Inclusion of *Lippia graveolens* essential oil in diets for *Oreochromis niloticus* juveniles: Effects on growth, feed utilization, hematobiochemical parameters, histomorphometry of the intestine and liver, digestive enzymes, and bacterial challenge.

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Abstract

Including essential oregano oils in fish diets can promote fish growth and improve their overall health. The aim of this study was to evaluate the effects of adding dietary supplements of *Lippia graveolens* essential oil (oregano EO) on the growth, food consumption, hematological parameters, as well as the intestinal and liver morphology, digestive enzymes, and bacterial challenges in *Oreochromis niloticus* juveniles. The study involved testing four different diet levels of EO (0, 300, 600, and 1200 mg/kg). After 30 days of feeding, the oregano EO supplementation has a positive effect on the growth and feed utilization parameters, especially with treatments of 300 and 1200 mg/kg diet, which, despite showing a lower trend of feed consumption, had a higher growth performance and feed utilization. In addition, there was no evidence of adverse changes in liver and intestinal histology, indicating that *L. graveolens* EO does not have a negative effect at concentrations ranging from 300 to 1200 mg/kg. However, it was observed that all treatments tended to increase liver enzyme activity. Moreover, it was found that the concentration of 1200 mg/kg positively impacted the increase of intestinal enzyme activity, particularly acid proteases, chymotrypsin, and lipase. All treated groups exhibited a reduction in mortality rates compared to the control. In conclusion this study demonstrated that incorporating *L. graveolens* EO in fish diets could enhance their overall health, promote growth performance, and improve their bacterial resistance.

Key words: essential oils, oregano, tilapia, aquaculture, *Aeromonas hydrophila*.

1. Introduction

Since 2000, aquaculture has been a rapidly expanding livestock activity in Guatemala. The sector has nearly doubled its annual production, reaching a figure of almost 22 MT, making Guatemala the second-largest Nile tilapia producer in Central America, according to the FAO

(2022). In this sense, the growth in the aquaculture industry has increased by aquaculture intensification, which has led to higher fish stocking densities (García-Pérez et al., 2021). Unfortunately, these practices can ultimately lead to a decline in animal performance and an increased susceptibility to bacterial-induced diseases. Consequently, antibiotics are employed indiscriminately against bacterial infections in most tilapia aquaculture operations and yield secondary benefits, such as growth stimulation (Romero et al., 2012). The long-term practice of using antibiotics in tilapia aquaculture has developed antibiotic-resistant bacteria strains (Becerril et al., 2012). Consequently, controlling bacterial outbreaks has become challenging, requiring extended treatment periods and more potent drugs. Additionally, certain medications may not be permitted for use in aquaculture (Reverter et al., 2014).

As a result, various issues arise concerning animal food safety, such as selecting the best medication to control aquaculture disease. Since antibiotics have been banned in many countries worldwide since 2008 (Lulijwa et al., 2020), research has been conducted to find a viable alternative that could reduce their usage in aquaculture. One promising option is using phytogenic additives in fish feed, which has positively impacted animal health and production (Dawood et al., 2022).

Oregano is widely recognized as one of the most used herbs in the aquaculture industry worldwide (Abdel-Latif et al., 2020; Özel et al., 2022a, 2022b; Zhang et al., 2020a). However, there are different genera and species of oregano that are distributed globally, including *Origanum onites* (known as 'Turkish oregano', 'Island oregano', or 'Cretan oregano'), *Oreganum vulgare* (known as 'European oregano' or 'Greek oregano'), *Coridohymus capitatu* (Spanish oregano), and *Lippia graveolens* (Mexican oregano) (Alarcon-Rojo et al., 2017; Leyva-López et al., 2017; Alagawany et al., 2020).

Recent studies have demonstrated that oregano's bactericidal and bacteriostatic effects are attributed to its high concentrations of phenolic compounds, particularly thymol and carvacrol. These compounds have shown an antimicrobial effect against both Gram-negative and Gram-positive bacteria, as highlighted by Leyva-López et al. (2017), Sutili et al. (2018), Alagawany et al. (2020, 2021), and Bautista-Hernández et al. (2021). For this reason, the essential oil of oregano and its main components, thymol or carvacrol, have been extensively researched for animal nutrition due to their easy extraction and application. Research has shown that when included in animal feed (Alarcon-Rojo et al., 2017), they can act in various ways, such as enhancing animal performance and acting as immunostimulants to prevent bacterial infections (Hayatgheib et al., 2020).

This study aimed to evaluate the impact of incorporating *Lippia graveolens* EO in the diet for *Oreochromis niloticus* juveniles on growth performance, feed utilization, hematobiochemical parameters, histomorphometry of the intestine and liver, digestive enzymes, and bacterial challenge.

2. Materials and Methods

2.1 Essential oil of *Lippia graveolens*

Lipia graveolens samples were collected from its natural habitat in the Zacapa department (ZAC: 14º 59' 50.2"N / 89º 40' 26.2"W) in October 2019. Afterward, they were oven-dried at 45 ºC for 48 h, then pulverized to a particle size between 1 and 3 mm using an electric grinder. The resultant powder was securely stored in an airtight bag at ambient temperature until use. Essential oil (EO) extraction was performed through steam distillation at the Laboratorio de Investigación de Extractos Vegetales, Facultad de Ingeniería, Universidad de San Carlos de Guatemala, employing a semi-industrial pilot plant for the process. Subsequent analysis to identify the chemical

constituents of the extracted essential oil was executed using Gas Chromatography/Mass Spectrometry (GC-MS) techniques at Instituto de Pesquisas de Productos Naturales, Río de Janeiro University, Brazil.

2.2 Experimental fish and management

Healthy tilapia fingerlings (220 fish of *O. niloticus* GIFT strain: 1.5 + 0.2 g) were obtained from a local tilapia breeding center. For transport, the fingerlings were securely placed in 50-liter polyethylene bags filled with a mixture composed of a 70:30 ratio of industrial oxygen to water. Upon arrival at the laboratory, the fish were acclimatized in a 500-liter cylindrical plastic tank, continuously aerated to maintain optimal conditions. Water quality within the tank was maintained within species-specific ranges, including dissolved oxygen levels exceeding 5 ppm, a pH value of 8.0 ± 0.1, water temperature maintained at 28.0 ± 0.5 ºC, nitrate nitrogen concentrations below 3 ppm, nitrite nitrogen levels under 20 ppm, and ionized ammonium concentrations not exceeding 1.5 ppm.

The acclimatization period was extended over 30 days. Throughout this phase, the fish were fed three times daily (8:00, 13:00, and 18:00 hs) with a commercial diet (MicroTek: 0.8 mm pellet size, 45% protein, and 12% of lipids, Silver Cup Tilapia, Mexico) until apparent satiation. To preserve water quality and initial volume, sediment was systematically extracted from the tank's bottom on a weekly basis, and the system was replenished with dechlorinated freshwater.

2.3 Preparation of diets and experimental design

To assess the effect of oregano EO on tilapia, four diets were formulated to be isonitrogenous (45% protein) and isolipidic (12% lipids). They were derived from a standard commercial feed (MicroTek: 400 µm pellet size, 45% protein, and 12% lipids, produced by Silver Cup Tilapia, México). While the control diet was free of any oregano EO, the other three diets were supplemented with 300, 600, and 1200 mg of EO per kilogram of feed. Given the minute quantities

of oregano EO incorporated into each diet, fish oil was employed as a vehicle at a concentration of 1%. The powdered tilapia feed and oregano EO were blended using a food mixer for 5 min. Subsequently, tap water was added until the mixture achieved a stiff, dough-like consistency. This dough was then processed through a mincer equipped with an 8 mm die to produce small pellets. To ensure homogeneous distribution of ingredients, this mincing procedure was repeated three times for each diet formulation. The pellets were oven-dried at 45 ,ºC for 24 h, then broken down into smaller pieces with sizes varying from 0.8 to 1.5 mm. Each formulated diet was then stored in a labeled plastic container at an ambient room temperature of 26.0 ± 2.0 ºC until use.

Following the acclimatization phase, fish with an average body weight of 13.64 ± 0.06 g were randomly allocated in a recirculating aquaculture system. This system comprised 12 aquaria of 70 L each, a biofilter, and a sedimentation tank. Three replicate aquaria were utilized for each diet, maintaining a stocking density of 15 fish per aquarium. The fish were hand-fed their respective experimental diets until the next day after stocking. The treatment groups were TCNT as a control group (0 mg/ kg oregano EO), T300, T600, and T1200, containing 300, 600, and 1200 mg/kg oregano EO, respectively. The feeding trial lasted 30 days, and fish were fed at apparent satiation at 7:00, 12:00, and 17:00 h. The levels of oregano EO incorporated into the diets were established based on findings reported by García-Perez et al. (2022).

Fish waste was siphoned daily, and 20% of each aquarium's water volume was replenished with dechlorinated freshwater. Water quality parameters were maintained within the recommended guidelines for tilapia culture: dissolved oxygen (4.72 ¬± 0.50 mg/L), pH (8.10 ± 0.08), water temperature (28.01 ± 0.33 ºC), turbidity (2.51 ± 0.77 NTU), nitrite nitrogen (2.05 ± 0.75 ppm), nitrate nitrogen (7.75 ± 3.50 ppm), ionized ammonium (1.06 ± 0.9 ppm), salinity (0.30 ± 0.0 ppt) and conductivity (585 ± 28.82 µS/cm).

At the beginning and end of the experiment, bromatological analyses of the feed and the whole fish without viscera were conducted following the methodologies prescribed by the Association of Official Analytical Chemists (2006). Analyzes were done at Laboratorio de Bromatología de la Facultad de Medicina Veterinaria y Zootecnia, Escuela de Zootecnia, Universidad de San Carlos de Guatemala.

The experimental protocol concerning the use of laboratory animals received formal approval from the Postgraduate Bioethics Committee (Comité de Bioética de Postgrado) of the Facultad de Medicina Veterinaria y Zootecnia, Universidad de San Carlos de Guatemala, under the reference number EEPVirtual.176.2021.

2.4 Growth performance and feed utilization

Upon completion of the feeding experiment, the fish's weight was recorded, and their food intake was quantified. The growth and feed utilization were then assessed by calculating the following parameters: weight gain (WG%), specific growth rate (SGRg/day), condition factor (CFg/cm^3), hepatosomatic index (HIS%) and viscerosomatic index (VSI%), food conversion ratio (FCR), feed intake (FIg / fish), feed efficiency ratio (FERg/g), protein efficiency ratio (PER%), protein retention efficiency (NPUα±%), energy efficiency ratio (EER%), and energy retention efficiency (ERE%). Calculations were done according to Gabriel et al. (2015) and Kim et al. (2016) using the formulas:

Weight gain (WGg) = average final body weight - average initial body weight

Specific growth rate (SGR%/day) = [(LN (average final body weight) - LN (average initial body weight)) / time] * 100

Condition factor (CFg/cm3) = [final body weight / (final body length)3] * 100

Hepatosomatic index (HSI%) = (wet liver weight / final body weight) * 100

Viscerosomatic index (VSI%) = (wet visceral weight / final body weight) * 100

Feed intake (FIg/per fish) = (dry feed intake / average of fish during experiment)

Feed efficiency ratio (FERg/g) = (weight gain / Feed intake)

Feed conversion ratio (FCR) = (Feed intake / Weight gain)

Protein retention efficiency ($NPU\alpha_{\pm\%}$) = [(final total body protein - initial total body protein) / total protein intake] * 100

Protein efficiency ratio (PER) = (weight gain / total amount of protein intake per fish)

Energy retention efficiency (ERE%) = [(final total body lipid - initial total body lipid) / total lipid intake] * 100

Energy efficiency ratio (EER%) = (weight gain / total amount of lipid intake per fish)

At the end, survival was calculated as: (SV%) = (Number of fish at the end / initial number of fish) * 100

2.5 Hematology and Plasma Chemistry

At the end of the feeding period, three fish were randomly chosen from each tank (n = 9 fish per treatment group). Blood samples were drawn from the fish's caudal vasculature using syringes treated with heparin without anesthesia. Following this, the fish were humanely euthanized in compliance with the guidelines outlined in the Aquatic Animal Health Code by the World Organization for Animal Health. The blood was subsequently separated for different tests. The first portion was moved into heparinized capillaries and centrifugated at room temperature at 1,2000 g for 5 minutes to determine the hematocrit (Hct%) using the microhematocrit technique. The second portion was centrifugated at room temperature at 3,000 x g for 10 minutes to isolate the plasma, and the samples were kept at -20 ºC. The third portion was utilized for a blood smear, which was fixed with methanol and stained with Giemsa dye to examine the shape and size of the

blood cells. The descriptive data were obtained by observing the shape of the cell and its nucleus. For erythrocytes, a complete description and 30 cell measurements were performed manually using the ImageJ program (NIH, Bethesda, MD). The measurements taken include the cell length (LAm), the cell's long axis (LA μm), the cell's short axis (SA μm), the long axis of the nucleus (N-LA μm), and the short axis of the nucleus (N-SA μm). The identified cell types were classified as Normal cells (CN%) and Abnormal cells (CAN%). Further classification was done within the abnormal cells as follows: Immature cell (IC%), Ghost cell (CTF%), Segmented cells (CTS%), and Poikilocyte-type cell (CTP%). For leukocytes, classification was performed on a total of 100 cells based on their cell type: Lymphocytes (LF%), monocytes (MO%), granulocytes (GR%), and thrombocytes (TR%).

The optimized UV method was utilized to analyze plasma samples with commercial kits from Wiener Lab and SpinReact. Total protein was determined as TPg / dL (SpinReact 1001290), glucose as GEg/L (Wiener Lab Cat. No. 1400106), aspartate aminotransferase as ASTU/L (Wiener Lab Cat. No. 1751302), alanine aminotransferase as ALTU/L (Wiener Lab Cat. No. 1761302) and creatinine as CREmg/L (Wiener Laboratory Cat. No. 1008149).

2.6 Histomorphometry of the intestinal and liver.

Upon completion of the feeding experiment, three fish from each tank (n = 9 fish per treatment) were randomly selected and sacrificed by the Aquatic Animal Health Code protocol. The liver and a section of the intestine (approximately 1 inch from the anterior end) were extracted from each fish. These samples were initially preserved in 10% formalin for 168 hours and stored in 70% ethanol. The samples underwent dehydration using a sequence of ethanol solutions with varying concentrations. Following this, they were clarified in xylol and embedded in paraffin blocks at 60 ± 1 ºC. Sections of 6 μm thickness were then stained with Harris hematoxylin and eosin solution (H&E). Post-staining, the slides were mounted in Entellan resin (Merck) and

examined under a trinocular microscope (Boeco BM-180) equipped with an AmScope camera (MU500). A total of 15 microphotographs were taken per animal, resulting in 135 per treatment for each tissue.

Microphotographs of intestine slide samples were taken at 100X magnification to measure the following variables: fold height (WIFμm), fold length (LIFμm), and number of goblet cells (GBN#/fold). In the case of the liver, slide samples were microphotographed at 400X magnification to measure the area of the hepatocyte (HPAμm^2). All the analysis were performed using Image J software taking special consideration of not measured twice the same cell-area.

2.7 Digestive enzyme determination.

The stomach and the anterior intestine were collected from the same fish that was sampled for the hematology and plasma chemistry analysis. The collected samples were kept frozen for 45 days at -20 ºC until all samples were lyophilized. The lyophilized stomach samples were macerated with 100 mM glycine-HCl pH 2, while the intestinal samples were macerated with 50 mM Tris-HCl pH 7.5. After maceration, all samples were centrifuged at 16,000 g at 4 ºC for 15 min. The supernatants were collected and stored at -80 ºC.

The soluble protein was quantified using the Bradford (1976) technique. The acid protease activity in the stomach samples was quantified using the Anson (1938) method, while the alkaline protease activity was determined according to Sarathet al. (1989). The trypsin activity was determined using the Erlanger et al. (1961) method, and chymotrypsin activity was determined using Del Mar et al. (1979) method. The leucine aminopeptidase activity was determined using Maroux et al. (1973) method. Finally, the lipase activity was determined according to Versaw et al. (1989) method.

The enzyme activity was determined using the following equations: units per ml (U/mL) = [Δabs × final reaction volume (mL)]/[ε × time (min) × extract volume (mL)]−1; specific activity (U/mg protein) = U mL/ mg of soluble protein.

2.8 Challenge with *Aeromonas hydrophila*

After the 30-day feeding experiment, 15 fish per treatment (n = 5 per replicate) were challenged with pathogenic *A. hydrophila* by intraperitoneal injection of 100 µL/g body weight containing 2.1 x 10^5 CFU (LD50 based on preliminary work). The fish were observed twice daily until the 18th day to record mortality and macro-clinical signs of infection. Dead and moribund fish were removed every day. The infection was confirmed by re-isolating the bacteria from the anterior kidney or, in the case of moribund fish, by taking a blood sample to confirm the pathogen. The bacteria were cultured in tryptic soy agar medium and incubated at room temperature for 24 hours to visualize and characterize *A. hydrophila* colonies. These colonies are Gram-negative and have a bacilli form.

2.9 Statistical Analysis

The results are presented as the mean ± standard error of the mean. Normality and homoscedasticity were tested for all variables using the Shapiro-Wilk and Levene tests, respectively. For data that showed normal distribution and homoscedasticity, a one-way analysis of variance (ANOVA) was conducted, followed by Fisher's Least Significant Difference (LSD) post hoc analysis ($p < 0.05$). In the case of data that violated any of the above assumptions, a nonparametric test of Kruskal-Wallis followed by Mann–Whitney–Wilcoxon test ($p < 0.05$) was performed (R Core Team, 2020).

3. Results

3.1 Composition of the essential oil of *Lippia graveolens*

For the essential oil of *Lippia graveolens* were identified 25 volatile components representing 99.75% of the total substances. The main compounds were thymol (73.03%), followed by *p*-cymene (7.08%), *E*-caryophyllene (4.20%) and *α*-humulene (2.52%). Other volatile components accounted for < 2% of the total essential oil composition (Table 1).

3.2 Growth performance and feed utilization

The initial fish weight (Wo) did not differ between treatments ($p > 0.22$). From the onset of the feeding regimen, all fish demonstrated high levels of appetite and readily accepted the administered diets. Upon concluding the feeding trial, fish reared on diets incorporating essential oil (EO) showed significantly higher survival rates compared to the control group ($p < 0.037$). Specifically, the T600 group exhibited the highest survival rate at 95.6%, followed by the T1200 (93.3%) and T300 (91.1%) groups; the control group recorded a survival rate of 82.2%. Although mortality commenced on the ninth day of the trial, the fish exhibited no external signs of disease. Including oregano EO in the diets for *O. niloticus* positively influenced growth performance and feed utilization metrics, which were significantly superior in the T300 and T1200 treatment groups compared to the control group ($p < 0.05$, Table 2). While fish in the T600 group showed no statistically significant improvements, there was a discernible trend toward enhanced growth and feed utilization indices compared to those fed the control diet. Overall, metrics such as Weight Gain (WG), Specific Growth Rate (SGR), Feed Conversion Ratio (FCA), Protein Efficiency Ratio (PER), and Net Apparent Protein Utilization (NPUα) were improved in fish fed EO supplemented diets. While the Feed Intake (FI) showed no significant difference between treatments ($p > 0.65$), there was an improvement in feed efficiency. This increment in feed efficiency was evidenced by a lower FCA and significantly higher values of NPUα, NEU, and PER in the T300 and T1200 treatments ($p < 0.05$).

3.4 Hematology and Plasma Chemistry

The effects of experimental diets on serum protein and glucose profiles (TP and GLU), liver enzymes (AST and ALT), and renal function (CRE) are presented in Figure 1. Among the treatments, significant changes were observed ($p < 0.05$). There is an increase in the value of AST, ALT, and GLU compared to the control group. On the other hand, the parameters TP, CR, and HCT tended to decrease compared to the control group.

Hematological variables are summarized in Table 3. For leukocyte cells, no significant changes ($p > 0.05$) were observed for granulocytes and thrombocyte cells, but for lymphocytes and monocytes, significant changes were observed between treatments; however, the trend is not clear due to value fluctuations. T1200 showed the lowest percentage of monocytes (1.11 ± 1.96 %) and the highest percentage of lymphocytes (54.11 ± 12.96 %).

In contrast, the T600 showed a lower percentage of lymphocytes (43.22 ± 6.67 %) and a higher percentage of monocytes (3.67 ± 2.60 %) than the T1200 treatment.

In terms of granulocytes and thrombocytes, there is no significant difference among the treatments, but a trend was observed for higher granulocytes and lower values of thrombocyte cells with the oregano EO treatment compared to the control group.

Meanwhile, a significant difference ($p < 0.05$) was observed between treatments for CAN, CI, and CN type cells, with CAN and CI showing the lowest and CN the highest percentage compared to the control group. No significant changes were determined for the rest of the cell types, such as CTF, CTS, and CTP ($p > 0.05$). Nevertheless, once again, there is a trend for higher percentages with this type of cell compared to the control group.

According to erythrocyte morphometry, the cell's long axis showed a significant difference ($p < 0.05$) among the treatments with oregano EO but not with the control group, with the lower

size found with treatment T600. The other measurements, such as SA, N_LA, and N_SA showed no significant difference ($p > 0.05$) (Table 4).

3.5 Histomorphometry of the intestinal and liver

The effects of experimental diets on the histomorphometry of the intestine and liver are presented in Table 5. Treatment T600 presented a higher hepatocyte area ($p < 0.05$) than other treatments. For the intestinal fold height and number of Goblet cells, T300 showed a higher value but was not significantly different from the control group. The width of intestinal folds and the number of goblet cells did not show significant changes ($p > 0.05$) between treatments.

3.6 Digestive enzyme activities

The effects of experimental diets on the activities of digestive enzymes are presented in Figure 2. Among the treatments, significant changes ($p < 0.05$) were observed. Fish fed 1200 mg/kg of oregano EO showed the highest activity of acid proteases, chymotrypsin, and lipase. Fish fed 600 mg/kg showed only higher activity for alkaline protease and chymotrypsin, and finally, the concentration of 300 mg/kg oregano EO did not improve any digestive enzyme activity.

3.7 Challenge with *Aeromonas hydrophila*

All treated groups showed higher survival than the control ($p < 0.05$); fish that received diets containing different concentrations of oregano EO exhibited increased resistance to *A. hydrophila*, with 93.3%, 86. 67% and 73.3% fish survival for the groups received 600, 300, and 1200 mg/kg of oregano EO in the diet, respectively (Figure 3). After the fifth day of the experimental challenge, in the control and T1200, most of the fish reduced their feeding rate; only with T600 and T300 did the fish continue feeding normally. After day 8, the control fish and those fed 1200 mg/kg oregano EO showed clinical signs of *A. hydrophila* infection, such as external bleeding, scale loss, skin ulcers, and broken caudal fin, and the fish started to die.

4. Discussion

Globally, the incorporation of oregano as a feed additive in tilapia culture has been documented to enhance various facets of aquaculture production, including growth promotion, immunological response, and increased resistance to diseases (Abdel-Latif et al., 2020; Alagawany et al., 2020; Alarcón-Rojo et al., 2017; Leyva-López et al., 2017). This beneficial impact is commonly attributed to secondary metabolites like thymol and carvacrol in oregano. However, the oregano essential oils contain a broader spectrum of compounds at lower concentrations (Teixeira et al., 2013), which could also enhance fish performance.

In the current study, terpenoids emerged as the predominant class of compounds, with thymol accounting for 73.03% of the essential oil derived from *L. graveolens*. Generally, *L. graveolens* EO is recognized for its potent antibacterial properties (García-Pérez et al., 2022). However, limited evidence supports its efficacy as a growth or health enhancer in aquaculture (García-Pérez et al., 2019). Given the limited range of metabolites in *L. graveolens*, it is plausible that the prominent presence of thymol significantly contributed to the observed outcomes in this study. It appears evident that the inclusion of EO in the diet enhances survival, growth metrics such as GW and SGR, and feed utilization indices including NPUα, PER, NEU, and EER in tilapia fingerlings. These findings are congruent with those reported for this species and others (Heluy et al., 2020; Morselli, Reis, et al., 2020; Zhang et al., 2020a; Zheng et al., 2009). Thymol, the primary terpenoid component of the EO, could be chiefly accountable for the observed positive effects on growth and feed efficiency. Our study indicates that the most optimal growth performance was observed at 1200 mg/kg, followed by 300 mg/kg and 600 mg/kg. The mechanism through which *L. graveolens* EO enhances digestion and metabolic processes could be attributed to activating certain specific genes and enzymes, as had been suggested by Ahmadifar et al. (2011). It's also recognized that the suitable dietary inclusion of other EO's and extracts not only promotes the genes

that induce growth hormone and insulin growth factor but also enhances the activity of various enzymes and intestinal health (Alagawany et al. 2020, Abarike et al. 2022, Dawood et al. 2022, Magouz et al. 2022).

The influence of *L. graveolens* EO inclusion levels on certain variables is not distinctly noticeable, as the 600 mg/kg EO treatment yielded results that were not significant from the control group, albeit with a tendency towards improvement. On the other hand, the 300 and 1200 mg/kg EO treatments demonstrated a higher performance.

This inconsistency aligns with the findings of Aanyu et al. (2018), who determined that dietary thymol up to 500 ppm did not improve somatic growth and feed utilization. Variations between studies could be due to the phytogenetic itself, the method of extraction and application, chnages in water temperature (26 – 28°C) and fish stocking density, as observed with *O. niloticus* reared at lower temperatures (<28°C) and higher densities (> 3 kg/m^3) (Margaret et al. 2021; Shourbela et al. 2021a). Additionally, it could be associated with a higher stress level in fish kept at higher densities due to lower mortality. Stress in aquatic animals can lead to increased energy reserve consumption, and this reallocation of metabolic energy can adversely affect other physiological processes like growth (Barton & Zwama, 1991; Wendelaar Bonga, 1997).

The fact that FI and, therefore, the appetite were not affected by the inclusion of *L. graveolens* EO's suggests that the best growth of *O. niloticus* juveniles found with diets supplemented with the EO's could probably be attributed to an improved bile and digestive enzyme secretion but also to an efficient utilization of nutrients or nutrient transport and absorption, and better protein, lipid, and energy metabolism as stated by Dawood et al. (2022) and Magouz et. Al. (2022). The latter agrees with the higher NPUα, PER, NEU, and EER values found in juveniles fed diets containing the EO's. It is essential to point out that these results could be affected by

differences in fish size since smaller animals have more active growth (Loum et al., 2013) having more efficient somatic growth than bigger animals.

Moreover, the elevated protein level in the diet used in our experiment could lead to improved feed utilization. Due to the high protein and lipid levels (high-energy diet), less feed is required to balance energy for growth and metabolism (Miller 2004), which could account for the low FCA observed in this study. Consequently, *L. graveolens* EO may function as a growth promoter, as reported by various authors who used different phytogenetics in diets, including thymol (Aanyu et al., 2020; de Souza et al., 2020; Magouz et al., 2021; Morselli, Baldissera, et al., 2020; Morselli, Reis, et al., 2020). However, it should also be highlighted that the effects of using phytogenic in fish diets are closely related to the environmental and biological characteristics of the fish species (Núñez-Torres et al., 2022). Therefore, obtaining clear results requires using *L. graveolens* in various aquatic species to be validated and evaluated under culture conditions.

Hematological and biochemical variables are indicators for assessing overall health, metabolic status, and disease tolerance, among other factors. In general, our data suggest that the addition of EO has enhanced the health and metabolic condition of the tilapia juvenile, thereby increasing its resistance to diseases. For instance, in this research, it was observed that the ALT and AST enzyme levels were higher compared to the control group, suggesting an enhancement in the metabolic state of the fish. As a result, it seems that including *L. graveolens* EO, for a 30-day feeding period, in the diet of juvenile tilapia is a safe supplement under the existing conditions. However, these levels of ALT and AST are slightly lower than those reported by other researchers who used thymol or other essential oils in tilapia and other aquaculture species (Alagawany et al., 2021; de Souza et al., 2020; Hoseini & Yousefi, 2019; Zargar et al., 2019). These discrepancies could be attributed to variations in EO levels, culture conditions, and feeding strategies.

Liver enzyme activity typically increases in response to drug exposure and toxic substances (Tennant & Center, 2008). Our findings suggest that the metabolite thymol has no adverse impact on the liver, a crucial organ in nutrient metabolism (Caballero et al., 2004). Additionally, there was no indication of detrimental effects on liver hepatocyte histology, confirming that *L. graveolens* does not negatively influence the fish at concentrations ranging from 300 to 1200 mg/kg. The elevated levels of ALT and AST in the liver could signify an increased utilization of dietary amino acids for growth and as a substrate for gluconeogenesis (Bibiano-Melo et al., 2006).

Protein and glucose are typically used as biochemical markers to assess the metabolic condition of fish. Across all treatments, fish did not result in hypoglycemia or hyperproteinemia in juvenile Nile tilapia, since the values remained within the reference range for the species (Hrubec et al., 2000). Higher glucose suggests chronic stress in experimental animals fed diets with EO oregano, a finding that may be associated with the higher stocking density at the end of the experiment found in these diets. Moreover, the lower glucose value of the TCNT may be influenced by non-specific stressors, such as the "fight or flight" reaction of fish, which requires considerable energy reserves such as describe Odhiambo et al., (2020). Thereby influencing glucose values and subsequently impacting essential physiological functions such as growth and health. The elevated creatinine levels observed in this study could be attributed to increased nitrogen waste from protein metabolism in all treatments, particularly the control and 1200 mg/kg treatments.

It is speculated that the kidney may be inefficient in removing nitrogen wastes in these treatments as the typical values for tilapia were exceeded (Hrubec et al., 2000). This kidney deficiency could potentially be linked to a high dietary protein content.

The observations from the blood cell count indicate an increase in lymphocyte frequency (LF) across all treatments compared to the control. This LF value suggests that the immune system is active, likely due to the high bacterial loads and reduced water quality that fish are exposed to in

high-density production systems, which can affect the lymphocyte ratio (Hrubec et al., 2000). Our study found a lower percentage of abnormal cells than the control group, implying that erythropoiesis was deficient in the control group. Elevated immature and other abnormal erythrocytes could account for the higher hematocrit (Hct) value observed in the control group. This abnormal erythrocyte presence could lead to inefficient oxygen transport by red blood cells throughout the body, as Vo et al. (2023) noted.

Several factors influence feed digestibility and nutrient absorption, one of which is the inclusion of dietary additives of plant origin. Phytoadditives, such as essential oils, can enhance digestion by boosting the production and secretion of intestinal enzymes or improving specific digestive structures' performance. Some of these structures include the height and length of intestinal folds, the number of goblet cells per fold, and the area of the hepatocytes (Vallado et al., 2017; Dawood et al., 2021). Although our study did not find any distinct effects of EO on intestinal histomorphometry, we did notice an increase in hepatocyte area in all treatments involving *L. graveolens* EO, suggesting high energy reserves.

Regarding digestive enzymes, a dietary concentration of 1200 mg/kg of EO demonstrated a positive impact by enhancing the activity of intestinal enzymes, particularly acid proteases, chymotrypsin, and lipase. This digestive enzyme increment could lead to improved absorption and utilization of nutrients such as amino acids and fatty acids. Similar outcomes were observed with the use of *Ocimum basilicum, Zingiber officinale, Thymus vulgaris, Aloe barbadensis*, and *Origanum vulgare* (Dotta et al., 2018; de Souza et al., 2019; Valladão et al., 2019; Zhang et al., 2020b; Chung et al., 2021). This increment in the activity of those enzymes could enhance the digestive system's ability to break down and absorb nutrients from feed efficiently. In summary, more proteins are available for growth, while more lipids for energy in all internal processes.

Moreover, all treatments that included *L. graveolens* EO in the diet exhibited lower mortality rates than the control group following the *A. hydrophila* challenge.

Although this study did not find any distinct benefits in terms of blood cell parameters, liver, and intestinal morphometry, it can be inferred that including *L. graveolens* EO in all diets may have influenced other immunological parameters. These parameters, such as total immunoglobulin, phagocytic activity, cytokine expression, and cellular immune response, could contribute to the improved survival rate observed after the bacterial challenge.

Our study demonstrated that the inclusion of *L. graveolens* EO could enhance the health and immune system of fish, as well as increase their resistance to bacteria, as found in other studies on phytochemicals (Brum et al., 2017; de Oliveira et al., 2020; de Souza et al., 2019a; Mohammadi et al., 2020). The proposed mechanism of action for EO is that it improves the digestibility of fish feed, leading to better nutrient absorption and utilization and, consequently, healthier, and more resilient fish (Dawood et al., 2022).

5. Conclusion

In conclusion, incorporating *Lippia graveolens* essential oils in the diet could serve as a more effective alternative to antibiotics for enhancing the growth performance and bacterial resistance of *O. niloticus*. While our experiments provided insights into growth, feed utilization, hemo-biochemical parameters, gastrointestinal and liver morphometry, digestive enzymes, and bacterial issues, the specific mechanisms remain unclear. As such, further research is required.

6. Data Availability

The supporting data for this study's findings can be obtained from the corresponding author, provided the request is reasonable.

7. Conflicts of Interest

The authors declare that they have no conflicts of interest.

8. Authors' Contributions

García-Pérez, J: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. Vissio, P: Supervision, Writing - Review & Editing. Pérez-Sirkin, D: Supervision, Writing - Review & Editing. Álvarez-González, C.A: Resources, Supervision, Writing - Review & Editing. Quiroz, C.A: Resources, Writing - Review & Editing. Ulloa-Rojas, J.B: Formal analysis , Writing - Review & Editing, Supervision.

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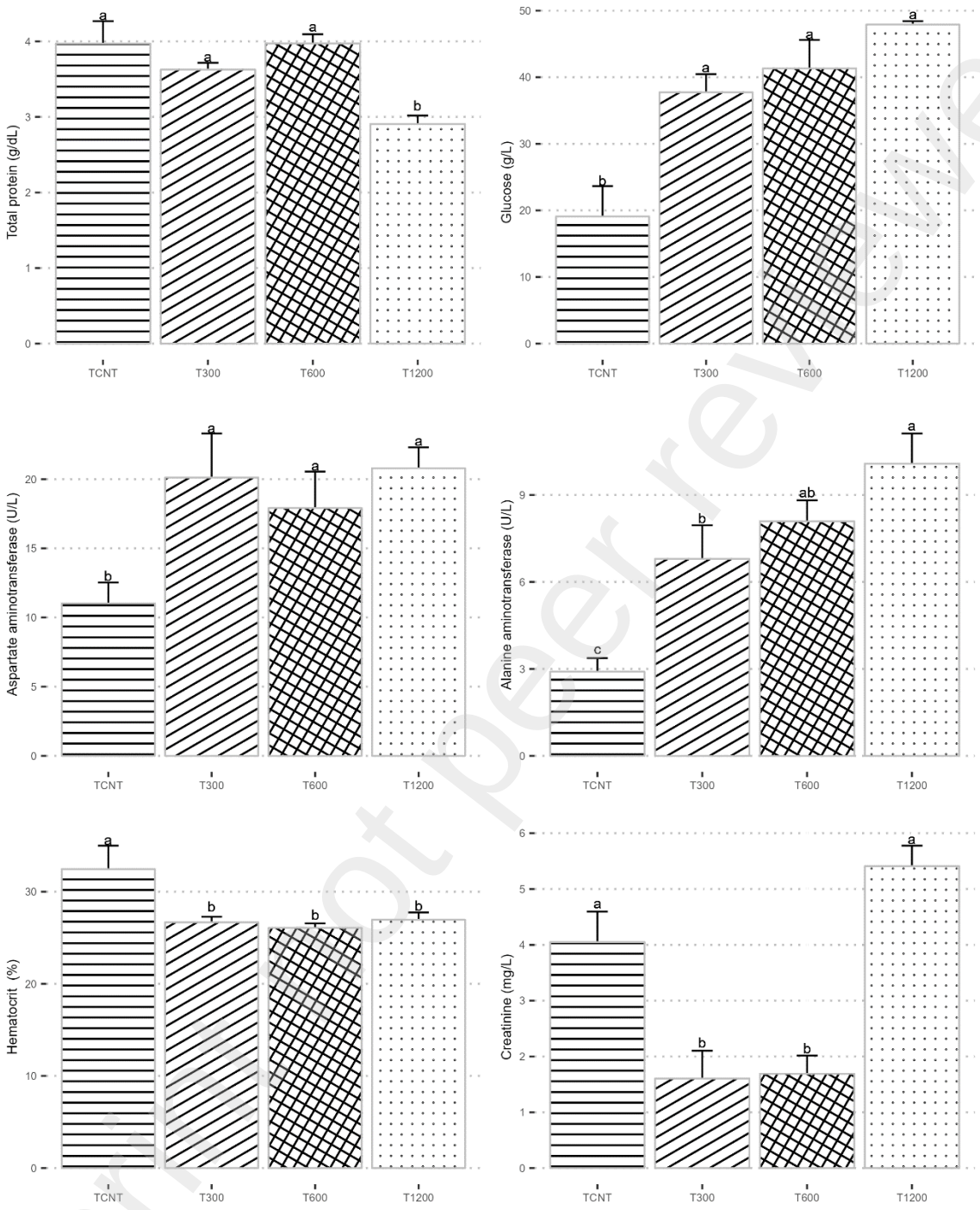

Figure 1. Biochemical parameters (mean ± SEM) from juvenile tilapia of the GIFT strain (*O. niloticus*) fed with different *L. graveolens* essential oil inclusion levels for 30 days. Treatment means with different superscripts were significantly difference ($p < 0.05$).

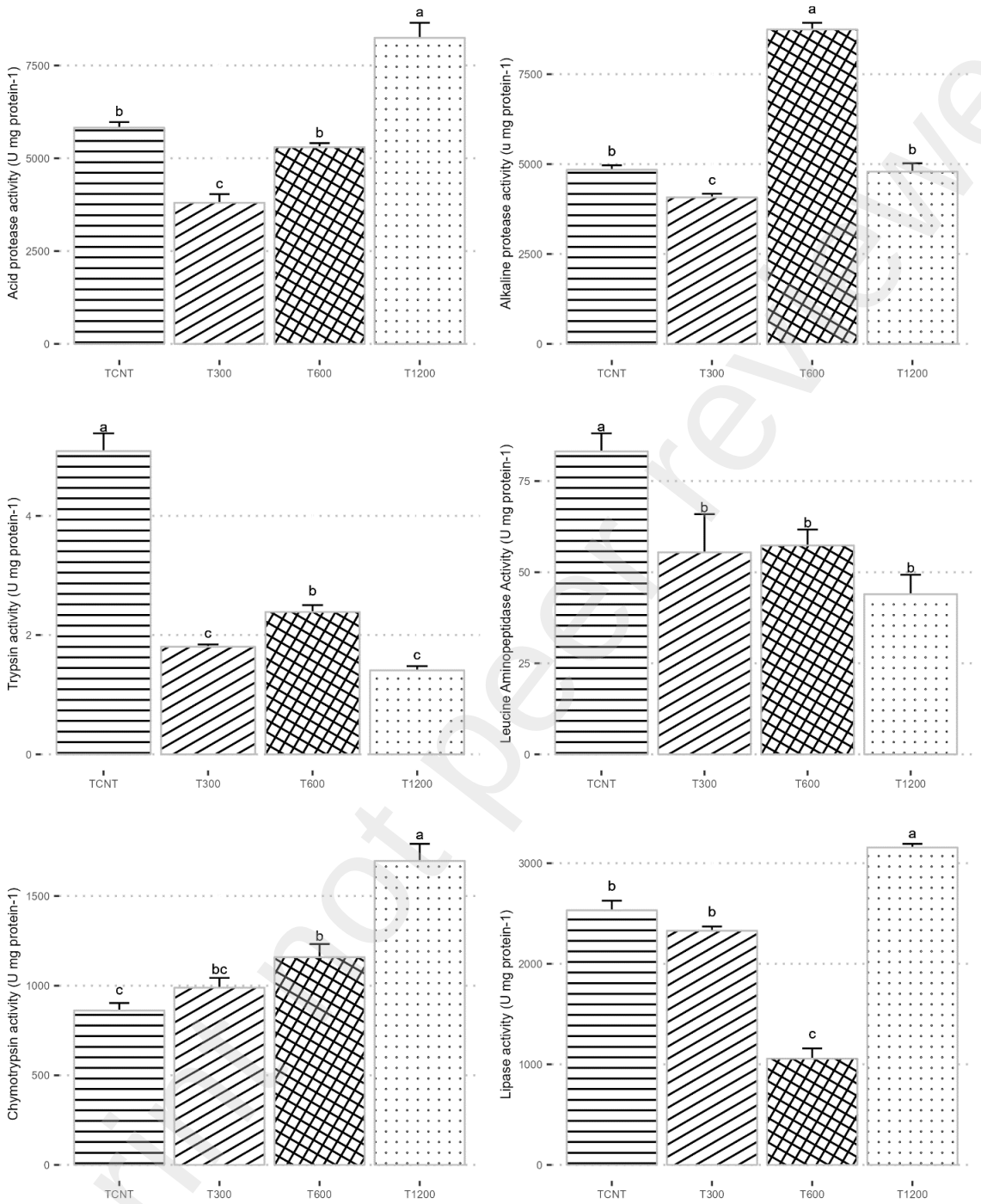

Figure 2. The activity of digestive enzymes (U mg protein^{-1}) (mean ± SEM) of juvenile tilapia of the GIFT strain (*O. niloticus*) fed different levels of essential of *L. graveolens* essential oil for 30 days. Treatments having different superscripts were significantly different ($p < 0.05$).

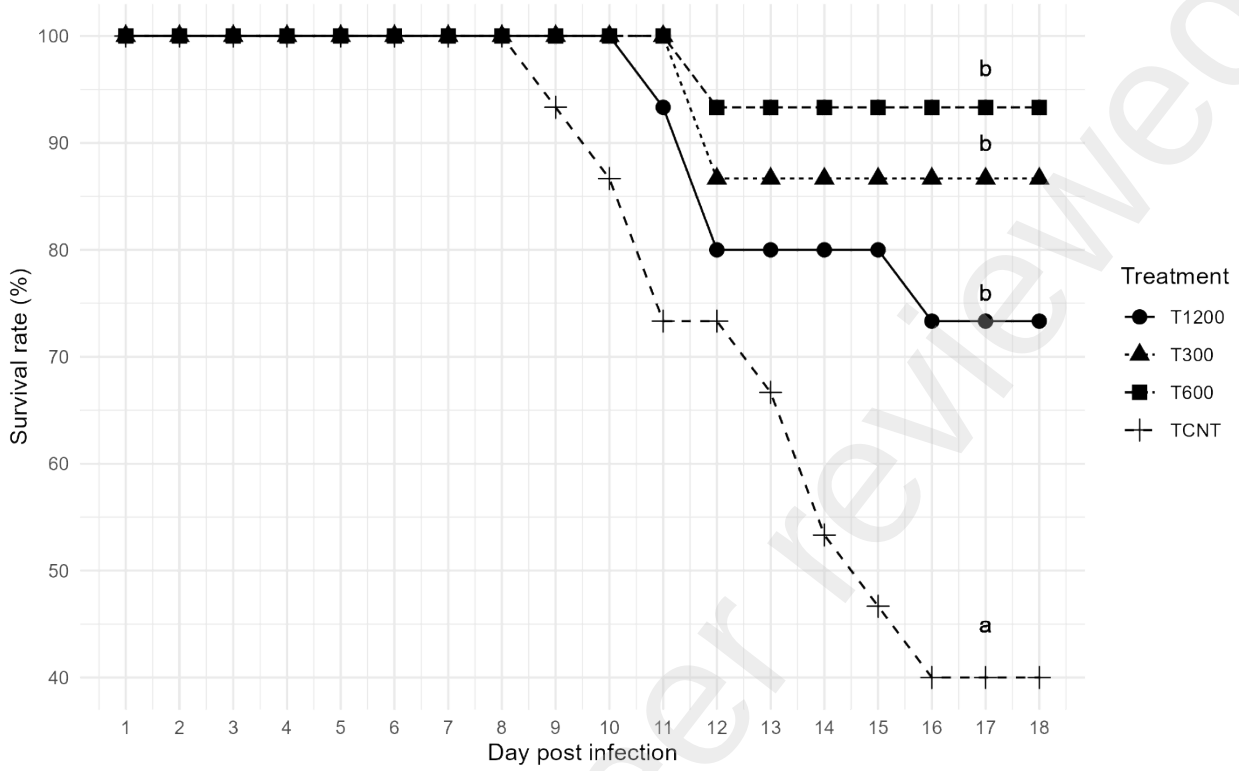

Figure 3. Survival rate obtained in different groups of juvenile tilapias of the GIFT strain (*O. niloticus*) fed with different essential oil inclusion levels of *L. graveolens* after challenge with *A. hydrophila*. Treatments having different letter were significantly different ($p < 0.05$).

Table 1. Composition of essential oil (%) in *L. graveolens* by steam distillation technique.

	NAME AS MASS SPECTRA	%	RT
1	*α*-Thujene	0.18	5.20
2	*α*-Pinene	0.14	5.40
3	Myrcene	1.67	6.90
4	*α*-Terpinene	0.79	7.83
5	*ρ*-Cymene	7.08	8.10
6	Limonene	0.30	8.25
7	1,8-Cineole	0.27	8.36
8	*γ*-Terpinene	1.90	9.28
9	*cis*-Sabinene hydrate	0.27	9.76
10	Linalool	0.46	10.90
11	*trans*-Sabinene hydrate	0.22	11.02
12	Umbellulone	0.20	14.00
13	*4-ol*-Terpinen	1.11	14.23
14	Thymol, methyl ether	0.78	16.35
15	*Z*-Ocimenone	0.18	18.76
16	Thymol	73.03	19.09
17	Carvacrol	0.70	19.54
18	*E*-Caryophyllene	4.20	24.6
19	*α-trans*-Bergamotene	0.96	25.24
20	*α*-Humulene	2.52	26.15
21	*β*-Bisabolene	1.13	28.45
22	*γ*-Cuprenene	0.11	29.82
23	Caryophyllene oxide	0.93	31.4
24	Humulene epoxide II	0.41	32.54
25	*5Z,9E*-Farnesyl acetone	0.21	42.99
	Total	99.75	

Note: %: percentage of total area; RT: retention time

Table 2. Growth performance and feeding utilization in juvenile tilapia of the GIFT variety (*O. niloticus*) fed different dietary *L. graveolens* essential oil for 30 days.

Parameters	TCNT	T300	T600	T1200	*p*
$SV_{\%}$	82.22 ± 2.22 b	91.11 ± 2.22 a	95.56 ± 2.22 a	93.33 ± 3.85 a	0.04
Wo_g	13.67 ± 0.01 a	13.68 ± 0.01 a	13.67 ± 0.00 a	13.68 ± 0.00 a	0.22
Wf_g	45.53 ± 1.84 c	53.12 ± 1.97 ab	49.75 ± 1.60 bc	55.22 ± 1.83 a	0.00
GW_g	31.86 ± 1.84 c	39.44 ± 1.97 ab	36.08 ± 1.60 bc	41.54 ± 1.83 a	0.00
$SGR_{\%/day}$	3.94 ± 0.14 b	4.45 ± 0.14 a	4.26 ± 0.11 ab	4.60 ± 0.11 a	0.00
CF_{g/cm^3}	1.69 ± 0.03 a	1.76 ± 0.03 a	1.72 ± 0.02 a	1.70 ± 0.01 a	0.16
$HSI_{\%}$	2.91 ± 0.13 a	2.70 ± 0.08 a	2.98 ± 0.13 a	2.70 ± 0.14 a	0.26
$VSI_{\%}$	10.45 ± 0.25 b	11.80 ± 0.40 a	11.17 ± 0.23 ab	11.55 ± 0.20 a	0.01
$FI_{g/fish}$	36.82 ± 1.76 a	37.21 ± 0.87 a	36.51 ± 0.73 a	35.11 ± 1.25 a	0.65
$FER_{g/g}$	87.04 ± 8.46 b	106.14 ± 3.28 ab	98.81 ± 4.66 b	118.25 ± 5.76 a	0.03
FCA	1.17 ± 0.11 a	0.94 ± 0.03 b	1.02 ± 0.05 ab	0.85 ± 0.04 b	0.04
$NPU\alpha_{\%}$	28.06 ± 2.79 c	38.00 ± 1.17 ab	33.51 ± 1.61 bc	43.47 ± 2.10 a	0.00
PER	1.96 ± 0.19 b	2.39 ± 0.07 ab	2.22 ± 0.10 b	2.66 ± 0.13 a	0.03
$NEU_{\%}$	49.52 ± 4.30 b	63.91 ± 1.91 a	56.26 ± 2.26 ab	61.50 ± 2.60 a	0.03
$EER_{\%}$	6.98 ± 0.68 b	8.51 ± 0.26 ab	7.92 ± 0.37 b	9.48 ± 0.46 a	0.031

Note: Values are mean ± SEM of triplicate groups of fish, values in each row with a different superscript were significantly different ($p < 0.05$). Where $SV_{\%}$ = survival; Wo_g and Wf_g = initial and final weight, WG_g = weight gain; $SGR_{\%/day}$ = specific growth rate; $CF_{g/cm3}$ = condition factor; $HSI_{\%}$ = hepatosomatic index; $VSI_{\%}$ = viscerosomatic index; $FI_{g/per\ fish}$ = feed intake; $FER_{g/g}$ = feed efficiency ratio; FCR = feed conversion ratio; $NPU_{\alpha\%}$ = protein retention efficiency; PER = protein efficiency ratio; $ERE_{\%}$ = energy retention efficiency; $EER_{\%}$ = energy efficiency ratio.

Table 3. Hematological parameters of juvenile tilapia of the GIFT strain (*O. niloticus)* fed different inclusion levels of *L. graveolens* essential oil for 30 days.

Parameters	TCNT	T300	T600	T1200	*p*
$LF_{\%}$	50.44 ± 11.05 ab	52.56 ± 13.15 ab	43.22 ± 6.67 b	54.11 ± 12.96 a	0.05
$MO_{\%}$	2.11 ± 2.32 ab	2.67 ± 3.35 ab	3.67 ± 2.60 a	1.11 ± 1.96 b	0.02
$GR_{\%}$	9.67 ± 5.61 a	6.33 ± 2.29 a	7.00 ± 4.72 a	6.89 ± 5.04 a	0.43
$TR_{\%}$	37.78 ± 10.73 a	38.44 ± 11.59 a	46.11 ± 8.95 a	37.89 ± 13.29 a	0.43
$CN_{\%}$	48.89 ± 5.41 b	62.22 ± 7.45 ab	67.04 ± 3.07 a	62.96 ± 1.49 4.95	0.03
$CAN_{\%}$	51.11 ± 5.41 a	37.78 ± 7.45 ab	32.96 ± 3.07 b	37.04 ± 4.95 ab	0.03
$IC_{\%}$	20.00 ± 3.77 a	17.41 ± 4.07 ab	9.26 ± 2.41 b	15.19 ± 3.43 ab	0.04
$CTF_{\%}$	3.33 ± 0.96 a	4.07 ± 1.45 a	3.33 ± 1.24 a	2.22 ± 0.96 a	0.74
$CTS_{\%}$	15.56 ± 2.15 a	11.11 ± 2.83 a	12.22 ± 2.78 a	11.48 ± 2.67 a	0.62
$CTP_{\%}$	12.22 ± 2.66 a	5.19 ± 2.16 a	8.15 ± 2.16 a	8.15 ± 3.05 a	0.29

Note: Values are mean ± SEM of triplicate groups of fish, where values in each row with a different superscript was significantly different ($p < 0.05$). Where LF% = lymphocytes; MO% = monocytes; GR% = granulocytes; TR% = thrombocytes; CN% = normal cells, CAN% = abnormal cells; IC% = immature cell; CTF% = ghost cell; CTS% = segmented cells and CTP% = Poikilocyte-type cell.

Table 4. Morphological erythrocytes analysis of juvenile tilapia of the GIFT variety (*O. niloticus*) fed with different inclusion levels of essential oil from *L. graveolens* for 30 days.

Parameters	TCNT	T300	T600	T1200	*p*
$^{1}LA_{\mu m}$	1.08 ± 0.01 ab	1.09 ± 0.01 ab	1.04 ± 0.02 b	1.10 ± 0.02 a	0.05
$^{2}SA_{\mu m}$	0.68 ± 0.01 a	0.70 ± 0.01 a	0.68 ± 0.01 a	0.67 ± 0.01 a	0.39
$^{3}N\_LA_{\mu m}$	0.45 ± 0.01 a	0.46 ± 0.00 a	0.45 ± 0.01 a	0.45 ± 0.01 a	0.50
$^{4}N\_SA_{\mu m}$	0.29 ± 0.01 a	0.31 ± 0.01 a	0.30 ± 0.01 a	0.30 ± 0.01 a	0.35

Note: Values are mean ± SEM of triplicate groups of fish, where each row with a different superscript was significantly different ($p < 0.05$). Where $LA_{\mu m}$ = cell's long axis; $SA_{\mu m}$ = cell's short axis; N-$LA_{\mu m}$ = long axis of the nucleus and N-$SA_{\mu m}$ = short axis of the nucleus.

Table 5. Morphological analysis (mean ± SEM) of the liver and intestine of juvenile tilapia of the GIFT variety (*O. niloticus*) fed different levels of essential oil from *L. graveolens* for 30 days.

Parameters	TCNT	T300	T600	T1200	*p*
$LIF_{\mu m}$	3.25 ± 0.24 ab	3.70 ± 0.13 a	3.41 ± 0.16 ab	3.20 ± 0.15 b	0.05
$WIF_{\mu m}$	0.46 ± 0.01 a	0.46 ± 0.01 a	0.45 ± 0.01 a	0.49 ± 0.02 a	0.28
$GBN_{\#/fold}$	9.74 ± 1.44 a	12.06 ± 1.48 a	11.29 ± 0.56 a	9.22 ± 1.00 a	0.31
$HPA_{\mu m}{}^{2}$	0.23 ± 0.01 b	0.25 ± 0.01 b	0.29 ± 0.02 a	0.24 ± 0.01 b	0.02

Note: Values are mean ± SEM of triplicate groups of fish, where each row with a different superscript was significantly different ($p < 0.05$). Where $LIF_{\mu m}$ = fold length; WIFμm = fold height; $GBN_{\#/fold}$ = number of goblet cells and $HPA_{\mu m2}$ = area of the hepatocyte.