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Research Article

Food supplementation with essential oil of *Lippia sidoides* for *Cyprinus carpio* koi as prevention against *Aeromonas hydrophila*

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ABSTRACT. This study aimed to evaluate the potential of *Lippia sidoides* essential oil as a food additive for *Cyprinus carpio* koi carp in improving the health parameters and resistance to infection by *Aeromonas hydrophila*. A total of 312 carp were divided into groups. Twenty-four animals were in the lethal dose 50% (LD50) test group. The remaining 288 were distributed in 24 experimental units (n = 12) divided into six treatments-*Lippia*$_{0.250\%}$, *Lippia*$_{0.125\%}$, *Lippia*$_{0.063\%}$, *Lippia*$_{0.031\%}$, alcohol, and control-with four replicates each. The animals were fed thrice daily with 5% of living biomass for 55 days. At the end of the period, five specimens were collected from each treatment to analyze the intestinal tract's zootechnical performance, hematological analysis, and microbiology. The remaining animals were challenged with *A. hydrophila*, and the mortality rate was monitored for 100 days. Thymol was the component with the highest concentration (76.6%) in the essential oil of *L. sidoides*. Fish fed *Lippia*$_{0.125\%}$ had the greatest post-challenge survival. Fish fed *Lippia*$_{0.063\%}$ showed increased zootechnical performance, and those fed *Lippia*$_{0.250\%}$ had the highest concentration of lactic acid bacteria in the intestine. Hematological analyses did not show significant differences among treatments. The authors suggest new studies with higher concentrations of *L. sidoides* essential oil in the diet of *C. carpio* than were used in this study.

Keywords: *Cyprinus carpio*; ornamental aquaculture; cyprinid; aquaculture; phytotherapy

INTRODUCTION

The international ornamental fish market moved millions of dollars annually, US$ 347.5 million in 2014, with Asian countries responsible for 57% of the export chain (Dey 2016). The countries in South America that lead the export of ornamental fish are Brazil, Colombia, and Peru (Moreau & Coomes 2007, Mancera-Rodríguez & Álvarez-León 2008, Anjos et al. 2009).

Ornamental fish production in Brazil is one of the most profitable sectors in fish farming because the fish are sold individually and have high economic value (Texeira 2015). Between 2006 and 2015, the state of Amazonas exported US$ 23.0 million ornamental fish mainly to the countries of Germany, Taiwan, USA, and Japan (Tribuzy-Neto et al. 2021).

The colored carp *Cyprinus carpio* koi variety, a freshwater fish, has added economic value because its market share is one of the highest among ornamental fish in the international consumer market (Yanuhar et al. 2021). Its market share is linked to its characteristics, such as its vivid colors, body shape, and easy

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adaptation to various cultivation methods (Laksono et al. 2021).

The success of ornamental fish farming is related to various factors that add value to fish, such as color, rarity, size, and vigor. For these characteristics to occur in the animals of the herd, a necessity is to, for example, have adequate management in the breeding process (Rodrigues et al. 2013).

A high stocking density of animals combined with inadequate water quality management can promote the spread of diseases caused by bacteria (Moraes & Martins 2004). Physiological stress and physical injuries are the precursors to disease and mortality in aquaculture (Rotmann et al. 1992). Moreover, according to Rottmann et al. (1992), although fish can adapt to environmental conditions, their energy reserves decrease, and a hormonal imbalance can occur, suppressing the immune system and increasing susceptibility to diseases. The appearance of diseases is related to the interaction of the pathogen, environment, and host. The stress caused by the variation in water quality, caused by the high density of stock, favors an increase in bacterial communities and other pathogens (Toranzo et al. 2009, Leira et al. 2016).

Bacterial diseases cause economic losses and can reach 100% mortality depending on the culture phase (Shiogiri et al. 2015). The species of the genus *Aeromonas* are bacteria mostly found in the aquatic environment and cause disease in farmed and wild fish (Cipriano 2001). The shape of gram-negative bacilli and motility characterize this genus. When this affects animals, it causes hemorrhagic septicemia, with the appearance of superficial lesions, focal hemorrhages (petechiae), and exophthalmos (Carraschi et al. 2011).

The pathogenicity of *Aeromonas hydrophila* is related to several factors but is mainly due to the production of extracellular proteins (Aanjur et al. 2021). *C. carpio* mortalities because of infection by *Aeromonas* spp. have been recorded (Yu et al. 2010), where the animals presented clinical signs of abdominal distension, bleeding points through the skin, hepatic congestion, and an enlarged spleen and kidney (Harikrishnan et al. 2003). In an experimental trial, they infected specimens of *C. carpio* with *A. hydrophila* (10^8 colony forming units, CFU mL^{-1}) and observed desquamation followed by hemorrhagic spots that progressed to epidermal lesions affecting the animals' muscles.

Treatment against bacterial infections is conducted by adding antibiotics to the water or feed (Moraes & Martins 2004). However, the excessive use of these antimicrobial agents favors the emergence of resistant bacteria (Smith et al. 2008). The worldwide and indiscriminate use of antibiotics is difficult to determine because each country has a regulatory agency and different drugs approved for use (Romero et al. 2012). Among the drugs most used during bacterial outbreaks in aquaculture are oxytetracycline, sulfadiazine, and florfenicol (Lulijwa et al. 2020). In Brazil, the only antibiotics authorized for use in aquaculture are oxytetracycline and florfenicol (Čížek et al. 2010, Sindan 2018), demonstrating the resistance of *Aeromonas* spp. strains isolated from *C. carpio* koi and observed that of the 79 strains isolated, 36 showed resistance to oxytetracycline.

In this context, developing bio-safe and ecological alternatives to antibiotics, such as probiotics and plant-based immunostimulants, has gained attention regarding fish management and health (Sahu et al. 2007).

Two alternatives to antibiotics are herbal products and probiotic bacteria because they are obtained exclusively from plant-based raw materials (Anvisa 2014). Herbal medicines are a technically viable alternative in the prophylaxis and prevention of pathogens to replace chemicals and antibiotics (Schalch et al. 2015). Among herbal medicines, essential oils extracted from plants have antimicrobial and antioxidant properties, are biodegradable, and act as immunostimulants (Figueiredo et al. 2014, Schalch et al. 2015). The antimicrobial activity of these herbal medicines is related to the terpenoid and phenol compounds present in their composition (Castro et al. 2011).

For example, the essential oil of *Lippia sidoides*, commonly known as alecrim-pimenta, contains approximately 60% thymol or a mixture of thymol and carvacrol (Souza et al. 2007). The antimicrobial power of this type of essential oil is related to its chemical affinity for the fatty acids, phospholipids, and lipopolysaccharides in the cell wall and cytoplasmic membrane, making them capable of crossing these barriers and causing porosity of the membranes; in bacteria, permeabilization of the membranes is associated with loss of ions, resulting in cell death (Bakkali et al. 2008).

This composition may vary according to the geographic location, climate, and oil extraction method (Castro et al. 2011). This study aimed to determine the minimum inhibitory concentration (MIC) of *L. sidoides* essential oil against *A. hydrophila* and its effects when supplemented in the diet, assessing zootechnical, hematological, and intestinal microbiota composition parameters of *C. carpio* koi.

MATERIALS AND METHODS

Obtention and composition of *Lippia sidoides* essential oil

The essential oil of *L. sidoides* was provided by Embrapa Western Amazônia (EMBRAPA), Manaus, Amazonas, Brazil (03°06'23.04"S, 60°01'35.14"W). The oil was extracted from the leaves in the Laboratory of Phytochemistry and Medicinal Plants at EMBRAPA by hydrodistillation using a Clevenger-type apparatus (Majolo et al. 2016). The product of this process was stored in 500 mL amber glass and placed in a -18°C freezer until the experimental diets were prepared.

This study used an Agilant (Palo Alto, USA) 7890B gas chromatograph equipped with an HP-5 capillary column (5% diphenyl-95%-dimethyl silicone, 30 m × 0.25 mm × 0. 24 µm) and an oven temperature from 60 to 240°C, using nitrogen gas as a carrier (1.5 mL min^{-1}) to analyze the chemical composition of the oil 1 µL of a 1% solution of the oil in dichloromethane (Merck Millipore, Darmstadt, Germany) was injected in split-flow mode (1:100; inlet at 250°C). The mass spectrum was obtained by the Agilent 5975C system and operated in electronic ionization mode at 70eV, using the injection mentioned above procedure. Helium gas was used as carrier gas (1.05 mL min^{-1}). Retention rates were calculated from the retention time of each oil component and n-alkanes (C7-C20). These components were identified by comparing the mass spectra obtained with data from the spectral library (Hoffmann & Stroobant 2007) and retention indices calculated and compared with published values (Adams 2007).

Minimum inhibitory concentration (MIC)

The strain of *A. hydrophila* (ATCC 7966) from the strain bank of the microbiology sector of the Laboratory of Pathology of Aquatic Organisms - AQUOS/UFSC was used to determine the MIC. The bacterial solution was previously cultured in BHI® Himedia broth and incubated at 28°C for 24 h. The stock solution of *L. sidoides* was made by diluting the oil in 8% grain alcohol. The assay was performed according to CLSI (2006) in triplicates, with positive, negative, and grain alcohol controls. The positive control consisted only of the bacteria grown in the culture medium; the negative, in turn, was an uninoculated culture medium, and the cereal alcohol control consisted of a culture medium inoculated with microorganisms in the presence of cereal alcohol. In a 96-well microplate, serial dilutions of the stock solution were performed at a factor of 1:2, previously containing 200 µL of the Luria-Bertani (LB) culture medium, after which 20 µL of *A. hydrophila* inoculum at 1×10^9 CFU concentration were added. The microplate was incubated at 28°C for 24 h, then read with the naked eye to determine the MIC.

Experimental design

For the experimental design, 288 colored *C. carpio* with an initial weight of 5.3 ± 1.6 g from the Vale dos Betas Ornamental Fish Farm Biguaçu, Santa Catarina, were used. The animals were transported to the Laboratory of Sanitation of Aquatic Organisms - AQUOS/UFSC and acclimatized for 10 days. After acclimatization, the fish were randomly distributed into 24 experimental units (n = 12). Each experimental unit contained 80 L and was connected to a water recirculation system equipped with mechanical filtration, biological filtration, an ultraviolet reactor, and constant aeration. The water quality parameters were recorded weekly with a LabconTest® colorimetric kit: pH (6.86 ± 1.4), temperature (25.15 ± 1.5°C), toxic ammonia (0.0014 ± 0.0013 mg L^{-1}), and dissolved oxygen (7.86 ± 0.62 mg L^{-1}). The temperature was measured using a thermometer (25.15 ± 1.5°C). The animals were fed for 55 days. The per day ration was supplemented with the essential oil of *L. sidoides* at various concentrations: *Lippia*$_{0.250\%}$, *Lippia*$_{0.125\%}$, *Lippia*$_{0.063\%}$, *Lippia*$_{0.031\%}$, alcohol, and control (in quadruplicate). The animal procedures were approved by the Ethics Committee on the Use of Animals of the Federal University of Santa Catarina (CEUA Nº 7572220419).

Preparation of experimental diets and food routine

For the preparation of the experimental diets, the commercial feed Puro Trato® Juvenil 40% crude protein, 2 mm pellet was used as a base, and the inclusion of the essential oil of *L. sidoides* was performed according to Dairiki et al. (2013), where dilution of the oil used Grain alcohol is used as an incorporation vehicle. For each kilogram of ration, 100 mL of grain alcohol was sprinkled with *L. sidoides* oil diluted at concentrations of 0.031, 0.063, 0.125, and 250%, and in the control ration, only grain alcohol was sprayed. The ration was dried at room temperature for 24 h and then stored at -18°C. The ration was weighed and stored at 4°C the day before supply until feeding. The experimental rations were offered for 55 days, three times a day, and with an amount corresponding to 5% of the total biomass of the tank. The amount of feed was adjusted according to the growth of the animals and measured by biweekly biometrics.

Growth performance

The specific growth rate (SGR) and feed conversion factor (FCF) were calculated by the following formulas (Fu et al. 2005):

Weight gain = initial weight - final weight (g)

SGR = 100 × (ln initial weight (g) - ln final weight (g)) / time (d)

FCF = feed consumption (g) / (initial weight (g) - final weight (g))

Hematological analyzes

After the experimental period, five fish were sampled per experimental unit for blood collection. The animals were anesthetized in eugenol solution (75 mg L^{-1}), and blood was collected by puncturing the caudal vein with a 3 mL syringe containing 10% EDTA. Blood extensions were made in duplicates for further staining in May-Grunwald/Giemsa/Wright for total and differential leukocyte and thrombocyte counts by the indirect method (Ishikawa et al. 2008). Hematimetric indices, hematocrit percentage, and total erythrocyte count were also determined (Ranzani-Paiva et al. 2013).

Lethal dose 50% 96 h for *Aeromonas hydrophila*

Seventy fish were distributed in four experimental units (n = 6 fish $pond^{-1}$) to determine the lethal dose of 50% (LD50). The animals were anesthetized with eugenol solution (75 mg L^{-1}) and challenged, via intraperitoneal injection, with 100 µL of bacterial solution at the following concentrations: 1×10^7, 1×10^8, and 1×10^9 CFU mL^{-1}. The control group received only sterile saline solution (0.65% NaCl). The animals were observed for 10 days every 8 h, and the treatment that presented a mortality rate of 50% in 96 h was defined as the dose to be used in the experimental infection performed at the end of the 55 days of feeding with a supplemented diet.

Experimental infection with *Aeromonas hydrophila*

At the end of the supplementation period and after the first data collection, the fish were submitted to experimental infection with the bacterium *A. hydrophila*. The microorganisms were cultured in BHI® Himedia broth, incubated at 28°C for 24 h, and centrifuged for 30 min at 1800 g. Next, the supernatant was discarded, and the pellet was resuspended in a sterile 0.65% saline solution. The bacterial concentration in the infection was 2×10^7 CFU, a concentration determined by the 96 h LD50 performed in the prior section. After being anesthetized with eugenol solution (75 mg L^{-1}), each fish received 100 µL bacterial inoculum via intraperitoneal (ip) injection. The fish were observed for 10 days, and mortality was recorded daily. Dead animals were immediately removed and discarded according to laboratory protocol. Clinical signs linked to infection caused by *A. hydrophila* were analyzed during the infection period. At the end of the infection period, the remaining animals were euthanized according to the UFSC Ethics Committee guidelines and discarded. For disease confirmation, through the postulate of Koch according to Evans (1976), samples of the brain, liver, and kidney were collected from fish that presented characteristic clinical signs of aeromoniosis, sown in BHI® Himedia medium, and incubated at 28°C for 24 h. Subsequently, the grown media were seeded on TSA agar medium and again incubated at 28°C for 24 h to characterize *A. hydrophila* bacterial colonies.

Microbiological analysis of the intestinal tract

The average portion of the intestine of five fish from each experimental unit was collected aseptically, macerated, and diluted 1:10 in 0.65% saline solution. The dilutions were seeded in Rogosa Sharpe Man Agar (MRS, HIMEDIA®) with aniline blue (10 g L^{-1}) for lactic acid bacteria count and on Soy Tryptone Agar (TSA, HIMEDIA®) for bacteria count-total heterotrophs. The dilutions were also seeded on Thiosulfate Citrate Bile Sucrose Agar (TCBS, HIMEDIA®) for quantification of Vibrionaceae and Cetrimide Agar (HIMEDIA®) for bacteria of the genus *Pseudomonas* spp. All plates were incubated at 30°C for 24 h, except the MRS plates, which were incubated at 35°C for 48 h (Jatobá et al. 2008).

Statistical analysis

The results from the tests performed were submitted to one-way ANOVA ($P < 0.05$). Levene's test was used to verify homoscedasticity and Shapiro Wilk's test for normality. Tukey's test was applied to separate the means when statistical differences were observed. For mortality after the challenge, the Keplen-Meier test was applied.

RESULTS

Composition of *Lippia sidoides* essential oil

In the composition of *L. sidoides* essential oil, thymol (76.6%) stood out, followed by ortho-cymene (6.3%) and beta-caryophyllene (5.0%) (Table 1).

Table 1. Composition of *Lippia sidoides* essential oil (%). RI: retention index.

Components	(%)	RI
Alpha-thujene	0.3	929
Alpha-pinene	0.1	937
Myrcene	1.1	992
Alpha-terpinene	0.7	1019
Ortho-cimene	6.3	1027
Limonene	0.4	1031
1.8-cineole	0.7	1034
Gamma-terpinene	2.0	1061
Ipsdienol	0.6	1148
Umbellulone	0.2	1176
4-terpinenol	1.0	1178
Alpha-terpineol	0.2	1191
Thymol-methyl-ether	1.0	1236
Thymol	76.6	1296
Alpha-copaene	0.4	1375
Beta-cariofilene	5.0	1417
Aromadendrene	0.4	1436
Alpha-humulene	0.3	1451
Ledene	0.3	1492
Delta-cadinene	0.3	1521
Caryophyllene oxide	0.7	1579
Total	98.6	

Minimum inhibitory concentration (MIC)

The observed results indicate the inhibitory activity of the essential oil of *L. sidoides* against *A. hydrophila* at all dilutions (Table 2). The growth of bacteria can be observed in all wells of the positive control and from the 0.016% dilution in the treatment with grain alcohol alone.

Zootechnical performance

After the 55 days of food supplementation, there was a significant increase in the growth of animals supplemented with *Lippia*$_{0.063\%}$ in the control, alcohol, and *Lippia*$_{0.031\%}$ Still, the other treatments had no significant difference (Table 3). There was also no significant difference between SGR and FCF treatments.

Hematological analyzes

The total erythrocyte count, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) showed no significant difference among supplemented and control groups. In hematocrit, it was observed that there was no statistical difference between the treated groups, but the fish supplemented with *Lippia*$_{0.031\%}$ (21.8 ± 5.42%) was different from the alcohol group (31.75 ± 6.46%) and *Lippia*$_{0.250\%}$ (32.72 ± 11.15%). There was no significant difference between the treated and untreated groups regarding total leukocytes. On the other hand, the number of basophils was lower in the supplemented animals (Table 4).

Microbiological analysis of the intestinal tract

In the microbiological analysis of the intestinal tract, there was no significant difference between the supplemented and non-supplemented animals for vibrionaceous bacteria and total heterotrophic bacteria. The highest concentration of lactic acid bacteria was observed in animals supplemented with *Lippia*$_{0.250\%}$ (5.65 $\log_{10}$ CFU g^{-1}), which was also the group with the highest concentration of bacteria of the genus *Pseudomonas* sp. (5.62 $\log_{10}$ CFU g^{-1}) (Fig. 1).

Mortality after challenge with *Aeromonas hydrophila*

After 55 days of supplementation, the animals were infected via intraperitoneal injection with an *A. hydrophila* solution at a concentration of 2×10^7 CFU g^{-1} defined in the LD50. The animals were observed for 9 days and showed clinical signs such as exophthalmos, anorexia, and hemorrhagic spots through the integument (Fig. 2).

The dead fish were removed. Next, the remaining animals were euthanized, and their organs were collected for Koch's postulate to confirm the bacterial infection. The highest survival rate was observed in fish supplemented with *Lippia*$_{0.125\%}$, and the lowest was in non-supplemented fish (Fig. 3).

DISCUSSION

Marco et al. (2012) and Brito et al. (2015) have reported that the component with the highest concentration in the essential oil of *L. sidoides* is thymol, as observed in this study, where thymol corresponds to 76.6%. Thymol is a phenolic monoterpene compound found in several essential oils (Yousefi et al. 2018), such as *Thymus ciliatus* (Kabouche et al. 2009). The variation in oil composition can be influenced by genetic, climatic, or vegetative factors (Guimarães et al. 2014). The production of secondary metabolites can be affected by environmental conditions such as seasonality, temperature, altitude, and rainfall (Gobbo-Neto & Lopes 2007). Due to these conditions, the composition of the essential oil depends on the plant's place of cultivation and the season in which it is harvested (Veras et al. 2017). This study observed that in plants grown in the southern region of Minas Gerais, there was a change in the composition of *L. sidoides* oil,

Table 2. Minimum inhibitory concentration of *Lippia sidoides* essential oil against *Aeromonas hydrophila.* (+) indicates bacterial growth, and (-) indicates no bacterial growth.

	4%	2%	1%	0.5%	0.250%	0.125%	0.063%	0.031%	0.016%	0.008%	0.004%	0.002%
L. sidoides + Grain alcohol	-	-	-	-	-	-	-	-	-	-	-	-
Grain alcohol	-	-	-	-	-	-	-	-	+	+	+	+
Positive control	+	+	+	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. Zootechnical performance parameters (mean ± standard deviation) of koi carp after 55 days of supplementation with *Lippia sidoides* essential oil at different concentrations. SGR: specific growth rate, FCF: feed conversion factor. Different letters in the same column indicate a significant difference between treatments by Tukey's test ($P < 0.05$).

Treatment	Final weight (g)	Weight gain (%)	SGR (%)	FCF
Control	9.39 ± 3.40^{b}	5.11 ± 2.34^{c}	0.60 ± 0.10	2.14 ± 0.58
Alcohol	14.94 ± 2.96^{a}	8.12 ± 1.66^{b}	0.62 ± 0.06	1.86 ± 0.23
Lippia$_{0.250\%}$	11.79 ± 1.83^{ab}	6.44 ± 1.83^{abc}	0.62 ± 0.05	2.01 ± 0.32
Lippia$_{0.125\%}$	11.17 ± 3.39^{ab}	6.16 ± 1.62^{abc}	0.65 ± 0.11	1.89 ± 0.35
Lippia$_{0.063\%}$	14.90 ± 2.77^{a}	8.74 ± 1.99^{a}	0.58 ± 0.08	2.09 ± 0.43
Lippia$_{0.031\%}$	9.31 ± 2.37^{b}	4.90 ± 1.70^{bc}	0.59 ± 0.09	2.29 ± 0.46

which had carvacrol (26.44%) and 1.8 cineole (22.63%) as the major components.

The antimicrobial activity of thymol is well understood; Majolo et al. (2017) tested the MIC of isolated *L. sidoides* and thymol against *Staphylococcus aureus* and observed that the same concentration (128 µg mL^{-1}) was effective against the pathogen. In this study, the antimicrobial activity of the essential oil was observed at all concentrations, with the lowest concentration of 0.002%, corresponding to 34.79 µg mL^{-1}. In contrast, Monteiro et al. (2020) observed that the lowest concentration that inhibited *A. hydrophila* growth was 1.250 µg mL^{-1}, and the essential oil contained 76.6% thymol. Nazzaro et al. (2013) tested the antimicrobial activity of *L. sidoides* against *A. hydrophila* isolated from tambaquis *Colossoma macropomum* from cultures Amazonas, and the lowest value was 625 µg mL^{-1}. The ability to inhibit the essential oil of *L. sidoides* is due to the presence of thymol in its composition, which can cause structural damage to the bacterial cell membrane, increasing the permeability of the cell, extravasating the cellular content, and promoting the coagulation of cytoplasm the bacterium causing damage to its functions (Nazzaro et al. 2013). Studies have stated that carp supplemented with oregano essential oil (4500 mg kg^{-1}) have increased activity of digestive enzymes, increased immune activity, and change in the intestinal microbiota structure of fish (Zhang et al. 2020). The authors associated the results with the benefits of oil supplementation and the change in the microbiological composition of the intestine. In this study, an increase in lactic acid bacteria was observed in fish supplemented with *Lippia*$_{0.250\%}$, and the probiotic potential of acid-lactic has been confirmed in the literature (Ringø & Gatesoupe 1998, Alishahi et al. 2018, Shabirah et al. 2019). Essential oils use several mechanisms that result in an unfeasible bacterial cell, such as interacting with proteins in the cell wall, impairing the transport of essential molecules, decreasing ATP synthesis, and causing porosity of the cell wall due to the interaction of the essential oil with the fatty acids present (Nazzaro et al. 2013). The mechanisms above suggest that the increase in the colonization of lactic acid bacteria in the intestinal tract of supplemented animals is related to the presence of *L. sidoides* essential oil, owing to the composition of the intestinal microbiota of fish being related to the type of feeding or form of preparation of the ration, generating the selection of specific bacteria.

Tripathi et al. (2004) described the reference values of hematological parameters of healthy-colored koi carp (weight, approximately 200 g; length, 18 cm). They observed that the number of erythrocytes in healthy animals was 1.81×10^{6} µL^{-1}. Our study observed the highest erythrocyte count in animals supplemented with alcohol alone (1.38×10^{6} µL^{-1}). However, common carp fingerlings supplemented with *Lippia citrodora*

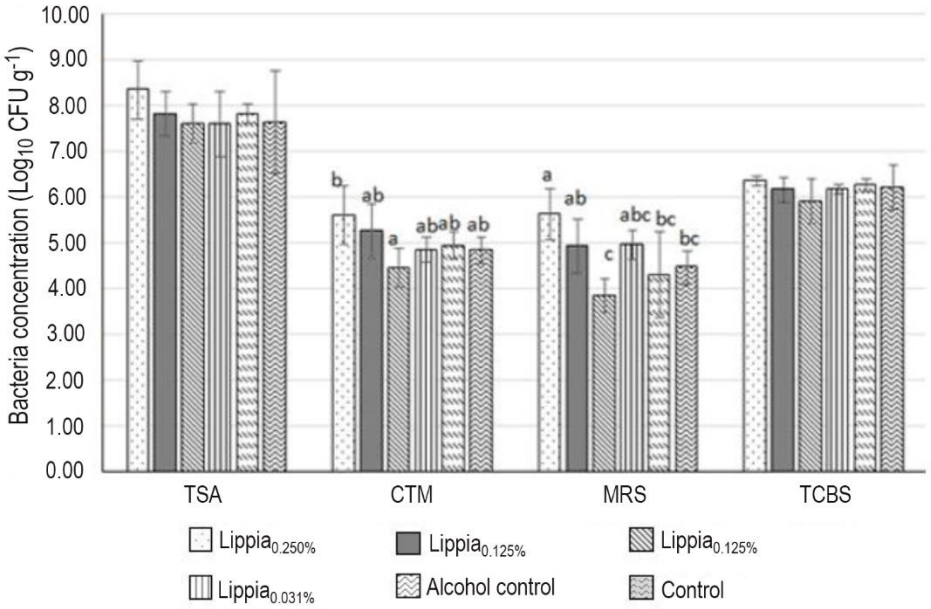

Figure 1. Bacterial concentration per gram of koi carp intestine supplemented with *Lippia sidoides* essential oil in different concentrations. TSA: total heterotrophic bacteria, CTM: *Pseudomonas* sp., MRS: total lactic acid bacteria, TCBS: Vibrionaceae. Different letters indicate a significant difference between treatments by Tukey's test ($P < 0.05$).

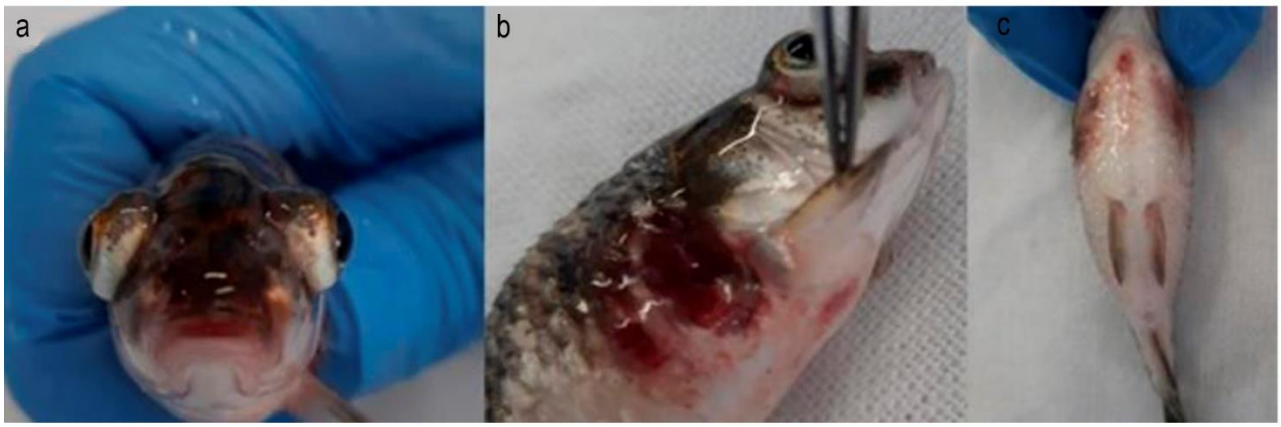

Figure 2. Specimens of koi carp with clinical signs after experimental infection with *Aeromonas hydrophila*. a) exophthalmos, b) bleeding ulceration, c) bleeding spots.

essential oil (0.15 mL and 0.30 mL kg^{-1}) for 30 days had a lower number of erythrocytes than the control did (Gholipourkanani et al. 2017). The percentage values of hematocrit were similar to the reference values described by Gholipourkanani et al. (2017); however, fish that received a diet supplemented with *Lippia*$_{0.031\%}$ had a lower percentage (21.8 ± 5.42%) than in their study. Brasil et al. (2019) did not observe statistical differences in the hematocrit values of colored carp fed with *L. sidoides* essential oil at concentrations of 0, 0.25, 0.50, and 1%, suggesting that supplementation with *L. sidoides* is not harmful to fish. Silver catfish (*Rhamdia quelen*) decreased hematocrit percentage when supplemented with *Lippia alba* powder, a finding related to the animals' decreased productive performance (Marasca et al. 2020).

This study did not observe a significant difference in the total leukocyte count. An increase in the total number of leukocytes of common carp supplemented with different concentrations of *Epilobium hirsutum* extract (0, 0.5, 1, and 3%) and the control group was observed. Still, there was no significant difference in the number of monocytes, lymphocytes, and neutrophils among the treated groups (Pakravan et al. 2011).

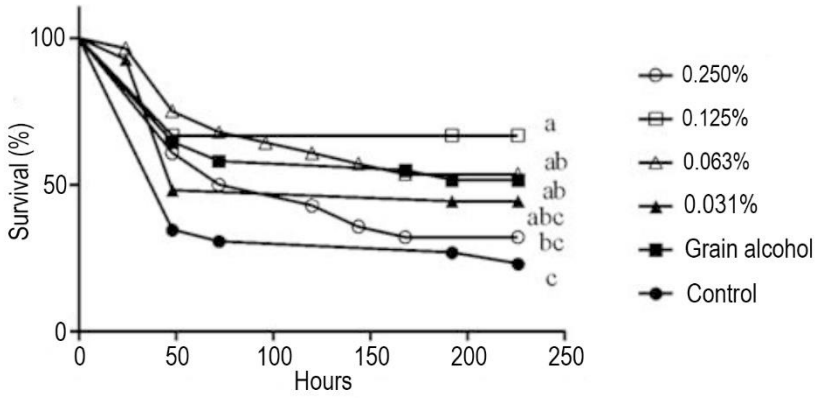

Figure 3. Mortality rate of koi carp supplemented with *Lippia sidoides* essential oil at different concentrations after experimental challenge with *Aeromonas hydrophila*.

Table 4. Hematological parameters (mean ± standard deviation) of koi carp after 55 days of supplementation with *Lippia sidoides* essential oil at different concentrations. MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, MCH: mean corpuscular hemoglobin. Different letters in the column indicate a significant difference between treatments by Tukey's test ($P < 0.05$).

Parameter	Control	Alcohol	*Lippia*$_{0.250\%}$	*Lippia*$_{0.125\%}$	*Lippia*$_{0.063\%}$	*Lippia*$_{0.031\%}$
Erythrocytes ($\times 10^6$ µL^{-1})	1.24 ± 0.43	1.38 ± 0.27	1.24 ± 0.29	1.32 ± 0.32	1.33 ± 0.28	1.12 ± 0.29
Hematocrit (%)	29.1 ± 8,1^{ab}	31.75 ± 6.46^{a}	32.72 ± 11.1^{a}	27.41 ± 8.32^{ab}	27.61 ± 9.21^{ab}	21.8 ± 5.42^{b}
Hemoglobin (g dL^{-1})	9.03 ± 2.09	9.45 ± 2.39	9.23 ± 1.97	10.85 ± 2.27	9.34 ± 1.34	8.89 ± 2.02
MCV (fL)	2.25 ± 1.16	2.29 ± 0.77	2.29 ± 0.98	1.84 ± 0.55	2.29 ± 1.17	1.95 ± 0.84
MCH (g gL^{-1})	0.30 ± 0.37	0.57 ± 0.29	0.33 ± 0.44	0.45 ± 0.46	0.55 ± 0.40	0.52 ± 0.40
MCHC (g dL^{-1})	23.10 ± 19.21	30.53 ± 14.30	17.36 ± 20.29	26.38 ± 21.48	30.02 ± 25.57	34.20 ± 23.0
Total leukocytes (10^3 µL^{-1})	43.50 ± 24.50	37.70 ± 12.10	46.40 ± 21.90	46.40 ± 20.10	39.40 ± 15.90	44.20 ± 21.10
Basophils ($\times 10^3$ µL^{-1})	0.10 ± 0.19^{b}	0.59 ± 0.62^{a}	0.34 ± 0.27^{ab}	0.52 ± 0.82^{ab}	0.30 ± 0.33^{ab}	0.33 ± 0.43^{ab}
Lymphocytes ($\times 10^3$ µL^{-1})	24.50 ± 15.20	25.10 ± 9.53	25.80 ± 10.70	25.90 ± 9.76	22.80 ± 6.95	19.90 ± 6.71
Neutrophils ($\times 10^3$ µL^{-1})	0.17 ± 0.41	0.03 ± 0.07	0.02 ± 0.06	0.02 ± 0.09	0.03 ± 0.09	0.01 ± 0.05
Monocytes ($\times 10^3$ µL^{-1})	19.00 ± 14.20	12.00 ± 6.87	20.3 ± 14.7	20.00 ± 15.00	20.00 ± 12.40	21.90 ± 14.70
Glucose (mg dL^{-1})	47.89 ± 22.23	42.4 ± 16.51	48.0 ± 14.58	42.73 ± 22.30	44.21 ± 19.40	36.95 ± 17.91

Similar results were observed when using extracts of *Oliviera decumbens* and *Satureja khuzestanica* at 0.5% in the carp diet (Khansari et al. 2013). The leukocyte count of the animals in this study might be related to the concentrations of *L. sidoides* being lower than those in the studies cited.

Fish supplemented with *Lippia*$_{0.063\%}$ showed the greatest weight gain (8.74 ± 1.99%) during the experimental period, different from that reported by Brasil et al. (2019): they observed that koi carp supplemented with 0.75% of the *L. sidoides* essential oil showed a reduction in weight gain in relation with the unsupplemented fish. Cornelius et al. (2013) observed that *Oreochromis niloticus* fed a diet supplemented with the lactic acid bacterium *Lactobacillus platarum* obtained greater weight gain and feed conversion than the control group. The weight gain of the animals treated with *Lippia*$_{0.063\%}$ is possibly related to the increase in the stimulus in their digestive function, providing the increase in digestive enzymes such as proteases, lipases, and amylase (Zhang et al. 2020) and the increase in the concentration lactic acid bacteria present in the intestines of animals.

This study observed the highest survival after the challenge in fish supplemented with *Lippia*$_{0.125\%}$. A similar result was reported in common carp juveniles supplemented with different levels of dehydrated (*Olea europea*) leaves, where animals supplemented with 1 g kg^{-1} showed an improved immune response and survival rate when challenged with *Edwardsiella tarda* (Zemheri-Navruz et al. 2019). Mohamad & Abasali (2010) evaluated supplementation with a mix of 10 plants, including *Thymus vulgaris*, *Mentha piperita*, and *Ocimum europea*, in common carp for 60 days.

Fish that received 1000 mg kg^{-1} showed 80.95% survival when challenged with *A. hydrophila*. The improved survival rate of the supplemented animals may be related to the essential oil improving the immune response of the fish.

CONCLUSION

The essential oil of *L. sidoides* improved the weight gain of the animals when supplemented with *Lippia*$_{0.063\%}$. However, the concentration of essential oil of *L. sidoides* that showed the best survival rate was 0.125% after challenging with *A. hydrophila*. Regarding the intestinal microbiota, fish supplemented with *Lippia*$_{0.250\%}$ showed the greatest concentration of lactic acid bacteria, which have probiotic potential. The authors suggest further studies on adding *L. sidoides* essential oil in the diet of koi carp, especially with higher concentrations of essential oil than those used in this study and immunological analysis.

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