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Assessing the effects of Guiera senegalensis, Pluchea odorata, and Piliostigma reticulatum leaf powder supplementation on growth, immune response, digestive histology, and survival of Nile tilapia (Oreochromis niloticus Linnaeus, 1758) juveniles before and after Aeromonas hydrophila infection

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Abstract

This study investigated the effects of including leaf powder from three medicinal plants collected in Senegal in the feeds of juvenile Nile tilapia (*Oreochromis niloticus*) on growth, survival, immunity, and digestive histology. Four experiments were conducted in triplicate, testing each plant alone or in combination at different inclusion levels: A) *Guiera senegalensis* at 0, 1, 2, 4 and 8%; B) *Pluchea odorata* at 0, 0.5, 1, 2 and 4%; C) *Piliostigma reticulatum* at 0, 1 and 2%; and D) a mixture of the three plants at 0, 1 and 2% per plant. After 28 days of feeding, fish underwent an experimental infection with *Aeromonas hydrophila* for 12 days. Immunological (bactericidal, lysozyme, and respiratory burst activities) and digestive histology analyses were performed following both feeding and infection trials. Except for *G. senegalensis*, the inclusion of medicinal plants, either alone or in combination, positively influenced the immune response at all inclusion levels. The diet containing 4% *P. odorata* increased plasma lysozyme and bactericidal activities without compromising feed conversion efficiency or growth. Despite the observed improvements in immunity, none of the dietary treatments enhanced survival following the infection. However, fish fed the 1% mixture diet exhibited healthier livers and intestines, characterized by reduced cell swelling and normal lipid deposits appearance, followed by the 2% mixture and the control groups. These results highlight the potential benefits of incorporating *P. odorata* and the plant mixture in the diets of Nile tilapia juveniles for enhancing their immune response and defense against *A. hydrophila* infections.

Introduction

Global aquaculture production has dramatically increased from 21.8 million tonnes of aquatic animals in the 1990s to 87.5 million tonnes in 2020 (FAO, 2022), mainly due to the improvement and intensification of production systems and the availability of high-quality aquafeeds. However, the intensification of aquaculture systems has led to an increase in diseases, which are currently one of the primary challenges faced by the industry. In fact, between 40 and 50% of losses in aquaculture production can be attributed to diseases (Bastos et al., 2017a; Assefa & Abunna, 2018). The increase of diseases has led to massive use of antibiotics, which, in turn, has caused serious threats such as the spread of drug-resistant pathogens, suppression of aquatic animal immunity, and adverse environmental effects (Allameh et al., 2016). As a result, international organizations (UN, FAO, WHO, OIE, EFSA) warn of the need to reduce the use of antibiotics and to develop alternatives based on biosecurity and preventive methods. Medicinal plants are considered suitable alternatives in this regard due to their ability to promote fish growth and enhance its immunity. They act as antibacterial and antiviral agents, strengthening the host immune system, and contain bioactive compounds such as terpenoids, tannins, alkaloids, and flavonoids, which possess antibacterial properties (Kari et al., 2022). In Africa, for instance, herbs and medicinal plants are extensively used for healthcare, with over 80% of the rural population relying on them (Jiofack et al., 2010). Moreover, they are not only cost-effective and efficient but also have fewer side effects and pose no environmental or hazardous risks (Citarasu, 2010; Van Hai, 2015). They are generally used individually or in combination with other plants or drugs for treating human and animal diseases (Che et al., 2013).

Their use in aquaculture has garnered significant global attention, with more than 60 species being studied for their potential in enhancing fish health and disease management, and ongoing scientific research continues to actively investigate this area (Caruso et al., 2013; Bulfon et al., 2015; Singh et al., 2020; Abdel-Tawwab & El-Araby, 2021).

The Nile tilapia (*Oreochromis niloticus* L., 1758) is one of the most widely farmed fish worldwide and plays a crucial role in improving nutrition and livelihoods, especially in developing countries (FAO, 2022), due to its rapid growth, easy reproduction, and resistance to handling. Despite its resistance, Nile tilapia remains susceptible to infectious diseases caused by viral or bacterial agents (Romana-Eguia et al., 2020). *Aeromonas hydrophila*, for instance, is one of the primary pathogenic bacteria in tropical aquaculture, affecting both marine and freshwater fish, including Nile tilapia. This opportunistic bacterium causes motile Aeromonas septicemia and includes symptoms such as cloacal hemorrhage, ascites, gastroenteric hemorrhage, and septicemic ulceration on the skin. Current antibiotic treatments have proven ineffective in managing this bacterial infection in aquaculture (Pang et al., 2015; Kari et al., 2022). In this context, several medicinal plants or their extracts have been tested for their antibacterial activities. Examples include *Murraya koenigii*, *Pandanus odoratissimus*, *Colocasia esculenta*, and *Euphorbia hirta*, which inhibit the growth of *A. hydrophila*, and *Prunus mume*, *Fructus toosendan*, *Artemisia argyi*, *Polygonum aviculare*, *Cephalanoplos segetum*, *Artemisia capillaries, Piper betle*, *Piper sarmentosum*, and *Piper nigrum*, which inhibit the bacterial activity of *A. hydrophila* (Kari et al., 2022). On the other hand, medicinal plants have also shown to enhance immune response and disease resistance in Nile tilapia (Tang et al., 2014; Sheikhlar et al., 2017; Kuebutornye & Abarike, 2020), as well as in other fish species (Nguyen et al., 2016; Nafiqoh et al., 2020; Maiti et al., 2023).

The objective of the present study was to evaluate the phytobiotic potential of three medicinal plants native to West Africa, namely *Guiera senegalensis*, *Pluchea odorata*, and *Piliostigma reticulatum*, in Nile tilapia farming. *Guiera senegalensis*, belonging to the Combretaceae family, is one of the most popular medicinal plants in West Africa. It has been extensively used in traditional medicine to treat various ailments, particularly malaria and intestinal disorders. Within the Poular community, *G. senegalensis* is commonly used in veterinary medicine, often in combination with *Heeria insignis* and *Crossopteryx febrifuga*, to enhance weight, reproductive capacity, and milk secretion of animals (Guèye, 2019). *Pluchea odorata*, a member of the Asteraceae family, is locally known as "soigne tout", which translates to “cure-all” in French. It is widely used in traditional medicine to treat headaches, allergies, fever, and muscle pain. Some studies have demonstrated the biological activity of *P. odorata* extracts, including antimicrobial (Perera et al., 2006; Pérez et al., 2007; Biabiany et al., 2013), antioxidant (Fernández & Torres, 2006; Perera et al., 2014), and anti-leishmanial (García et al., 2011) properties, thereby validating its traditional uses. *Piliostigma reticulatum* (D.C.) Hochst, locally known as "Nguiguis" in Wolof, is an evergreen shrub belonging to the Caesalpiniaceae family (Leguminosae). It naturally occurs in the Sudano-Sahelian and Sudanese regions, ranging from Mauritania and Senegal in the west to Sudan in the east. The leaves of *P. reticulatum* are employed in pharmacopeia to treat various diseases (Yelemou et al., 2007; Babajide et al., 2008; Arbonnier, 2009). Phytochemical analyses of *P. reticulatum* leaf extracts have revealed the presence of antimicrobial substances that act against bacteria such as *Staphylococcus*

aureus and *Escherichia coli*, as well as fungi like *Aspergillus niger* and *Candida albicans* (Babajide et al., 2008). In the present study, different dietary inclusion levels of each individual medicinal plant and in combination were evaluated to assess their effects on growth performance, immunity, histopathology, and survival of Nile tilapia juveniles after a 28-day feeding trial followed by a 12-day infection challenge with *A. hydrophila*.

Materials and methods

Medicinal plant collection and experimental diets

The medicinal plants used in this study (*G. senegalensis, P. odorata*, and *P. reticulatum*) were selected based on their frequent use in traditional medicine in Senegal. Fresh leaves of *G. senegalensis* were collected at Nguekokh (Thies) (14°30'11.8"N 17°00'09.4"W), *Pluchea odorata* at Mbour (Thies) (14°24'06.5"N 16°57'30.7"W), and *P. reticulatum* at the botanical garden of the faculty of medicine at Cheikh Anta Diop University (Dakar) (14°41'13.0"N 17°28'01.6"W). The leaves were washed and sun-dried for 72 h before being transformed into a powder using a mortar. The resulting powder was then sieved to obtain a homogeneous consistency and stored until further processing.

Four independent experiments were carried out to evaluate different inclusion levels of the three plants individually and in combination. For each experiment, varying doses of plants were added to a commercial diet (extruded Carp-Coul 2, Le Gouessant, Lamballe-Armor, France; 32% protein, 9% lipid, 20.5% carbohydrate) to formulate the experimental diets. In experiment A, *G. senegalensis* was incorporated into the diet at inclusion levels of 1, 2, 4, and 8% of dry matter (DM). In experiment B, *P. odorata* was included in the diet at 0.5, 1, 2, and 4% DM. In experiment C, *P. reticulatum* was included at 1 and 2% DM. Experiment D tested two mixtures, consisting of the three plants at inclusion levels of 1 and 2% DM for each plant, respectively. Each experiment included a control diet without any medicinal plants.

The corresponding amount of the commercial diet and plant leaf powder were mixed using a mixer to manufacture the different experimental diets. To create a malleable paste, approximately 40% water per kg of feed produced was added. Additionally, 25 mL of sunflower oil per kg of feed was added to the feed paste. The feed paste was processed into spaghetti filaments using a Santos meat grinder. The spaghetti filaments were manually spread on trays and then placed in a dryer at 35°C for approximately 18 h. After drying, the spaghetti filaments were broken into small pellets and stored at 4°C until use.

The proximate composition of the experimental diets, including crude protein, lipids, ash, and dry matter, was analyzed following the procedures of the Association of Official Analytical Chemists (AOAC). The samples were dried to constant weight at 105°C for 24 h to determine the moisture content and calculate the dry matter. Crude protein (total nitrogen x 6.25) was determined using the micro-Kjeldahl method (Kjeltec System 1002 distillation unit, Tecator, Hoeganaes, Sweden). Lipids were extracted using the Soxhlet method, and ash content was determined by incinerating the samples in a muffle furnace at 550°C for 6 h. Results were expressed as a percentage of dry matter (% DM) (Table 1).

Table 1
Proximate composition of the different experimental diets (in % DM)

Experiment	Diet	Dry matter	Ash	Protein	Lipid	Fiber
A	0% G. senegalensis	92.5	10.3	32.6	9.0	4.7
	1% G. senegalensis	88.7	10.0	30.7	8.8	4.8
	2% G. senegalensis	92.2	10.3	31.8	9.4	5.8
	4% G. senegalensis	92.7	10.2	31.5	9.2	5.5
	8% G. senegalensis	89.7	9.8	29.8	8.8	6.1
B	0% P. odorata	92.9	10.3	32.8	9.1	4.7
	0.5% P. odorata	92.8	10.1	32.5	9.0	4.7
	1% P. odorata	92.5	10.3	32.6	9.2	4.9
	2% P. odorata	92.6	10.2	31.8	9.3	5.1
	4% P. odorata	92.5	10.2	31.6	9.4	5.4
C	0% P. reticulatum	94.0	10.3	33.6	9.0	4.7
	1% P. reticulatum	94.3	10.3	34.0	9.2	5.3
	2% P. reticulatum	93.4	10.3	32.9	9.0	5.2
D	0% Mixture	94.0	10.3	33.6	9.0	4.7
	1% Mixture	94.0	10.3	31.4	9.3	5.7
	2% Mixture	93.9	10.3	31.2	9.3	6.0

Fish rearing and sampling

The four experiments were conducted at the approved aquatic experimental platform PLATAX of the Institut des Sciences de l'Evolution de Montpellier (ISEM) c/o IRSTEA, located in Montpellier, France (14°38'46.4"N 3°52'28.2"E). The experiments were conducted in compliance with the Guidelines of the European Union Council (2010/63/EU) on the protection of animals used for scientific purposes and received approval from the French Ministry of Higher Education and Research (project authorization APAFIS#28283-2020112412125734 v2). Juveniles of Nile tilapia were distributed in 250-l aquariums connected to a recirculation aquaculture system with mechanical and biological filters, following a randomized design. Each diet was tested in triplicate for 28 days. The temperature of the water tanks was maintained at 28 ± 1°C using a 2-kW heater and a temperature controller. Physico-chemical parameters, including temperature, oxygen, and pH, were monitored and recorded twice a day for each aquarium.

Every 15 days, the weights of the fish were measured to adjust the feed ration and assess growth and feed efficiencies. The specific rearing conditions for each of the experiments are summarized in Table 2.

Table 2
Rearing conditions of the four experiments (*n* = 3)

Experiment	Number of fish	Initial wet Weight per fish (g)	Water temperature (°C)	Dissolved oxygen (mg/l)	pH
A	450	12.90 ± 0.40	26.7 ± 0.3	4.1 ± 0.1	8.0 ± 0.1
B	525	15.79 ± 0.36	27.9 ± 0.1	3.8 ± 0.3	8.4 ± 0.1
C	315	22.23 ± 0.59	26.7 ± 0.5	4.1 ± 0.2	8.0 ± 0.1
D	315	22.23 ± 0.59	26.7 ± 0.5	4.1 ± 0.2	8.0 ± 0.1

Fish were manually fed at a rate of 3% of their biomass twice daily, at 09:00 a.m. and 04:00 p.m., throughout the duration of the experiment. The aquarium bottoms were cleaned daily in the morning prior to feeding and the water volume was readjusted as necessary.

At the end of the 28-day feeding period, 6 fish were randomly selected from each aquarium and anaesthetized using eugenol (0.8 μL/L). Approximately 500 μL of blood was collected from the tail vein of each fish using a 1 mL heparinized syringe. Two blood samples per aquarium were immediately used for respiratory burst activity analysis, while the remaining 4 samples were rapidly centrifuged at 3000*g* at 4°C for 10 min to separate the plasma. The plasma from these samples was collected in Eppendorf tubes and stored at -20°C until further analyses of plasma lysozyme and bactericidal activities. For experiments C and D, two fish per aquarium were sampled for histological analysis of the digestive system. The liver and intestine were removed and fixed overnight in a 4% buffered formalin solution (pH 7.4).

Growth, survival, and feed efficiency

All fish were individually weighed every two weeks to determine the average weight gain (AWG, in g), the specific growth rate (SGR, in %/day), and the feed conversion rate (FCR). Survival rate (SR, in %) was calculated at the end of the 28-day feeding trial and after the infection challenge. The equations used to calculate each parameter were as follows:

$$\mathrm{AWG\,(g)} = \mathrm{Wf} - \mathrm{Wi}$$

,

$$\mathrm{SGR}\left(\frac{\%}{\mathrm{day}}\right) = \frac{\ln \mathrm{Wf} - \ln \mathrm{Wi}}{\ln \mathrm{WiT}} * 100,$$

$$\mathrm{FCR} = \frac{\mathrm{Totalweightofthefeedgiven\,(g)}}{\mathrm{Fishwetgain\,(g)}},$$

$$\mathrm{SR} = \frac{\mathrm{Numberoffinalfish}}{\mathrm{Numberofinitialfish}}*100,$$

where Wf and Wi are the average final and initial weights, respectively, and T is time in days.

Immunological analyses

Respiratory Burst Activity

The production of oxygen radicals by blood phagocytes during respiratory burst activity was measured using the colorimetric test with nitroblue tetrazolium (NBT). Fresh heparinized blood was used for the NBT assay, in which a 0.2% NBT solution (N5514, Sigma, France) was added. The mixture was then incubated for 30 min at 25°C. Following incubation, 50 µL of this mixture was combined with 1 mL of N, N-dimethyl (D455, Merck, France) in a glass tube and centrifuged at 3000*g* for 5 min. The optical density of the supernatant was measured at 540 nm using a DR 3900 spectrophotometer (Hach, Colorado USA). The values were expressed as mg NBT to formazan/ml.

Bactericidal Activity of Plasma

The bactericidal activity of plasma was assessed using the *Aeromonas hydrophila* strain ATCC 35654 according to Leaño et al. (2003). Equal volumes of plasma (50 µL) and the bacterial suspension (10^4 CFU/mL) were mixed and incubated at 30°C for 1 h. A positive control was prepared by substituting the plasma with sterile Phosphate-buffered saline (PBS). After incubation, the mixture was diluted with Tryptic Soy Broth (TSB) (22092, Sigma, France) at a ratio of 1:10. Then, 100 µL of this mixture was plated onto Tryptic Soy Agar (TSA) and incubated at 30°C for 24 h. The bactericidal activity was determined as follows:

$$\mathrm{Bactericidalactivity\,(\%)} = \left(1 - \frac{\mathrm{Testcount}}{\mathrm{C+count}}\right)*100$$

,

where C + is positive control count.

Plasma Lysozyme Activity

Plasma lysozyme activity was determined by turbidometric assay according to Caruso & Lazard (1999), with a minor modification. Instead of NaH_2PO_4, PBS at pH 6.2 was used. Approximately 25 µL of plasma were added to 175 µL of *Micrococcus lysodeikticus* (M3770, Sigma, France) suspended in PBS pH 6.2. Two absorbance measurements were performed at 450 nm using an Elisa reader (ELx808, Biotek, USA), one immediately after mixing and the other after 4.5 min. One unit of lysozyme activity was defined as the amount of plasma lysozyme causing a decrease in absorbance of 0.001/min at 450 nm.

Infection challenge

The *Aeromonas hydrophila* strain (Reference: 8581) used in this study was provided by the “Laboratoire Vétérinaire de Hérault” (Montpellier, France) and was originally isolated from a diseased carp (*Cyprinus carpio*). The strain was cultured at 30°C for 24 h in TSB (22092 500, Sigma) to achieve a final concentration of 7.10^8 CFU/mL. At the end of the 28-day feeding period of the 4 trials, 22 (trial A) and 25 individuals per aquarium (trials B, C, and D) were anaesthetized with eugenol (0.8 µL/L) and then subjected to an intramuscular injection on the left side of their bodies. Each fish received 0.1 mL (trials A and B) or 0.2 mL (trials C and D) of *A. hydrophila* at a concentration of 5.10^7 CFU/mL (trial A), 4.10^9 CFU/mL (trial B) or 5.10^8 CFU/mL (trials C and D). Different doses of the bacterium were tested in a preliminary experiment to determine the lethal dose (LD50) for the bacterial challenge. As negative controls, at least one individual per aquarium received 0.1 or 0.2 mL of sterile TSB (n = 20). The fish were fed the same diets during the challenge period (12 days). Mortality was monitored twice daily.

At the end of the infection challenge, two fish per aquarium were also sampled in trials C and D for histological analysis of the digestive system. The liver and intestine were removed and fixed overnight in a 4% buffered formalin solution (pH 7.4).

Histological analyses

The fixed livers and intestines were dehydrated with graded series of ethanol and embedded in paraffin using an automatic tissue processor (STP120, Myr, France). Paraffin blocks were prepared and cut into 3 µm sections using an automatic microtome (Leica, RM 2235RT, USA). The paraffin sections were kept at 40ºC overnight. Following this, the samples were deparaffinized using a graded series of xylene substitute and stained with hematoxylin and eosin for general morphological observations. The histological preparations were examined under a microscope equipped with a camera (Leica, DM6 B, Germany). The number of hepatocytes and lipid deposits in the liver, as well as the number of enterocytes and goblet cells in the intestine, were counted in 6 randomly chosen fields per specimen (50x50 µm and 100 µm per field, respectively). The length of the intestinal folds was measured in 6 randomly chosen fields per specimen (100 µm per field). Measurements on the histological slides were performed with the ImageJ software (Schneider et al., 2012), and the data were expressed as the mean ± S.D. Additionally, histopathological observations were conducted on specimens from experiment D.

Statistical analysis

The Shapiro-Wilk and Levene tests where initially conducted to check for normality distribution and homogeneity of variances, respectively. For AWG, FCR, SGR, SR, and histological analyses differences among regimes were analyzed using One-way ANOVA, followed by the Tukey test to determine significant differences between groups. Immunological analyses were assessed using the Kruskal-Wallis test to examine differences among the experimental regimes. Mortality rates between treatments were compared using the Chi-square analysis, while survival curves were analyzed using the Kaplan-Meier survival analysis. The Mann-Whitney U test was used to compare the interaction of hepatocyte, lipid

deposition, mucosal cell, and enterocyte numbers before and after infection. Statistical significance was determined at P values < 0.05.

Results

Feeding efficiency, growth, and survival

The feeding efficiency and growth of Nile tilapia juveniles from the four experiments are shown in Fig. 1. The inclusion of *G. senegalensis* up to 4% in the diet had no significant effect on FCR, growth, or survival of juvenile Nile tilapia at the end of the 28-day feeding trial. However, at 8% dietary inclusion level, there was a significant increase in FCR and a decrease in fish growth and SGR compared to the control group (Fig. 1A). Among the tested inclusion levels of *P. odorata*, AWG was not affected. The only significant differences were observed in FCR and SGR between the 1% and 4% inclusion levels, with the 4% inclusion level showing a higher FCR and lower SGR (Fig. 1B). The 2% *P. reticulatum* diet significantly increased FCR and decreased SGR and AWG compared to the control diet (Fig. 1C), while the mixture of plants significantly increased the FCR and decreased the SG and AWG in a dose-dependent manner (Fig. 1D). Survival rate was above 97% in all experiments and was not significantly affected by any of the tested diets (P > 0.05).

Immunological analyses

The immunological parameters of the fish from the four experiments are presented in Fig. 2. None of the dietary inclusion levels of *G. senegalensis* tested had a significant effect on the analyzed immunological parameters of the fish (Fig. 2A). Fish fed the 1, 2, and 4% *P. odorata* diets showed similar bactericidal activity of the plasma, which was higher compared to the groups fed the 0% and 0.5% *P. odorata* diets (Fig. 2B). Regarding the lysozyme activity of the plasma, a significant difference was observed between the fish fed the control diet and those fed the 4% *P. odorata* diet (Fig. 2B). However, there were no significant differences among the diets in terms of respiratory burst activity as assessed by NBT assay (Fig. 2B). The inclusion of *P. reticulatum* in the diet resulted in a significant dose-dependent increase in the bactericidal activity of the plasma. On the contrary, there were no significant differences among the diets in terms of plasma lysozyme and respiratory burst activities (Fig. 2C). For the plant-mixture diets, the bactericidal activity of the plasma significantly increased in a dose-dependent manner (P < 0.05, Fig. 2D). Plasma lysozyme activity increased significantly in the 1% mixture group compared to the control (P < 0.05, Fig. 2D). However, there were no significant differences among the diets in terms of respiratory burst activity (P > 0.05, Fig. 2D).

Infection challenge

In experiment A using *G. senegalensis*, the first mortalities occurred the day following the infection. The survival rates were 89% in the groups fed 2% *G. senegalensi*s, 92% in the groups fed 4 and 8% *G. senegalensi*s, 96% in the groups fed 0% *G. senegalensi*s and 99% in the groups fed 1% *G. senegalensi*s.

No mortalities were recorded after two days of infection in fish fed the 8% *G. senegalensis* diet, three days post-infection in the control, 1% and 2% *G. senegalensis* groups, and eight days post-infection in fish fed 2% *G. senegalensis*. However, these differences in survival among dietary groups were not statistically significant (Fig. 3A).

In experiment B using *P. odorata*, the control group displayed a tendency of higher mortality rate shortly after the infection, with survival decreasing from 91% on day 1 to 85% on day 3 post-infection, and then remaining stable. The survival rates of the groups fed diets containing *P. odorata* at 0.5, 1, 2, and 4% inclusion levels decreased from 99 to 93%, from 96 to 88%, from 95 to 85%, and from 97 to 92%, respectively, between day one and eight post-infection. However, no significant differences were found among the dietary treatments ($P > 0.05$, Fig. 3B).

In experiment C using *P. reticulatum*, the first mortalities were recorded on the first day after infection, and no mortalities were recorded beyond 6, 7, and 8 days post-infection in groups fed 2, 1 and 0% *P. reticulatum*, respectively. At the end of the infection challenge, the survival rates were 57% in the control and 1% *P. reticulatum* groups, and 67% in the 2% *P. reticulatum* group, although the differences were not statistically significant ($P > 0.05$, Fig. 3C). Some of the infected fish showed one or more typical signs of aeromonosis, including skin ulcers, reddening at the base of the fins, hemorrhages, discoloration, and abnormal swimming behavior.

After the infection challenge of experiment D, there were no significant differences ($P > 0.05$) in the survival rate among the dietary treatments after 12 days post-infection. The fish fed the 1% mixture, 2% mixture, and the control diets recorded survival rates of 69%, 63%, and 57%, respectively. The mortalities occurred during the first week after infection (Fig. 3D).

Histological analyses

The results of the histological analyses performed in experiments C and D are shown in Fig. 4. Following the 28-day feeding trials with *P. reticulatum* and the plant mixtures, none of the evaluated histological features were significantly affected by the medicinal plants ($P > 0.05$, Figs. 4B, D), except for the 2% *P. reticulatum* dietary group, which exhibited a lower number of hepatic lipid deposits compared to the control group ($P < 0.05$, Fig. 4A). After the infection challenge, the groups fed the control and 1% *P. reticulatum* diets showed a decreased number of hepatic lipid deposits (Fig. 4C). For the rest of the histological features, there were no significant differences between the pre- and post-infection challenges in any of the dietary groups tested ($P > 0.05$, Figs. 4A, C). After the infection challenge in experiment D, histological sections of the liver from fish fed the control diet showed congestion of leukocytes, hepatocyte degeneration, a decreased number of hepatocytes, increased lipid deposits, and degeneration of blood vessels (Fig. 5A). Fish fed the 1 and 2% mixture diets displayed lesions including vessel degeneration, congestion, and degenerated hepatocytes (Fig. 5B, C). Sections of the intestines of the control group revealed mucosal cell degeneration, leukocyte infiltration, degeneration of the intestinal epithelium, leukocyte congestion, and edema. In contrast, the groups fed the 1 and 2% mixture exhibited healthier intestinal mucosa with some signs of edema and mucosal cell degeneration (Fig. 5A', B', C').

Discussion

The present study showed that survival during the 28-day feeding period was not influenced by any of the tested medicinal plants compared to the control. A meta-analysis focusing on the treatment durations of plant-enriched diets ranging from 1 to 16 weeks and their effects on various fish parameters reported that 4 weeks was the most common duration. It also revealed that administration for relatively short periods (e.g. 2–4 weeks) was as effective as longer supplementation periods (≥ 8 weeks) (Reverter et al., 2020). The effects on growth varied depending on the plant species, dietary inclusion level, and whether the plants were included individually or in combination. Inclusion levels of *G. senegalensis* and *P. odorata* up to 4%, as well as *P. reticulatum* up to 1% had no negative effect on growth. However, higher inclusion levels of these plants, or their combination at 1 or 2% inclusion levels, led to increased FCR and decreased SGR and growth. These findings suggest that the combination of the three plants reduced the nutritional quality of the feed, potentially due to the overall higher inclusion levels of medicinal plants (3 and 6%, respectively), interactions between components of the three plants, and/or increased presence of antinutritional factors (Lech & Reigh, 2012; Ali et al., 2016). Anti-nutritional factors, such as phytate, reduce mineral bioavailability and protein digestibility by forming phytic acid-protein complexes that inhibit nutrient absorption (Francis et al., 2001). Studies conducted on Nile tilapia with other medicinal plants at various inclusion levels, such as *Lagenaria siceraria* seed powder up to 1%, *Salvadora persica* or *Centella asiatica* leaf powders up to 2%, *Portulaca oleracea* leaf powder up to 3%, *Moringa oleifera* leaf powder up to 5%, or a combination of *Stachytarpheta jamaicensis* and *Garcinia kola* at 3.5% inclusion levels each, have also shown no significant impact on growth parameters such as weight gain or SGR (Ekanem et al., 2017; Abdel-Razek et al., 2019; Lebda et al., 2019; Abd El-Gawad et al., 2020; Srichaiyo et al., 2020; Radwan et al., 2022). Reduced growth at higher inclusion levels of several medicinal plants has been also reported in Nile tilapia and other fish species, likely associated with the increased content of phytochemicals (Dada & Ikuerowo, 2009; Yilmaz et al., 2014; Kapinga et al., 2018; Nafiqoh et al., 2020; Adeniyi et al., 2021). However, the literature indicates that powdered plants remain the most used material for fish feed supplementation due to their low costs, higher accessibility, easy use, and their relative safety compared to other extracts, such as ethanolic and methanolic extracts or essential oils (Reverter et al., 2020).

The immune response of the fish exhibited variations depending on the specific medicinal plant and level of dietary inclusion, which can be attributed to the varying amount and nature of active compounds present in each plant. In fish, lysozyme acts as a first line of defense against bacteria and is considered an important biomarker of the immune system (Mahmoud et al., 2017). It catalyzes the β-1,4 linked glycoside bonds found in both Gram-positive and Gram-negative bacterial cell wall peptidoglycans, leading to the breakdown and lysis of the bacterial cell wall. Among the three tested medicinal plants, only *P. odorata* stimulated lysozyme activity in the fish when included at a 4% dietary level. This stimulatory effect was also observed in fish fed the combination of the three plants, most likely attributed to the presence of *P. odorata*. Similarly, in Nile tilapia, juveniles of similar weight exhibited significantly improved lysozyme activity when fed licorice (*Glycyrrhiza glabra*) root powder at dietary inclusion levels ranging from 0.5 to 2% (Abdel-Tawwab & El-Araby, 2021). Licorice root powder also increased respiratory

burst activity in fish at the same inclusion levels (Abdel-Tawwab & El-Araby, 2021). Conversely, none of the three medicinal plants tested in the present study influenced the respiratory burst activity. The response of respiratory burst activity has been shown to vary depending on factors such as the type and level of medicinal plant dietary inclusion, fish species, and development stage (Basha et al., 2013; Adeshina et al., 2017; Nafiqoh et al., 2020). Plasma bactericidal activity is a lysin mechanism known for its role in killing and eliminating pathogenic organisms in fish (Ellis, 2001). Similar to lysozyme activity, plasma bactericidal activity increased in fish fed *P. odorata* at all tested inclusion levels and with the mixture of the three plants, indicating that these dietary supplements enhanced the humoral immune elements in the serum. The enhancement of bactericidal activity observed in this study has been previously reported with other plants and fish species (Abdel-Tawwab et al., 2010; Awad & Austin, 2010; Mohamad & Abasali et al., 2010; Talpur & Ikhwanuddin, 2013; Ngugi et al., 2015).

The histology of the digestive system is a reliable marker for assessing the nutritional condition in fish and the adequacy of experimental diets (Castro-Ruiz et al., 2021; 2022). Additionally, histopathological examination is widely employed to study the pathological alterations caused by chemicals or biological infectious agents (Camargo & Martinez, 2007). It is desirable that medicinal plants have the property to increase growth performance, biochemical parameters, and immunological responses in fish without adversely affecting the gastrointestinal tract at histological level (Yilmaz et al., 2014; Nofouzi et al., 2017; Abdel Rahman et al., 2019). In the present study, none of the histological features analyzed in the liver and intestine of the fish were affected by the tested medicinal plants, except for the 2% inclusion level of *P. reticulatum*, which showed a decrease in the number of hepatic lipid deposits compared to the control group.

Research indicates that flavonoid-rich plant extracts can modulate fat deposition in rats and fish by affecting energy and lipid metabolism (Song et al., 2016; Kim et al., 2017; Yao et al., 2020). Specifically, in wild grass carp (*Ctenopharyngodon idellus*), Lotus leaf extract has been found to reduce fat accumulation in liver and muscle by inhibiting fatty acid synthesis and promoting lipid breakdown and export in a dose-dependent manner (Yao et al., 2020). Given the significant phenolic content of *P. reticulatum* (Boualam et al., 2021), it is suggested that it may inhibit liver lipid accumulation in fish at a 2% dietary inclusion level. Regarding the infection challenge, none of the tested diets showed a favorable effect on the survival rate after infection compared to the control group. Similar results have been reported in Nile tilapia juveniles fed up to 3% inclusion levels of *Brazilian propolis* for 30 days and subsequently infected with *A. hydrophila* for 15 days (Orsi et al., 2017), as well as in juvenile *Clarias gariepinus* fed *P. betle*, *Psidium guajava*, and *Tithonia diversifolia* at 8% inclusion level each (Nafiqoh et al., 2020). Nonetheless, the histological results evidenced the observed bactericidal effect of the medicinal plants. Intracellular infectious bacteria depend on host lipid deposits to acquire nutrients and lipids for immune evasion (Libbing et al., 2019). Following infection, the control group exhibited fusion of hepatic lipid deposits due to the hepatocyte degeneration, most likely caused by the bacteria. The control group also showed hyperplasia of mucosal cells, along with degeneration of the epithelium, leukocyte infiltration, and atrophy enterocytes upon infection with *A. hydrophila*. Similar histopathological changes in the liver of Nile tilapia following *A. hydrophila* infection have been reported previously (Abu-Elala et al.,

2016). In contrast, the groups fed the mixture diets, especially at 1% inclusion, mitigated intestinal and hepatic damage, showing healthier intestines and livers with reduced cell swelling and normal appearance of the lipid deposits compared to the control group.

Conclusion

This study evaluated for the first time the effects of dietary supplementation with *G. senegalensis*, *P. odorata*, and *P. reticulatum*, either individually or in combination, on the growth, immunity, and survival of juvenile Nile tilapia after a 28-day feeding trial followed by a 12-day infection challenge with *A. hydrophila*. The results showed that growth, FCR, and SGR were not negatively affected compared with the control, except for 8% *G. senegalensis*, 2% *P. reticulatum*, and the 1% and 2% mixture dietary groups, which displayed higher FCR and lower growth and SGR than the control group. With the exception of *G. senegalensis*, plasma bactericidal activity was improved in a dose-dependent manner by supplementation with *P. odorata*, *P. reticulatum*, and the plant mixture. Plasma lysozyme activity was enhanced with 4% *P. odorata* and the 1% mixture. NBT assay results and survival rates after infection were not affected by plant supplementation. The diet containing the 1% mixture demonstrated better protection of the intestinal mucosa after infection compared with the control. These results highlight the potential benefits of incorporating 4% *P. odorata* and the 1% mixture of plants into the diet of juvenile Nile tilapia to improve their immune response and defense against bacterial infection.

Declarations

Author Contribution

Conceptualization: J.F., M.J.D., D.C., M.D., S.S.; Data curation: P.M.N.; Formal Analysis: P.M.N., S.S.; Investigation: P.M.N., M.C., E.P., S.H., S.K.L.F.; Methodology: P.M.N., J.F., M.J.D., D.C., M.D., S.S.; Project administration: S.S.; Resources: M.J.D., S.S.; Supervision: J.F., M.J.D., S.S.; Validation: P.M.N., S.S.; Visualization: P.M.N.; Writing – original draft: P.M.N.; Writing – review & editing: J.F., M.J.D., D.C., M.D., S.S.

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Figures

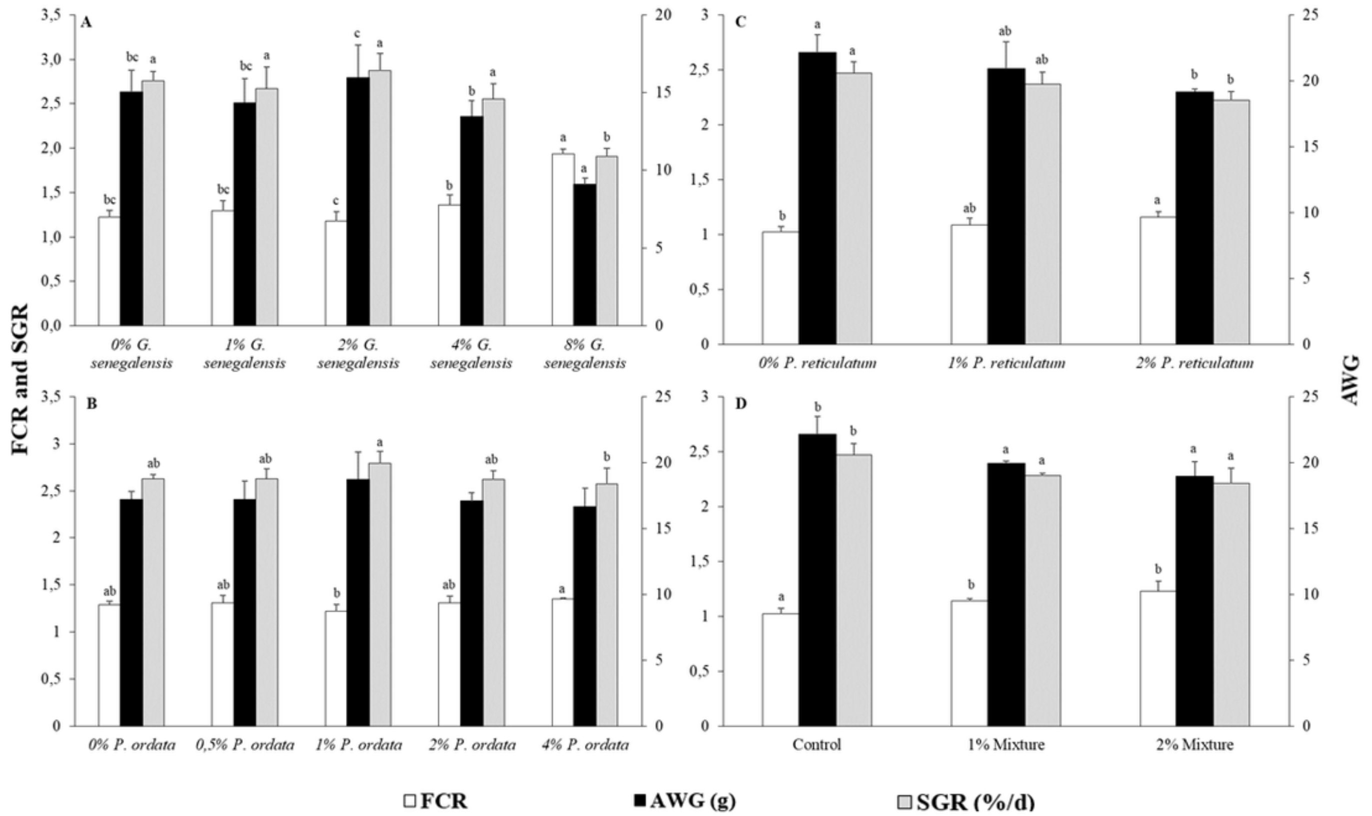

Figure 1

Growth and feed efficiency parameters of Nile tilapia juveniles fed for 28 days on the different experimental diets (*n=3*). **A)** Groups fed 0, 1, 2, 3, 4, and 8% *G. senegalensis* dietary inclusions; **B)** Groups fed 0, 0.5, 1, 2, and 4% *P. odorata* dietary inclusions; **C)** Groups fed 0, 1, and 2% *P. reticulatum* dietary inclusions; **D)** Groups fed 0, 1, and 2% mixture dietary inclusions. Different letters indicate statistically significant differences among dietary groups (P < 0.05). FCR, feed conversion ratio; AWG, average weight gain; SGR, specific growth rate

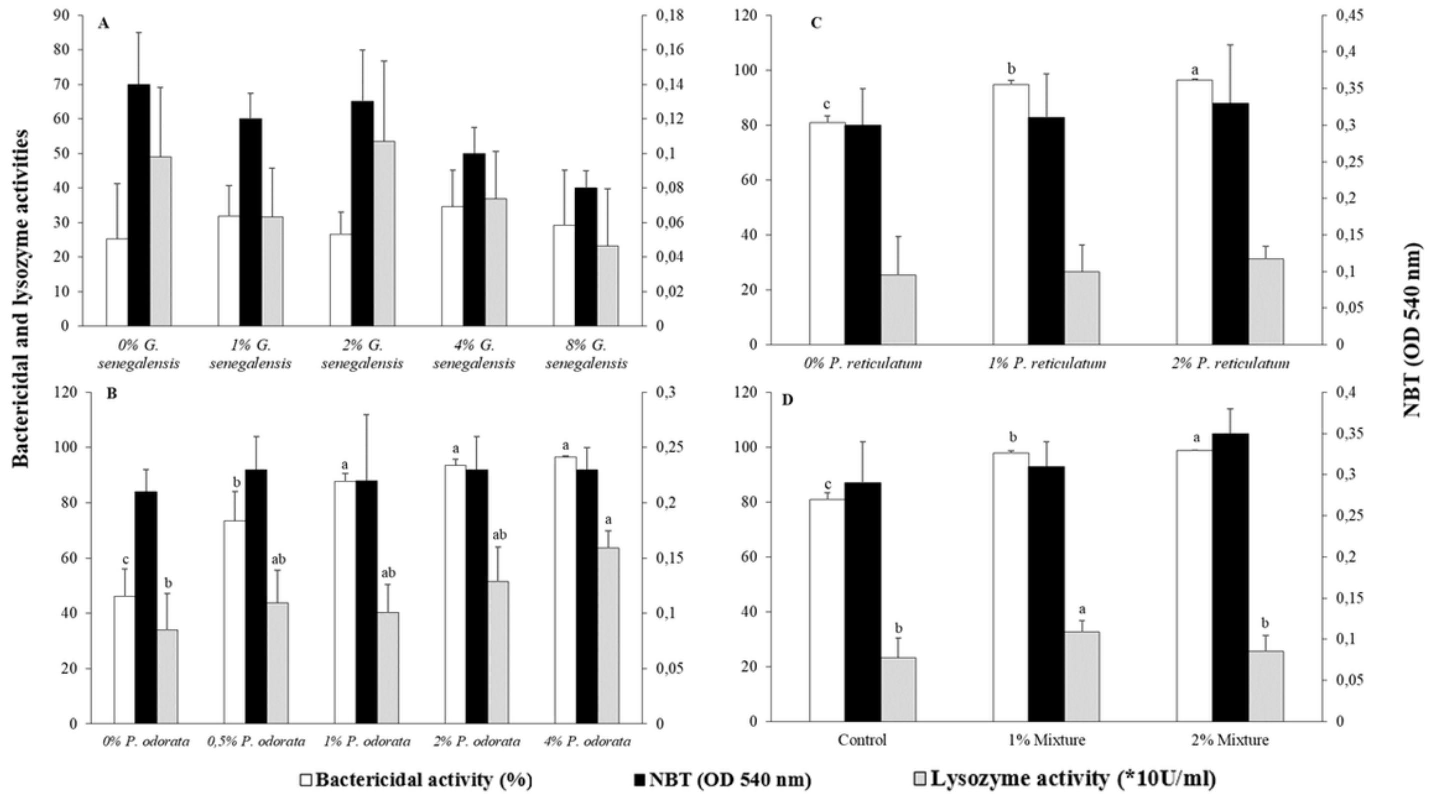

Figure 2

Bactericidal and lysozyme activities of plasma and NBT activity of Nile tilapia juveniles fed for 28 days fed on the different experimental diets (*n*=6). **A)** Groups fed 0, 1, 2, 3, 4, and 8% *G. senegalensis* dietary inclusions; **B)** Groups fed 0, 0.5, 1, 2, and 4% *P. odorata* dietary inclusions; **C)** Groups fed 0, 1, and 2% *P. reticulatum* dietary inclusions; **D)** Groups fed 0, 1, and 2% mixture dietary inclusions. Different letters indicate statistically significant differences among dietary groups (P<0.05)

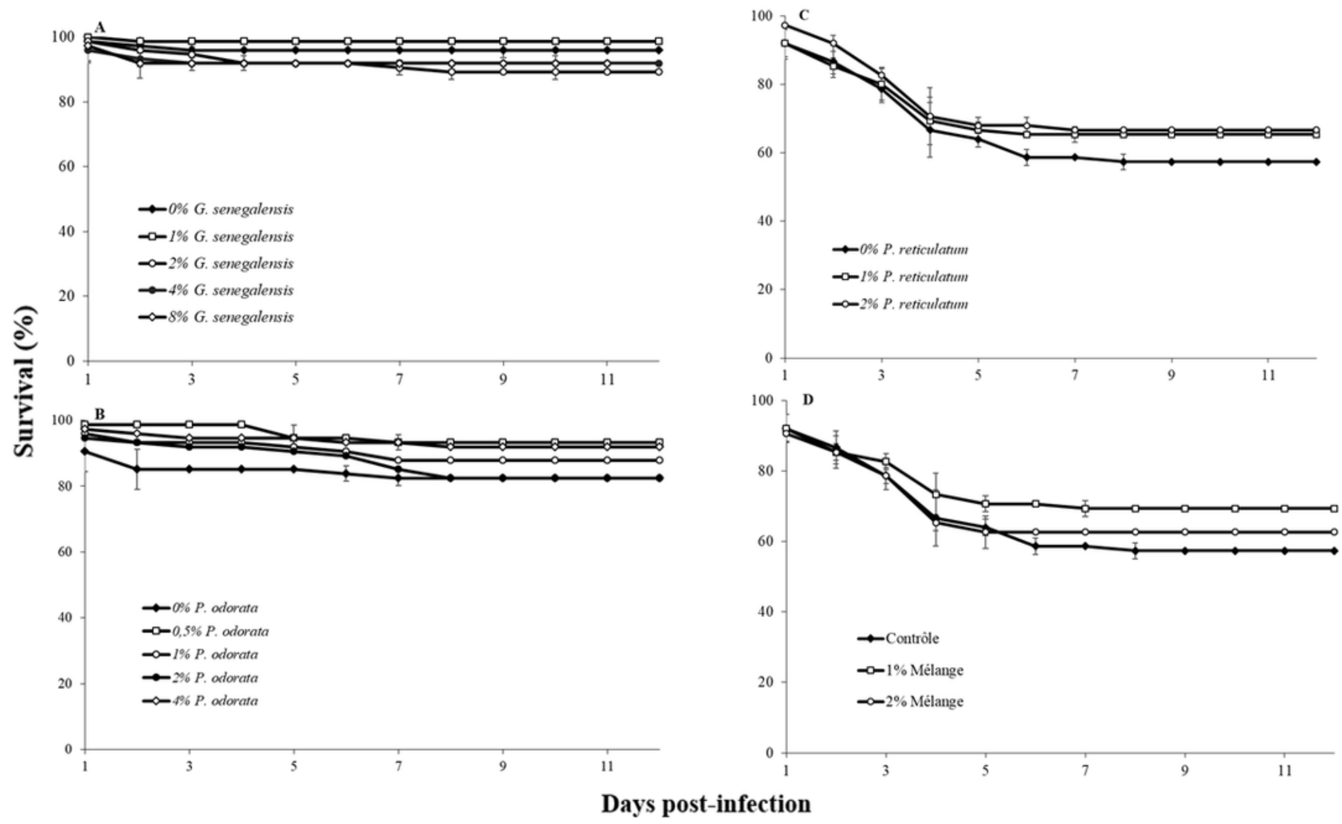

Figure 3

Survival rate 12 days post-infection with *Aeromonas hydrophila* of Nile tilapia fed during 28 days with different experimental diets A, B, C, D (*n*=3)

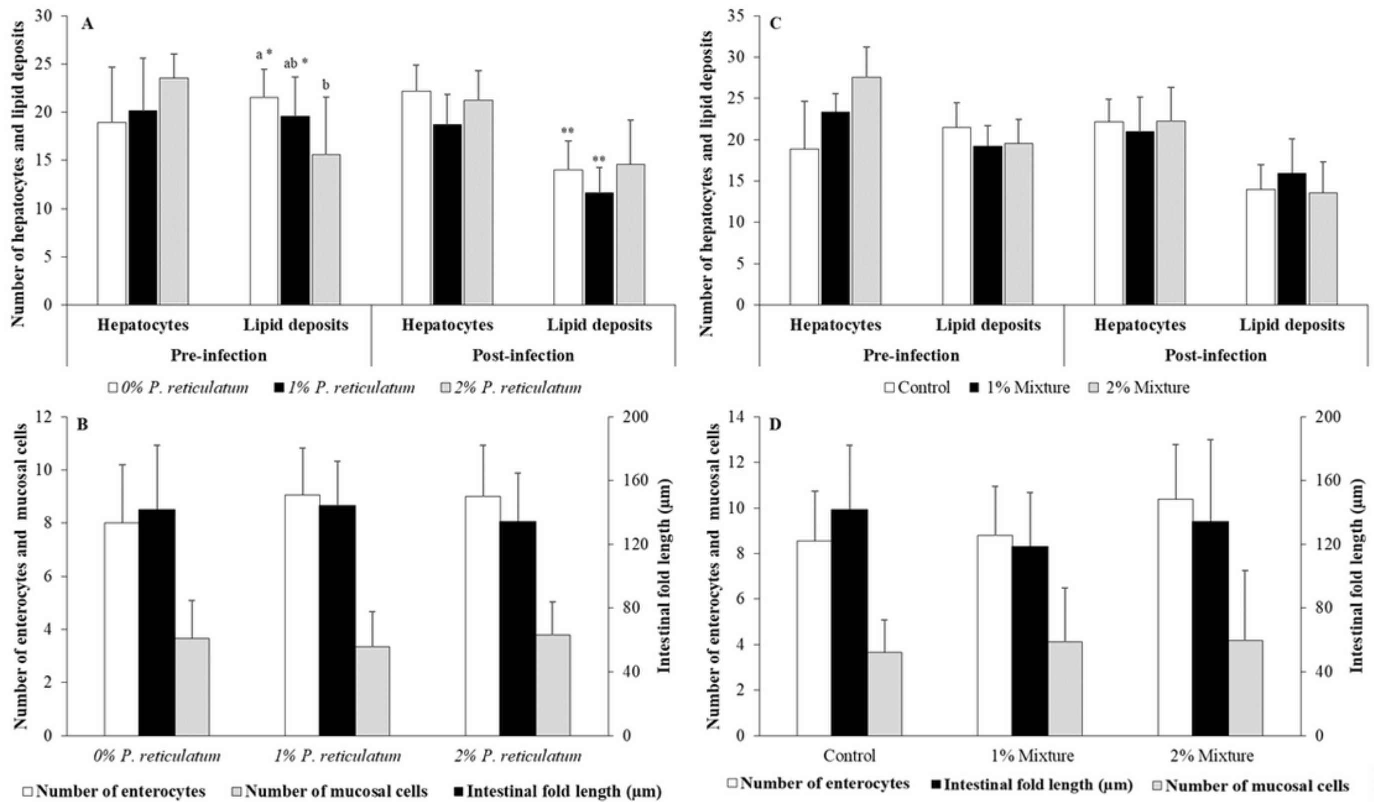

Figure 4

Histological analyses performed in the liver and intestine of Nile tilapia juveniles fed for 28 days with different experimental diets (*n*=6). **A**) Number of hepatocytes and hepatic lipid deposits of fish after being fed 0, 1, and 2% *P. reticulatum* diets for 28 days and at 12 days post-infection. **B**) Number of enterocytes and mucous cells and length of intestinal folds after 28 day-feeding period with 0, 1, and 2% *P. reticulatum* diets. **C**) Number of hepatocytes and hepatic lipid deposits of fish after being fed the 0, 1, and 2% mixture diets for 28 days and at 12 days post-infection. **D**) Number of enterocytes and mucous cells and length of intestinal folds after a 28 day-feeding period with 0, 1, and 2% mixture diets. Different letters indicate differences statistically significant among experimental groups and different asterisks indicate differences in the histological features before and after the infection challenge within a dietary group (P < 0.05)

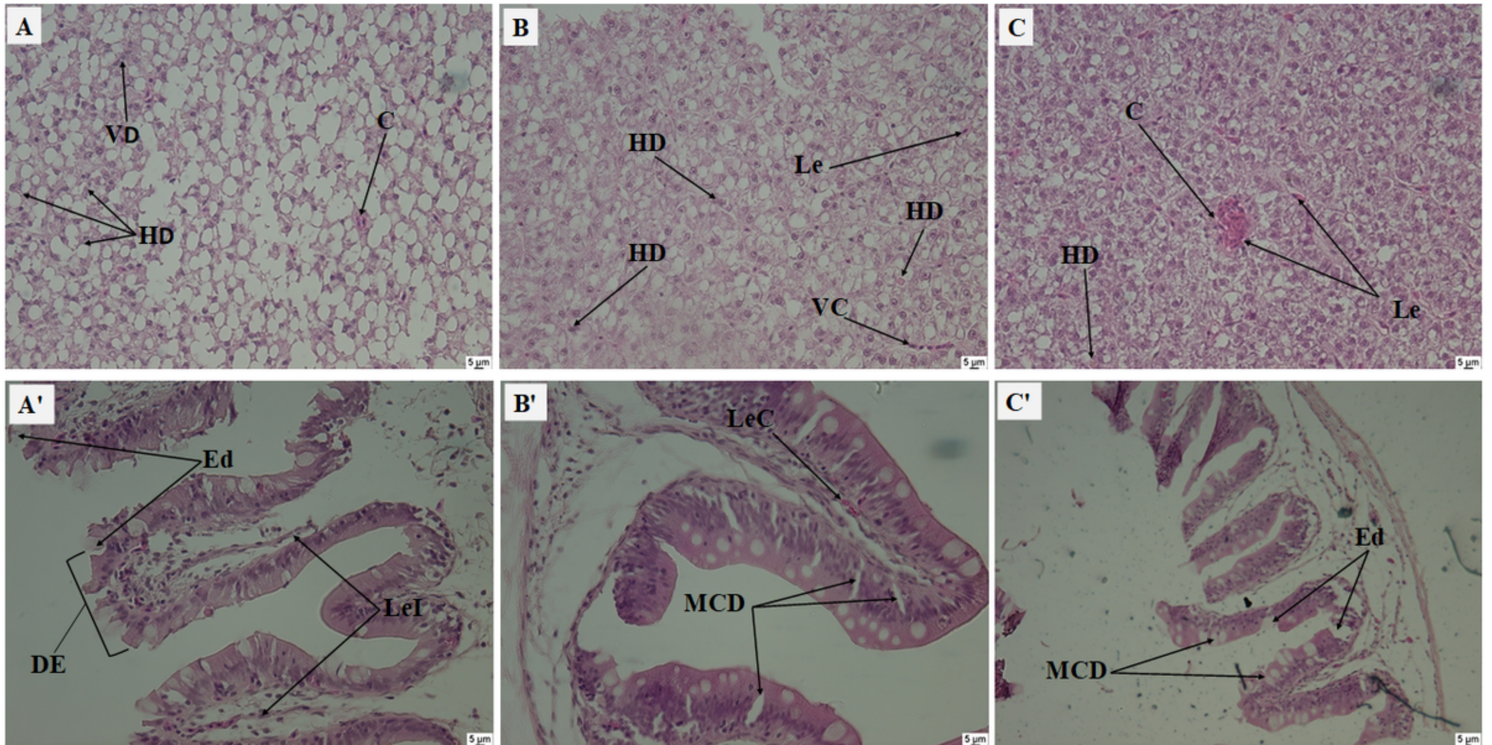

Figure 5

Histological sections of livers (A, B, C) and intestines (A', B', C') from juvenile Nile tilapia fed 3 different diets for 28 days and infected with *A. hydrophila*for 12 days. **A**) Control group, **B**) 1% mixture group, and **C**) 2% mixture group (H & E staining, x40). VD, vessel degeneration; C, congestion; DH, hepatocyte degeneration; Le, leucocyte; MCD, mucosal cell degeneration; LeI, leukocyte infiltration; DE, degeneration of the intestinal epithelium; LeC, leukocyte congestion; Ed, edema