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Cymbopogon flexuosus essential oil as an additive improves growth, biochemical and physiological responses and survival against *Aeromonas hydrophila* infection in Nile tilapia

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Abstract: The objective of the present study was to evaluate growth, biochemical, hematological and intestinal enzymes responses and survival of Nile tilapia juveniles fed a diet containing the essential oil of lemongrass *Cymbopogum flexuosus* (EOCF) and infected by *Aeromonas hydrophila*. Five diets were evaluated (in quadruplicate) with increasing levels of EOCF (0.0 - control; 0.25; 0.50; 1.0 and 2.0 mL kg diet-1). On day 45, eight fish per treatment were sampled and blood, liver and intestine samples were taken. Others eight fish per treatment were infected with A. *hydrophila* followed by a 15-day period of observation. Citral is the main constituent of EOCF. The inclusion of 2.0 mL EOCF kg diet-1 increased specific growth rate and survival after A. *hydrophila* infection and decreased feed conversion ratio of Nile tilapia. In general, higher concentrations of EOCF in the diet reduced plasma glucose and triglycerides levels, and increased plasma amino acids, alanine aminotransferase (ALT) and hepatic ALT levels, hematological parameters, and the activity of intestinal enzymes. It was concluded that the inclusion of 2.0 mL EOCF kg diet-1 improved growth performance, biochemical and physiological responses and decreased mortality of Nile tilapia after A. *hydrophila* infection.

Key words: citral, diet, glucose, hematological, intestinal enzymes, lemongrass.

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is one of the major species of freshwater fish produced in the world (FAO 2018). On the other hand, the intensification of production in intensive systems can trigger the occurrence of fish diseases caused by opportunistic agents (Martins et al. 2008). In this context, the involvement of *Aeromonas hydrophila* in sickness is usually associated with other conditions, and its pathogenicity appears to be related to environment stress in debilitated hosts (Janda & Abbott 2010). De Souza et al. (2018) found the following clinical signs preceding Nile tilapia mortalities by *A. hydrophila* infection: exophthalmos, dermatitis, ascites, cutaneous hemorrhages, total destruction of the caudal peduncle with exposure of the musculature, erosion of the fins, apathy, and reduced appetite.

Synthetic antibiotics have commonly been used to treat infections in fish. This can trigger selective pressure for the emergence of bacterial resistance, introducing risks to the environment and public health (Bueno et al. 2017, De Souza et al. 2017, Klatte et al. 2017). Moreover, based on consumer expectations, the aquaculture industry is expected to reduce the use of synthetic growth-promoting agents in fish diets because of the risk caused to humans by chemical residues in fish (Harikrishnan et al.

2011). In this regard, essential oils (alternative antimicrobial agents) have been showing potential to be used in fish production (Sutili et al. 2018). They are efficient, inexpensive, biodegradable and rapidly metabolized, with a low risk of accumulation in tissues (Teixeira et al. 2017, Da Cunha et al. 2018). Essential oils may affect the host and act directly on bacterial cells, causing changes in the morphology and lipid profile of bacterial cell membranes, increasing membrane permeability and leading to disruption with cytoplasmic leakage (Nazzaro et al. 2013). Essential oils can be used as nutritional additives in fish diets, showing promising results for health and welfare, such as increased resistance against bacterial infection (Zheng et al. 2009, De Souza et al. 2019b), improved activity of intestinal enzymes (De Souza et al. 2019a), intestinal microbiota (Mahmoud et al. 2004, Navarrete et al. 2010) and immunity (Sutili et al. 2016, Ghafari Farsani et al. 2018). In addition, analysis of biochemical, hematological and enzymatic variables can reveal physiological dysfunctions in fish (Hrubec & Smith 2001), helping in the diagnosis and prognosis of fish diseases.

However, previous studies have found divergent results on the contribution of essential oils in the diet to fish growth. Increased growth and feed conversion ratio was found with the addition of the essential oils of oregano (*Origanum heracleoticum*) and sweet basil (*Ocimum basilicum*) in the diet of channel catfish (*Ictalurus punctatus*) and Nile tilapia, respectively (Zheng et al. 2009, De Souza et al. 2019a). On the other hand, supplementation with the essential oils of *Ocimum americanum* or *Lippia alba* in the diet did not increase growth in red drum (*Sciaenops ocellatus*) or silver catfish (*Rhamdia quelen*), respectively (Souza et al. 2015, Sutili et al. 2016).

Cymbopogon flexuosus (Stapf.) (Poaceae) is an aromatic herb, commonly known as lemongrass. Some studies find that essential oil of *C. flexuosus* (EOCF) have shown its effectiveness as an anesthetic (Limma-Netto et al. 2016), antifungal (Kumar et al. 2009), anticancer and antioxidant agent (Sharma et al. 2009) and presented *in vitro* antimicrobial response (Oussalah et al. 2006, Ahmad & Viljoena 2015). In addition, a recent study demonstrated that EOCF showed high *in vitro* inhibitory activity against *Aeromonas* spp. (De Souza et al. 2018). However, despite the therapeutic potential of EOCF, studies related to its use as a nutritional additive in fish have not yet been reported in the literature.

Therefore, based on the aforementioned biochemical, physiological and antimicrobial activities, EOCF with different concentrations was incorporated into the diet and fed to Nile tilapia with the aim to investigate its effects on the growth, survival, biochemical, intestinal enzymes and hematological responses and prevention against *A. hydrophila* infection.

MATERIALS AND METHODS

Essential oil from Cymbopogon flexuosus

Leaves of *C. flexuosus* cultivated in Três Passos, RS, Brazil, were collected in August 2015. EOCF was extracted from fresh leaves of the plant by hydrodistillation for 2 h using a Clevenger-type apparatus (European Pharmacopoeia 2007). Chemical compounds were performed by chromatographic analysis using and Agilent 7890A gas equipment coupled to Agilent 5975C mass selective detector (GC-MS), where were identified 92.48% of the compounds (geranial - 48.89%; neral - 40.32, cis-Verbenol - 2.38 and, camphene - 0.89%) of the EOCF. The identification of the constituents was realized by comparison of retention indices (NIST 2008).

Animals

Sex-reversed males Nile tilapia juveniles (Gift lineage) were purchased from Fish Farm Bebedouro — Companhia de Desenvolvimento dos Vales do São Francisco e do Parnaíba (CODEVASF), Petrolina, PE, Brazil. The fish (5.63 ± 0.18 g; 6.37 ± 0.03 cm; six juveniles per tank) were housed in continuously aerated 90 L tanks (useful volume 80 L; stocking density 0.44 kg m^{-3}), with a semi-static system and biological filters in the Laboratory of Nutrition of the Universidade Federal do Vale do São Francisco (UNIVASF), Petrolina, PE. Before the experiment, the animals underwent a ten-day adaptation period. The experimental design was completely randomized with five treatments and four replicates. The total length of the experiment was 60 days. The experimental protocol (number 14/2014) was approved by the Ethical Committee of the Instituto de Biologia of the Universidade Federal da Bahia.

In order to remove excess feces and feed residues, the tanks were cleaned siphoning. The water parameters remained stable throughout the experimental period. The physical-chemical parameters of the water: pH (pH meter Waterproof, HI 98130), temperature (digital thermometer Incoterm), and dissolved oxygen (oximeter Linelab DO Eco) were monitored daily; un-ionized ammonia and alkalinity (kit Alfatecnoquímica, Florianópolis, SC) were monitored weekly. Throughout the experiments, water quality was maintained at a temperature of 25.9 (± 0.14) °C, pH 6.4 (± 0.22), 6.0 (± 0.18) mg O_2 L^{-1} of dissolved oxygen, 0.05 (± 0.01) mg NH_3 L^{-1} of total ammonia and an alkalinity of 50 (± 0.10) mg $CaCO_3$ L^{-1}.

Diets and growth performance

Five diets were formulated (36.90% crude protein and 4,072 Kcal kg^{-1} digestible $energy^{-1}$) based on De Souza et al. (2019a). The ingredients (Table I) were purchased locally, weighed scale, finely ground and manually homogenized. The amount of ingredients is the same in all treatments and the difference occurred by the addition of EOCF according to each treatment. Different concentrations of the EOCF (0 - control, 0.25, 0.5, 1.0, or 2.0 mL kg $diet^{-1}$) were added to the mixture together with soya oil and, after that, water. The

Table I. Composition of experimental diets of control group supplied to Nile tilapia.

Ingredients	(%)
Soya meal	52.0
Corn meal	25.5
Wheat meal	10.0
Fish meal	9.75
Soya oil	0.83
NaCl	0.50
Dicalcium phosphate	0.50
Vitamins and minerals*	0.50
Vitamin C	0.20
Calcium propionate	0.20
Butylated hydroxytoluene	0.02

*** Vitamin and mineral mix (guaranteed levels per kg of product) — folic acid: 1,200 mg; nicotinic acid: 20 g; BHT: 5 mg; vitamin A: 2,400 UI; vitamin D3: 600 UI; vitamin E: 30 UI; vitamin K3: 3 mg; vitamin C: 60 g; pantothenic acid: 10 mg; biotin: 200 mg; cholin: 100 g; Inositol: 25 g; Vitamin B1: 4 mg; Vitamin B2: 4 mg; Vitamin B12: 8 mg; Vitamin B2: 4 mg; Vitamin B6: 3 mg; Co: 80 mg; Fe: 10 g; Se: 100 mg; Cu: 3,500 mg; Mn: 10 mg; Zn: 24 mg; I: 160 mg.**

mixture was pelleted and then dried in a forced air circulation oven at 35.5 °C for 24 h. The pellets were stored under refrigeration at -20 °C in glass containers with hermetically sealed caps and they fractionated into diameters compatible with the fish mouth size. The juveniles received the experimental diets three times a day (7:30 and 12:00 a.m. and 4:30 p.m.; divided into three approximately equal parts) at a level of 5% of their body weight. The biomass in each tank was assessed weekly to adjust the pellet size and the amount of feed offered.

The following animal performance parameters were analyzed at the end of the experiment: weight gain (WG) = final body weight - initial body weight; feed conversion ratio (FCR) = consumed feed/weight gain; specific growth rate (SGR) = 100 x (ln final weight - ln initial weight)/days of experiment; and survival (S) = (final fish number x 100)/initial fish number.

Sample collection and chemical analysis

On day 45, two fish from each tank (n = 8 per treatment) were randomly sampled and blood, liver and intestinal samples were taken. The animals were removed from the tank and sedated with benzocaine hydrochloride (30 ppm) for blood collection (1.5 mL) from caudal vasculature a sterile syringe with 10 µL of heparin (5000 UI). Prior to blood collection, the animals underwent a fast of 24 h.

The blood was divided amongst the different analyses. Two blood aliquots were collected. The first aliquot (about 0.5 mL) was used for hematological analyses and the second aliquot (about 1.0 mL) was used for plasma analyses. Both aliquots were transferred to 2.5 mL polyethylene tubes for further analysis.

For hematological analyses, samples were subsequently transferred to heparinized capillaries and centrifuged (Microspin, model Spin 1000) at 12.000 x g for 5 min, to determine the hematocrit (Hct) by the microhematocrit method. Erythrocyte (Er) counts were performed in a Neubauer chamber with the aid of a binocular optical microscope, after dilution 1:200 in Natt and Herrick solution. Hemoglobin (Hb) concentration was determined according to Collier (1944), using a spectrophotometer at 540 nm. Calculations of estimated hematimetric indices were performed according to the following equations: mean corpuscular volume (MCV) = Hct*10/Er(*10^6 µL), expressed as fL; mean corpuscular hemoglobin (MCH) = Hb*10/Er, expressed as pg and; mean corpuscular hemoglobin concentration (MCHC) = Hb*100/Hct, expressed as g dL^{-1}.

The second aliquot of blood was centrifuged at 4 °C at 3000 x g (15 min) to separate the plasma. The samples were stored under constant refrigeration at -20 °C. After blood collection, the animals were euthanized with a lethal dose of benzocaine hydrochloride (300 ppm) and section of the spinal cord in order to collect the intestine and liver. Determinations of plasma triglycerides, total protein, and alanine aminotransferase (ALT) levels were performed using commercial Kits (Labtest TM kits; Vista Alegre, MG, Brazil) in a semi-automatic biochemical analyzer (DolesTM, model D-250). For plasmatic amino acid concentrations determination, a 1 mm glycine standard was used, having 0.1 ninhindrin in isopropyl alcohol as substrate, and the readings were performed using spectrophotometer at 570 nm (adapted from Copley 1941). Plasma glucose levels were determined enzymatically by glucose oxidase/glucose peroxidase in a BT 3000 apparatus (Wiener Lab, Rosario, Argentina) according Teixeira et al. (2017). Plasma lysozyme activity (µg mL^{-1}) was measured using a turbidimetric assay according to Ellis (1990), as described in detail by De Souza et al. (2019b).

The liver and total intestine tract samples were collected and preserved at -80 °C until analysis. Liver and total intestine tract samples (100 mg) were homogenized in buffer (10 mM phosphate per 20 mM tris-pH 7.0) using a mechanical homogenizer (Marconi MA039) before centrifugation at 600 x g for 3 min at 4 °C. The supernatant was centrifuged again at 6000 x g for 8 min for hepatic ALT and intestinal enzyme analysis.

Hepatic ALT activity was measured using commercial Kits (Labtest TM kits; Vista Alegre, MG, Brazil) and determined spectrophotometrically at 545 nm. The intestinal amylase and lipase activities were determined spectrophotometrically at 405 nm using commercial Kits (Labtest TM kits; Vista Alegre, MG, Brazil). Intestinal protease activity was determined according to Walter (1984), where the enzymatic activity was defined as the amount of enzyme needed to catalyze the formation of 1 mg tyrosine min^{-1}. Determination of hepatic glycogen was determined spectrophotometrically at 480 nm as described in detail by De Souza et al. (2019a).

Antimicrobial activity

Aeromonas hydrophila isolates from the bacterial collection of the Laboratory of microbiology and animal immunology at UNIVASF were obtained as described by De Souza et al. (2018). The *A. hydrophila* strain used in the current study was confirmed by polymerase chain reaction (PCR) and sequencing (Oliveira et al. 2012).

On day 45 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 = 8 per treatment). The bacterial inoculum was diluted in sterile saline solution (0.85 g per 100 mL) at a concentration of 10^8 colony forming unit per mL. Mortality caused by *A. hydrophila* was observed and recorded in each group for 15 additional days. The fish were maintained under the same experimental conditions of feed management and water quality.

Statistical analysis

The results are expressed as the means ± standard error of the mean (S.E.M.). Levene's test demonstrated the homogeneity of data variances. The data were compared using one-way analysis of variance (ANOVA). The effects of EOCF on the variables were evaluated based on linear regression. If no significant relationship was found, the differences between treatment means were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Tukey test (De Souza et al. 2019a). The significance was set at a critical level of 95% ($p < 0.05$).

RESULTS

Growth performance

A positive linear effect ($p < 0.05$) was observed between the EOCF and SGR and survival post-infection and a negative linear effect ($p < 0.05$) was observed between the EOCF and FCR. Initial weight, final weight, final length, WG, and survival pre-infection did not present linear regression or differ among treatments (Table II).

Biochemical and physiological variables

The regression ($p < 0.05$) demonstrated that the dietary increase of EOCF proportionally increased activities of intestinal lipase and protease enzymes in juveniles. In addition, Nile tilapias fed a diet containing 2.0 mL EOCF kg $diet^{-1}$ had a significantly higher activity of intestinal amylase enzyme than those in the other treatments ($p < 0.05$) (Table III).

Another linear effect ($p < 0.05$) demonstrated that the dietary increase of EOCF proportionally increased plasma amino acids and hepatic ALT

Table II. Growth performance and survival of Nile tilapia fed with diets containing different concentrations of the essential oil of *Cymbopongon flexuosus* (EOCF).

	EOCF (mL kg diet^{-1})				
Variables	**0.0 - control**	**0.25**	**0.5**	**1.0**	**2.0**
In weight	5.92±0.28	5.69±0.12	5.85±0.25	5.29±0.06	5.29±0.11
Fn weight	16.04±0.97	15.70±0.74	15.60±0.73	15.00±0.84	15.90±0.97
Fn length	9.69±0.10	9.73±0.10	9.74±0.16	9.66±0.12	9.70±0.19
WG	10.12±1.02	10.01±0.85	9.75±0.95	9.56±0.80	10.61±0.86
SGR	2.21±0.17	2.25±0.15	2.18±0.19	2.32±0.11	2.43±0.09
FCR	2.09±0.09	2.06±0.08	2.09±0.14	2.02±0.07	1.93±0.04
Sur pre-infection	95.83±4.17	100±0.00	100±0.00	95.83±4.17	95.83±4.17
Sur post-infection	12.50±25.00	12.50±25.00	25.00±28.87	37.50±25.00	62.50±25.00

In weight (initial weight), Fn weight (final weight) and WG (weight gain) are expressed in g, Fn length (final length) is expressed in cm, SGR (specific growth rate) is expressed as % day^{-1} and, Sur (survival) is expressed as %. FCR = feed conversion ratio. Equations: In weight, Fn weight, Fn length, WG, and Sur pre-infection: no regression; SGR: y = 2.191 + (0.116x), R^2 = 0.84; FCR: y = 2.099 - (0.081x), R^2= 0.92; Sur post-infection: y = 10.313 + (26.250x), R^2 = 0.98. Data are presented as the means ± SEM. The means obtained showed no significant differences (Tukey's test, p > 0.05, n =4 tanks per treatment).

in juveniles. Additionally, plasma glucose and triglycerides levels were significantly higher in the control group in comparison with the other treatments and the addition of 0.25 mL EOCF kg diet^{-1} significantly increased plasma triglycerides levels compared with those receiving 2.0 mL EOCF kg diet^{-1} (p < 0.05). Plasma ALT was significantly higher in fish fed a diet containing 1.0 or 2.0 mL EOCF kg diet^{-1} than in control group (p < 0.05). Plasma total protein, lysozyme activity, and hepatic glycogen did not present linear regression or differ among treatments (Table IV).

A positive linear effect (p < 0.05) was observed between dietary EOCF and hematocrit, erythrocyte and, Hemoglobin concentrations. The hematimetric indices did not present linear regression or differ among treatments (Table V).

DISCUSSION

Citral, in the present study, was the most abundant component of EOCF. Citral is a monoterpene comprising a mixture of geranial (α-citral) and neral (ß-citral) isomers (Teixeira et al. 2017), which has been known for its immunomodulatory, anti-inflammatory, antiseptic, antimicrobial and fungistatic properties (Bachiega & Sforcin 2011). In summary, citral (89.21% in the present study) has potential for use as a dietary supplement to treat infections and improve health in fish, because citral can suppress oxidative stress through induction of the endogenous antioxidant proteins, according verified by Zeppenfeld et al. (2017) in study with *Aloysia triphylla* essential oil (50.19% of citral). Previous studies verified the addition of 2.0 mL *Aloysia triphylla* essential oil kg diet^{-1} (50.19% of citral) increased silver catfish growth, but it did not affect zebrafish (*Danio rerio*) growth (Zeppenfeld et al. 2016, Zago et al. 2018). The present study revealed that 2.0 mL EOCF kg diet^{-1} improved SGR and FCR of Nile tilapia, which demonstrates the potential of EOCF (with citral as the major component) in growth performance of fish. The growth-enhancing effect of EOCF could be correlated with their antibacterial effect (Al-Sagheer et al. 2018) as it is verified by the *in vivo* (our study) and *in vitro* (De Souza et al. 2018) inhibition

Table III. Activity of intestinal enzymes (UI mg protein^{-1}) of Nile tilapia fed with diets containing different concentrations of the essential oil of *Cymbopongon flexuosus* (EOCF).

	EOCF (mL kg diet^{-1})				
Variables	**0.0 - control**	**0.25**	**0.5**	**1.0**	**2.0**
Amylase	0.27± 0.03^{b}	0.27±0.03^{b}	0.26±0.02^{b}	0.24±0.02^{b}	0.39±0.03^{a}
Lipase	2.21±0.13	2.20±0.18	2.42±0.15	2.67±0.06	2.68±0.08
Protease	83.02±4.43	85.42±2.57	84.46±2.56	90.20±3.31	89.93±4.83

Equations: Lipase: y = 2.240 + (0.262x), R^2 = 0.78; Protease: y = 83.944 + (3.549x), R^2 = 0.74. Data are presented as the means ± SEM. Different letters in the same row indicate statistical differences between treatments (Tukey's test, p < 0.05; n = 8 fish per treatment).

of *A. hydrophila*. The antimicrobial properties of EOCF together with the biochemical and physiological responses found in the current study can positively influence fish performance.

Indeed, the reduction of Nile tilapia mortality after infection by *A. hydrophila*, in the current study, with the addition of 2.0 mL EOCF kg diet^{-1} may have been linked to its chief component, citral (De Souza et al. 2018) or an interaction of the different components of the essential oil with citral. The inclusion of essential oils in fish diets can provide greater resistance and survival after bacterial infection (Nazzaro et al. 2013). The mechanism of action of essential oils for bacterial death involves disruption of bacterial cell membranes (leaving them more permeable to the release of molecules and ions), inhibition of enzyme synthesis and/or absorption of nutrients (Bakkali et al. 2008, De Souza et al. 2017). Citral affected the cell membrane of *Cronobacter sakazakii*, as evidenced by decreased intracellular ATP concentration, reduced pH, and cell membrane hyperpolarization (Shi et al. 2016). Additionally, the antibacterial activity of the essential oil of *Cymbopogon citratus* is mainly due to its citral active constituents (Oliveira et al. 2010).

Essential oil components also act on innate immune system in fish (e.g. plasma lysozyme activity) (Carbone & Faggio 2016). Essential oils can indirectly influence lysozyme release (De Souza et al. 2019a), because lysozyme concentration, and therefore activity, is greatest at the digestive tract mucosa-lumen interface and lysozyme limit microbial attachment and invasion into the mucosa (Mason & Huffnagle 2009). In the present study, differences in plasma lysozyme activity were not verified, however.

In addition, the present study revealed that the higher level of EOCF (2.0 mL kg diet^{-1}) added to the Nile tilapia diet increased activity of intestinal enzymes. This could be related to the better efficiency of a diet, because they act directly on the digestion and assimilation of nutrients (De Souza et al. 2019a). In previous studies, the addition of carvacrol or thymol to rainbow trout (*Oncorhynchus mykiss*) and *Cymbopogon citratus* and *Pelargonium graveolens* essential oils to Nile tilapia diet regulated intestinal microbial (Giannenas et al. 2012; Al-Sagheer et al. 2018), demonstrating that essential oils can enhance the activity of intestinal enzymes.

Despite the improved activity of intestinal amylase, 2.0 mL EOCF kg diet^{-1} reduced plasma glucose and triglycerides levels in Nile tilapia in the present study. This suggests that the products generated by the catabolism of these metabolites were available to the fish as an energy source (Copatti et al. 2015), possibly

Table IV. Biochemical variables of plasma and liver of Nile tilapia fed with diets containing different concentrations of the essential oil of *Cymbopongon flexuosus* (EOCF).

Plasmatic	**EOCF (mL kg diet^{-1})**				
Variables	**0.0 - control**	**0.25**	**0.5**	**1.0**	**2.0**
Glucose	99.33±5.47^{a}	66.60±5.77^{b}	66.20±10.50^{b}	63.8±5.3^{b}	70.00±4.74^{b}
Triglycerides	277.10±13.09^{a}	163.72±7.05^{b}	118.33±9.24^{bc}	129.46±11.58^{bc}	104.62±9.77^{c}
Total proteins	2.24±0.21	1.74±0.23	2.02±0.15	2.17±0.17	2.22±0.15
Amino acids	17.33±1.60	25.59±1.85	25.92±1.71	25.02±2.35	33.40±1.67
ALT	1.59±0.24^{b}	2.58±0.58^{ab}	3.65±0.56^{ab}	3.82±0.83^{a}	3.86±0.46^{a}
Lysozyme	0.19±0.03	0.18±0.03	0.13±0.02	0.14±0.02	0.15±0.03
Hepatic variables	**0.0 – control**	**0.25**	**0.5**	**1.0**	**2.0**
Glycogen	9.64±0.89	10.0±0.97	10.57±0.77	9.57±1.46	8.89±0.99
ALT	0.33±0.04	0.38±0.09	0.33±0.08	0.66±0.12	1.19±0.12

Glucose, triglycerides, and glycogen are expressed as mg dL^{-1}, total proteins is expressed as g dL^{-1}, amino acids concentration is expressed as µmol mL^{-1}, ALT is expressed as UI, and lysozyme is expressed as µg mL^{-1}. Equations: Plasma glucose, triglycerides, total proteins, ALT, and lysozyme and Hepatic glycogen: no regression; Plasma amino acids: y = 20.732 + (6.293x), R^2 = 0.76; Hepatic ALT: y = 0.238 + (0.453x), R^2 = 0.94. Data are presented as the means ± SEM. Different letters in the same row indicate statistical difference between treatments (Tukey's test, $p < 0.05$; n = 8 fish per treatment).

resulting in an energy-sparing effect on plasma total protein and hepatic glycogen levels, since these parameters, commonly used to assess the metabolic state of fish tissues, were not altered. Hepatic glycogen synthesis and degradation are regulated to maintain plasma glucose levels as required to meet the needs of the organism (Berg et al. 2002).

Transamination and deamination processes have been studied in fish as an important tool to evaluate their use of proteins (Adams et al. 1996). Excess amino acids, which cannot be stored in the same way as carbohydrates (e.g. glycogen) or fats (e.g. triglycerides) are therefore made available for protein synthesis and/or oxidized to generate energy (Moraes & Almeida 2014). Possibly, the addition of 2.0 mL EOCF kg diet^{-1} may have stimulated transamination, glycogenolysis, gluconeogenesis and lipolysis in order to maintain homeostasis in the fish. Hence, it is likely that the addition of 2.0 mL EOCF kg diet^{-1} affects energy metabolism.

According to our results, the addition of 2.0 mL EOCF kg diet^{-1} of Nile tilapia juveniles increased plasma amino acid levels, which could be related to elevated plasma and hepatic ALT levels. An elevation of ALT levels could be related to liver damage (De Souza et al. 2019a). However, previous studies verified that silver catfish fed with diet containing citral-rich (2.0 mL essential oil of *Aloysia triphylla* kg diet^{-1}) reduced lymphocyte and neutrophil counts and stress response (thiobarbituric acid-reactive substances, lipid hydroperoxide, superoxide dismutase, catalase and non-protein thiols) (Santos et al. 2017, Zeppenfeld et al. 2017) which demonstrates that EOCF with citral could improve some physiological effects, but it could also cause damage to the hepatic tissue. So, it is suggested that more research with antioxidant

Table V. Hematological variables of Nile tilapia fed with diets containing different concentrations of the essential oil of *Cymbopongon flexuosus* (EOCF).

	EOCF (mL kg diet^{-1})				
Variables	**0.0 - control**	**0.25**	**0.5**	**1.0**	**2.0**
Hct	17.83±1.58	17.67±1.38	20.67±0.67	21.83±1.49	24.83±1.08
Er	2.31±0.15	2.46±0.24	2.94±0.10	2.91±0.24	3.20±0.13
Hb	9.85±0.93	10.77±1.35	11.94±0.43	12.14±1.09	14.29±1.14
MCV	77.46±7.25	72.19±4.24	70.68±2.80	77.33±7.60	78.45±5.73
MCH	42.59±2.54	46.80±8.00	40.96±2.28	43.32±5.21	44.36±2.50
MCHC	55.77±2.98	63.51±10.47	58.02±2.56	58.03±8.17	58.55±6.16

Hct (Hematocrit) is expressed as %, Er (erythrocyte) concentration is expressed as 10^6 µL^{-1}, Hb (hemoglobin) concentration and MCHC (mean corpuscular hemoglobin concentration) are expressed as g dL^{-1}, MCV (mean corpuscular volume) is expressed in fL and MCH (mean corpuscular hemoglobin) is expressed in pg. Equations: MCV, MCH and, MCHC: no regression; HCT: y = 17.830 + (3.648x), R^2 = 0.93; Er: y = 2.455 + (0.412x), R^2 = 0.78; Hb: y = 10.256 + (2.056x), R^2 = 0.94. Data are presented as the means ± SEM. The means obtained showed no significant differences (Tukey's test, p > 0.05, n = 8 fish per treatment).

and histological studies should be carried out to determine if EOCF or citral triggers liver damage.

Finally, hematological variables reflect body processes and serve as important indicators of fish health, but no studies have been carried out to assess the effect of EOCF in the diet on fish hematological values. In the present study, the addition of 2.0 mL EOCF kg diet^{-1} increased hematocrit, erythrocyte and hemoglobin values in Nile tilapia, which contributed to the avoidance of anemia or reduction of red blood cell or oxygen supply at the cellular level (Tavares-Dias 2015). In addition, the increased hemoglobin values observed in our study may be related to the reduced plasma glucose and triglycerides levels, since a greater oxygen supply is required to catabolize these substrates for energy generation. Hence, improvements in hematological variables verified in the current study in Nile tilapia juveniles fed 2.0 mL EOCF kg diet^{-1} may have contributed to their increased growth and survival against *A. hydrophila* infection.

CONCLUSIONS

In conclusion, the present study indicates that EOCF has potential as a feed additive for inclusion in fish diets. The findings demonstrated that the best EOCF inclusion concentration was 2.0 mL kg diet^{-1} that improved growth performance, metabolic, hematological and intestinal enzyme responses and survival after *A. hydrophila* infection in Nile tilapia juveniles. However, more studies are needed to confirm your benefits, because EOCF caused an elevation in plasma and hepatic ALT.

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REFERENCES

ADAMS SM, HAM MS, GREELEY MS, LEHEW RF, HINTON DE & SAYLOR F. 1996. Downstream gradient in bioindicator responses: Point source contaminant effects on fish health. Can J Fish Aquat Sci 53: 2177-2187.

AHMAD A & VILJOENA A. 2015. The *in vitro* antimicrobial activity of *Cymbopogon* essential oil (lemon grass) and its interaction with silver ions. Phytomedicine 22: 657-665.

AL-SAGHEER AA, MAHMOUD HK, REDA FM, MAHGOUB SA & AYYAT MS. 2018. Supplementation of diets for *Oreochromis niloticus* with essential oil extracts from lemongrass (*Cymbopogon citratus*) and geranium (*Pelargonium graveolens*) and effects on growth, intestinal microbiota, antioxidant and immune activities. Aquacult Nutr 24: 1006-1014.

BACHIEGA TF & SFORCIN JM. 2011. Lemongrass and citral effect on cytokines production by murine macrophages. J Ethnopharmacol 137: 909-913.

BAKKALI F, AVERBECK S, AVERBECK D & IDAOMAR M. 2008. Biological effects of essential oils - Review. Food Chem Toxicol 46: 446-475.

BUENO I, WILLIAMS-NGUYEN J, HWANG H, SARGEANT JM, NAULT AJ & SINGER RS. 2017. Impact of point sources on antibiotic resistance genes in the natural environment: A systematic review of the evidence. Anim Health Res Rev 18: 112-127.

CARBONE D & FAGGIO C. 2016. Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. Fish Shellfish Immunol 54: 12-178.

BERG JM, TYMOCZKO JL & STRYER L. 2002. Biochemistry. 5th ed., New York: W.H. Freeman, New York, USA, 1100 p.

COLLIER HB. 1944. The standardizations of blood haemoglobin determinations. Can Med Assoc J 50: 550-552.

COPATTI CE, BOLNER KCS, DE ROSSO FL, LORO VL & BALDISSEROTTO B. 2015. Tolerance of piava juveniles to different ammonia concentrations. Semina: Ciênc Agrár 36: 3991-4002.

COPLEY NG. 1941. Alloxan and ninhydrin Test. Analyst 66: 492-493.

DA CUNHA JA, HEINZMANN BM & BALDISSEROTTO B. 2018. The effects of essential oils and their major compounds on fish bacterial pathogens - A review. J Appl Microbiol 125: 328-344.

DE SOUZA RC, DA COSTA MM, BALDISSEROTTO B, HEINZMANN BM, SCHMIDT D, CARON BO & COPATTI CE. 2017. Antimicrobial and synergistic activity of essential oils of *Aloysia triphylla* and *Lippia alba* against *Aeromonas* spp. Microb Pathog 113: 29-33.

DE SOUZA EM, DE SOUZA RC, DA COSTA MM, PINHEIRO CG, HEINZMANN BM & COPATTI CE. 2018. Chemical composition and evaluation of the antimicrobial activity of two essential oils. Bol Inst Pesca 44: e321.

DE SOUZA EM, DE SOUZA RC, MELO JFB, DA COSTA MM, DE SOUZA AM & COPATTI CE. 2019a. Evaluation of the effects of *Ocimum basilicum* essential oil in Nile tilapia diet: Growth, biochemical, intestinal enzymes, haematology, lysozyme and antimicrobial challenges. Aquaculture 504: 7-12.

DE SOUZA RC, DE SOUZA EM, DA COSTA MM, MELO JFB, BALDISSEROTTO B & COPATTI CE. 2019b. Dietary addition of the essential oil from *Lippia alba* to Nile tilapia and its effect after inoculation with *Aeromonas* spp. Aquacult Nutr 25: 39-45.

ELLIS AE. 1990. Lysozyme assays. In: Stolen JS, Fletcher TC, Anderson DP, Roberson BS & Muiswinkel WB (Eds), Techniques in fish immunology. Fair Haven: SOS publications, Fair Haven, USA, p. 101-103.

EUROPEAN PHARMACOPOEIA. 2007. European Pharmacopoeia, 6th ed., Strasbourg, France. European Directorate for the Quality of Medicines.

FAO – FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. 2018. The state of world fisheries and aquaculture 2018 - Meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO.

GHAFARI FARSANI H, GERAMI MH, FARSANI MN, RASHIDIYAN G, MEHDIPOUR N, GHANAD M & FAGGIO C. 2018. Effect of different levels of essential oils (*Satureja hortensis*) in diet on improvement growth, blood biochemical and immunity of angelfish (*Pterophyllum scalare* Schultze, 1823). Nat Prod Res 24: 1-6.

GIANNENAS I, TRIANTAFILLOU E, STAVRAKAKIS S, MARGARONI M, MAVRIDIS S, STEINER T & KARAGOUNI E. 2012. Assessment of dietary supplementation with carvacrol or thymol containing feed additives on performance, intestinal microbiota and antioxidant status of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 350: 26-32.

HARIKRISHNAN R, BALASUNDARAM C & HEO MS. 2011. Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. Aquaculture 317: 1-15.

HRUBEC TC, SMITH SA & ROBERTSON JL. 2001. Age-related changes in hematology and plasma chemistry values

of hybrid striped bass (*Morone chrysops* X *Morone saxatilis*). Vet Clin Pathol 30: 8-15.

KLATTE S, SHAEFER HC & HEMPEL M. 2017. Pharmaceuticals in the environment – A short review on options to minimize the exposure of humans, animals and ecosystems. Sust Chem Pharm 5: 61-66.

JANDA JM & ABBOTT SL. 2010. The genus *Aeromonas*: Taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23: 35-73.

KUMAR A, SHUKLA R, SINGH P & DUBEY NK. 2009. Biodeterioration of some herbal raw materials by storage fungi and aflatoxin and assessment of *Cymbopogon flexuosus* essential oil and its components as antifungal. Int Biodeterior Biodegradation 63: 712-716.

LIMMA-NETTO JD, SENA AC & COPATTI CE. 2016. Essential oils of *Ocimum basilicum* and *Cymbopogon flexuosus* in the sedation, anesthesia and recovery of tambacu (*Piaractus mesopotamicus* Male X *Colossoma macropomum* Female). Bol Inst Pesca 42: 727-733.

MAHMOUD BSM, YAMAZAKI K, MIYASHITA K, IL-SHIK S, DONG-SUK C & SUZUKI T. 2004. Bacterial microflora of carp (*Cyprinus carpio*) and its shelf-life extension by essential oil compounds. Food Microbiol 21: 657-666.

MARTINS ML, MIYAZAKI DMY & MOURIÑO JLP. 2008. *Aeromonas caviae* during mortality on Nile tilapia and supplementation with vitamin C in the diet. Bol Inst Pesca 34: 585-590.

MASON KL & HUFFNAGLE GB. 2009. Control of mucosal polymicrobial populations by innate immunity. Cell Microbiol 11: 1297-1305.

MORAES G & ALMEIDA LC. 2014. Nutrição e aspectos funcionais da digestão de peixes. In: Baldisserotto B, Cyrino JEP & Urbinati EC (Eds), Biologia e fisiologia de peixes neotropicais de água doce. Jaboticabal: Funep/ Unesp, Jabotical, Brazil, p. 233-252.

NAVARRETE P, TOLEDO I, MARDONES P, OPAZO R, ESPEJO R & ROMERO J. 2010. Effect of *Thymus vulgaris* essential oil on intestinal bacterial microbiota of rainbow trout, *Oncorhynchus mykiss* (Walbaum) and bacterial isolates. Aquacult Res 41: 667-678.

NAZZARO F, FRATIANNI F, DE MARTINO L, COPPOLA R & DE FEO V. 2013. Effect of essential oils on pathogenic bacteria. Pharmaceuticals 6: 1451-1474.

NIST. 2008. EPA, NIH mass spectral library and search, analysis programs. Hoboken, NJ: J. Wiley & Sons.

OLIVEIRA MMM, BRUGNERA DF, CARDOSO MG, ALVES E & PICCOLI RH. 2010. Disinfectant action of *Cymbopogon* sp. essential oils in different phases of biofilm formation by *Listeria monocytogenes* on stainless steel surface. Food Control 21: 549-553.

OLIVEIRA STL, VENERONI-GOUVEIA G & DA COSTA MM. 2012. Molecular characterization of virulence factors in *Aeromonas hydrophila* obtained from fish. Pesq Vet Bras 32: 701-706.

OUSSALAH M, CAILLET S, SAUCIER L & LACROIX M. 2006. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella typhimurium*, *Spaphylococcus aureus* and *Listerria monocytogenes*. Food Control 18: 414-420.

SANTOS AC DOS, SUTILI FJ, HEINZMANN BM, DA CUNHA MA, BRUSQUE ICM, BALDISSEROTTO B & ZEPPENFELD CC. 2017. *Aloysia triphylla* essential oil as additive in silver catfish diet: Blood response and resistance against *Aeromonas hydrophila* infection. Fish Shellfish Immunol 62: 213-216.

SHARMA PR, MONDHE DM, MUTHIAH S, PAL HC, SHAHI AK, SAXENA AK & QAZI GN. 2009. Anticancer activity of an essential oil from *Cymbopogon flexuosus*. Chem Biol Interact 179: 160-168.

SHI C, SONG K, ZHANG X, SUN Y, SUI Y, CHEN Y, JIA Z, SUN H, SUN Z & XIA X. 2016. Antimicrobial activity and possible mechanism of action of citral against *Cronobacter sakazakii*. Plos one 11: e0159006.

SOUZA CF ET AL 2015. *Rhamdia quelen* (Quoy & Gaimard, 1824), submitted to a stressful condition: Effect of dietary addition of the essential oil of *Lippia alba* on metabolism, osmoregulations and endocrinology. Neotrop Ichithyol 13: 707-714.

SUTILI FJ, GATLIN DM, HEINZMANN BM & BALDISSEROTTO B. 2018. Plant essential oils as fish diet additives: benefits on fish health and stability in feed. Rev Aquacult 10: 716-726.

SUTILI FJ, VELASQUEZ A, PINHEIRO CG, HEINZMANN BM, GATLIN DM & BALDISSEROTTO B. 2016. Evaluation of *Ocimum americanum* essential oil as an additive in red drum (*Sciaenops ocellatus*) diets. Fish Shellfish Immunol 56: 155-161.

TAVARES-DIAS M. 2015. Parâmetros sanguíneos de referência para espécies de peixes cultivados. In: Tavares-Dias M & Mariano WS (Eds), Aquicultura no Brasil: Novas perspectivas. São Carlos: Pedro and João, São Carlos, Brazil, p. 11-30.

TEIXEIRA RR, DE SOUZA RC, SENA AC, BALDISSEROTTO B, HEINZMANN BM, COUTO RD & COPATTI CE. 2017. Essential oil of *Aloysia triphylla* in Nile tilapia: Anaesthesia, stress

parameters and sensory evaluation of fillets. Aquacult Res 48: 3383-3392.

WALTER HE. 1984. Proteinases: Methods with hemoglobin, casein and azocoll as substrates. In: Bergmeyer HU (Ed), Methods of enzymatic analysis. Weinheim: Verlag Chemie, Weinheim, Germany, p. 270-277.

ZAGO DC ET AL. 2018. *Aloysia triphylla* in the zebrafish food: Effects on physiology, behavior, and growth performance. Fish Physiol Biochem 44: 465-474.

ZEPPENFELD CC, HERNANDEZ DR, SANTINON JJ, HEINZMANN BM, DA CUNHA MA, SCHMIDT D & BALDISSEROTTO B. 2016. Essential oil of *Aloysia triphylla* as feed additive promotes growth of silver catfish (*Rhamdia quelen*). Aquacult Nutr 22: 933-940.

ZEPPENFELD CC ET AL. 2017. *Aloysia triphylla* essential oil as food additive for *Rhamdia quelen* - Stress and antioxidant parameters. Aquacult Nutr 23: 1362-1367.

ZHENG ZL, TAN JYW, LIU HY, ZHOU XH, XIANG X & WANG KY. 2009. Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*). Aquaculture 292: 214-218.

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