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Dietary copaiba oil and propolis improve innate immunity, intestinal integrity and resistance to streptococcosis in *Oreochromis niloticus*

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Abstract

Copaiba oil and propolis are natural compounds with recognized immunostimulatory and antimicrobial properties and have emerged as promising functional feed additives for sustainable aquaculture. This study evaluated the effects of dietary supplementation with copaiba oil and propolis, alone or in combination, on innate immunity, intestinal morphology, and resistance to *Streptococcus agalactiae* in Nile tilapia. A total of 270 juveniles (27.0 ± 1.5 g) were randomly distributed into nine dietary treatments, each consisting of three replicate tanks with 10 fish per tank and fed experimental diets for 12 weeks. Leukocyte respiratory burst activity and serum lysozyme activity were significantly enhanced after nine weeks of supplementation, particularly in the 1OC2PROP group, whereas complement activity remained unchanged. Dietary supplementation also improved intestinal morphology, with increased villus height and goblet cell density, especially in fish receiving propolis-containing diets. Following challenge with *S. agalactiae*, the non-supplemented control group showed 100% cumulative mortality (no survivors), whereas cumulative mortality decreased to 50% in fish fed the 1OC2PROP diet. Relative percent survival ranged from 20% to 50%, with the highest protection observed in fish receiving combined supplementation with 1% copaiba oil and 2% propolis. The protection observed together with the stimulation of innate immune responses and improvements in intestinal morphology suggests that these phytogenic compounds promote a multifaceted host response that enhances resistance to streptococcosis. No severe hepatic histopathological alterations were observed, indicating good tolerability of the tested supplementation levels and supporting their application as sustainable functional feed additives for Nile tilapia culture.

1. Introduction

Aquaculture has expanded rapidly worldwide and currently represents an important source of animal protein for human consumption (FAO, 2026). Among farmed fish species, Nile tilapia (*Oreochromis niloticus*) stands out due to its fast growth, efficient feed utilization, adaptability to intensive production systems, and broad market acceptance (El-Sayed 2006; Ng and Romano 2013; FAO 2026). However, production intensification has increased exposure to stressors such as high stocking densities, handling, and water-quality fluctuations, impairing physiological homeostasis, immune competence, and disease resistance (Moraes and Martins 2004; Zimmerman et al. 2004). Under these conditions, bacterial diseases remain a major constraint, with *Streptococcus agalactiae* among the most important pathogens affecting Nile tilapia, causing high morbidity, mortality, and economic losses in intensive farming systems (Abdallah et al. 2024).

Traditionally, bacterial diseases in aquaculture have been controlled through the use of antibiotics and chemotherapeutic agents. However, the indiscriminate use of these compounds has raised serious concerns regarding the development of antimicrobial resistance, environmental contamination, and risks to animal and human health (Sørum 2006); Santos and Ramos 2018; Kovalakova et al. 2020). As a result, the search for sustainable and environmentally friendly alternatives to antibiotics has become a priority in modern aquaculture.

In this context, functional feeds containing immunomodulatory compounds have gained increasing attention as sustainable strategies to enhance fish health, innate immune responses, physiological resilience, and disease resistance, without the drawbacks associated with chemotherapeutic agents (Gatlin 2002; Moraes and Moraes 2009; Chagas et al. 2014; Acunha et al. 2023). Among these compounds, plant-derived products and natural extracts such as propolis (Dotta et al. 2015; Alrashada et al. 2023) and copaiba oil (Mello et al. 2024) have been widely investigated due to their bioactive properties and low environmental impact. Propolis, a resinous substance produced by bees from plant exudates, is rich in phenolic compounds and flavonoids and exhibits antimicrobial, antioxidant, and immunomodulatory activities, with evidence of improved hematological parameters, immune responses, and resistance to stressors and pathogens in Nile tilapia (Dotta et al. 2015; Alrashada et al. 2023). Similarly, copaiba oil, obtained from species of the genus *Copaifera*, has been traditionally used for its anti-inflammatory and antimicrobial properties, largely attributed to sesquiterpenes such as β-caryophyllene (Gertsch et al. 2008; Pieri 2009; Mello et al. 2024).

Understanding the potential additive or synergistic effects of these natural compounds is essential for supporting their use as functional feed additives in sustainable aquaculture systems. Therefore, the present study aimed to evaluate the effects of dietary supplementation with copaiba oil and propolis, alone or in combination, on hematological and innate immune responses, intestinal histomorphometry, and resistance to *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*), providing insight into their potential application as sustainable health-promoting feed additives in aquaculture.

2. Materials and Methods

2.1 *Experimental fish and treatments*

A total of 270 juvenile Nile tilapia (*Oreochromis niloticus*; 27.0 ± 1.5 g / 13.0 ± 0.8 cm) were obtained from a commercial fish farm in the Jaboticabal region, São Paulo, Brazil. Fish were acclimated for 15 days in fiberglass tanks supplied with continuous-flow artesian well water and supplemental aeration. During acclimation and throughout the experimental period, fish were fed a commercial pelleted diet containing 36% crude protein and 4,000 kcal kg^{-1} gross energy, offered twice daily at 3% of total biomass. Biometric evaluations were performed every three weeks, and feed allowance was subsequently adjusted based on the updated tank biomass to maintain a feeding rate equivalent to 3% of body weight throughout the experiment. The study was conducted in accordance with Brazilian animal welfare standards established by the National Council for the Control of Animal Experimentation (CONCEA). It was approved by the Institutional Ethics Committee on Animal Use of São Paulo State University (UNESP) under protocol no. 0004509/23.

Following acclimation, all fish were individually weighed and measured and then randomly assigned to 27 fiberglass tanks (250 L) at a density of 10 fish per tank. The experiment followed a completely randomized design consisting of nine dietary treatments, each with three replicate tanks (n = 30 fish per treatment). Fish were allocated to ensure similar initial biomass among experimental units and were fed their respective experimental diets for 12 weeks. Each tank was considered an independent experimental unit for all feeding, immunological, histological, and health-related analyses. The dietary treatments

consisted of a non-supplemented control group and eight supplemented groups receiving copaiba oil, propolis extract, or their combinations, as follows: control (CTL); 0.5% copaiba oil (0.5OC); 1.0% copaiba oil (1.0OC); 1% propolis (1PROP); 2% propolis (2PROP); 0.5% copaiba oil + 1% propolis (0.5OC1PROP); 0.5% copaiba oil + 2% propolis (0.5OC2PROP); 1.0% copaiba oil + 1% propolis (1.0OC1PROP); and 1.0% copaiba oil + 2% propolis (1.0OC2PROP). Supplement concentrations were calculated based on the percentage of dry weight inclusion in the diet.

The experimental diets were prepared using a commercial pelleted feed as the basal matrix and supplemented with copaiba oil obtained as an oleoresin from *Copaifera duckei* (Amazonian origin) and an alcoholic green propolis extract derived from *Baccharis dracunculifolia* (Table 1). Copaiba oil concentrations were selected based on preliminary palatability trials, which indicated that inclusion levels above 1.0% reduced feed intake. The additives were incorporated into the diets (Skov 2012), with modifications, and homogeneously mixed to ensure uniform distribution. All diets were prepared weekly and stored under refrigeration at 4°C until use (Mello 2024).

Water quality parameters were monitored regularly using a multiparameter probe (Horiba U-50) and remained within the optimal range for Nile tilapia throughout the trial: dissolved oxygen, 6.20 ± 0.35 mg L^{-1}; temperature, 28.87 ± 0.42°C; pH, 7.51 ± 0.12; and electrical conductivity, 158.20 ± 5.40 µS cm^{-1}.

2.2 *In vitro antibacterial assay*

An *in vitro* antibacterial assay was performed to evaluate the bactericidal activity of the combined use of copaiba oil and propolis against *Streptococcus agalactiae* and *Aeromonas hydrophila* using a previously described disk diffusion method, with adaptations (Abdollahzadeh et al. 2014). Bacterial suspensions were prepared in brain heart infusion broth and standardized by spectrophotometry at 625 nm.

Aliquots of 100 µL of each bacterial suspension were spread on brain heart infusion agar plates. Sterile paper disks (6 mm diameter) were placed on the agar surface and impregnated with 8 or 10 µL of copaiba oil, propolis extract, or their combination. Oxytetracycline disks (30 µg) were used as a positive control, and sterile disks without additives served as a negative control. Plates were incubated at 28°C for 24–72 h, and inhibition zones were measured using a digital caliper. The degree of bacterial inhibition was classified as absent (–), mild (+), partial (++), or total (+++). All assays were performed in triplicate.

2.3 *Hematological and Innate Immune Analyses*

At weeks 9 and 12, five fish per treatment were randomly selected across the three replicate tanks for blood sampling by caudal vein puncture (3.0 mL blood per fish). Following blood collection, the fish were euthanized for subsequent tissue sampling and histopathological analyses. An aliquot (2.0 mL) containing 10% EDTA was used for hemogram and quantification of reactive oxygen species (ROS). The remaining aliquot was centrifuged at 1,200 × g for 5 min at 4°C to obtain serum, which was stored at − 80°C until analysis (Claudiano et al. 2019).

2.3.1 Hematological analyses

A whole-blood aliquot was used to determine hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV = Ht × 10 / RBC), mean corpuscular hemoglobin (MCH = Hb × 10 / RBC), and mean corpuscular hemoglobin concentration (MCHC = Hb × 100 / Ht), as described by (Tavares-Dias et al. 1999). Erythrocyte counts (RBC) were determined using the indirect method (Witeska 2022). Leukocyte and thrombocyte counts were also determined indirectly from blood smears stained with a rapid panoptic kit, according to previously established methodologies (Tavares-Dias et al 1999; Witeska 2022).

2.3.2 Leukocyte respiratory burst, serum lysozyme, and alternative complement pathway activity (hemolytic assay)

Leukocyte respiratory burst activity was determined using a nitroblue tetrazolium (NBT) reduction assay, which quantifies the production of reactive oxygen species (ROS) by blood leukocytes through a turbidimetric method (Marzocchi-Machado et al. 1999; Castro et al. 2014). Serum lysozyme activity was determined by a turbidimetric assay based on the lysis of *Micrococcus lysodeikticus*, following previously described methodologies with minor adaptations (Marzocchi-Machado et al. 1999; Castro et al. 2014).

Alternative complement pathway activity was determined by a hemolytic assay. Serum samples were serially diluted in 8 mM TEA-EDTA buffer containing 2 mM Mg^{2+} and 0.1% gelatin (1:2.3, 1:3, 1:4, 1:5, 1:9, 1:12.3, and 1:19). Subsequently, 40 µL of red blood cell suspension was added to each dilution and absorbance was measured at 700 nm using a SpectraMax M5 Microplate Reader® after incubation at 37°C. Heat-inactivated serum (56°C for 30 min) was used as the negative control. Complement activity was expressed as the change in absorbance (ΔOD = initial absorbance − final absorbance) (Claudiano et al. 2019).

2.4 Intestinal histomorphometry and histopathological analyses

Intestine, liver, spleen, and kidney samples were collected and fixed in buffered formalin for 24 h, transferred to 70% ethanol, dehydrated through a graded ethanol series, embedded in paraffin, sectioned at 5 µm, and processed according to standard histological procedures. Sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (BX51, Olympus, Tokyo, Japan) equipped with a digital camera (DP72, Olympus). Periodic acid–Schiff (PAS) staining was used to evaluate hepatic glycogen and neutral polysaccharide content in liver sections, whereas Alcian Blue (pH 2.5) staining was used to identify acidic mucosubstances and goblet cells in the intestinal mucosa (Suvarna et al. 2013). Tissue measurements and cell counts were performed using CellSens Standard 1.5 software (Olympus). For intestinal morphometry, a portion of the proximal intestine (3-cm segment distal to the pylorus) was processed for histological evaluation. Ten well-oriented villi were measured per fish, and the mean value was used for statistical analysis (Mello et al., 2013).

2.5 Determination of lethal dose (LD_{50}) and Bacterial challenge

To determine the median lethal dose (LD_{50}–96 h) of *S. agalactiae*, four fiberglass tanks (250 L) containing 10 fish each (average weight of 80 g) were used. Fish were inoculated intraperitoneally with bacterial suspensions at concentrations of 10^6, 10^7, and 10^8 CFU mL^{-1}, while the control group received sterile phosphate-buffered saline (PBS). Mortality was recorded daily for 96 h, and the LD_{50} was calculated based on cumulative mortality data (Marcusso et al. 2021).

After 12 weeks of dietary supplementation, 20 fish from each treatment were randomly selected and equally distributed into two challenge tanks (10 fish per tank), resulting in two replicate tanks per group. Fish were then inoculated intraperitoneally with *S. agalactiae* (5.03×10^7 CFU mL^{-1}) at a dose of 1 mL per 100 g of body weight. A control group was inoculated with sterile PBS only. Fish were monitored every 12 h for morbidity and mortality until no further deaths were observed.

2.6 Statistical analysis

Data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. Parametric data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test when significant differences were detected. Non-parametric data were analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparison test. Statistical significance was set at $P < 0.05$. Survival after the infectious challenge was analyzed using the Kaplan–Meier method, and survival curves were compared using the log-rank (Mantel–Cox) test. Relative percent survival (RPS) was calculated as: RPS (%) = [1 − (% mortality in the treated group / % mortality in the challenged control group)] × 100. All analyses were performed using RStudio software.

3. Results

3.1 *In vitro antibacterial activity*

The *in vitro* antibacterial assay demonstrated inhibition of both *Aeromonas hydrophila* and *Streptococcus agalactiae* by copaiba oil and propolis (Fig. 1). Mean inhibition zones of approximately 1.5 ± 1.0 cm for *A. hydrophila* and 1.3 ± 0.2 cm for *S. agalactiae* were observed, with consistently greater inhibition against *S. agalactiae*. As shown in Fig. 1A-B, the antibacterial activity was classified according to inhibition intensity. Based on these results, *S. agalactiae* was selected for the *in vivo* challenge test (Fig. 1C).

3.2 *Hematological and Innate Immune Responses*

No changes were observed in the erythrogram and differential leukocyte counts of fish supplemented for 12 weeks with diets containing copaiba oil, propolis extract, or their combinations (Table 2). However, total leukocyte counts increased significantly in fish fed the 0.5OC and 0.5OC1PROP diets compared to the control group (Table 2; $P < 0.05$).

Respiratory burst activity of leukocytes differed significantly among treatments at week 9, with fish fed the diet containing 1.0% copaiba oil combined with 2% propolis (1OC2PROP) exhibiting the highest activity ($p < 0.05$; Fig. 2A). However, in week 12, no significant differences were detected among treatments, suggesting normalization of respiratory burst activity over time ($p > 0.05$; Fig. 2B).

Similarly, serum lysozyme activity showed significant differences among treatments at week 9 ($P = 0.0183$), indicating a transient stimulation of innate immune responses during the initial phase of dietary supplementation (Fig. 2C). Higher mean values were observed in fish fed diets containing propolis alone or in combination with copaiba oil, particularly in the 1OC2PROP treatment ($p < 0.05$). However, no significant differences were detected at week 12 ($P > 0.05$; Fig. 2D). Serum complement activity did not differ significantly among treatments at either sampling time ($P > 0.05$, Fig. 2E–F).

3.3 *Histopathological evaluation and Intestinal histomorphometry*

Histopathological evaluation of liver samples revealed similar levels of hepatic steatosis in both the control and supplemented groups, with mild cytoplasmic vacuolation being similarly distributed among treatments (Fig. 3A). No evident differences in hepatic architecture were observed among treatments, and hepatocytes maintained normal morphology and organization. In addition, periodic acid–Schiff (PAS) staining demonstrated a normal distribution of glycogen within hepatocytes, indicating that the tested dietary treatments did not induce detectable metabolic disturbances or hepatotoxic effects (Fig. 3B).

At week 9, fish receiving diets containing propolis, particularly in synergistic combination with copaiba oil, showed numerically greater villus height and a higher number of goblet cells compared with the control group (Table 3 and Figs. 3C – D). Villus height (VH) and villus width (VW) measurements are illustrated in Fig. 3C, while goblet cells are indicated by arrows in Fig. 3D. This trend persisted at week 12, when fish fed the 1PROP and 1OC2PROP diets exhibited the highest goblet cell counts, with significant increases relative to the control group (Table 3; $P < 0.05$). Villus width showed only minor variation among treatments, whereas villus height was consistently greater in treatments containing propolis, either alone or in combination with copaiba oil, without evident morphological alterations.

3.4 *Streptococcosis*

Infection was confirmed by positive blood culture in all *Streptococcus agalactiae*-inoculated fish, with the non-supplemented challenged control group exhibiting 100% cumulative mortality, whereas the PBS-injected unchallenged group maintained 100% survival (Fig. 4). Dietary supplementation significantly enhanced resistance against the bacterial challenge, conferring biologically relevant protection with relative percent survival (RPS) values of 20% for the 0.5OC and 1PROP groups, 25% for the 1OC and 0.5OC2PROP groups, 30% for the 2PROP and 0.5OC1PROP groups, and 35% for the 1OC1PROP group. Notably, fish fed the 1OC2PROP diet achieved the highest survival rate (50%), which was significantly greater than that of the non-supplemented challenged control group ($P < 0.05$), thereby confirming the efficacy of the treatments in mitigating streptococcosis (Fig. 4).

4.Discussion

The present study demonstrates that dietary supplementation with propolis, particularly when combined with copaiba oil, promoted an integrated improvement in host health by simultaneously enhancing innate immune responses, preserving intestinal integrity, and increasing resistance to *Streptococcus agalactiae*. Rather than acting on a single physiological process, the observed responses indicate that these phytogenic additives modulated multiple defense mechanisms that ultimately contributed to greater resilience against bacterial infection. Similar integrated responses have been reported for functional feed additives, whose benefits are generally expressed through coordinated improvements in immune competence, intestinal function, and disease resistance rather than through uniform changes in all physiological parameters (Panserat et al. 2009; de la Cruz-Cervantes et al. 2018; Hernandez-Contreras et al. 2023). Collectively, these findings reinforce the concept that phytogenic compounds promote fish health through multifactorial mechanisms involving immunomodulation, reinforcement of the intestinal barrier, and indirect regulation of the intestinal microbial environment by their antimicrobial bioactive constituents (Panserat et al. 2009; de la Cruz-Cervantes et al. 2018; Hernandez-Contreras et al. 2023; Mello et al., 2024).

One of the most relevant findings of the present study was the superior protective effect observed in fish receiving the combined supplementation of copaiba oil and propolis, particularly the 1OC2PROP diet, which resulted in the highest relative percent survival following the *S. agalactiae* challenge. This response suggests that the two phytogenic compounds did not act independently but rather promoted complementary physiological mechanisms that collectively enhanced host resistance. Although the precise interactions between these additives remain to be elucidated, propolis is known to contain phenolic compounds with antioxidant, immunomodulatory, and mucosal protective properties, whereas copaiba oil is rich in terpenes with recognized anti-inflammatory and antimicrobial activities (Mello et al. 2024). Therefore, their combined supplementation may have strengthened both systemic immune competence and local intestinal defenses while simultaneously reducing bacterial pressure through the antimicrobial activity of their bioactive constituents. Similar improvements in disease resistance following dietary supplementation with functional feed additives have been reported even in the absence of marked hematological changes (Ibrahem et al. 2010), and enhanced immune responses have also been described in Nile tilapia supplemented with *Uncaria tomentosa* (Yunis-Aguinaga et al. 2015). Together, these observations reinforce the concept that phytogenic additives exert pleiotropic effects by simultaneously modulating immune function, intestinal integrity, and resistance to bacterial infections (Chakraborty and Hancz 2011; Firmino et al. 2021; Salomón et al. 2021).

The enhancement of leukocyte respiratory burst activity and serum lysozyme activity observed after nine weeks of dietary supplementation indicates that copaiba oil and propolis stimulated key components of the innate immune system in Nile tilapia. Rather than representing isolated immunological responses, these parameters reflect complementary defense mechanisms that participate in the early recognition and elimination of invading pathogens. Activated phagocytes generate reactive oxygen species capable of destroying microorganisms through the respiratory burst (Secombes and Fletcher 1992; Abdallah et al. 2024), whereas lysozyme contributes to bacterial clearance by hydrolyzing peptidoglycans in the cell wall, particularly of Gram-positive bacteria such as *Streptococcus agalactiae* (Saurabh and Sahoo 2008). The simultaneous stimulation of both responses suggests that dietary supplementation increased the capacity of fish to mount an efficient early antimicrobial response, which may have contributed to the greater resistance observed following bacterial challenge. Similar modulation of innate immunity has been reported in fish receiving functional dietary immunostimulants (Amar et al. 2001; Acunha et al. 2023; Magnadóttir 2006; Uribe et al. 2011).

The transient nature of these immunological responses is particularly noteworthy. Although respiratory burst and lysozyme activities returned to baseline levels after 12 weeks of supplementation, fish remained more resistant to *S. agalactiae*, indicating that continuous immune activation was not required to maintain the protective effects. Instead, the early stimulation of innate immunity, together with the antimicrobial properties of copaiba oil and propolis and improvements in intestinal morphology, likely provided sufficient physiological conditioning to enhance host resilience during the subsequent bacterial challenge. Similar associations between transient immune activation and increased resistance to *S. agalactiae* have been reported in Nile tilapia receiving phytogenic immunostimulants (Yunis-Aguinaga et al. 2015). This temporal pattern also supports the concept that dietary immunomodulators promote immune preparedness rather than chronic stimulation. In vertebrates, immune activation is normally followed by regulatory mechanisms that restore homeostasis while preserving the capacity to respond efficiently to future infectious challenges (Tort 2011). Therefore, the normalization of respiratory burst and lysozyme activities should be interpreted as evidence of physiological adaptation and immune balance rather than loss of biological efficacy.

The stable complement activity observed throughout the experimental period further reinforces the hypothesis that dietary supplementation selectively modulated specific components of innate immunity rather than inducing generalized immune activation. Although the complement system represents an essential humoral defense mechanism (Harpf et al. 2020), its responsiveness to dietary immunomodulators is highly dependent on the nature of the additive, supplementation period, and host physiological status (Magnoni et al. 2017). In the present study, the absence of changes in complement activity, despite the stimulation of respiratory burst, lysozyme activity, and enhanced disease resistance, suggests that copaiba oil and propolis promoted a balanced immunomodulatory response by activating defense mechanisms directly involved in pathogen elimination while preserving overall immune homeostasis. Such selective modulation is particularly desirable for long-term dietary supplementation because it may reduce the risk of unnecessary immune activation and its associated energetic costs.

Immunomodulators incorporated into fish diets frequently induce a short-term enhancement of innate immune parameters, followed by immune homeostasis as the organism adapts to prolonged exposure (Acunha et al. 2023). Similar observations have been reported in rainbow trout fed carotenoid-supplemented diets, which exhibited increased complement and lysozyme activities without sustained changes in other immune indices (Amar et al. 2001). Dietary β-glucan supplementation has also been shown to enhance immune responses in fish in a time-dependent manner (Matos et al. 2025). Likewise, dietary supplementation with propolis and *Aloe barbadensis* did not induce significant hematological alterations in Nile tilapia despite the observed antiparasitic efficacy (Dotta et al. 2015). Respiratory burst activity has been identified as a sensitive functional marker of innate immune activation during the early immune response in *Oreochromis niloticus* (Abdallah et al. 2024). Collectively, these findings reinforce the hypothesis that natural immunomodulators

and phytogenic extracts can promote beneficial biological effects and adaptive immunomodulation without inducing systemic, hematological, or immune overstimulation, a response that is particularly desirable in long-term feeding trials.

Regarding intestinal morphology, supplementation with propolis, either alone or in combination with copaiba oil, increased villus height and goblet cell density, indicating improved intestinal integrity and mucosal protection, corroborating the findings obtained with other functional compounds, such as dietary mannanoligosaccharides, in the same species (Schwarz et al. 2010; Muzzolon et al. 2021). Similarly, increased villus length and enhanced immune activity have been observed in fish supplemented with *Uncaria tomentosa* (Yunis-Aguinaga et al. 2015).

These intestinal improvements observed in treatments containing propolis may be associated with the antioxidant and mucosal protective effects of its phenolic compounds, which support epithelial integrity, innate immune responses, and immune modulation (Muzzolon et al. 2021). Combined with the anti-inflammatory properties of copaiba oil terpenes (Mello 2024), these effects likely explain the enhanced innate immunity and the superior protection observed in the 1OC2PROP group (RPS = 50%), which surpassed all isolated treatments (RPS = 20–35%). This pattern suggests additive or synergistic interactions between the bioactive compounds of copaiba oil and propolis. Similar levels of protection have been reported for other phytogenic additives, including dietary ginger (*Zingiber officinale*), which achieved RPS values of 46.7–61.6% against *Aeromonas hydrophila* (Şahan et al., 2016), and phytogenic blends that markedly improved survival following *Lactococcus garvieae* challenge in rainbow trout (*Oncorhynchus mykiss*) (Yilmaz et al., 2024). Collectively, these findings support the biological relevance of the protective effects observed in the present study.

The superior protection observed in the 1OC2PROP group highlights the potential of combining phytogenic compounds to enhance host resilience against bacterial diseases. Although injectable vaccines may achieve survival rates approaching 100% under homologous challenge conditions (Marcusso et al., 2021; Coaguila-Dávila et al., 2024), vaccine efficacy can vary considerably according to the pathogen, antigenic matching, and formulation. For example, bacterin-based vaccines against *A. hydrophila* have yielded RPS values ranging from only 25.61% to 37.05%, whereas formulations associated with oral boosters reached 74.20% (Pereira et al., 2015). Similarly, a formalin-inactivated bacterin administered to pacu (*Piaractus mesopotamicus*) resulted in an RPS of 31.33% (Vaz Farias et al., 2020). In contrast, phytogenic additives provide a continuously acting and multifaceted prophylactic effect by stimulating innate immunity, reinforcing intestinal barrier function, and exerting antimicrobial activity.

In addition, the diets demonstrated clinical safety, as evidenced by the absence of relevant clinical, behavioral, and hematological alterations, as well as the absence of severe hepatic lesions in fish fed diets supplemented with copaiba oil and propolis, indicating that the tested concentrations were well tolerated. Similar findings have been reported in Nile tilapia supplemented with *Uncaria tomentosa*, in which no histological alterations were detected in vital organs (Yunis-Aguinaga et al. 2015). Likewise, the absence of deleterious hematological and tissue effects has been reported in tilapia supplemented with other natural extracts (Dotta et al. 2015). Taken together, the present findings demonstrate that copaiba oil and propolis improved immune responsiveness, intestinal morphology, and resistance to *S. agalactiae* without causing adverse hematological or histopathological effects, supporting their use as safe functional feed additives in Nile tilapia culture.

5. Conclusion

Overall, the present study demonstrates that dietary supplementation with copaiba oil and propolis promotes a coordinated physiological response characterized by immunomodulation, improved intestinal integrity, and enhanced resistance to streptococcosis in Nile tilapia. The combination of 1% copaiba oil and 2% propolis provided the greatest protection, achieving a relative percent survival of 50% without causing adverse hematological or histopathological effects. These findings indicate that the combined use of copaiba oil and propolis represents a safe, sustainable, and continuously acting prophylactic strategy to improve fish health and resilience in intensive aquaculture systems.

Declarations

FUNDING

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COMPETING INTERESTS

The authors declare that they have no relevant financial or non-financial interests to disclose.

Author Contribution

Janaina Graça de Oliveira Carvalho: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. Hugo Leandro dos Santos: Investigation, Data curation. João Pedro Silva Ramos: Investigation, Data curation. Cleni Mara Marzocchi Machado: Methodology, Supervision, Formal analysis. Gustavo da Silva Claudiano: Conceptualization, Methodology, Supervision, Formal analysis, Writing – review & editing, Co-supervision. Julieta Rodini Engracia de Moraes: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.All authors reviewed the manuscript, approved the final version, and agree to be accountable for all aspects of the work.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL

This study was conducted at the Faculty of Agricultural and Veterinary Sciences (FCAV), Department of Pathology, Reproduction and One Health (DPRSU), in accordance with the guidelines for the presentation of scientific works of São Paulo State University (2000) and the ethical principles for animal experimentation established by the Institutional Animal Care and Use Committee. The experimental procedures were approved by the Ethics Committee on Animal Experimentation under certificate number 0004509/23.

CLINICAL TRIAL NUMBER: NOT APPLICABLE.

References

1. Abdallah ESH, Metwally WGM, Abdel-Rahman MAM, Albano M, Mahmoud MM (2024) Streptococcus agalactiae infection in Nile tilapia (*Oreochromis niloticus*): a review. Biology 13:914
2. Abdollahzadeh E, Rezaei M, Hosseini H (2014) Antibacterial activity of plant essential oils and extracts: the role of thyme essential oil, nisin, and their combination to control *Listeria monocytogenes* inoculated in minced fish meat. Food Control 35:177–183
3. Acunha RMG, Sanches DS, Almeida RGS, Oliveira F, Soares MP, Davalo MRS et al (2023) The use of immunomodulators in fish feed: a review. Res Soc Dev 12:e11312440964
4. Alrashada YN et al (2023) Dietary propolis improves the growth performance, redox status, and immune response of Nile tilapia upon a cold-stress challenge. PLoS ONE 18:e0293727
5. Amar E, Kiron V, Satoh S, Watanabe T (2001) Influence of various dietary synthetic carotenoids on bio-defense mechanisms in rainbow trout (*Oncorhynchus mykiss*). Aquac Res 32:162–173
6. Castro MP, Claudiano GS, Petrillo TR, Shimada MT, Belo MAA, Marzocchi-Machado CM, Moraes JRE, Manrique GW, Moraes FR (2014) Acute aerocystitis in Nile tilapia bred in net cages and supplemented with chromium carbochelate and *Saccharomyces cerevisiae*. Fish Shellfish Immunol 36:284–290
7. Chagas E, Majolo C, Boijink C, Chaves F, Hashimoto G, Figueredo A, Martins ML (2014) Use of essential oils and extracts in the treatment of fish diseases. In: Maldi RR, Campos CM, Lizama MAP, Takemoto RM (eds) Pathology and health of aquatic environments. Editora Massoni, Maringá, p 269
8. Chakraborty SB, Hancz C (2011) Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in finfish culture. Rev Aquac 3:103–119
9. Claudiano GS et al (2019) Hematological and immune changes in *Piaractus mesopotamicus* in the sepsis induced by *Aeromonas hydrophila*. Fish Shellfish Immunol 88:259–265
10. Coaguila-Dávila J, Fernandez-Espinel C, Flores-Dominick V, Gonzalez-Callirgos L, Medina-Morillo M, Yunis-Aguinaga J (2024) Experimental vaccination against *Aeromonas hydrophila* in *Colossoma macropomum*: bacterial characterization, lethal doses, and mortality. Aquac Rep 36:102118. https://doi.org/10.1016/j.aqrep.2024.102118
11. de la Cruz-Cervantes JA, Benavides-González F, Sánchez-Martínez JG, Vázquez-Sauceda MDLL, Ruiz-Uribe AJ (2018) Propolis in aquaculture: a review of its potential. Rev Fish Sci Aquac 26:337–349
12. Dotta G, Brum A, Jerônimo GT, Maraschin M, Martins ML (2015) Effect of dietary supplementation with propolis and *Aloe barbadensis* extracts on hematological parameters and parasitism in Nile tilapia. Rev Bras Parasitol Vet 24:66–71
13. El-Sayed AFM (2006) Tilapia culture. CABI, Wallingford
14. FAO (2026) The State of World Fisheries and Aquaculture 2026 – Blue Transformation: turning vision into impact. Rome, FAO
15. Firmino JP, Vallejos-Vidal E, Balebona MC, Ramayo-Caldas Y, Cerezo IM, Salomón R, Tort L et al (2021) Diet, immunity, and microbiota interactions: an integrative analysis of the intestine transcriptional response and microbiota modulation in gilthead seabream (*Sparus aurata*) fed an essential oils-based functional diet. Front Immunol 12:625297
16. Gatlin DM (2002) Nutrition and fish health. In: Halver JE, Hardy RW (eds) Fish nutrition, 3rd edn. Academic, London, pp 671–702
17. Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ, Altmann KH et al (2008) β-Caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci U S A 105:9099–9104
18. Harpf V, Rambach G, Würzner R, Lass-Flörl C, Speth C (2020) Candida and complement: new aspects in an old battle. Front Immunol 11:1471
19. Hernández-Contreras Á, Teles A, Salas-Leiva JS, Chaves-Pozo E, Tovar-Ramírez D (2023) Feed additives in aquaculture. In: Arsenos G, Giannenas I (eds) Sustainable use of feed additives in livestock. Springer Nature Switzerland, Cham, pp 811–846
20. Ibrahem M, Fathi M, Mesalhy S, El-Aty AA (2010) Effect of dietary supplementation of inulin and vitamin C on the growth, hematology, innate immunity, and resistance of Nile tilapia (*Oreochromis niloticus*). Fish Shellfish Immunol 29:241–246
21. Kovalakova P, Cizmas L, McDonald TJ, Marsalek B, Feng M, Sharma VK (2020) Occurrence and toxicity of antibiotics in the aquatic environment: a review. Chemosphere 251:126351

22. Magnadóttir B (2006) Innate immunity of fish (overview). Fish Shellfish Immunol 20:137–151
23. Magnoni LJ et al (2017) Dietary supplementation of heat-treated Gracilaria and Ulva seaweeds enhanced acute hypoxia tolerance in gilthead seabream (*Sparus aurata*). Biol Open 6:897–908
24. Marcusso PF, Claudiano GS, Yunis-Aguinaga J, Marinho-Neto FA, Eto SF, Fernandes DC, Rosolem MC, Salvador R, Moraes JRE, Moraes FR (2021) Immunogenicity in Oreochromis niloticus vaccinated with sonicated antigens against streptococcosis. Fish Shellfish Immunol 115:134–141. https://doi.org/10.1016/j.fsi.2021.04.030
25. Marzocchi-Machado CM, Polizello ACM, Azzolini AECS, Lucisano-Valim YM (1999) The influence of antibody functional affinity on the effector functions involved in the clearance of circulating immune complexes anti-BSA IgG/BSA. Immunol Invest 28:89–101
26. Matos EJA, Nascimento CCO, Costa DCB, Silva MA, Corrêa CF, Hisano H, Boscolo WR, Furuya WM (2025) Inclusion of ora-pro-nóbis (*Pereskia aculeata*) leaf meal in diets for Nile tilapia: effects on growth performance, intestinal morphometry and economic viability. Aquac Nutr
27. Mello H, Moraes JRE, Niza IG, Moraes FR, Ozório ROA, Shimada MT, Engracia Filho JR, Claudiano GS (2013) Efeitos benéficos de probióticos no intestino de juvenis de Tilápia-do-Nilo. Pesq Vet Bras 33:724–730. https://doi.org/10.1590/S0100-736X2013000600011
28. Mello NP, Espinoza FCR, Claudiano GS, Yunis-Aguinaga J, Carvalho JGO, Silva JEA, Oliveira ECP, Moraes JRE (2024) Copaiba oil's bactericidal activity and its effects on health and zootechnical performance for Nile tilapia after oral supplementation. Sci Rep 14:17405
29. Moraes FR, Martins ML (2004) Predisposing conditions and main diseases of teleost fish in intensive aquaculture. In: Cyrino JEP, Urbinati EC, Fracalossi DM (eds) Special topics in intensive tropical freshwater aquaculture. TecArt, São Paulo, pp 343–383
30. Moraes FR, Moraes JRE (2009) Nutraceuticals in inflammation and wound healing of fish of zootechnical interest. In: Tavares-Dias M (ed) Management and health of cultured fish. Embrapa, Macapá, pp 625–723
31. Muzzolon A, Bicudo AJA, Oldoni TLC, Sado RY (2021) Dietary brown propolis extract modulated nonspecific immune system and intestinal morphology of pacu (*Piaractus mesopotamicus*). Braz Arch Biol Technol 64:e21200787
32. Ng WK, Romano N (2013) A review of the nutrition and feeding management of farmed tilapia throughout the culture cycle. Rev Aquac 5:220–254
33. Panserat S, Hortopan GA, Plagnes-Juan E, Kolditz C, Lansard M, Skiba-Cassy S, Esquerré D et al (2009) Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. Aquaculture 294:123–131
34. Pereira GV, Silva BC, Nascimento Vieira F, Seiffert WQ, Ushizima TT, Mouriño JLP, Martins ML (2015) Vaccination strategies with oral booster for surubim hybrid (*Pseudoplatystoma corruscans* × *P. reticulatum*) against haemorrhagic septicaemia. Aquac Res 46:1831–1841
35. Pieri FA, Mussi MC, Moreira MAS (2009) Copaiba oil (*Copaifera sp*.): history, extraction, industrial applications and medicinal properties. Rev Bras Plantas Med 11:465–472
36. Şahan A, Özütok S, Kurutaş EB (2016) Determination of some hematological parameters and antioxidant capacity in Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) fed ginger (*Zingiber officinale* Roscoe) and challenged with *Aeromonas hydrophila*. Turk J Fish Aquat Sci 16:197–204
37. Salomón R, Reyes-López FE, Tort L, Firmino JP, Sarasquete C, Ortiz-Delgado JB, Quintela JC et al (2021) Medicinal plant leaf extract from sage and lemon verbena promotes intestinal immunity and barrier function in gilthead seabream (*Sparus aurata*). Front Immunol 12:670279
38. Santos L, Ramos F (2018) Antimicrobial resistance in aquaculture: current knowledge and alternatives to tackle the problem. Int J Antimicrob Agents 52:135–143
39. Saurabh S, Sahoo PK (2008) Lysozyme: an important defence molecule of fish innate immune system. Aquac Res 39:223–239
40. Schwarz KK, Furuya WM, Natali MRM, Michelato M, Gualdezi MC (2010) Mannanoligossacaride in diets for juvenile Nile tilapia. Acta Sci Anim Sci 32:197–203
41. Secombes CJ, Fletcher TC (1992) The role of phagocytes in the protective mechanisms of fish. Annu Rev Fish Dis 2:53–71
42. Skov PV, Kjaer MA, Bruun SW, Rasmussen RS, Holm J (2012) Effects of dietary supplementation of oregano (*Origanum vulgare*) essential oil and probiotic *Pediococcus acidilactici* on growth, immune response, and fillet quality of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 350–353:124–129
43. Sørum H (2006) Antimicrobial drug resistance in fish pathogens. In: Aarestrup FM (ed) Antimicrobial resistance in bacteria of animal origin. ASM, Washington, DC, pp 213–238
44. Suvarna KS, Layton C, Bancroft JD (2013) Bancroft's theory and practice of histological techniques, 7th edn. Elsevier, Toronto
45. Tavares-Dias M, Martins ML, Silva ED, Moraes FR, Perecin D (1999) Hematologia de teleósteos brasileiros com infecção parasitária. I. Variáveis do *Leporinus macrocephalus* Garavello & Britski, 1988 (Anastomidae) e *Piaractus mesopotamicus* Holmberg, 1887 (Characidae). Acta Sci Biol Sci 21:337–342
46. Tort L (2011) Stress and immune modulation in fish. Dev Comp Immunol 35:1366–1375
47. Uribe C, Folch H, Enríquez R, Moran G (2011) Innate and adaptive immunity in teleost fish: a review. Vet Med 56:486–503
48. Vaz Farias TH, Arijo S, Medina A, Pala G, Prado EDR, Montassier HJ, Pilarski F, Belo MAA (2020) Immune responses induced by inactivated vaccine against *Aeromonas hydrophila* in pacu, *Piaractus mesopotamicus*. Fish Shellfish Immunol 101:186–191. https://doi.org/10.1016/j.fsi.2020.03.059
49. Witeska M, Kondera E, Ługowska K, Bojarski B (2022) Hematological methods in fish: not only for beginners. Aquaculture 547:737498
50. Yilmaz S, Ergün S, Yilmaz E, Ahmadifar E, Yousefi M, Abdel-Latif HMR (2024) Effects of a phytogenic diet on growth, haemato-immunological parameters, expression of immune- and stress-related genes, and resistance of *Oncorhynchus mykiss* to *Lactococcus garvieae* infection. Aquaculture 587:740845. https://doi.org/10.1016/j.aquaculture.2024.740845
51. Yunis-Aguinaga J, Claudiano GS, Marcusso PF, Manrique WG, Moraes JRE, Moraes FR, Fernandes JB (2015) *Uncaria tomentosa* increases growth and immune activity in *Oreochromis niloticus* challenged with *Streptococcus agalactiae*. Fish Shellfish Immunol 47:630–638

52. Zimmerman S, Fitzsimmons K (2004) Intensive tilapia farming. In: Cyrino JEP, Urbinati EC, Fracalossi DM (eds) Special topics in intensive tropical freshwater aquaculture. TecArt, São Paulo, pp 239–266

Tables

Table 1 – Ingredient composition and proximate nutritional composition of the basal commercial diet.

Ingredients %	
Soybean meal	17.11
Soy protein concentrate (60% CP)	14.11
Poultry by-product meal	18.00
Ground corn	18.58
Wheat bran	9.00
Rice bran	8.00
Meat meal	6.69
Proteinose	3.45
Dicalcium phosphate	0.75
Premix	0.60
Soybean oil	3.00
Antifungal (Filax)	0.20
Salt	0.30
DL-Methionine	0.17
Antioxidant (Banox)	0.05
Calculated chemical composition *(%)	
Mixture	9.36
Crude protein	36
Total lipid	9.18
Fiber	3.02
Starch	25
Mineral matter	8.97
Calcium	2.36
Match	1.4
Digestible energy (kcal.kg $^{-1}$)	4,000.00
Digestible protein	35.97
Total lysine	2.7
Total methionine + cysteine	0.95
Tryptophone	0.39
Threonine	1.35

Table 2. Hematological parameters and differential leukocyte counts of Nile tilapia (*Oreochromis niloticus*) after 12 weeks of dietary supplementation with copaiba oil and propolis.

	CTL	0.5OC	1OC	1PROP	2PROP	0.5OC1PROP	0.5OC2PROP
Erythrocytes(10^6/mL)	2.68±1.41	3.25±0.67	2.45±1.02	2.58±0.89	3.03±0.72	2.56±0.34	2.74±0.52
Hb(g/dL)	8.50±3.37	9.38±4.33	9.85±2.82	9.00±3.40	11.00±3.00	8.81±1.45	11.62±3.35
VCM(fL)	107.51±19.56	84.65±7.99	119.26±18.20	122.86±23.87	115.86±23.51	107.80±19.17	118.56±21.04
HCM(pg/cel)	31.78±5.90	27.21±13.29	37.65±18.45	33.05±12.21	36.71±10.80	31.16±5.77	44.07±12.28
CHCM(%)	34.18±10.04	31.88±15.19	33.38±12.35	29.08±9.89	29.90±4.20	29.18±5.13	35.06±12.01
Leukocytes(mL)	121837±34878	268991±34901*	190094±91660	191250±74831	233820±67150	208158±52618	186561±40289
Neutrophil(%)	25.5±5.1	20.9±5.3	17.8±2.7	19.4±2.5	17.5±9.7	13.9±8.3	19.6±8.9
Lymphocyte(%)	36.2±0.5	50.3±1.3	46.3±2.2	51.4±9.1	42.7±8.0	47.6±6.6	24.5±9.1
Monocyte(%)	20.2±2.1	19.6±0.3	31.3±5.5	25.4±2.3	26±8.3	28.4±8.2	19.5±7.9
Eosinophil (%)	8.8±5.7	3.7±3.6	1.5±1.3	1.2±0.7	5.8±3.4	3.5±3.3	6.2±1.2
Plasma Cell(%)	7.4±7.9	5.8±3.4	2.9±2.7	8.3±4.6	6.5±7.2	5.9±4.7	7.9±2.0

(1) F: test statistic. p: critical values. Different * in the same column indicate significant differences among treatments (P < 0.05).Hb: hemoglobin concentrat
corpuscular volume (fL); MCH: mean corpuscular hemoglobin (pg cell^{-1}); MCHC: mean corpuscular hemoglobin concentration (%).

Table 3. Intestinal histomorphometry of Nile tilapia supplemented with copaiba oil and propolis for 9 and 12 weeks.

	9 weeks								
	CTL	0.5OC	1OC	1PROP	2PROP	0.5OC1PROP	0.5OC2PROP	1OC1PROP	1OC2PROP
Villus length (µm)	237.6±47.5	202.9±47.1	228.5±50.5	309.9±28.8	245.8±58.3	353.5±51.3	285.8±41.8	298.1±39.6	330.2±54.8
Villus width (µm)	46.3±11.7	45.4±9,71	38.4±15.2	53.5±11.6	51.8±15.4	52.4±11.5	53.5±5.9	49.4±11.3	54.05±9.1
Number of Goblet cells	52.1±15.4	68.3±18.5	66.3±15.7	79.5±121	54.1±14.2	58.7±15.8	66.9±14.8	71.7±14.5	75.9±11.8
	12 weeks								
	CTL	0.5OC	1OC	1PROP	2PROP	0.5OC1PROP	0.5OC2PROP	1OC1PROP	1OC2PROP
Villus length (µm)	278.8±39.8	288.4±28.9	280.1±57.1	390.7±41.6	262.5±45.3	268.8±82.1	264.1±32.8	253.4±25.8	306.3±10.2
Villus width (µm)	463.9±12.7	532.1±9.5	47.6±6.9	61.4±10.1	59.9±12.1	49.1±7.8	44.1±8.9	50.1±11.7	59.6±10.26
Number of Goblet cells	60.8±12.1	61.8±12.5	59.6±14.8	83.9±10.8a	69.8±9.8	68.7±6.8	72.8±10.8	75.9±14.8	81.8±12.8a

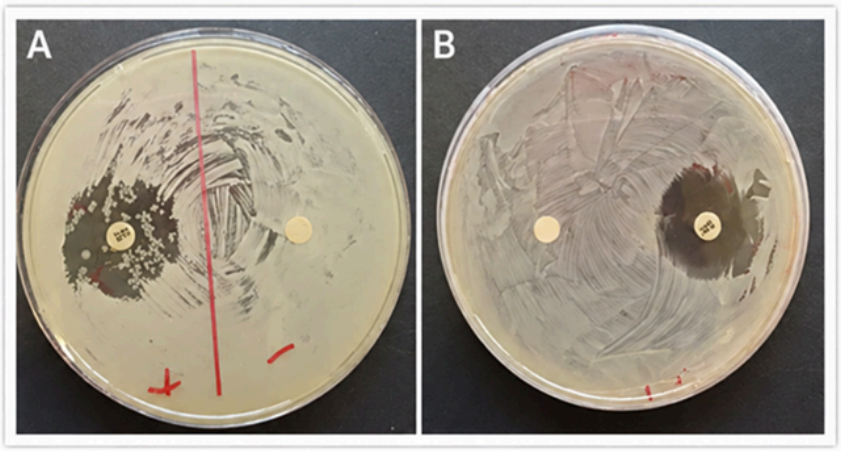
Figures

C

Inhibition of bacterial growth.

Bacteria	Inhibition
Streptococcus agalactiae	++
Aeromonas hydrophila	–

Inhibition: ++ strong inhibition; – no inhibition.



Antibacterial activity.
(A) *Streptococcus agalactiae* showing inhibition (++).
(B) *Aeromonas hydrophila* showing no inhibition (–).

Figure 1

In vitro antibacterial activity of the combined use of copaiba oil and propolis against fish pathogenic bacteria.

(A) *Streptococcus agalactiae* showing bacterial growth inhibition (++).

(B) *Aeromonas hydrophila* showing no inhibitory activity (−).

(C) Classification of inhibition intensity observed in the disk diffusion assay.

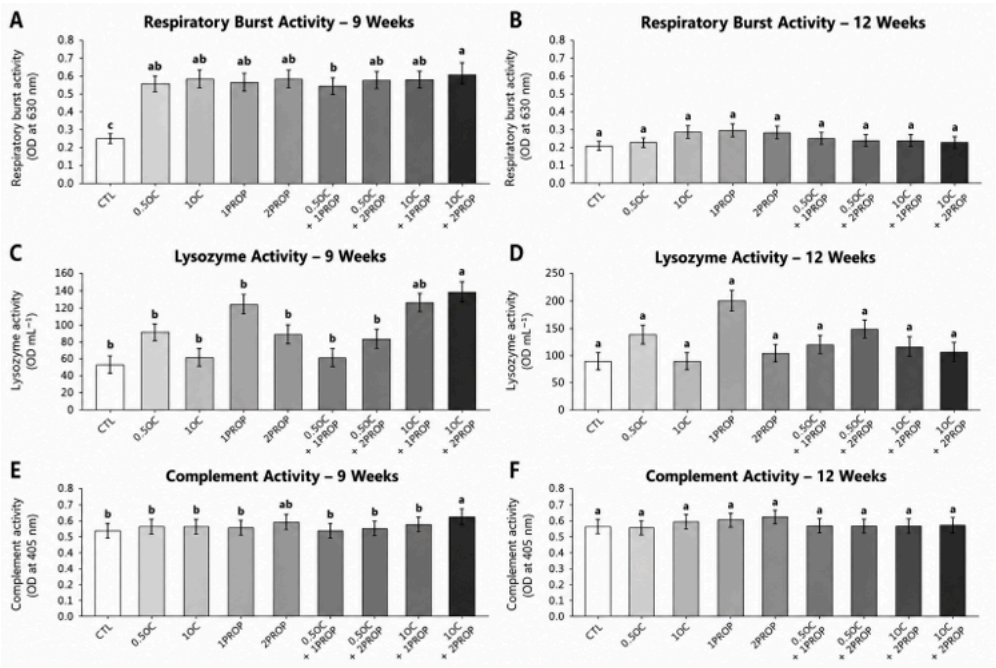


Figure 2

Respiratory burst activity, serum lysozyme activity, and alternative complement pathway activity in Nile tilapia after 9 and 12 weeks of dietary supplementation with copaiba oil and propolis, supplied alone or in combination. (A, B) Respiratory burst activity; (C, D) serum lysozyme activity; (E, F) complement activity at 9 and 12 weeks, respectively. Different letters indicate significant differences among treatments according to Tukey's test ($P < 0.05$).

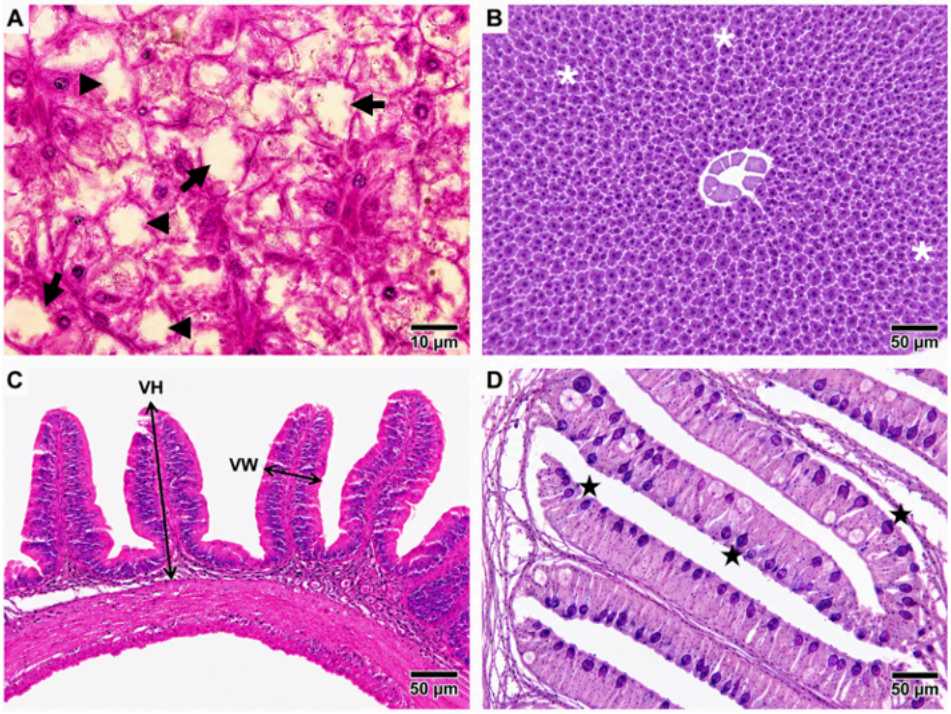


Figure 3

Histological and histomorphometric evaluation of Nile tilapia tissues after dietary supplementation with copaiba oil and propolis. (A) Liver section showing mild hepatic steatosis (arrowheads), similarly distributed among treatments. (B) PAS staining demonstrates preserved hepatic glycogen distribution; white asterisks indicate PAS-positive glycogen deposits. (C) Representative photomicrograph illustrating villus height (VH) and villus width (VW) measurements in the proximal intestine. (D) Intestinal goblet cells (colored in bright purple) indicated by stars. H&E staining.

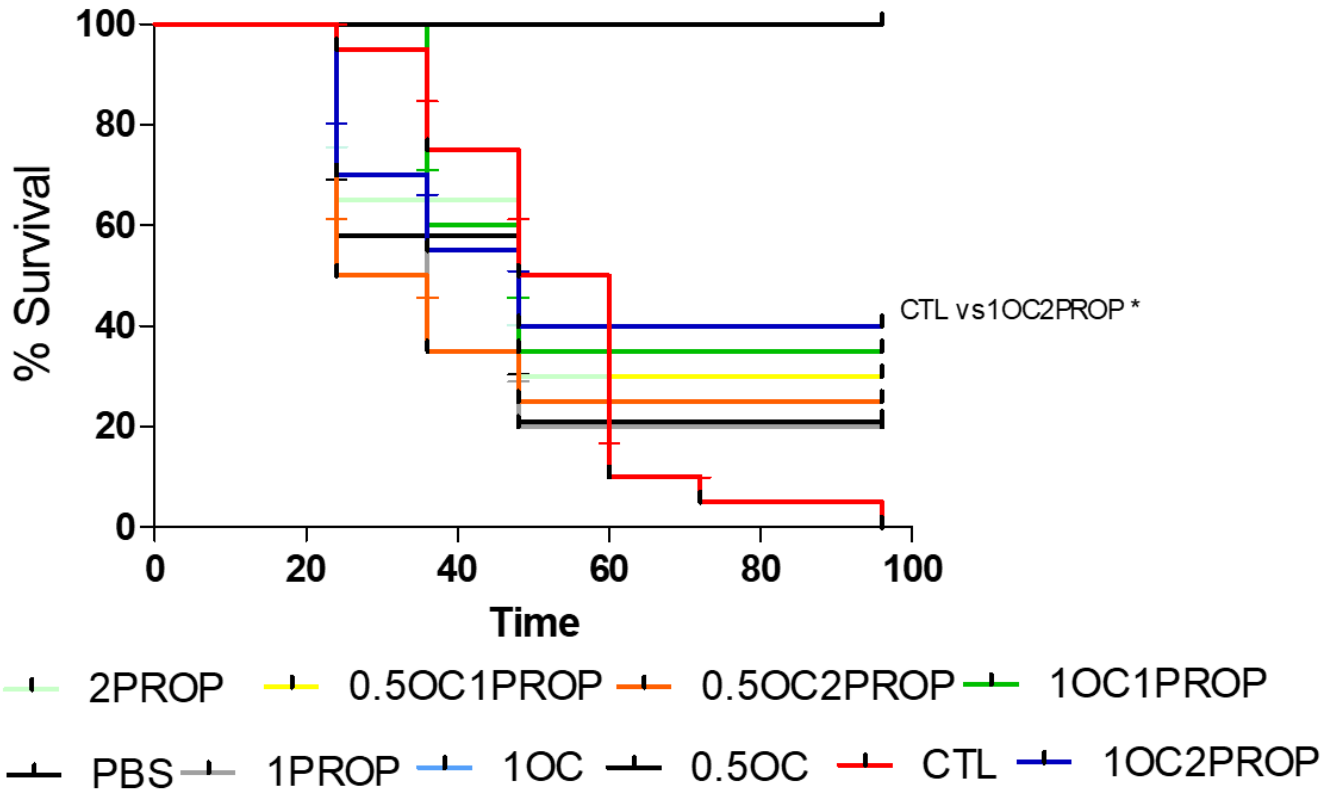


Figure 4

Kaplan–Meier survival curves of Nile tilapia (*Oreochromis niloticus*) experimentally challenged with *Streptococcus agalactiae* after 12 weeks of dietary supplementation. Treatments were: CTL, non-supplemented challenged control; 0.5OC, 0.5% copaiba oil; 1OC, 1% copaiba oil; 1PROP, 1% propolis; 2PROP, 2% propolis; 0.5OC1PROP, 0.5% copaiba oil + 1% propolis; 0.5OC2PROP, 0.5% copaiba oil + 2% propolis; 1OC1PROP, 1% copaiba oil + 1% propolis; and 1OC2PROP, 1% copaiba oil + 2% propolis. PBS represents non-infected fish inoculated with sterile phosphate-buffered saline. Significant differences among survival curves were determined using the log-rank test ($P < 0.05$).