauer chamber according to the following formulae:

$$\text{Leucocytes}\left(10^3\ \mu\text{L}\right) = (\text{leucocyte number} * \text{erythrocyte number})/2,000\ \text{erythrocytes};$$
$$\text{Thrombocytes}\left(10^3\ \mu\text{L}\right) = (\text{thrombocyte number} * \text{erythrocyte number})/2,000\ \text{erythrocytes}.$$

The total thrombocyte and leukocyte count and the differential leukocytes, lymphocytes, neutrophils, monocytes, basophils, and eosinophils were performed by staining air-dried blood smears with May–Grünwald–Giemsa–Wright (Rosenfeld 1947). An optical microscope performed the observations at 100× magnification.

To determine respiratory burst activity, 100 µL of blood was added to 100 µL of 0.2% nitro blue tetrazolium solution (Sigma, St. Louis, MO, USA). The final solution was homogenized and then incubated for 30 min at 25 °C. After incubation, we performed a second homogenization. Then, 50 µL from the solution was added to 1 ml of N, N-dimethyl formamide (Sigma). This solution was then homogenized and centrifuged at 3000× g for 5 min. The optical density of supernatant was determined on a spectrophotometer (Biospectro SP-220, Curitiba, Brazil) at 540 nm (Biller-Takahashi et al. 2013).

A semi-automated biochemical analyzer (Doles®, model D250, Goiânia, GO, Brazil) was used for the plasma biochemical analyses. The plasma obtained was used to determine glucose (mg dL^{-1}), cholesterol (mg dL^{-1}), triglycerides (mg dL^{-1}), aspartate aminotransferase (AST) (U L^{-1}), total proteins (g dL^{-1}), and albumin (g dL^{-1}) (Labtest®; Lagoa Santa, MG, Brazil) by the colorimetric enzymatic method using commercial kits; measurements were made with a spectrophotometer (Biospectro SP-220).

Aeromonas hydrophila challenge

Aeromonas hydrophila isolated from the bacterial collection of the Laboratory of Microbiology and Animal Immunology of UNIVASF were obtained as described by Felix e Silva et al. (2022). The *A. hydrophila* strain (IF2) used in the current study was determined by 16S ribosomal DNA (rDNA) polymerase chain reaction (PCR) and sequencing previously performed by Freire et al. (2019).

The lethal dose for 50% of the individuals (LD_{50}) was determined by testing different *A. hydrophila* concentrations (10^6, 10^7, and 10^8 colony forming units (CFU) mL^{-1}). The bacterial inoculum was diluted in sterile saline solution (0.85 g per 100 mL) at 1×10^8 CFU mL^{-1}. Mortality caused by *A. hydrophila* was observed and recorded in each group every 12 h for eight additional days. Then, on day 91 of the experiment, 0.2 mL of *A. hydrophila* solution was inoculated intramuscularly in the laterodorsal right side of each fish in the experimental groups ($n = 10$ per tank).

Before the *A. hydrophila* challenge, all fish were healthy and without apparent signs of clinical symptoms of the disease. After this challenge, the fish were maintained under

the same experimental conditions of feed management and water quality. Blood collection and hematological and plasmatic analysis were performed on all surviving fish on day 98, as detailed in item 2.6.

Statistical analysis

The data and results are expressed as the mean ± standard error of the mean. We analyzed the normality of the residuals of the data (Shapiro–Wilk at a at 0.05) and the homogeneity of variances (Levene at a at 0.05). The data showed homogeneous variances and were compared using a one-way analysis of variance (ANOVA) followed by the Tukey post hoc test ($p < 0.05$). Since the treatments were quantitative independent variables with graded levels of CLE in the diet, significant results ($p < 0.05$) were compared using orthogonal polynomial contrasts. All values were used to determine the linear or quadratic effects of the different treatments tested. The best model was based on the *p*-value and R^2 value. The data of hematological and plasma analyses after the *A. hydrophila* challenge that did not show homogeneous variances were analyzed using a Mann–Whitney non-parametric test ($p < 0.05$) and did not differ between treatments.

Results

Composition of Citrus limon extract

Without needing previous isolation, the nuclear magnetic resonance spectroscopic technique identifies metabolites in complex mixtures, like plant extracts. Using this technique, we can observe signals of chemical shifts characteristic of the presence of primary and secondary metabolites. The ^{1}H NMR spectrum of the CLE revealed signals in all spectral regions (Fig. 1). Expressive signals in the aromatic region at 5.50–7.50 ppm may be associated with the presence of phenolic compounds (mainly flavonoids). On the other hand, the existence of signals in the region between 3.00 and 5.50 ppm was attributed to the presence of carbohydrates (polysaccharides), which were the major chemical constituents in the extract due to the high intensity of the signals (Fig. 1). This information is confirmed by the observation of ^{13}C NMR spectra (Figs. 1–2S, supplementary information) where were observed the presence of less intense signals in the region of 170–100 ppm, and more intense signals in the region of 60–100 ppm, which is characteristic of the presence of carbohydrates.

Two-dimensional NMR experiments (^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC) (Figs. 3S–6S, supplementary information) were performed; however, there was extensive overlap of signals in all spectral regions, making the identification of specific metabolites in the extract complex. The results presented in the NMR analysis are in accordance with the results obtained by mass spectrometry in the negative (Fig. 2A) and positive (Fig. 2B) modes. In Fig. 2 A, the presence of sugars could be observed by the ions at *m/z* 341 (maltose), 179 (glucose), and 161 (which is a result of the loss of water from the glucose molecule).

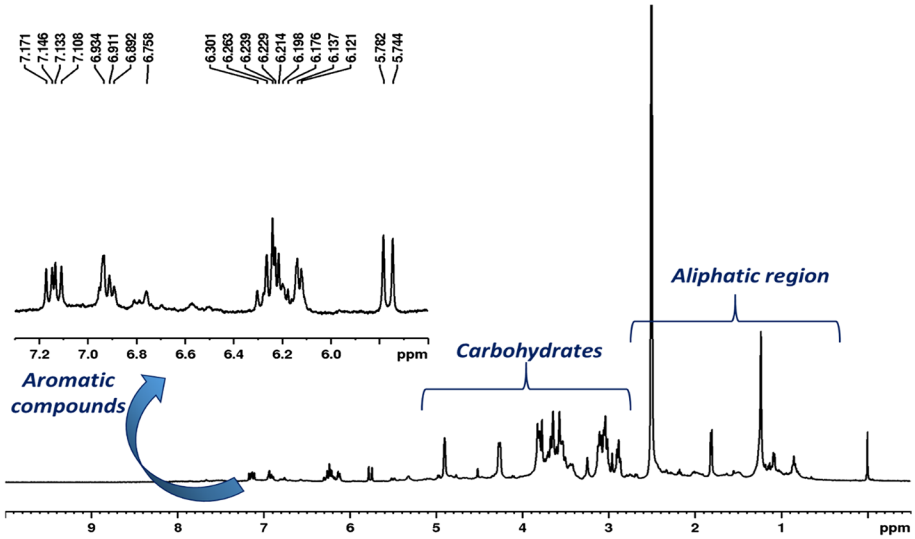

Fig. 1 ^{1}H NMR spectrum of the probiotic (400 MHz, DMSO-d_6)

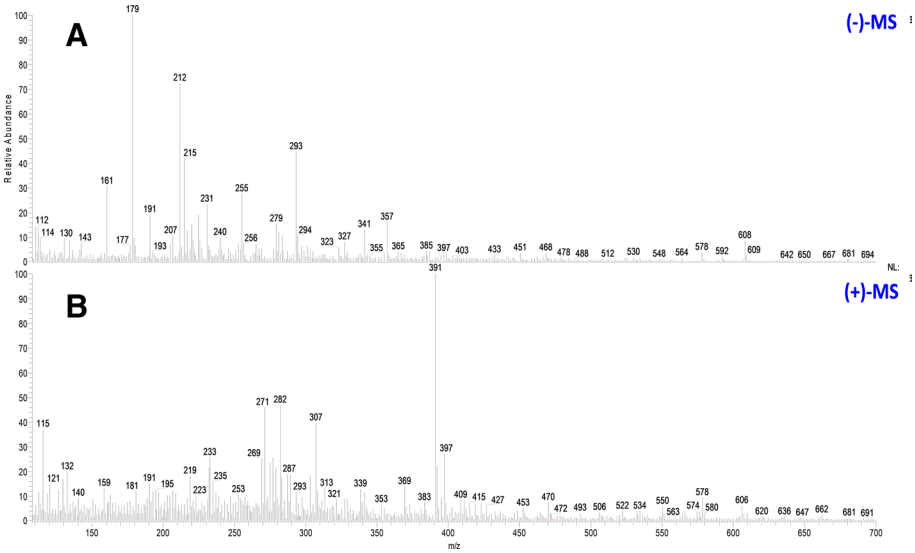

Fig. 2 Mass spectrum using the APCI technique in negative mode (**A**) and positive mode (**B**)

Growth performance

Fish supplemented with 0.4 g CLE kg diet^{-1} showed final weight, weight gain, and SGR significantly higher than fish supplemented with other diets (except for 0.2 g CLE kg^{-1}) ($p < 0.05$). For these same variables, the control group presented values significantly lower than treatments with content between 0.2 and 1.6 g CLE kg^{-1} ($p < 0.05$). The SGR for the

control group was significantly lower than the fish fed 3.2 g CLE kg^{-1} ($p < 0.05$). In addition, for final weight and weight gain, fish fed 0.2 g CLE kg^{-1} showed values significantly higher than fish fed 1.6 and 3.2 g CLE kg^{-1} ($p < 0.05$). The FCR was significantly higher in the control group compared to other treatments and significantly lower in the treatments 0.2 and 0.4 g CLE kg $diet^{-1}$ compared to treatments containing CLE between 1.6 and 3.2 g kg $diet^{-1}$ ($p < 0.05$) (Table 2).

Linear regression analysis showed that the dietary increase in CLE caused a proportional decrease in HSI ($y = 2.919 - (0.146x)$; $R^2 = 0.10$). Initial weight, initial length, HSI, and VSI did not differ between treatments ($p > 0.05$). There was no fish mortality before the *A. hydrophila* challenge (Table 2).

Biochemical, hematological, and immune analysis before the A. hydrophila challenge

Fish supplemented with 3.2 g CLE kg $diet^{-1}$ had significantly higher total thrombocyte, total leukocyte, lymphocyte, and monocyte values than fish supplemented with other diets (except 0.2 g CLE kg^{-1} for total leukocytes and lymphocytes and 1.6 g CLE kg^{-1} for lymphocytes and monocytes) ($p < 0.05$) (Table 3).

Linear regression analysis showed that the dietary increase in CLE caused a proportional increase in plasma albumin levels ($y = 2.711 + (0.161x)$; $R^2 = 0.15$), respiratory burst ($y = 0.246 + (0.0136x)$; $R^2 = 0.09$), total leukocytes ($y = 29.173 + (8.963x)$; $R^2 = 0.24$), and lymphocytes values ($y = 9.260 + (3.796x)$; $R^2 = 0.21$). According to the quadratic regression, treatments containing between 0.2 and 1.6 g CLE kg $diet^{-1}$ showed lower and higher values for erythrocytes ($y = 3.403$—$(1.222x) + (0.422x^2)$; $R^2 = 0.20$) and MCV ($y = 121.957 + (56.486x)$—$(19.911x^2)$; $R^2 = 0.20$), respectively. The quadratic regression analysis also verified that fish supplemented with 3.2 CLE kg $diet^{-1}$ had the highest number of total thrombocytes ($y = 96.662$—$(58.523x) + (27.272x^2)$; $R^2 = 0.51$) and monocytes ($y = 1.340 + (0.253x) + (0.161x^2)$; $R^2 = 0.39$) (Table 3).

Table 2 Growth performance (mean ± SEM) of striped catfish (*Pangasius hypophthalmus*) fed diets containing different *Citrus limon* extract (CLE) concentrations for 90 days

Variables	CLE (g kg $diet^{-1}$)					
	0.0	0.2	0.4	0.8	1.6	3.2
IW	1.77 ± 0.02	1.75 ± 0.01	1.72 ± 0.05	1.73 ± 0.05	1.69 ± 0.03	1.78 ± 0.05
FW	30.76 ± 1.59^{d}	55.86 ± 1.34^{ab}	62.52 ± 2.06^{a}	48.03 ± 1.16^{bc}	43.82 ± 3.01^{c}	39.30 ± 1.75^{cd}
WG	28.99 ± 1.61^{d}	54.10 ± 1.34^{ab}	60.81 ± 2.10^{a}	46.30 ± 1.18^{bc}	42.13 ± 2.99^{c}	37.52 ± 1.71^{cd}
SGR	3.17 ± 0.07^{d}	3.84 ± 0.02^{ab}	3.99 ± 0.07^{a}	3.69 ± 0.05^{bc}	3.61 ± 0.07^{bc}	3.44 ± 0.04^{c}
FCR	1.16 ± 0.06^{a}	0.62 ± 0.02^{d}	0.55 ± 0.02^{d}	0.70 ± 0.04^{cd}	0.80 ± 0.03^{bc}	0.90 ± 0.04^{b}
HSI	3.09 ± 0.29	2.82 ± 0.16	2.91 ± 0.16	2.69 ± 0.13	2.56 ± 0.11	2.54 ± 0.12
VSI	4.93 ± 0.38	6.23 ± 0.70	5.83 ± 0.50	5.13 ± 0.68	4.31 ± 0.32	5.29 ± 0.39
Mortality	0.0	0.0	0.0	0.0	0.0	0.0

IW (initial weight), FW (final weight), and WG (weight gain) are expressed in g. HSI (hepatosomatic index), VSI (viscerosomatic index), and survival are expressed in %. SGR (specific growth rate) is expressed in % per day^{-1}. FCR, feed conversion ratio. Different letters indicate a statistical difference between treatments (Tukey's test, $p < 0.05$). $N = 3$ tanks per treatment, except for HSI and VSI, where $N = 9$ fish per treatment

Table 3 Biochemical, hematological, and immune variables (mean ± SEM) of striped catfish (*Pangasius hypophthalmus*) fed diets containing different *Citrus limon* extract (CLE) concentrations for 90 days

Variables	CLE (g kg diet^{-1})					
	0.0	0.2	0.4	0.8	1.6	3.2
AST	50.20 ± 2.74	44.81 ± 5.31	36.97 ± 2.83	42.63 ± 4.55	56.38 ± 5.46	46.79 ± 3.11
Glucose	92.78 ± 3.43	104.92 ± 7.06	108.80 ± 6.27	102.97 ± 5.86	101.88 ± 4.03	96.06 ± 4.67
Triglycerides	656.68 ± 27.55	647.73 ± 25.26	659.22 ± 44.64	674.60 ± 36.94	674.20 ± 36.23	677.62 ± 26.35
Cholesterol	219.03 ± 5.67	218.33 ± 5.13	229.49 ± 7.87	232.02 ± 5.53	234.62 ± 5.22	230.01 ± 7.86
Total proteins	3.84 ± 0.44	3.75 ± 0.20	3.93 ± 0.42	3.96 ± 0.28	4.04 ± 0.30	3.36 ± 0.19
Albumin	2.69 ± 0.22	2.59 ± 0.15	2.89 ± 0.13	2.95 ± 0.15	2.92 ± 0.15	3.12 ± 0.08
Hematocrit	37.38 ± 0.92	38.62 ± 1.08	37.38 ± 0.60	37.62 ± 0.62	38.62 ± 0.53	37.12 ± 0.61
Hemoglobin	9.06 ± 0.57	10.14 ± 0.57	9.78 ± 0.32	9.23 ± 0.70	8.04 ± 0.48	9.25 ± 0.57
Erythrocytes	3.76 ± 0.42	2.78 ± 0.27	2.89 ± 0.36	2.76 ± 0.33	2.61 ± 0.24	3.80 ± 0.21
MCV	107.17 ± 10.47	148.45 ± 14.82	145.19 ± 19.67	152.16 ± 20.36	157.62 ± 15.36	100.11 ± 6.22
MCH	25.78 ± 2.82	38.71 ± 4.08	38.07 ± 5.15	36.91 ± 5.54	32.09 ± 2.51	24.60 ± 1.35
MCHC	24.16 ± 1.18	26.40 ± 1.68	26.21 ± 0.94	24.46 ± 1.63	20.78 ± 1.09	25.08 ± 1.82
Respiratory burst	0.22 ± 0.01	0.26 ± 0.02	0.27 ± 0.02	0.25 ± 0.01	0.26 ± 0.01	0.29 ± 0.01
Thrombocytes	107.95 ± 9.98^{b}	94.14 ± 19.94^{b}	50.76 ± 13.09^{b}	66.50 ± 11.94^{b}	83.29 ± 14.93^{b}	186.48 ± 15.81^{a}
Leukocytes	32.98 ± 4.42^{b}	37.87 ± 8.72^{ab}	32.96 ± 8.03^{b}	22.27 ± 4.98^{b}	45.31 ± 3.99^{b}	60.07 ± 5.79^{a}
Lymphocytes	10.30 ± 2.33^{b}	14.49 ± 2.81^{ab}	10.60 ± 3.07^{b}	5.18 ± 1.40^{b}	15.84 ± 2.50^{ab}	22.68 ± 3.83^{a}
Monocytes	1.29 ± 0.37^{b}	1.17 ± 0.34^{b}	1.97 ± 0.42^{b}	1.34 ± 0.30^{b}	2.22 ± 0.33^{ab}	3.79 ± 0.52^{a}
Neutrophils	20.74 ± 3.89	29.45 ± 9.39	19.44 ± 5.83	15.36 ± 3.76	27.09 ± 3.76	32.64 ± 3.59

AST (aspartate aminotransferase) is expressed as U L^{-1}. Glucose, triglycerides, and cholesterol are expressed as mg dL^{-1}. Total proteins, albumin, and globulin are expressed as g dL^{-1}. Hematocrit is expressed as %. Erythrocytes are expressed as × 10^{6} µL^{-1}. Hemoglobin concentration and MCHC (mean corpuscular hemoglobin concentration) are expressed as g dL^{-1}. MCV (mean corpuscular volume) is expressed as fL. MCH (mean corpuscular hemoglobin) is expressed as pg. Thrombocytes, leukocytes, lymphocytes, monocytes, and neutrophils are expressed as 10^{3} µL. Different letters indicate a statistical difference between treatments (Tukey's test, $p < 0.05$). $N = 9$ fish per treatment

Plasma AST, glucose, triglycerides, cholesterol, total proteins levels, hematocrit, hemoglobin, MCH, MCHC, and neutrophils values did not show a regression nor a significant difference among the treatments. Basophils and eosinophils were not found (Table 3).

Biochemical, hematological, and immune analysis after the A. hydrophila challenge

There was a linear effect as the concentration of CLE in the diet increased, with a reduction in plasma total proteins ($y = 0.156 + (0.0291x)$; $R^2 = 0.37$) and number of neutrophils ($y = 34{,}089.475 + (16{,}537.470x)$; $R^2 = 0.26$). Plasma AST and glucose levels, respiratory burst, hematocrit, hemoglobin, erythrocyte, MCV, MCH, MCHC, thrombocyte, leukocyte, lymphocyte, and monocyte values did not show a regression, nor a significant difference among the treatments. Basophils and eosinophils were not found (Table 4).

Table 4 Mortality and biochemical, hematological, and immune variables (mean ± SEM) of striped catfish (*Pangasius hypophthalmus*) fed diets containing different *Citrus limon* extract (CLE) concentrations for 90 days following a bacterial challenge for 8 days

	CLE (g kg diet^{-1})					
Variables	0.0	0.2	0.4	0.8	1.6	3.2
AST	34.33 ± 3.58	12.22 ± 5.70	15.13 ± 1.76	19.21 ± 8.30	20.66 ± 6.33	10.48 ± 1.42
Glucose	83.84 ± 19.27	92.32 ± 17.98	101.65 ± 11.03	98.66 ± 11.62	92.97 ± 2.88	89.04 ± 8.39
Total proteins	4.21 ± 0.90	4.64 ± 0.26	3.43 ± 0.43	3.09 ± 0.65	3.38 ± 0.94	3.31 ± 0.21
Hematocrit	43.33 ± 2.73	46.00 ± 4.04	36.33 ± 1.98	44.00 ± 2.08	40.50 ± 1.32	36.33 ± 3.28
Hemoglobin	12.78 ± 1.30	13.93 ± 1.64	8.42 ± 1.28	10.21 ± 1.13	10.97 ± 1.22	9.96 ± 0.54
Erythrocytes	2.82 ± 0.80	2.94 ± 0.36	2.40 ± 0.32	3.66 ± 0.53	2.67 ± 0.47	2.86 ± 0.36
MCV	178.69 ± 44.72	165.50 ± 34.91	175.30 ± 38.64	125.67 ± 19.32	164.90 ± 25.22	133.18 ± 24.55
MCH	51.13 ± 10.78	50.51 ± 12.06	35.67 ± 3.08	29.66 ± 7.10	42.96 ± 4.15	35.86 ± 4.56
MCHC	29.37 ± 1.53	30.03 ± 0.93	23.17 ± 3.23	23.44 ± 3.20	26.89 ± 2.22	27.79 ± 2.56
Respiratory burst	0.13 ± 0.01	0.14 ± 0.01	0.18 ± 0.02	0.21 ± 0.02	0.22 ± 0.02	0.23 ± 0.01
Thrombocytes	84.81 ± 23.85	101.00 ± 16.13	54.07 ± 15.72	62.93 ± 28.23	82.35 ± 28.71	48.81 ± 10.18
Leukocytes	62.25 ± 22.02	35.54 ± 10.07	51.80 ± 10.37	120.81 ± 29.15	67.26 ± 9.31	103.03 ± 24.30
Lymphocytes	15.95 ± 6.50	8.37 ± 0.70	11.78 ± 2.01	19.10 ± 4.42	9.92 ± 4.37	10.84 ± 4.08
Monocytes	8.41 ± 3.61	5.05 ± 1.38	3.28 ± 1.28	4.18 ± 1.18	5.98 ± 2.51	4.22 ± 1.86
Neutrophils	33.74 ± 10.68	20.98 ± 3.02	35.09 ± 9.73	92.70 ± 18.12	47.86 ± 14.75	86.55 ± 18.98
Mortality	86.66 ± 3.33	90.00 ± 5.77	80.00 ± 5.77	86.66 ± 6.67	83.33 ± 6.67	86.66 ± 6.67

AST (aspartate aminotransferase) is expressed as U L^{-1}. Glucose is expressed as mg dL^{-1}. Total proteins are expressed as g dL^{-1}. Hct (hematocrit) and mortality are expressed as %. Ery (erythrocytes) is expressed as × 10^{6} µL^{-1}. Hg (hemoglobin concentration) and MCHC (mean corpuscular hemoglobin concentration) are expressed as g dL^{-1}. MCV (mean corpuscular volume) is expressed as fL. MCH (mean corpuscular hemoglobin) is expressed as pg. Thrombocytes, leukocytes, lymphocytes, monocytes, and neutrophils are expressed as 10^{3} µL. $N = 3$ fish in 0.2 g CLE kg diet^{-1}. $N = 4$ fish in 0.0, 0.8, and 3.2 g CLE kg diet^{-1}. $N = 5$ fish in 1.6 g CLE kg diet^{-1}. $N = 6$ fish in 0.4 g CLE kg diet^{-1}

Discussion

Bioactive ingredients in plant extracts are very complex, and the exact mechanisms by which phytochemicals improve growth response in fish are yet to be elucidated. We detected flavonoids and polysaccharides in the composition of CLE that must have contributed to fish development (González-Molina et al. 2010) in the present study. In addition, CLE could promote growth and stimulate juveniles' appetites as it contains these bioactive compounds. In general, flavonoids and polysaccharides are widely known for their beneficial influences on overall performance and feeding efficiency (Ahmadifar et al. 2021; Morante et al. 2021; Souza et al. 2021). Phytochemicals and their metabolic products also provide health benefits such as improved intestinal morphometry (with consequent improvement in feed digestion and nutrient absorption) (Mohamed et al. 2021) and production of selective growth factors and fermentation substrates for beneficial intestinal microbiota while acting as selective inhibitors of harmful intestinal bacteria, thereby exerting prebiotic-like effects and contributing to growth performance in fish (Zheng et al. 2009;

Van Doan et al. 2019). As the present study did not examine intestinal physiology, future studies are suggested to investigate how the plant additives may influence the gut microbiome and morphology that could link to growth performance in fish.

Polyphenolic compounds have also been reported to promote DNA, RNA, and protein synthesis, stimulate growth hormone and insulin-like growth factor 1 production and function, besides actions on the hypothalamus-pituitary–gonad axis and other anabolic effects in fish, resulting in growth increases (Citarasu 2010; Chakraborty et al. 2014; Mohamed et al. 2021). Flavonoids can decrease animal stress and enhance hepatopancreatic function (Li et al. 2019). Dietary carbohydrate utilization by fish is varied and appears to be related to the complexity of carbohydrates (Ighwela et al. 2015). They can result in a protein-sparing effect, directing the use of proteins for fish growth and reducing the elimination of nitrogenous residues, besides being their main energy source (Felix e Silva et al. 2020). In the current study, glucose and maltose were the most important carbohydrates. Among the important nutrients that provide energy to maintain the cell's vital processes, glucose stands out as a source of carbohydrates (De Souza et al. 2021). Maltose is the least common disaccharide in nature, and when incorporated into the diet, it was efficiently utilized and contributed to body mass in Nile tilapia (Ighwela et al. 2015). Therefore, the bioactive compounds present in CLE may have contributed to improving striped catfish growth, where the 0.4 g CLE kg diet^{-1} treatment had the best results.

Interestingly, the effect of CLE was influenced by its concentration in the diet. Concentrations between 0.2 and 1.6 g CLE kg diet^{-1} demonstrated better growth results than the control group (not supplemented with CLE). Disregarding the control group, the maximum and minimum benefits of CLE for zootechnical parameters were found with 0.4 and 3.2 g kg diet^{-1}, respectively. Only the treatment with the highest concentration of CLE (3.2 g kg diet^{-1}) did not differ from the control group in terms of these parameters, except for FCR, whose values indicated better feed utilization. The FCR demonstrates that juveniles from the control group use food energy less effectively than the other groups for growth. In addition, adequate levels of dietary CLE (mainly 0.2 and 0.4 g kg diet^{-1}) increased the conversion of food into muscle protein (Marchão et al. 2022). In general, the treatment 0.4 g CLE kg diet^{-1} showed greater stability in assessing physiological (erythrocytes and MCV) and immunological parameters (total leukocytes and lymphocytes) compared to the treatment 0.2 g CLE kg diet^{-1}.

Differences in the growth response to a phytochemical may be due to differences in fish species' abilities to metabolize a particular diet substance (Chakraborty et al. 2014). As seen in our results, excessive concentrations of the herbal extract can harm fish development. This could have happened because an increase in the concentration of CLE in diets can trigger anti-lipogenic and diuretic effects (eliminate excess fluid) that limit fish growth (Beltrán et al. 2017; Rahman et al. 2019); therefore, a concentration of 3.2 g CLE kg diet^{-1} should be avoided in fish supplementation.

In line with our results, previous studies on adding lemon to fish diets also promoted growth in different species. This was verified with diets supplemented with dehydrated lemon peel powder for gilthead seabream (*Sparus aurata*) (1.5–3.0%) (Beltrán et al. 2017) and rainbow trout (*Oncorhynchus mykiss*) (0.5–2.5%) (Chekani et al. 2021) and with essential oil extract from lemon peels for Nile tilapia (*Oreochromis niloticus*) (0.75–1.0%) (Mohamed et al. 2021) and ningu (*Labeo victorianus*) (10.0–80.0 g kg^{-1}) (Ngugi et al. 2017). Diets enriched with dried lemon peels for rohu (*Labeo rohita*) (1.0–5.0 g kg^{-1}) (Harikrishnan et al. 2020) and with lemon pomace powder for carp (*Cyprinus carp*) (Laein et al. 2021) also improved weight gain and feed conversion ratio. Limonene from *Citrus* peels (200–600 ppm) in Nile tilapia diets increased growth and gene expression levels of

insulin growth factor 1 (Aanyu et al. 2018). In contrast, there was no weight gain in diets supplemented with lemon peels (1% and 2%) for Nile tilapia and African catfish (*Clarias gariepinus*) (Rahman et al. 2019). Therefore, the effects of diets with lemon compounds may vary according to the experimental conditions and species studied.

Improvements in health conditions can also lead to faster growth in fish. In the present study, phytochemicals, such as flavonoids and polysaccharides in the diet provided to the striped catfish, are considered health-promoting by their antioxidant activity (capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxyl radicals) and positive modulation of the cellular and tissue redox balance (Liu 2003), and their stimulation of immunity (specific and non-specific) by modulating the functions of the immune cells related to immunity expression genes, thereby increasing antibody production (Citarasu 2010; Chakraborty et al. 2014; Gabriel and González-Redondo 2021). Flavonoids also have vasodilatory, antibacterial, antiviral, antiallergic, anti-inflammatory, and antimutagenic actions (Chakraborty and Hancz 2011). In addition, polysaccharides are an important medicinal product, playing a major role in preventing and controlling infectious microbes in aquaculture (Wang et al. 2016) because they are prebiotic substances boosting immune responses. They are widely accepted as a nutritional component for regulating intestinal microbiota and health conditions in fish (Mohan et al. 2019). Polysaccharides are in the highest capacity for transporting bio information, and monosaccharides can connect at different points to form a vast range of diverged or straight structures (Sharon and Lis 1993). In this sense, the evaluation of variations in physiological parameters is a tool that can monitor fish health and nutrition, facilitating the identification of conditions that affect growth performance (Ngugi et al. 2017; Souza et al. 2021). Therefore, the benefits of CLE for improving growth in striped catfish should be evaluated together with our results for biochemical, hematological, and immunological parameters.

In the present study, dietary supplementation of CLE showed mixed effects on these parameters. On the one hand, it did not affect hematocrit, hemoglobin, and MCHC values. We believed this could indicate that the oxygen supplementation in the tissues was not influenced by the different treatments (Tavares-Dias et al. 2007). However, to verify that the oxygen transport at the tissue level remains the same, an analysis of the oxygen consumption rate should also have been performed, which was not done in the present study, and we suggest that it be evaluated in future studies. On the other hand, an increase in erythrocytes and MCV values was also observed in the fish that received dietary CLE. Erythrocytes transport hemoglobin in the blood and an increase in MCV indicates an increase in the size of red blood cells (Presa et al. 2022). Consequently, oxygen in the tissues must have been increased in the fish fed diets supplemented with CLE, which is in line with our results for zootechnical parameters. In addition, body composition variables (HSI and VSI) and plasma AST, total proteins, triglycerides, cholesterol, and glucose levels were not influenced by diets supplemented with CLE, demonstrating that there was no change in the use of lipid, amino acid, and glycogen reserves, as well as general energetic status by the fish (e.g., HSI and VSI) (Melo et al. 2006; Souza et al. 2020; Morante et al. 2021).

Similarly, no differences were found in plasma glucose levels of African catfish, in hematological parameters of rainbow trout fed dehydrated lemon peel powder (Rahman et al. 2019; Chekani et al. 2021), nor in plasma AST, cholesterol, and total protein levels of carp fed lemon pomace powder (Laein et al. 2021). In contrast, plasma glucose levels were increased in gilthead seabream and Nile tilapia fed diets supplemented with dehydrated lemon peel powder (Beltrán et al. 2017; Rahman et al. 2019). An increase in plasma total protein levels was found in rohu receiving dried lemon peels in their diet (Harikrishnan et al. 2020). Dried lemon peels in the diet for ningu caused an increase in erythrocyte,

hematocrit, hemoglobin, MCV, MCHC, and plasma total protein levels and a decrease in plasma glucose, cholesterol, and triglyceride levels (Ngugi et al. 2017). The essential oil extracted from lemon peels added to the diet of Nile tilapia increased plasma total protein levels and decreased plasma AST, cholesterol, triglycerides, and glucose levels (Mohamed et al. 2021). The responses found in our study and previous studies may even differ in some hematological and biochemical parameters. So, we believed that the greater stability of these parameters in fish fed with 0.2 to 1.6 g CLE kg diet^{-1} is related to better physiological homeostasis, which contributed to the growth gain observed in the juveniles of this study.

The fish immune response parameters analyzed in our study (e.g., respiratory burst, total leukocyte count, and differential leukocyte count) are commonly evaluated by other researchers (Ngugi et al. 2017; Harikrishnan et al. 2020; Mohamed et al. 2021). In the current study, before bacterial infection, CLE increased plasma albumin levels, the number of total thrombocytes, total leukocytes, lymphocytes, and monocytes, and respiratory burst, indicating an improvement of the immune defense response in juveniles. Leukocyte levels constitute the first line of defense against any invader. The increased leukocyte count results in a high proportion of monocytes and lymphocytes, indicating a primary innate immune response against diseases (Harikrishnan et al. 2020). Respiratory burst activity is associated with the cytokine release and inflammatory response (Ngugi et al. 2017). Thrombocytes connect the innate immune system with the adjustment system and involve great potential for phagocytosis (Oliveira et al. 2019). Plasma albumin levels and the previously mentioned parameters are also indicators of immunity status in fish (Laein et al. 2021). Therefore, the elevated immunological parameters in striped catfish fed CLE may be due to the defense system's enhancement, which also contributed to improving growth performance.

The benefits of using CLE in a fish diet can be attributed to its bioactive compounds acting in immune strengthening. Flavonoids and polysaccharides in herbal extracts are known to support the inhibition or suppression of the oxidation process (Kenari et al. 2014), promote immune function, and attenuate the inflammatory response (Wang et al. 2016; Gabriel and González-Redondo 2021), making them proficient candidates for the development of immunomodulators in aquaculture. Flavonoids such as those found in CLE may contribute to immune defenses and have antibacterial activity, including an aggregating effect on bacterial cells, which reduces the surface area of the population and limits oxygen consumption and nutrient absorption (Ahmad et al. 2015).

The increased values of these parameters point toward the improved immunological status of fish, as was previously determined with other diets with added *C. limon*. An improvement in humoral and cellular immunity and expression of some immune-related genes was verified in gilthead seabream fed dehydrated lemon peel (Beltrán et al. 2017). Increased plasma albumin and total leucocyte values, lysozyme activity, phagocytic activity, and other immunological parameters occurred in ningu and rohu fed dried lemon peels added to their diets and in Nile tilapia that received essential oil extracts from lemon peels in their diet (Ngugi et al. 2017; Harikrishnan et al. 2020; Mohamed et al. 2021). These alterations were similar to the diet of rainbow trout fed dehydrated lemon peel powder after crowding stress (Chekani et al. 2021). In addition, diets supplemented with other *Citrus* species have also shown promising results in promoting fish growth and immunity, as detailed in a recent review by Kesbiç et al. (2022). This reinforces the importance of *Citrus* as a food additive capable of improving fish development and health.

Our findings before the challenge with *A. hydrophila* indicate an increase in the defense mechanisms of juveniles against bacterial infections due to the increase in phagocytic activity (respiratory burst), thrombocytes, and leukocytes. Despite this, the

different treatments of the present study did not influence the survival rate of fish after infection by *A. hydrophila*. Except for a reduction in plasma total proteins levels and number of neutrophils, the addition of CLE in the fish diet did not cause significant changes in the metabolic, physiological, and immune profiles of striped catfish after bacterial inoculation. Therefore, the benefits we found for growth promotion, immunity enhancement, and physiological homeostasis of striped catfish fed CLE before microbial challenge were insufficient to improve their survival after infection by *A. hydrophila*.

In contrast to our study, in Nile tilapia and African catfish supplemented with diets with dehydrated lemon peel, there was a decrease in mortality after infection by *A. hydrophila* (Rahman et al. 2019). Dried lemon peels in diets for ningu and rohu reduced mortality caused by *A. hydrophila* and *Aeromonas sorbia*, respectively (Ngugi et al. 2017; Harikrishnan et al. 2020). In addition, dietary supplementation with many plant polysaccharides reduced mortality in crucian carp (*Carassius mmuneus*) and grass carp (*Ctenopharyngodon mmune*) after a challenge with *A. hydrophila* (Kenari et al. 2014; Gou et al. 2018). Another study (in vitro) verified that lemon peel essential oil induced a more potent antibacterial effect in six fish pathogens, including *A. hydrophila* (Öntaş et al. 2016). In our study, although CLE improved the immunity of striped catfish, it did not increase their survival and, in general, did not change the biochemical, hematological, and immunological parameters after the antimicrobial challenge.

Conclusion

CLE presented flavonoids and polysaccharides as the main classes of chemical constituents, influencing the beneficial effects of CLE after the 90-day supplementation period on fish growth and health. Including 0.4 g CLE kg diet^{-1} in fish feed is recommended for improving the growth performance and immune resistance of striped catfish in intensive culture. Despite this, diets with CLE were not effective in improving the survival of juveniles after infection by *A. hydrophila*.

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Author contribution J.S.M.: carried out the experiments and biometric and biochemical analyses and contributed to the results and discussion. E.V.C., F.M.A.S., L.M.D., V.L.A.S., and J.R.G.S.A.: chemical composition analysis, figures, supplementary material, writing of M&M and results about chemical composition and collaboration on the discussion. M.T.D.: hematological and immunological analyzes, revision, and collaboration on the discussion. C.E.C.: statistical analysis, revision, writing, and final text. J.F.B.M.: conception and design, revision and collaboration on the discussion. All the authors have read and approved the manuscript.

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Declarations

Ethics approval The study was conducted under the rules of conduct for the use of animals in teaching and research and current legislation; This study was carried out according to the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Committee on Ethics in the Use of Animals (CEUA) of the Universidade Federal do Vale do São Francisco (UNIVASF), Petrolina, Brazil, under the protocol number 0001/270121.

Competing interests The authors declare no competing interests.

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