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**PROPHYLACTIC POTENTIAL OF ESSENTIAL OILS AGAINST *Aeromonas* spp. IN
*Colossoma macropomum***

RAYZA LIMA ARAÚJO

Manaus – Amazonas

July, 2026

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Advisor: Edsandra Campos Chagas

Thesis presented to Postgraduate Program in
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RESUMO

O objetivo desse trabalho foi identificar e avaliar o potencial patogênico de espécies de *Aeromonas* associadas a sistemas semi-intensivos de cultivo semi-intensivo de tambaqui (*Colossoma macropomum*) no estado do Amazonas (Brasil), bem como investigar a atividade antimicrobiana de nove óleos essenciais (*Croton sacaquinha*, *Cymbopogon nardus*, *Curcuma longa*, *Lippia origanoides*, *Mentha arvensis*, *Ocimum basilicum*, *Ocimum micranthum*, *Piper hispidum* e *Piper marginatum*) e seu potencial profilático contra a infecção por *Aeromonas hydrophila*. No capítulo 1 foi realizada uma revisão de literatura abrangendo o estado atual da aquicultura e do cultivo de tambaqui, a ocorrência de aeromonose na aquicultura, o uso de antimicrobianos e óleos essenciais no cultivo de organismos aquáticos, e a aplicação de óleos essenciais no cultivo de tambaqui. O capítulo 2 teve como objetivo isolar e caracterizar cepas de *Aeromonas* em tambaqui cultivados por meio do isolamento, identificação molecular e avaliação do seu potencial patogênico com base na presença de genes de virulência e em testes de patogenicidade *in vivo*. Foram isoladas cinco cepas de *A. hydrophila* e uma cepa de *A. jandaei*. As cepas de *Aeromonas* apresentaram resultado positivo para a presença de pelo menos sete fatores de virulência. Os peixes submetidos ao experimento de determinação da dose letal (DL) apresentaram despigmentação da pele, ulcerações, letargia e hipo/anorexia em todos os grupos inoculados, enquanto os animais do grupo controle não apresentaram sinais clínicos. As DL₁₀, DL₅₀, DL₉₀ e DL₉₉ foram estabelecidas em $9,1 \times 10^6$, $2,9 \times 10^7$, $9,1 \times 10^7$ e $2,3 \times 10^8$ UFC mL⁻¹, respectivamente. O capítulo 3 teve como objetivo determinar a composição química e a ação antimicrobiana de nove óleos essenciais (OEs) frente a cepas de *A. jandaei* e *A. hydrophila* isoladas de tambaqui. Os componentes majoritários identificados nos OEs de *C. sacaquinha*, *C. nardus*, *C. longa*, *L. origanoides*, *M. arvensis*, *O. basilicum*, *O. micranthum*, *P. hispidum* e *P. marginatum* foram β-selineno (14,5%), geraniol (53,3%), ar-turmerona (30,2%), carvacrol (43,3%), mentol (88,5%) metil chavicol (33,9%), óxido de piperitona (36,2%), γ-terpineno (37,1%), e δ-3-careno (13,1%), respectivamente. Os OEs de *L. origanoides* (9,2 mg.mL⁻¹), *C. nardus* (10 mg.mL⁻¹), *M. arvensis* e *P. marginatum* (15 mg.mL⁻¹) apresentaram atividade antimicrobiana mais expressiva; enquanto todos os OEs testados apresentaram atividade bactericida frente às cepas de *Aeromonas*. O Capítulo 4 teve como objetivo avaliar os efeitos da suplementação dietética com os óleos essenciais de *C. nardus*, *L. origanoides* e *M. arvensis* sobre o desempenho de crescimento, os parâmetros hematológicos e imunológicos, e a sobrevivência de tambaqui após desafio com *A. hydrophila*. A suplementação dietética com os óleos essenciais promoveu respostas distintas em juvenis de tambaqui, melhorando o

desempenho zootécnico, a eficiência alimentar e a resistência à infecção por *A. hydrophila*. *C. nardus* destacou-se por aumentar o ganho de peso final e melhor conversão alimentar, enquanto *L. origanoides* e *M. arvensis* proporcionaram maior sobrevivência após o desafio bacteriano, sem comprometer a homeostase hematológica e bioquímica dos peixes.

Palavras-chave: Tambaqui; Aeromonose; Ensaio de patogenicidade, Fitobióticos; Suplementação dietética.

ABSTRACT

The aim of this study was to identify and evaluate the pathogenic potential of *Aeromonas* species associated with semi-intensive farming systems of tambaqui (*Colossoma macropomum*) in the state of Amazonas, Brazil, as well as to investigate the antimicrobial activity of nine essential oils (*Croton sakaquina*, *Cymbopogon nardus*, *Curcuma longa*, *Lippia origanoides*, *Mentha arvensis*, *Ocimum basilicum*, *Ocimum micranthum*, *Piper hispidum* e *Piper marginatum*) and their prophylactic potential against *Aeromonas hydrophila* infection. Chapter 1 consisted of a literature review addressing the status of aquaculture and tambaqui farming, the occurrence of aeromoniasis in aquaculture, the use of antimicrobials and essential oils in the cultivation of aquatic organisms, and the application of essential oils in tambaqui farming. Chapter 2 aimed to isolate and characterize *Aeromonas* strains from farmed tambaqui through bacterial isolation, molecular identification, and assessment of their pathogenic potential based on the presence of virulence genes and *in vivo* pathogenicity assays. Five strains of *A. hydrophila* and one strain of *A. jandaei* were isolated. The *Aeromonas* strains tested positive for at least seven virulence factors. Fish subjected to the lethal dose (LD) determination assay exhibited skin depigmentation, ulcerations, lethargy, and hypo/anorexia in all inoculated groups, whereas fish in the control group showed no clinical signs. The LD₁₀, LD₅₀, LD₉₀, and LD₉₉ values were established at 9.1×10^6 , 2.9×10^7 , 9.1×10^7 , and 2.3×10^8 CFU mL⁻¹, respectively. Chapter 3 aimed to determine the chemical composition and antimicrobial activity of nine essential oils (EOs) against strains of *A. jandaei* and *A. hydrophila* isolated from tambaqui. The major compounds identified in the EOs of *C. sakaquina*, *C. nardus*, *C. longa*, *L. origanoides*, *M. arvensis*, *O. basilicum*, *O. micranthum*, *P. hispidum*, and *P. marginatum* were β -selinene (14.5%), geraniol (53.3%), ar-turmerone (30.2%), carvacrol (43.3%), menthol (88.5%), methyl chavicol (33.9%), piperitone oxide (36.2%), γ -terpinene (37.1%), and δ -3-carene (13.1%), respectively. The EOs of *L. origanoides* (9.2 mg mL⁻¹), *C. nardus* (10 mg mL⁻¹), *M. arvensis*, and *P. marginatum* (15 mg mL⁻¹) exhibited the most pronounced antimicrobial activity, whereas all tested EOs demonstrated bactericidal activity against *Aeromonas* strains. Chapter 4 aimed to evaluate the effects of dietary supplementation with the essential oils of *C. nardus*, *L. origanoides*, and *M. arvensis* on growth performance, hematological and immunological parameters, and the survival of tambaqui following challenge with *A. hydrophila*. Dietary supplementation with essential oils promoted distinct responses in juvenile tambaqui, improving growth performance, feed efficiency, and resistance to *A. hydrophila* infection. *C. nardus* was the most effective in enhancing final weight and feed

conversion ratio, whereas *L. origanoides* and *M. arvensis* provided the highest survival rates following the bacterial challenge, without compromising the hematological and biochemical homeostasis of the fish.

Keywords: Tambaqui; Aeromoniasis.; Pathogenicity assay, Phytobiotics; Dietary supplementation.

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CHAPTER I – INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW

1. INTRODUCTION

In 2024, global aquaculture production reached a record 142 million tons, accounting for the largest share of aquatic animals produced for the second time, representing more than 59% of food destined for human consumption (FAO, 2026). According to data from the Ministry of Fisheries and Aquaculture, aquaculture production in Brazil was approximately 880,000 tons in 2024, with an 11.2% increase over 2023 production, and a cumulative growth of 25.3% in the four-year period 2021-2024 (MPA, 2025). Fish farming stands out as the fastest-growing animal production activity in recent years, with a 9.8% increase over the previous year.

Brazilian fish farming reached a historic milestone in 2025, producing 1,011,540 metric tons of farmed fish, representing a 4.41% increase compared with 2024 (PEIXEBR, 2026). Tambaqui is the most widely farmed native fish species in Brazil. However, unlike the overall Brazilian aquaculture sector, which has been driven primarily by tilapia farming, tambaqui production has declined over the past three years, with the lack or inadequacy of health management programs representing one of the main bottlenecks in its production chain.

The demand for fish consumption has made aquaculture one of the most viable alternatives for fish production and perhaps the only one to supply sufficient fish to the human population (SHEN *et al.*, 2021). Within this context, fish farming has increasingly sought to optimize its productivity, while at the same time there is an increase in diseases related to cultivation, which cause significant economic losses and can represent a risk to the health of consumers (ASSEFA; ABUNNA, 2018). According to Lieke *et al.* (2020), high stocking densities and poor quality of culture water are the determining factors for the appearance of diseases that are rapidly taken to pathogenic levels.

Bacterial infections in fish farming, in general, are caused by opportunistic pathogens, endogenous to the environment, that affect fish with already compromised immune systems (MZULA *et al.*, 2021). Among the bacterial diseases related to significant productivity losses in fish farms, those caused by the genus *Aeromonas* stand out (HAYATGHEIB *et al.*, 2020; HOAI *et al.*, 2019; SOTO-DÁVILA *et al.*, 2022). This genus is composed of gram-negative rods belonging to the Aeromonadaceae family, ubiquitous in aquatic environments (CHANDRARATHNA *et al.*, 2018).

The main species of this genus associated with infection in farmed fish is *Aeromonas hydrophila*. This bacterium's natural habitat is aquatic environments (BARGER *et al.*, 2021), and it is metabolically diverse, fast-growing, and capable of exploiting a wide range of hosts (PEATMAN *et al.*, 2018). In fish, it can cause septicemia, known as Motile *Aeromonas* Septicemia (MAS), resulting in high mortality rates in fish farms (CLAUDIANO *et al.*, 2019; RAN *et al.*, 2018; ZHANG, X. *et al.*, 2020).

Currently, there is widespread use of antibiotics, prebiotics, and probiotics for prevention and control of bacterial diseases in commercial fish farming (AMENYOGBE *et al.*, 2020). The prolonged and indiscriminate use of antibiotics in commercial fish farms generates strong selective pressure, favoring resistant bacteria and the transfer of resistance genes in the aquatic environment (HOSSAIN *et al.*, 2022). Furthermore, these compounds are discarded into the environment, contaminating water and sediments, constituting a public health problem (MILIJASEVIC *et al.*, 2024). In several production systems, the use of these drugs is observed in a manner incompatible with technical recommendations, including prophylactic administration, off-label use, inadequate dosages, insufficient treatment periods, and the absence of prior etiological diagnosis (STRÖM *et al.*, 2019). According to Rasul *et al.* (2025), these practices compromise the therapeutic effectiveness of antimicrobials and reduce their ability to control diseases.

Given this scenario, essential oils (EOs) are emerging as a promising and sustainable alternative in the prevention and treatment of these diseases, reducing the use of synthetic antimicrobials in aquaculture and tissue accumulation, and resistance development due to their biodegradability (KOLYGAS *et al.*, 2025; RAJENDRAN *et al.*, 2026). Several studies show the action of essential oils against pathogenic fish bacteria such as *Aeromonas*, *Vibrio*, *Streptococcus*, and *Pseudomonas*, often with low Minimum Inhibitory Concentration (MIC) (ANASTASIOU *et al.*, 2019; KLÜGA *et al.*, 2021; KOLYGAS *et al.*, 2025). There are also antiparasitic effects, for example, against *Ichthyophthirius* and *Gyrodactylus*, and improved growth, immunity and disease resistance when included in feed (DAWOOD *et al.*, 2021, 2022).

Considering the increasing sanitary challenges faced by tambaqui farming and the limitations associated with the conventional use of antimicrobials, there is a need to identify pathogenic bacterial strains affecting cultured fish and to develop sustainable prophylactic strategies. In this context, the characterization of *Aeromonas* strains isolated from tambaqui and the evaluation of the antimicrobial and prophylactic potential of essential oils may contribute to the development of alternative approaches for disease

prevention and health promotion in aquaculture, supporting improved productive performance and increased resistance of fish to bacterial infections. (SOUZA *et al.*, 2019; WICKRAMANAYAKE *et al.*, 2023).

2. OBJECTIVES

2.1.General Objective

Identify *Aeromonas* species in semi-intensive tambaqui (*Colossoma macropomum*) farming systems in the State of Amazonas (Brazil) and evaluate their pathogenic potential, as well as to investigate the antimicrobial activity of nine essential oils and their prophylactic potential against bacterial infection caused by *Aeromonas hydrophila*.

2.2.Specific Objectives

- To profile *Aeromonas* strains present in tambaqui fish farms in four municipalities of the Manaus Metropolitan Region (Amazonas, Brazil), through isolation, species-level identification using biochemical tests and molecular biology techniques;
- To verify the bactericidal and bacteriostatic action, through evaluation of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration, of essential oils from the species *Croton sacaquinha*, *Ocimum basilicum*, *Ocimum micranthum*, *Curcuma longa*, *Cymbopogon nardus*, *Lippia origanoides*, *Piper hispidum*, *Piper marginatum* and *Mentha arvensis*;
- To evaluate the prophylactic potential of the essential oil that performed best in the previous stage, when administered in the diet of tambaqui fish cultivated under controlled conditions;
- To evaluate whether the inclusion of essential oils in the diet of tambaqui fish promotes increased survival after experimental challenge with a strain of *Aeromonas hydrophila*;
- To monitor the zootechnical indicators and the overall health of the animals during all stages of the experiment.

3. LITERATURE REVIEW

3.1. Aquaculture and tambaqui farming

According to data presented in *The State of World Fisheries and Aquaculture* report published by the Food and Agriculture Organization of the United Nations (FAO) in 2026, aquaculture has consolidated itself as the segment that most drives the increase in the global supply of aquatic foods, reaching 53% of the world's aquatic animal production, equivalent to 103 million tons (FAO, 2026).

FAO emphasizes that the expansion of aquaculture will be indispensable to feed the world's population, but this growth needs to occur with efficient use of resources, technological innovation, biosecurity, social inclusion, and environmental sustainability. Furthermore, according to the document, the volume of algae and aquatic animals produced in 2024 generated an estimated economic value of US\$391 billion directly in production units, demonstrating not only its nutritional relevance but also its global economic impact.

Boyd *et al.* (2020) highlight that global aquaculture has become the fastest-growing food production technology in the world, continuously expanding its global contribution to food security and the supply of aquatic protein. The authors state that the aquatic organism production chain has entered a new phase of development, mainly based on sustainability, where productive growth needs to occur in conjunction with efficient use of natural resources and reduction of environmental impacts.

Aligned with the global trend of sector expansion, Brazil has shown continuous growth in aquaculture production, reaching 880,000 tons in 2024, reporting growth of approximately 11.2% compared with 2023 and generating revenues of R\$ 10,761 million (MPA, 2025). Furthermore, according to the Statistical Bulletin of Fisheries and Aquaculture from the Ministry of Fisheries and Aquaculture, continental aquaculture accounted for 82.25% of total national production in 2024, and fish farming stands out as the most structured segment and responsible for sustaining the expansion of Brazilian aquaculture activity.

Data from the Brazilian Fish Farming Yearbook (PEIXE BR, 2026) indicate that in 2025 Brazil reached the milestone of one million tons of farmed fish, representing growth of 4.41% compared with the production volume recorded in 2024. This increase reflects the expansion of tilapia production, which accounts for nearly 70% of all farmed fish produced nationally. Tilapia (*Oreochromis niloticus*) remains the main species cultivated in the country, followed by tambaqui (*Colossoma macropomum*), which stands

out as the leading native farmed species, particularly in the Northern region, where the production of round fish is concentrated, consolidating its role as an economic foundation of family-based fish farming.

The tambaqui is a Neotropical fish (preferred temperature range of 25 to 34 °C), potamodromous, benthopelagic, frugivorous-omnivorous, frequently exceeding 1.0 m in total length and 30 kg (HILSDORF *et al.*, 2022). This species is widely distributed throughout the Amazon and Orinoco river basins, occurring naturally in Brazil, Venezuela, Colombia, Peru, and Bolivia (ARAÚJO-LIMA; GOULDING, 1997) .

According to Gomes *et al.* (2019), tambaqui is the most produced fish in the state of Amazonas, due to its rapid growth, robustness, and excellent zootechnical performance, being characterized as an extremely fertile and robust native species that easily adapts to polyculture systems. In Amazonas state, fish farming is mainly practiced through two production modalities: excavated ponds and stream channel systems, generally operated on an artisanal scale, with relatively few commercial-scale production units (PANTOJA-LIMA *et al.*, 2015). According to the same authors, fish farming is also practiced in reservoir ponds and net cage systems, although at lower intensity.

Despite the sector's growth, native fish production registered a slight decrease in 2025 (0.63%) compared to the previous year (PEIXE BR, 2026). This result indicates a concerning trend of gradual but continuous production decline over the last three years, reinforcing the importance of implementing good management practices and biosecurity measures in order to increase the productive efficiency and competitiveness of the tambaqui production chain.

In Amazonas, aquaculture production is predominantly based on the farming of round fish species, with Rio Preto da Eva, Manacapuru, Careiro, Itacoatiara, and Presidente Figueiredo being the municipalities with the largest production areas in excavated ponds, all belonging to the Metropolitan Region of Manaus (Amazonas, Brazil) (PEIXE BR, 2026). In the state, the decline in productivity was even more pronounced, with a production volume of 12,390 tons in 2025, a number almost 20% lower compared to the previous year. Valenti *et al.* (2021) report that the Brazilian aquaculture chain still has important weaknesses, highlighting productive and sanitary bottlenecks such as the need for enhanced biosecurity, increasing occurrence of bacterial and viral diseases, sanitary challenges in intensive production systems, and the need to consolidate epidemiological surveillance programs.

In this context, strengthening native fish farming in Brazil is closely associated with the sustainability of the sector, since cultivating these species reduces ecological risks related to the introduction and dissemination of exotic species, due to their compatibility with the local biota. Furthermore, this activity plays an important socioeconomic role, being largely supported by family-based production systems predominantly run by small rural producers (LIMA *et al.*, 2020; NOBILE *et al.*, 2020).

3.2. Aeromoniasis in aquaculture

The high regional demand for tambaqui consumption, combined with the technical and economic viability of its commercial-scale production, has driven the intensification of farming systems for this species in the Northern Brazil (LIMA *et al.*, 2025). However, despite the expansion of the activity, insufficient adoption of appropriate management practices remains one of the main obstacles to the sustainable development of tambaqui farming in the region (CRAVEIRO *et al.*, 2019). Factors related to water quality, daily animal management, feeding strategies, slaughter procedures, production infrastructure, as well as fish acquisition, transporting, and handling practices, directly influence animal welfare and, consequently, zootechnical performance, productivity, and the health status of production systems (ABD EL-HACK *et al.*, 2022; FRUSCELLA *et al.*, 2026; ZHANG *et al.*, 2024).

When these factors are not properly managed, fish become more susceptible to bacterial infections and parasite infestation, reducing feed efficiency and growth performance, and potentially leading to mortality (WANJA *et al.*, 2020). The production of heterogeneous-sized fish, susceptibility to bacterial contamination, and the occurrence of parasitic infections are among the constraints identified in the production chain of *C. macropomum* production, which may be mitigated through the adoption of good management practices both on farms and in processing facilities (VALENTI *et al.*, 2021).

Various biological agents coexist harmoniously with fish in aquatic environment and are responsible for several opportunistic diseases that mainly emerge due to poor water quality, which favors pathogen proliferation (MOHD-ARIS *et al.*, 2019). This increase in pathogenic populations, combined with fish immunosuppression resulting from stress induced by poor water quality and inadequate management, leads to increased environmental infection pressure and consequently to outbreaks of diseases commonly observed in fish farming (DAI *et al.*, 2023; ELGENDY *et al.*, 2023). It is estimated that about 50% of losses recorded in fish farms worldwide are caused by bacterial infections,

resulting in low growth, high mortality, and inadequate meat quality of farmed fish (ALGAMMAL *et al.*, 2020).

Among the pathogens frequently associated with severe infections in cultured fish, bacteria of the genus *Aeromonas* stand out (NHINH *et al.*, 2021). These microorganisms are natural components of aquatic microbiota and are known for their remarkable ability to inhabit a wide range of hosts (NEIL *et al.*, 2024). This group is composed of non-sporulating, generally motile, Gram-negative bacilli (ABDELLA *et al.*, 2024).

Bacteria of this group are described as opportunistic pathogens that primarily affect fish with compromised immune systems (LEANOVICH *et al.*, 2025), causing infections of varying severity depending on the infectious bacterial dose and the host immune response (MAJEED *et al.*, 2023). Akmal *et al.* (2020) recently reported increasing recognition of their importance as pathogens of fish and their frequent association with severe infections in aquaculture systems.

To date, the genus *Aeromonas* comprises 35 recognized species (FERNÁNDEZ-BRAVO; FIGUERAS, 2020). Species of *Aeromonas*, particularly *A. hydrophila*, *A. salmonicida*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. jandaei*, are frequently associated with severe infections and mass mortality events in fish farms involving different species across multiple geographic regions (DOS SANTOS *et al.*, 2023a; LAGADEC *et al.*, 2024; OLADELE *et al.*, 2021; VILLA *et al.*, 2023).

Among the aforementioned species, *Aeromonas hydrophila* stands out as the most frequently isolated species in aquaculture worldwide, as well as for its ability to infect multiple hosts (PESSOA *et al.*, 2020). Kari *et al.* (2022) emphasized that this species is frequently reported as the causative agent of Motile *Aeromonas* Septicemia (MAS) in aquatic animals, which, once infected, may exhibit symptoms such as ascites, gastroenteric hemorrhage, septic skin ulceration, exophthalmia, scale protrusion, tail rot, and fin rot (ARIEDE *et al.*, 2020; MARINHO-NETO *et al.*, 2019; SOTO-RODRIGUEZ *et al.*, 2018). Clinical signs are quite variable and depend on factors such as strain virulence, fish resistance to infection, and associated stress conditions (SEMWAL *et al.*, 2023).

Several authors have reported the occurrence of *A. hydrophila* in farmed tambaqui in Brazil, particularly in Amazon. Valladão *et al.* (2016) and Gallani *et al.* (2020) reported that, although *C. macropomum* is the most widely cultivated native fish species in South America, studies conducted in recent years on bacterial diseases affecting farms of this species have documented mortality outbreaks, especially in intensive farming regions.

Studies indicate that infections caused by *Aeromonas jandaei* in fish represent an emerging threat to aquaculture (ABARIKE *et al.*, 2022). This species has been isolated from specimens of tilapia (ASSANE *et al.*, 2021a), tambaqui (MIELKE *et al.*, 2022) and pirarucu (*Arapaima gigas*) (PROIETTI-JUNIOR *et al.*, 2021), all presenting typical clinical signs of aeromoniasis during mortality outbreaks.

According to Dong *et al.* (2021), aerolysin, a cytolytic enterotoxin, has been identified as the most aggressive virulence factor in pathogenic strains of *A. hydrophila*, and is encoded by the *aerA* gene. Chakraborty *et al.* (1987) reported that mutant strains of *A. hydrophila* lacking the gene encoding aerolysin exhibited significantly reduced virulence compared with wild-type strains when evaluated using a mouse model. These findings identified aerolysin as a promising target for reducing bacterial virulence.

Pessoa *et al.* (2020) conducted molecular characterization and virulence trait assessment of *Aeromonas* spp. isolated from farmed tambaqui in Brazil. The authors detected a predominance of *A. hydrophila*, representing 41% of the isolates. The study also identified genetic diversity among virulence factors, with protease production predominating in the evaluated strains. Some of *A. hydrophila* strains harbored all virulence genes (β -hemol, *ser* and *lip*) and produced all enzymes evaluated (hemolytic-HEM, lipolytic-LIP, and proteolytic-PROT activity) suggesting high virulence potential and significant risks to tambaqui production.

According to Muduli *et al.* (2021), most *Aeromonas* strains possess several virulence factors, including flagella, adhesins, hemolysins, proteases, lipases, DNases, enterotoxins, and aerolysin, enabling colonization and destruction of host cells. In this context, fish-pathogenic *Aeromonas* species are described as multidrug-resistant, hemolytic, and biofilm-forming (FERNÁNDEZ-BRAVO; FIGUERAS, 2020). Among the major virulence genes commonly associated with bacterial species of this genus, we can mention aerolysin (*aerA*), flagellin (*fla*), lipase (*lip*), elastase (*ela*), cytotoxic enterotoxin (*act*), thermolabile cytotoxic enterotoxin (*alt*), and hemolysin (*hlyA*), all of which facilitate the pathogenic process (MEDINA-MORILLO *et al.*, 2023). According to El-Bahar *et al.* (2019) and Tartor *et al.* (2021), *Aeromonas* strains isolated from fish frequently carry these virulence genes.

The pathogenicity of *A. hydrophila* is attributed to the release of multiple virulence factors, including enterotoxin, hemolysin, and adhesion-related factors that contribute to bacterial attachment to the host gastrointestinal tract, where they promote hemolysis and enterotoxin production (HAFEZ *et al.*, 2018). In general, the virulence

potential of *Aeromonas* is complex, multifactorial, dependent on host susceptibility, and remains incompletely understood (LI *et al.*, 2023).

3.3. Use of antimicrobials in aquaculture and its limitations

The intensification of aquaculture systems in recent decades has significantly contributed to increased productivity and the expansion of the global supply of fish products (VERDEGEM *et al.*, 2023). However, intensive production systems characterized by high-density monocultures, combined with inadequate management and biosecurity practices and fluctuations in water quality, have favored the increased occurrence and spread of infectious diseases, particularly those of bacterial origin (IRSHATH *et al.*, 2023). In this context, antimicrobials have become widely used as one of the main sanitary control strategies in aquaculture (SANTOS; RAMOS, 2018).

According to Hemamalini *et al.* (2022), despite their historical importance in disease control, the use of antimicrobials in aquaculture presents significant limitations that compromise their long-term efficiency and sustainability. Under commercial production conditions, the efficacy of these compounds may be reduced by several factors, including administration without prior etiological diagnosis, inappropriate selection of active ingredients, use at subtherapeutic doses, insufficient treatment duration, and application under environmental conditions that limit the animals' response (ALHUSEIN *et al.*, 2024; HOSSAIN *et al.*, 2022). The findings reported by Rasul *et al.* (2025) highlight an important limitation of conventional sanitary strategies in aquaculture, particularly regarding the extra-label (off-label) use of antimicrobials. The authors observed widespread use of aquaculture medicinal products, including antimicrobials originally developed for other veterinary applications and subsequently used in fish without specific approval for aquaculture, a practice that has become increasingly common among producers. Consequently, in addition to limited clinical effectiveness, these practices may mask management deficiencies and reduce the efficiency of conventional sanitary measures.

Another critical aspect associated with the continuous and inappropriate use of antimicrobials concerns the development of antimicrobial resistance (YUAN *et al.*, 2023). Recent scientific evidence indicates that frequent exposure of bacterial populations to sublethal concentrations of antibiotics exerts selective pressure on microorganisms present in farmed animals and the aquatic environment, favoring the survival and proliferation of resistant strains (PEPI; FOCARDI, 2021). According to Watts *et al.*

(2017) and Zhao *et al.* (2025), this phenomenon represents a growing challenge for aquaculture, as it progressively reduces the therapeutic options available for disease treatment and increases the likelihood of sanitary failures in production systems.

Wang *et al.* (2021) emphasized that, in addition to selecting resistant bacteria, aquaculture environments may act as reservoirs and dissemination points for antimicrobial resistance genes. As pointed out by Yuan *et al.* (2023), only 20 to 30% of antibiotics added to the farming system are absorbed by fish. The remainder is excreted or remains in uneaten feed, reaching sediments and adjacent water bodies, altering natural microbial communities and promoting horizontal gene transfer among different bacterial groups via plasmids, transposons, and integrons (CHEN *et al.*, 2022). Thus, the impacts associated with the inappropriate use of these compounds extend beyond the farming environment and become part of a broader problem related to the One Health concept, which recognizes the interdependence among animal, human, and environmental health (AHMAD *et al.*, 2023).

Given this scenario, there is a growing need to develop alternative prophylactic strategies capable of reducing dependence on antimicrobials without compromising the productive performance and health status of cultured organisms (SILVA *et al.*, 2021). Among the emerging approaches, improvements of biosecurity practices, nutritional management, and the use of vaccines, probiotics, prebiotics, synbiotics, immunostimulants, and bioactive compounds of natural origin stand out (KILIÇ; GÜLTEKIN, 2024). According to Lim *et al.* (2025), these strategies aim to strengthen the physiological and immunological mechanisms in animals, thereby improving immune response, growth performance, disease resistance, and tolerance to stressors.

In this context, medicinal plants and their secondary metabolites, such as essential oils, have received increasing attention as potential functional additives in aquaculture, since they contain antimicrobial compounds capable of combating a broad range of bacteria responsible for diseases in aquatic animals (BONDAD-REANTASO *et al.*, 2023). In a review conducted by Dawood *et al.* (2022), the authors highlighted that, in addition to exhibiting antimicrobial activity against several aquaculture-relevant pathogens, essential oils may exert antioxidant, gut microbiota-modulating, and immunostimulatory effects, contributing to increased fish resistance against infectious agents. Therefore, the development and validation of prophylactic approaches based on modulating the physiological and immune responses of organisms represent a promising

perspective for promoting more sustainable aquaculture systems with reduced dependence on the conventional use of antimicrobials (GADHIYA *et al.*, 2025).

3.4. Use of essential oils in aquaculture

Essential oils (EOs) are commonly defined as complex mixtures of volatile organic compounds produced by the secondary metabolism of aromatic plants (DE SOUSA *et al.*, 2023), with important ecological functions related to defense and interaction with the environment, and may also play roles in pollinators attraction and responses to environmental stress (AQEEL *et al.*, 2023; CHANG *et al.*, 2022). In recent decades, these compounds have attracted increasing interest in aquaculture due to their potential as functional additives capable of modulating physiological processes, improving productive performance, and strengthening the immune response of cultivated organisms (SUTILI *et al.*, 2018). The need to reduce the indiscriminate use of antimicrobials and the search for more sustainable strategies to promote aquatic animal health are among the main factors driving this interest (CUNHA *et al.*, 2018).

The biological activity of essential oils is directly related to their chemical composition, which may vary widely depending on the plant species, environmental cultivation conditions, plant developmental stage, extraction method, and storage conditions (MIRI, 2025). According to De Sousa *et al.* (2023), the components of essential oils possess distinct primary metabolic precursors and are generated through different biosynthetic pathways. The authors further state that these compounds may be classified into two main groups: a major group composed predominantly of terpenes and terpenoids, especially monoterpenes and sesquiterpenes; and a secondary (non-terpenoid) group consisting of phenolic compounds and oxygenated molecules, which frequently account for the pharmacological effects observed. Compounds such as carvacrol, thymol, geraniol, citronellal, menthol, linalool, and eugenol have been widely described in the literature due to their antimicrobial, antioxidant, and immunomodulatory properties (GUIMARÃES *et al.*, 2019; KHWAZA; ADERIBIGBE, 2025; KWIATKOWSKI *et al.*, 2022).

Although the presence of major compounds is frequently used to characterize an essential oil, studies have demonstrated that their biological effects result from interactions among multiple chemical constituents (ABDELMOHSEN; ELMAIDOMY, 2025). According to Nguyen and Karboune (2023), in addition to the contribution of predominant compounds, substances present at lower concentrations may act

synergistically or antagonistically, significantly modifying the final activity of the oil. This phenomenon demonstrates that the observed biological response cannot always be attributed exclusively to the most abundant component, making comprehensive chemical characterization necessary for adequate interpretation of experimental results (MORSY, 2017).

EOs have been widely used as bactericidal, virucidal, fungicidal, and antiparasitics agents and also exhibit anti-inflammatory, immunostimulatory, anesthetic, and antioxidant properties (BRANDÃO *et al.*, 2022; GHAFARIFARSANI *et al.*, 2023; KUMAR *et al.*, 2022; OLIVEIRA *et al.*, 2024; SANTOS *et al.*, 2020). Some of these oils are considered effective and low-toxicity alternatives (DAWOOD *et al.*, 2022), and can be used alone or in combination with synthetic chemical compounds (ALJAAFARI *et al.*, 2021; ASSANE *et al.*, 2021b; SHARMA *et al.*, 2020).

Among the main mechanisms of action attributed to essential oils, antimicrobial activity stands out, acting primarily through disruption and permeabilization of the cell membrane, resulting in leakage of essential intracellular components (BORDÓN *et al.*, 2025). In other words, the hydrophobic constituents of EOs insert into the lipid bilayer, disrupt phospholipid organization, and increase membrane permeability, leading to loss of membrane integrity and leakage of ions (K^+ , protons), proteins, nucleic acids, and other vital cellular constituents, ultimately resulting in cell death (NOUI MEHIDI *et al.*, 2024). Additionally, EOs may act by inhibiting protein synthesis, energy production, and enzymatic activity (FALLEH, 2025), or by reducing bacterial virulence mechanisms through inhibition of biofilm formation and interference with quorum sensing-dependent cell communication systems, thereby contributing to reduced pathogen infectivity (MARTÍNEZ *et al.*, 2021).

Another relevant mechanism refers to the immunomodulatory effects of essential oils in farmed fish (SUTILI *et al.*, 2018). Studies have demonstrated that dietary supplementation with these compounds can influence parameters associated with innate and adaptive immunity, promoting changes in phagocytic activity, leukocyte concentration and distribution, cytokine production, complement system activity, and synthesis of antimicrobial molecules (ABDEL-LATIF *et al.*, 2020; DAWOOD *et al.*, 2022; GHAFARIFARSANI *et al.*, 2022). Ghani *et al.* (2024) and Huang *et al.* (2021) reported that, at the molecular level, such responses appear to involve modulation of signaling pathways associated with the inflammatory and immune responses, including

Toll-like receptors (TLRs), nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinases (MAPK).

In Nile tilapia, the canonical NF- κ B pathway is conserved and essential for both innate immune responses and T-cell-mediated responses (including IL-17A production and defense against bacterial infections), demonstrating its central role in fish immunity (WEI *et al.*, 2021). Firmino *et al.* (2021) evaluated the effects of dietary supplementation with garlic essential oil, carvacrol, and thymol on the modulation of the skin transcriptional immune response, bacterial growth capacity in skin mucus, and the general health status of gilthead seabream juveniles (*Sparus aurata*). The authors observed that the dietary inclusion of these compounds promoted changes in the expression of multiple pro- and anti-inflammatory mediators, demonstrating an immunomodulatory effect on barrier tissues. The results suggested that these bioactive compounds acted through regulation of mechanisms involved in inhibiting activation of signaling pathways mediated by Toll-like receptors (TLRs), including MAPK and NF- κ B pathways, recognized for their central role in inflammatory responses. This modulation indicated more refined control of inflammatory processes, contributing to maintenance of immune homeostasis and strengthening of innate immunity associated with mucosal surfaces.

In the context of dietary administration, the effects of essential oils may also be associated with modulation of the intestinal environment, altering the abundance of several bacterial genera and reducing pathogen adhesion (ZAMINHAN-HASSEMER *et al.*, 2022). The interaction between bioactive compounds and the gastrointestinal tract may promote maintenance of mucosal integrity, influence intestinal microbiota composition, and improve digestive and absorptive processes (CARDOSO *et al.*, 2024; ZHANG *et al.*, 2020). According to Lee *et al.* (2021) , the intestine represents an important immunological organ in fish, harboring diffuse lymphoid tissue (GALT), which coordinates innate and adaptive immunity, regulates microbiota, and protects against enteric pathogens. Thus, positive changes in this system may result in greater resistance to infectious agents and improved zootechnical performance (AMOAHA *et al.*, 2023).

Essential oils from certain botanical families have been recognized for their biological activity against various organisms and are already being tested in aquaculture (BRASIL *et al.*, 2019; YAZGAN *et al.*, 2019). The antimicrobial activity of essential oils from species belonging to the families Verbenaceae (*Lippia organoides*), Piperaceae (*Piper hispidum* and *Piper marginatum*), Lamiaceae (*Mentha arvensis*), and Poaceae (*Cymbopogon nardus*) has already been evaluated in previous studies, demonstrating

antimicrobial activity against *Aeromonas* strains isolated from tambaqui (CHAGAS *et al.*, 2020; GALLANI *et al.*, 2020; MAJOLO *et al.*, 2019, 2022). However, no studies were found evaluating the antimicrobial activity of essential oils from *Ocimum micranthum* (Lamiaceae), *Croton sakaquinha* (Euphorbiaceae) and *Curcuma longa* (Zingiberaceae) against *Aeromonas* spp. strains isolated from tambaqui.

It is important to emphasize the need for *in vivo* experiments involving essential oils that demonstrate promising properties in laboratory assays. Furthermore, the large-scale application of these products in aquaculture remains partially limited due to factors such as instability, low solubility, dose standardization, and costs, among others (DAWOOD *et al.*, 2022; KANT; KUMAR, 2022). Aqeel *et al.* (2023) highlight that the development of encapsulation technologies, process optimization, and the establishment of clear regulatory frameworks are key factors to enable the broad commercial application of these compounds. Studies have demonstrated the effectiveness of natural compounds in aquaculture when administered through different routes, including dietary incorporation, therapeutic baths, and synergistic use with antimicrobials, without evidence of toxicological alterations in cultured fish (FIRMINO *et al.*, 2021; MONTEIRO *et al.*, 2021). These approaches contribute to reducing the use of synthetic chemical compounds, thereby promoting the development of more resilient and environmentally sustainable aquaculture systems (KOLYGAS *et al.*, 2025).

3.5.Application of essential oils to tambaqui fish

The use of essential oils in aquaculture has emerged as a promising approach for promoting health status, improving productive performance, and reducing dependence on conventional antimicrobials (REVERTER *et al.*, 2021). In economically important species, such as tambaqui, recent studies have demonstrated that dietary supplementation with plant-derived bioactive compounds may influence different physiological processes, including growth, metabolism, immune response, antioxidant capacity, intestinal integrity, and resistance to pathogens (LOPES *et al.*, 2020; PEREIRA JÚNIOR *et al.*, 2024; SANTOS *et al.*, 2023). In addition to dietary administration, applications through therapeutic baths (DOS SANTOS *et al.*, 2023b; REIS *et al.*, 2025) and their use as anesthetics and sedatives have also been investigated (FARIAS *et al.*, 2024; FERREIRA *et al.*, 2022), expanding the potential of these compounds across different stages of aquaculture management.

Among the most consistently reported effects is the improvement in zootechnical performance. Different plant species used sources of essential oils have demonstrated the ability to stimulate growth and optimize nutrient utilization in tambaqui. Supplementation with sweet orange essential oil (*Citrus sinensis*), at concentrations ranging from 400 and 800 mg kg⁻¹ of feed, promoted increases in final weight and weight gain in fingerlings and reduced mortality after experimental challenge with *Aeromonas hydrophila*. (PEREIRA-JÚNIOR *et al.*, 2024). Similar results were observed with *Lippia sidoides* essential oil (0.5 mL kg⁻¹), which improved productive performance, increased hepatic and muscular energy reserves, and promoted favorable changes in hematological parameters of juvenile tambaqui (COPATTI *et al.*, 2022). Similarly, supplementation with *Nectandra grandiflora* essential oil (0.5 mL kg⁻¹) promoted greater weight gain, increased feed intake, and improvements in intestinal morphology (SANTOS *et al.*, 2023), whereas *Croton sonderianus* (1.5 mL kg⁻¹) resulted in a linear increase in growth and improvements in hematological indicators (PEREIRA *et al.*, 2024). Positive effects were also reported in tambaqui fed diets supplemented with lemongrass essential oil (*Cymbopogon citratus*), with increased weight gain and enhanced intestinal digestive enzyme activity observed at the concentration of 1.5 mL of *C. citratus* essential oil per kilogram of feed (COPATTI *et al.*, 2022).

The benefits observed in growth appear to be related not only to increased feed intake but also to modulation of energy metabolism and improved digestive efficiency. Several studies have demonstrated that supplementation with essential oils may stimulate intestinal enzymatic activities, including amylase, lipase, and alkaline protease, thereby enhancing nutrient digestion and absorption (WANG *et al.*, 2025; YOUSEFI *et al.*, 2025). Concurrently, positive changes in intestinal morphology, such as increased villus height and width and enlargement of intestinal folds, have frequently been associated with increased absorptive surface area and improved intestinal health (*gut health*), factors directly related to zootechnical performance (LI *et al.*, 2019).

In addition to metabolic effects, increasing attention has been directed toward the ability of essential oils to modulate immune mechanisms in tambaqui. Several studies have demonstrated that these compounds act as natural immunostimulants, promoting enhancement of innate immunity and increased resistance to sanitary challenges. Supplementation with *Citrus sinensis*, for example, was associated with increased numbers of thrombocytes, lymphocytes, monocytes, and neutrophils, as well as enhanced phagocytic activity and reduced mortality following infection with *Aeromonas*

hydrophila. (PEREIRA-JÚNIOR *et al.*, 2024). Similarly, Copatti *et al.* (2022) reported that *Lippia sidoides* essential oil increased plasma lysozyme activity and improved hematological indicators at doses ranging from 1.0 and 1.5 mL kg⁻¹ in tambaqui specimens. Monteiro *et al.* (2021) also evaluated the effects of tambaqui feed supplementation with *L. sidoides* essential oil and, corroborating the previously mentioned study, observed positive effects on enhancement of the nonspecific immune response, particularly through increased leukocyte respiratory burst activity and greater survival following bacterial challenge.

Evidence available in the literature indicates that the potential of essential oils has already been extensively investigated in aquaculture systems, especially against bacterial pathogens of sanitary relevance, such as *Aeromonas*, *Vibrio*, *Streptococcus*, *Photobacterium*, *Yersinia*, and *Tenacibaculum*, using approaches ranging from whole essential oils to nanostructured formulations and major bioactive compounds. (CUNHA *et al.*, 2018; KOLYGAS *et al.*, 2025). Majolo *et al.* (2017) investigated the chemical composition and antimicrobial activity of the essential oil of three *Lippia* species against strains of *A. hydrophila* isolated from farmed fish. Their findings confirmed the antimicrobial activity of the essential oils of *L. alba*, *L. origanoides*, and *L. sidoides* against *A. hydrophila*, with *L. sidoides* exhibiting the strongest inhibitory effect. In this context, Chagas *et al.* (2020) investigated the chemical composition of the essential oils of *Mentha arvensis* and *Mentha piperita*, as well as their antimicrobial potential against twelve *Aeromonas* spp. strains isolated from farmed tambaqui. The authors observed that both oils showed antimicrobial activity against the bacterial isolates evaluated; however, *M. arvensis* essential oil demonstrated greater efficacy.

Similarly, Gallani *et al.* (2020) tested the minimum inhibitory concentration of different classes of substances against *A. hydrophila* strains isolated from tambaqui presenting clinical signs of septicemia. The essential oils of *Cinnamomum zeylanicum* and *Eugenia caryophyllata* exhibited the highest bactericidal activity against *A. hydrophila* in tambaqui and were classified as promising treatment alternatives. The authors explained that the classification of these substances as bactericidal is mainly related to the mechanism of action of essential oils on microorganisms, characterized by membrane permeabilization culminating in swelling and disruption of bacterial cells.

Therefore, knowledge regarding the chemical composition and biological mechanisms of essential oils is essential for understanding their application as functional additives in aquaculture. Integrating the chemical characteristics of these compounds with

their physiological effects enables a broader understanding of their potential as sustainable tools for promoting health and immunocompetence in cultured fish, particularly in economically important species such as tambaqui.

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CHAPTER II – Molecular identification and pathogenic potential of *Aeromonas* spp.

5. MATERIAL AND METHODS

5.1. Collection and Isolation

Apparently healthy specimens of tambaqui (*C. macropomum*) were collected from semi-intensive systems during the fattening phase at nine fish farms in the municipalities of Presidente Figueiredo (3), Itacoatiara (3), and Manacapuru (3) in the Manaus Metropolitan Region (Amazonas, Brazil). A total of 90 fish (10 fish per farm) were transported alive to the Microbiology and Water Analysis Laboratory of the Federal Institute of Education, Science, and Technology of Amazonas (IFAM), Presidente Figueiredo Campus, for microbiological analysis.

Fish were euthanized with an overdose of benzocaine. Fragments of the cranial kidney from each fish were collected separately under aseptic conditions in a laminar flow hood, immediately inoculated onto MacConkey Agar (Difco) culture medium, and incubated at 29 °C for 24 hours. After the growth of colorless, pink, or greenish colonies, one colony was transferred to TSA Agar (Difco) and incubated at 29 °C for 24 h. Based on this growth, catalase, oxidase, and Gram staining tests were performed. Upon confirmation of the presence of pure Gram-negative coccobacilli, positive for catalase and oxidase, the isolates suggestive of *Aeromonas* spp. were stored in TSB Broth (Difco) with 20% sterile glycerol at -80 °C.

All experimental procedures were conducted under a protocol approved by the Animal Ethics Committee of the Federal Institute of Education, Science and Technology of Amazonas (IFAM) (Protocol nº 1741.0810/2020).

5.2. Molecular identification of bacterial isolates

Subsequently, each bacterial isolate was inoculated onto TSB Broth (Difco) and incubated at 29 °C for 18 h. After the incubation periods, 1.0 mL of the bacterial culture was used to extract DNA from the isolates. The Dneasy Blood and Tissue kit (Qiagen) was used according to the manufacturer's instructions. Quantification was performed by fluorimetry using the QuBit 4.0 instrument (ThermoFisher).

The PCR reaction consisted of: 2.5 µL of 10X buffer (10 mM Tris-HCl, 50 mM KCl); 0.2 µL of 25 mM DNTP; 1.0 µL of 50 mM MgSO₄; 0.2 µL of Taq High Fidelity (Platinum®Taq DNA Polymerase, Life Technologies, NY, USA); 2.0 µL of each primer

(10 pmol); 25 ng of template DNA; ultrapure water q.s.p. (sufficient for a 25 μ L reaction). The amplification program used was: 94 °C for 2 min, 35 cycles at 94 °C for 30 seconds, 55 °C (specific for annealing of each primer) for 30 s, 68 °C for 1.5 min, and 68 °C for 10 min for final extension. The primers used for the bacterial 16S rRNA gene were: 8F (AGA GTT TGA TYM TGG CTC AG) and 907R (CCG TCA ATT CMT TTR AGT TT), as described by Ben-Dov *et al.* (2006), Felske *et al.* (1997) e Lane *et al.* (1985). The amplicons generated (900 bp) were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The run was performed following the recommendations of Sambrook (2001).

The PCR products were purified using the MinElute Kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. Subsequently, the purified PCR products were quantified using QuBit 4.0, and genetic sequencing was performed using 50 ng/ μ L of sample. The sequencing methodology was recommended by Ref. (SANGER *et al.*, 1977). Sequencing was performed at the Multi-User Laboratory for Large-Scale Sequencing and Gene Expression (UNESP, Jaboticabal, Brazil). The obtained nucleotide sequences were visualized using the "Phred/Phrap/Consed" software (GORDON *et al.*, 1998).

Sequences longer than 400 bases and with a Phred quality score above 20 were selected using the Contgen program. The fragment was compared with sequences deposited in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>), requiring 100% coverage and an identity of 98% or higher with the deposited template to confirm the sequence's specificity. Subsequently, the sequences from this study were deposited in the NCBI GenBank database to obtain accession numbers.

5.3. Virulence and pathogenicity

Virulence and pathogenicity were confirmed by the presence of 12 virulence factor genes: lateral flagellum B (*LafB*), nuclease (*Nuc*), aerolysin (*aerA*), aerolysin (*Aero*), hemolysin (*hlyA*), lipase (*Lip*), glycerophospholipid cholesterol acyltransferase (GCAT), serine protease (*Ser*), cytotoxic enterotoxin (*act*), cytolytic enterotoxin (*ast*), cytolytic/cytotonic enterotoxin (*alt*), and type III secretion system (T3SS) (*ascV*). The presence of virulence genes was assessed by PCR using gene-specific primers. DNA extraction and the 25 μ L PCR reaction were performed as described in Section 5.2, and the primers and PCR conditions are described in Table 1.

Table 1. Primer sequences used for the detection of virulence genes in *Aeromonas* strains isolated from tambaqui (*C. macropomum*).

	Genes	Sequence Fw primer	Sequence Rw primer	Amplicon (bp)	Reference
Flagella	Lateral Flagella B (<i>LafB</i>)	GACCAGCAAGGAT AGTGGGTTGGAG	AAGCACCATCGCG TTGGTATAAGG	624	Nam and Joh (2004)
Nuclease	Nuclease (<i>Nuc</i>)	CAGGATCTGAACCG CCTCTATCAGG	GTCCCAAGCTTCG AACAGTTTACGC	504	Nam and Joh (2004)
Haemolysin	Aerolysin (<i>Aero</i>)	GAGCGAGAAGGTG ACCACCAAGAAC	TTCCAGTCCCACC ACTTCACTTCAC	417	Nam and Joh (2004)
	Aerolysin (<i>aerA</i>)	TTTGGAACCCATTT CTCGTGTGGC	TCGAAGTAGTCCG GGAAGGTCTTGG	1,236	Heuzenroeder <i>et al.</i> (2003)
	Haemolysin (<i>hylA</i>)	CCACGCAAATTCAT CACG	ATCCTTGTTCCACC TCGAC	1,079	Heuzenroeder <i>et al.</i> (2003)
Lipase	Lipase (<i>Lip</i>)	GACCCCCTACCTGA ACCTGAGCTAC	AGTGACCCAGGAA GTGCACCTTGAG	155	Nam and Joh (2004)
	Glycerophospholipid- cholesterol-acyltransferase GCAT	CTCCTGGAATCCCA AGTATCAG	GGCAGGTTGAACA GCAGTATCT	237	Nerland (2007)
Protease	Serine protease (<i>Ser</i>)	ACGGAGTGCGTTCT TCCTACTCCAG	CCGTTCATCACAC CGTTGTAGTCG	211	Nam and Joh (2004)
Cytolytic enterotoxin	<i>act</i>	AGAAGGTGACCAC CAAGAACA	AACTGACATCGGC CTTGAACTC	232	Kingombe <i>et al.</i> (2010)
	<i>ast</i>	TCTCCATGCTTCCC TTCCACT	GTGTAGGGATTGA AGAAGCCG	331	Sen and Rodgers (1992)
	<i>alt</i>	TGACCCAGTCCTGG CACGGC	GGTGATCGATCAC CACCAGC	442	Sen and Rodgers (1992)
Type III secretion system (TTXX)	<i>ascV</i>	GTAARCAGATGAGT ATCGATGG	GAGACSCGGGTGA CGATAAT	331	Yu <i>et al.</i> (2000)

5.4. *In vivo* experimental challenge

For the *in vivo* experiment, a strain identified as *Aeromonas* in step 5.2 and exhibiting the highest number of virulence genes in step 5.3 was selected. After a 10-day acclimatization period, 90 healthy juvenile tambaqui (70.41 ± 0.57 g, 16.50 ± 0.27 cm) were divided into five groups. All groups had six fish, and the experiment was conducted in triplicate, totaling 18 animals per group.

The *in vivo* experiment was conducted at the Embrapa Western Amazon facilities, in 300-L polyethylene tanks with constant water flow and aeration, and thermostats set to 30 °C. Water temperature (31.59 ± 0.295 °C), pH (5.30 ± 0.278), and dissolved oxygen (4.94 ± 0.165 mg L⁻¹) were measured daily in all groups and were suitable for tambaqui maintenance.

The selected strain was reactivated in 20 mL of TSA Agar (Difco). Single colonies were transferred to TSB Broth (Difco), and the bacterial suspensions were incubated (24 h at 29 °C). Next, an aliquot of the bacterial suspension was diluted in 0.85% saline until the desired optical density (OD) reading was reached (ThermoScientific Spectrophotometer) at 600 nm (OD 600), according to a methodology adapted from Gallani *et al.* (2020).

Following this methodology, five groups were established for the challenge. Groups G1, G2, G3, and G4 corresponded to final concentrations of 2.9×10^7 ; 5.2×10^7 ; 9.3×10^7 ; and 1.2×10^8 CFU mL⁻¹ of the selected strain, respectively. Group G0 (negative control) received an injection of 0.85% saline solution. For the challenge test, the fish were anesthetized with 100 mg L⁻¹ of benzocaine (Acros) and then inoculated intraperitoneally with 0.1 mL of the respective solution per 10 g of fish weight. Clinical signs of the disease and fish mortality were recorded twice daily for 15 days following the challenge. The lethal doses LD₁₀, LD₅₀, LD₉₀, and LD₉₉ were calculated according to the methodology proposed by Lei and Sun (2018).

Recently deceased animals were collected for microbiological examinations, following standard microbiological procedures. The cranial kidney was used for re-isolation of colonies on TSA Agar (24 h at 28 °C). To confirm bacterial identity, bacterial DNA extraction and PCR followed by sequencing were performed as described in section 5.2.

6. RESULTS

A total of 180 strains were isolated from MacConkey agar plates, and following biochemical testing, 33 strains were selected as likely *Aeromonas* spp. for molecular biology testing. The selected strains were Gram-negative, oxidase and catalase positive. Table 2 presents the identified genera and species, with their respective GenBank accession numbers, as well as the similarity percentage for each strain. One strain of *A. jandaei* and five strains of *A. hydrophila* were confirmed. Satisfactory results could not be obtained for the molecular identification of seven strains.

Table 2. Molecular identification of bacterial isolates from tambaqui (*C. macropomum*).

Strain	Strain ID	% similarity to the type strain
A-01	<i>Chromobacterium alkanivorans</i>	99.45
A-02	<i>Citrobacter freundii</i>	99.34
A-03	<i>Chromobacterium rhizoryzae</i>	99.59
A-04	<i>Enterobacter sichuanensis</i>	98.28
A-06	<i>Escherichia fergusonii</i>	99.63
A-07	<i>Citrobacter freundii</i>	99.85
A-08	<i>Aeromonas jandaei</i>	96.49
A-09	<i>Plesiomonas shigelloides</i>	99.86
A-14	<i>Klebsiella quasipneumoniae</i> subsp. <i>similipneumoniae</i>	99.62
A-17	<i>Citrobacter braackii</i>	99.79
A-18	<i>Pseudomonas otitidis</i>	99.61
A-19	<i>Enterobacter quasihomaechei</i>	100
A-22	<i>Citrobacter cronae</i>	98.48
A-26	<i>Enterobacter cloacae</i>	97.97
A-28	<i>Morganella morganii</i>	99.74
A-29	<i>Citrobacter freundii</i>	99.84
A-30	<i>Aeromonas hydrophila</i>	100
A-31	<i>Bacillus thuringiensis</i>	98.92
A-32	<i>Citrobacter freundii</i>	99.85
A-33	<i>Citrobacter freundii</i>	99.79

A-34	<i>Aeromonas hydrophila</i>	99.78
A-35	<i>Citrobacter pasteurii</i>	96.59
A-37	<i>Aeromonas hydrophila</i>	99.75
A-38	<i>Aeromonas hydrophila</i>	99.75
A-39	<i>Morganella morganii</i>	97.21
A-44	<i>Aeromonas hydrophila</i>	100

The partial 16S rRNA gene sequence (409–874 bp) of strain A-08 showed 96.49% similarity with several *A. jandaei* sequences deposited in GenBank, including those isolated from the intestine of the fish *Micropterus salmoides* in China and from the tambaqui *C. macropomum* in the Brazilian Amazon. In contrast, the partial 16S rRNA gene sequences from strains A-30, A-37, A-38, and A-44 showed 100% and 99.75% similarity to various *A. hydrophila* sequences deposited in GenBank, including those isolated from *Oreochromis niloticus* tilapia in Uganda, strains isolated from the spleen and kidney of diseased farmed *Brycon cephalus* fish in Peru, strains isolated from the intestinal mucosa of grass carp *Ctenopharyngodon idella* in China, and strains isolated from tambaqui farmed in the same region as the present study.

The species of the other strains identified in this study are also listed in Table 2, notably *Citrobacter freundii*, which had the highest isolation frequency (5/26), as well as *Plesiomonas shigelloides*, *Pseudomonas otitidis*, and *Morganella morganii*, all of which are emerging pathogens in aquaculture. Distribution of virulence genes among *Aeromonas jandaei* and *Aeromonas hydrophila* strains isolated from farmed tambaqui are shown in Table 3. The *A. jandaei* strain (A-08) exhibited 9/12 of the virulence genes analyzed; the *A. hydrophila* isolates exhibited 10/12 (A-30) and 9/12 (A-34, A-37, A-38, A-44) virulence genes. Seven virulence factors—*aerA*, *Ser*, *Lip*, GCAT, *act*, *Nuc*, and *alt*—were detected in all strains. The lateral flagellum B gene (*LafB*) was identified in only one *A. hydrophila* strain (A-44).

Table 3. Distribution of virulence genes among *Aeromonas jandaei* and *Aeromonas hydrophila* strains isolated from tambaqui (*C. macropomum*).

Strains	Genes											
	aerA	Ser	Lip	GCAT	hlyA	act	LafB	Nuc	Aero	ast	alt	ascV
<i>A. jandaei</i>												
A-08	+	+	+	+	+	+	-	+	-	+	+	-
<i>A. hydrophila</i>												
A-30	+	+	+	+	+	+	-	+	+	+	+	-
A-34	+	+	+	+	+	+	-	+	+	+	+	-
A-37	+	+	+	+	+	+	-	+	-	-	+	+
A-38	+	+	+	+	-	+	-	+	-	+	+	+
A-44	+	+	+	+	+	+	+	+	-	-	+	-

The *A. hydrophila* strain A-30 was selected for the pathogenicity assay because it tested positive for the greatest number of the virulence genes tested. Healthy tambaqui specimens inoculated with this strain in the *in vivo* pathogenicity assay, developed the following external clinical signs shown in Figure 1: skin ulcers with deep lesions and reddish edges (1-a), erosion and necrosis of the caudal, dorsal, and anal fins (1-b), and skin depigmentation and necrosis in the ventral region (1-c).



Figure 1. External clinical signs in tambaqui (*Colossoma macropomum*) experimentally infected with *Aeromonas hydrophila*.

Fish in the control group (G0), inoculated with 0.85% sterile saline solution, exhibited dark gray skin and a black ventral region extending to the lateral line, typical of the species. This was the only group that did not exhibit behavioral changes, feeding and swimming normally after the challenge. No mortality was recorded in this group.

Fish in Group 1 (G1) inoculated with 2.9×10^7 CFU mL⁻¹ of *A. hydrophila* exhibited moderate infection. The fish swam normally but did not feed for a few days after inoculation. The characteristic coloration of the species' integument was visible as normal, except for slight darkening. Some ulcerative lesions in different skin regions and the onset of corrosion lesions on the fins were observed starting on the second day post-inoculation. Mortality was recorded on days 1 and 2 (9/18).

Fish in group 2 (G2), inoculated with 5.2×10^7 CFU mL⁻¹, and in group 3 (G3), inoculated with 9.3×10^7 CFU mL⁻¹ of *A. hydrophila*, exhibited severe infection and did not feed during the 14-day experiment. On the first day after challenge, skin lesions were observed, mainly in the inoculated area and on the fins. Some animals exhibited extreme lethargy and swam at the bottom of the water. Mortality in these groups was recorded from the first to the third day, with 14/18 for G2 and 15/18 for G3.

Fish in group 4 (G4), inoculated with 1.2×10^8 CFU mL⁻¹ of *A. hydrophila*, exhibited very severe infection. The symptoms were similar to those observed in groups 2 and 3, but the lethality of the dose was higher and occurred over a shorter period of time. Mortality was recorded on days 1 and 2 (17/18). The sole surviving fish in this group at the end of the experiment exhibited skin depigmentation with lesions covering its entire body, as well as necrosis and erosion of the fins.

Figure 2 shows the cumulative mortality of tambaqui infected with *A. hydrophila*. Starting on the 4th day, mortality ceased at all tested concentrations. The colonies isolated from the challenged fish showed the same identity and similarity (corresponding to 100% *A. hydrophila*) as those initially isolated.

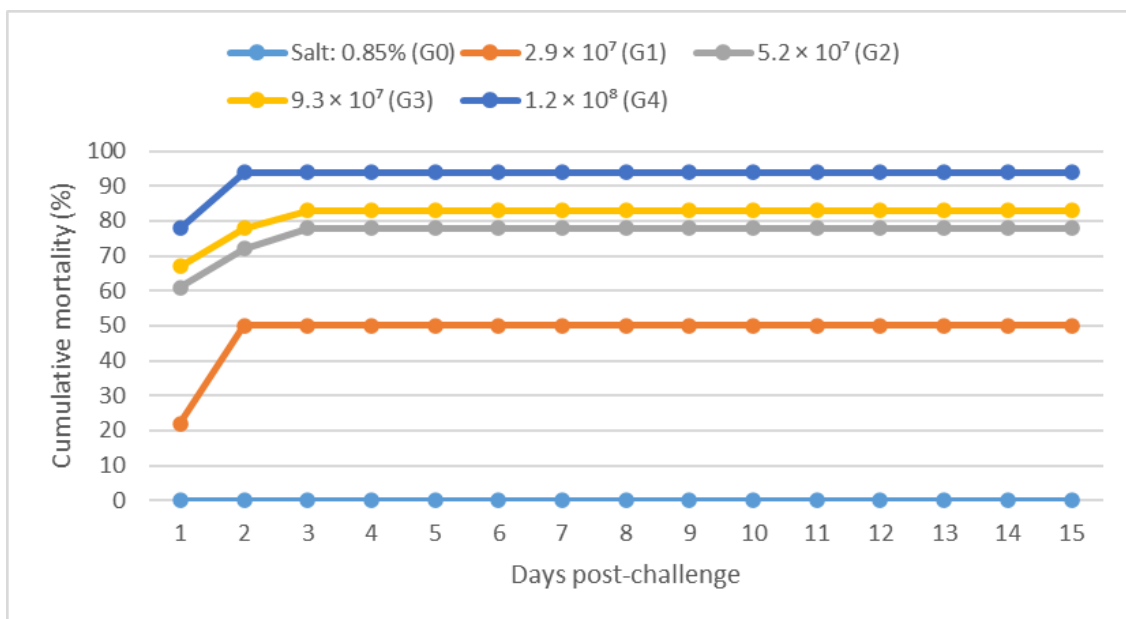


Figure 2. Experimental infection of tambaqui (*Colossoma macropomum*) with *Aeromonas hydrophila*. Cumulative mortality over 15 days following intraperitoneal inoculation of bacteria at four increasing concentrations (CFU mL⁻¹) and control.

Based on the mortality data in Figure 2, the lethal doses for the tested *A. hydrophila* strain were calculated, with LD₁₀ (10% mortality) equivalent to 9.1×10^6 ; LD₅₀ (50% mortality) equivalent to 2.9×10^7 ; LD₉₀ (90% mortality) equivalent to 9.1×10^7 ; and LD₉₉ (99% mortality) equivalent to 2.3×10^8 CFU mL⁻¹.

7. DISCUSSION

Molecular identification conducted in this study classified approximately 3% (1/33) of the isolated tambaqui strains as *Aeromonas jandaei*, while about 15% (5/33) were identified as *Aeromonas hydrophila*. These bacteria stand out as significant pathogens in aquaculture, frequently associated with septicemia, specifically Motile *Aeromonas* Septicemia (MAS), in freshwater fish, causing significant economic losses in the sector (AISIAH *et al.*, 2020).

Pellin *et al.* (2023) investigated the occurrence of *Aeromonas* in tambaqui from farming systems located in regions near the fish farming area evaluated in the present study. The authors observed a predominance of *A. jandaei* isolates (92%), while *A. hydrophila* accounted for only 4% of the total isolates. Sebastião *et al.* (2022), when investigating the occurrence of *Aeromonas* in asymptomatic tambaqui raised in the same region (Amazonas, Brazil), reported an isolation rate of *Aeromonas* spp. of 57.1%. In addition, the authors identified other isolated bacteria, including *Plesiomonas shigelloides* and representatives of the genera *Chromobacterium* and *Pseudomonas*, results similar to those observed in the present study.

Yu *et al.* (2024), while conducting a similar study with freshwater farmed fish in China, reported a comparable isolation rate of *A. hydrophila* (11.54%), using the 16S rRNA gene for molecular identification. The same authors did not report isolation of *A. jandaei*, with *A. veronii* considered the most frequent species in fish farms in that region. Kumar *et al.* (2022), when investigating the prevalence of *Aeromonas* strains in farmed fish in different regions of India, reported a high isolation rate of bacteria of this genus (83.1%). However, they observed a low isolation frequency of *A. hydrophila* (3/309) and *A. jandaei* (2/309), corresponding to less than 1% for both species.

Zhang *et al.* (2019) identified *Aeromonas* spp. from strains isolated from northern snakehead fish (*Channa argus*) farmed in China. The authors reported a higher percentage of bacterial isolates belonging to this genus (67.6%), with a 24.6% isolation rate for *A. hydrophila*.

Studies show that increased detection of potentially pathogenic bacteria is associated with environmental imbalance, host stress, and high organic load in the environment. These conditions favor the transition of commensal or opportunistic bacteria to pathogenic behavior (SHEPPARD, 2022). According to Abdella *et al.* (2024), the virulence of *Aeromonas* is directly associated with environmental culture conditions. Variations in water temperature and pH influence the metabolic activity and growth rate

of *A. hydrophila*, modulating its pathogenicity. High ammonia concentrations can intensify infections by compromising host immunity and promoting bacterial proliferation. According to these authors, oxygen levels, host physiological signals, and diet formulation influence the susceptibility of aquatic organisms to infection, serving as determining factors in the ecology, physiology, and pathogenicity of *A. hydrophila* in aquaculture.

It is observed that most studies have focused on the detection of *Aeromonas* strains in farmed fish exhibiting clinical signs of disease or a moribund condition, generally associated with infectious outbreaks in aquaculture systems with high mortality rates (KAUR *et al.*, 2024; MURSALIM *et al.*, 2022; SALEH *et al.*, 2021; TAHA, 2021). Under these circumstances, the animals already exhibit clinical, behavioral, and physiological signs characteristic of the disease, resulting in a high frequency of isolation of strains belonging to this bacterial genus. Adah *et al.* (2024) investigated the prevalence of *Aeromonas* spp. strain isolation in African catfish (*Clarias gariepinus*) farmed in Nigeria, comparing the isolation frequency between clinically ill and apparently healthy individuals. The authors observed a higher isolation rate (40.9%) in strains from fish exhibiting clinical signs consistent with aeromonosis, compared to asymptomatic fish (25%), corroborating the aforementioned statement. The study also demonstrated a statistically significant difference in the prevalence of *Aeromonas* species between clinically healthy fish and diseased fish.

The detection of *Plesiomonas shigelloides*, *Pseudomonas otitidis*, and species of *Chromobacterium* in farmed fish suggests the presence of opportunistic microorganisms that are naturally associated with the aquatic environment but have the potential to cause infection under predisposing conditions. *P. shigelloides* is frequently isolated from freshwater ecosystems and aquatic organisms; it is considered opportunistic and, under certain circumstances, associated with health issues in fish (MOHAMMED *et al.*, 2026). Although *P. otitidis* is not among the classic pathogens of fish farming, the genus *Pseudomonas* includes species recognized as pathogenic to fish, indicating that its occurrence may reflect environmental imbalances or immune compromise in the hosts (TEE *et al.*, 2024). Similarly, *Chromobacterium* species, although rarely described as primary pathogens in fish, are common in tropical aquatic environments and can act as opportunistic agents (BATISTA; DA SILVA NETO, 2017). Together, these findings reinforce the importance of microbiological monitoring and proper water quality

management, since environmental changes can favor the pathogenic expression of environmentally ubiquitous bacteria in aquaculture systems.

Although there are no previous reports of *Citrobacter freundii* isolation in farmed tambaqui, this bacterium has been widely reported in aquaculture systems, frequently associated with infectious outbreaks and mortality events in fish farms. Gong *et al.* (2023) isolated and identified *C. freundii* as the etiological agent responsible for a disease outbreak in black bass (*Micropterus nigricans*) farms in Guangdong Province, China. According to the authors, infected fish exhibited clinical signs characteristic of septicemia, including acute hemorrhage and abdominal distension, highlighting the pathogenic potential of this species in farmed aquatic organisms. *Citrobacter freundii* was also isolated from moribund tilapia (*Oreochromis niloticus*) during a mortality outbreak at a fish farm in India, in association with *Pseudomonas aeruginosa*, *Aeromonas juvenii*, and *Aeromonas veronii* (RAJ *et al.*, 2019).

Additionally, the detection of enterobacteria such as *Enterobacter* spp., *Klebsiella quasipneumoniae*, *Citrobacter* spp., *Escherichia fergusonii*, and *Morganella morganii* underscores the public health significance of aquaculture environments, as these microorganisms are widely recognized as human opportunistic pathogens, frequently associated with nosocomial infections and antimicrobial resistance (PATERSON, 2006; PELLI, 2017).

The presence of *Plesiomonas shigelloides*, a bacterium with zoonotic potential associated with waterborne or foodborne gastroenteritis, is also noteworthy (JIANG *et al.*, 2017; SELIM *et al.*, 2022). The genus *Plesiomonas* is widely distributed in aquatic environments, acting as an opportunistic pathogen in fish and representing a potential source of infection for humans. In this context, several studies have been conducted to investigate the possible transmission of this pathogen through fish products intended for human consumption, particularly via the ingestion of raw or undercooked fish and seafood (CORTÉS-SÁNCHEZ *et al.*, 2021). These findings highlight the interface between animal health, environmental health, and human health, reinforcing the need for continuous microbiological monitoring in aquaculture systems from the perspective of the One Health concept, especially in regions of intensive production (LAMY *et al.*, 2022; ZIARATI *et al.*, 2022).

Hamza *et al.* (2020) isolated strains belonging to the Enterobacteriaceae family in integrated agriculture and aquaculture systems in Egypt. Samples were obtained from internal organs (liver, spleen, and kidney) of fresh and apparently healthy tilapia (*O.*

niloticus) specimens, with the isolation of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* reported; the latter was also identified in the present study.

Among all bacterial species isolated in this study, only *Bacillus thuringiensis* is Gram-positive, consisting of spore-forming bacilli. This species is widely distributed in the environment, commonly found in soil, the rhizosphere, and decomposing organic matter, and can reach aquatic environments via surface runoff and particle transport (ARGÔLO-FILHO; LOGUERCIO, 2013; ICHIMATSU *et al.*, 2000; IRIARTE *et al.*, 2000).

Bacterial virulence factors play a central role in pathogenesis, acting in the processes of adhesion, invasion, replication, and evasion of the host's immune responses (DENZER *et al.*, 2020). These mechanisms contribute to the establishment of infection, promote tissue damage, compromise the host's defense systems, and, in more severe cases, can lead to the death of the host (DE SILVA *et al.*, 2021).

Aeromonas strains possess a wide range of virulence factors, including adhesion properties, lipopolysaccharides, siderophores, toxins, colonization, extracellular secretions, iron acquisition, and quorum sensing mechanisms. Furthermore, *Aeromonas* spp. possess various virulence genes that encode these different pathogenic properties, whose expression can be modulated and intensified under conditions of environmental stress, favoring the establishment and progression of infection (MAJEED *et al.*, 2023). Given that *Aeromonas* species are widely distributed in freshwater habitats, aquatic organisms are constantly at risk of infection, especially in intensive aquaculture systems (LI *et al.*, 2021; SURESH *et al.*, 2023). In this context, most of the virulence factors described for this genus have been reported in farmed fish, often associated with disease outbreaks and high-mortality events (MULIA *et al.*, 2024; PRADHAN *et al.*, 2023; TOOBA *et al.*, 2024).

The six *Aeromonas* strains analyzed in the present study tested positive for at least nine of the twelve putative virulence genes investigated, with one *A. hydrophila* strain (A-30) exhibiting ten detectable genes. Among the genes evaluated, *aerA*, *Ser*, *Lip*, *GCAT*, *act*, *Nuc*, and *alt* were identified in all *A. jandaei* and *A. hydrophila* strains analyzed. The *hlyA* gene was absent in only one *A. hydrophila* strain (A-38). In turn, the *LafB* gene had the lowest frequency, being detected exclusively in one *A. hydrophila* strain (A-44), followed by the *aero* and *ascV* genes, identified in only two *A. hydrophila* strains.

Pellin *et al.*(2023), when investigating *Aeromonas* strains isolated from tambaqui fish farmed in the same region as the present study, reported a lower frequency of detection for the *aerA*, *ast*, and *act* genes. Only 32% of the analyzed strains tested positive for at least one of the evaluated genes, with *ast* being the most frequently identified, while the *aerA* gene was not detected in any of the strains. In general, the detection of virulence genes in isolates from healthy animals is usually lower compared to strains associated with pathological conditions.

Jung-Schroers *et al.*(2019) investigated the presence of virulence factors in different *Aeromonas* species isolated from the internal organs of farmed fish exhibiting clinical signs of bacterial disease. The authors observed a high prevalence of virulence-associated gene fragments in most of the analyzed species, particularly in *A. hydrophila*, a finding similar to that observed in the present study. In both studies, marked variability was observed in the distribution of genes among the isolates, with no uniform pattern of occurrence or exclusivity of these determinants to a single species. Furthermore, positivity for the *Ser*, *Lip*, and *Nuc* genes was observed in all isolates evaluated in both studies.

Assane *et al.*(2021) performed phenotypic and genotypic characterization of *Aeromonas jandaei* isolates from farmed tilapia (*O. niloticus*) involved in a mass mortality outbreak in Brazil. The authors reported the detection of at least four virulence factors, including the hemolysin aerolysin (*aerA*) and nuclease (*Nuc*) genes, findings similar to those of the present study. According to Sheikh *et al.*(2023), degradative enzymes such as protease and lipase are encoded by the *aerA* gene, causing the degradation of cellular tissues and the formation of holes in the intestine and stomach, followed by hemorrhage and septicemia in the organ. According to the authors, lesions in the skin and muscles due to protein secretion have also been shown to have a significant effect on infected fish due to aerolysin activity, with *aerA* being one of the most frequently detected genes.

According to studies by Lee *et al.*(2023), the *hylA*, *ast*, *aerA*, and *act* genes, detected in the strains of the present study, encode cytotoxic and cytotoxic enterotoxins, as well as exotoxins. Furthermore, according to the authors, the *Aero*, *aerA*, and *act* genes are associated with hemolytic activity in *Aeromonas* strains, encoding toxins capable of causing lysis of red blood cells (erythrocytes) and damage to different types of host cells. These toxins act primarily by forming pores or degrading the cell membrane, resulting in cell rupture and the release of its contents.

Monitoring virulence determinants in *Aeromonas* isolates is essential not only to support the adoption of more effective therapeutic strategies, but also for the establishment of appropriate measures for outbreak management, tracing sources of infection, and risk assessment. Thus, it contributes to the development and implementation of more efficient preventive and control strategies, aiming to strengthen fish farming and reduce economic losses throughout the fish production chain.

The pathogenicity of *Aeromonas* spp. is multifactorial in nature, being related to various aspects, such as the expression and regulation of virulence genes, host-pathogen interactions, and the environmental conditions to which the organisms are subjected (TAO *et al.*, 2026). In this context, the detection of genes associated with virulence does not necessarily imply the effective expression of pathogenic potential in the host. Thus, complementary studies are necessary, including experimental assays that evaluate different bacterial doses and concentrations, as well as the manifestation of clinical signs of the disease.

Historically, virulence has been assessed based on quantifiable host outcomes, such as mortality. In this context, the most widely used measure is the lethal dose capable of causing the death of 50% of infected hosts (LD₅₀). Determining the LD₅₀ allows for comparison between different microorganisms, using host mortality as a clear and objective outcome (CASADEVALL, 2017).

The LD₅₀ (2.9×10^7 CFU/mL) observed in the present study was lower than the LD₅₀ of *A. hydrophila* strains isolated from pacu *Piaractus mesopotamicus* (2.1×10^8 CFU mL⁻¹) (CLAUDIANO *et al.*, 2020) and from the pirarucu *Arapaima gigas* (1.8×10^8 CFU mL⁻¹) (DIAS *et al.*, 2016). However, it was higher than the LD₅₀ of *A. hydrophila* isolated from farmed fish as defined by Azzam-Sayuti *et al.*(2021) at 1.0×10^5 CFU mL⁻¹, by Putri *et al.*(2022) at 1.0×10^6 CFU mL⁻¹ and by Bakiyev *et al.*(2022) at 2.89×10^6 CFU mL⁻¹.

Gallani *et al.*(2020), in investigating the virulence and pathogenicity profile of *Aeromonas hydrophila* isolated from an outbreak of hemorrhagic septicemia in farmed tambaqui in the same region as the present study, determined LD₁₀, LD₅₀, LD₉₀, and LD₉₉ values which were higher than those observed in this study. In pathogenicity assays, it is considered that the lower the LD₅₀ value, the greater the lethal potential of the evaluated strain. Although the strains analyzed in the present study were isolated from apparently healthy fish, the lower lethal dose values observed suggest a higher virulence and pathogenic potential compared to the strain described by Gallani.

8. CONCLUSION

The present study provided comprehensive information on the virulence and pathogenic potential of *Aeromonas* strains isolated from apparently healthy farmed tambaqui. Considering the decline in productivity observed in the tambaqui production chain in recent years, health monitoring of farmed fish in the region is essential. In this context, it is essential that fish farmers remain vigilant regarding the possibility of aeromonosis outbreaks, recognizing that the adoption of proper management practices and continuous monitoring of water quality are effective measures to reduce the risk of virulence factors manifesting. Furthermore, the results obtained in this study provide relevant information that can support the development of strategies for the prevention, control, and treatment of this disease in farmed tambaqui.

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CHAPTER III – Antimicrobial activity of essential oils against *Aeromonas* spp. isolated from tambaqui (*Colossoma macropomum* Cuvier, 1816)

10. MATERIAL AND METHODS

10.1. Collection of plant material and essential oil extraction

Leaves, inflorescences and/or rhizomes of *Croton sacaquinha*, *Cymbopogon nardus*, *Curcuma longa*, *Lippia origanoides*, *Mentha arvensis*, *Ocimum basilicum*, *Ocimum micranthum*, *Piper hispidum* and *Piper marginatum* were collected from mature plants belonging to the Medicinal Plant Collection of Embrapa Amazônia Ocidental, located in Manaus, AM, Brazil. The plant material was left to air dry at room temperature until constant weight was achieved.

Following this, the EO were extracted by hydrodistillation using a Clevenger apparatus. For each extraction, 500 g of dried leaves, inflorescences and/or rhizomes from each plant species were placed in a 12,000 mL round-bottom flask connected to a heating mantle, with distilled water added until the plant material was fully immersed. Once the heating mantle was activated and the EO began to distill, the process was considered to have started. At the end of the extraction, the volume of EO obtained was measured, collected, and stored in amber glass vials sealed with caps and stoppers, and kept frozen until chemical composition analysis.

10.2. Chemical composition of essential oils

Samples of the EO from *Croton sacaquinha*, *Cymbopogon nardus*, *Curcuma longa*, *Lippia origanoides*, *Mentha arvensis*, *Ocimum basilicum*, *Ocimum micranthum*, *Piper hispidum* and *Piper marginatum* were sent to Embrapa Agroindústria de Alimentos, where fractionation and chemical characterization were performed using gas chromatography on an Agilent 6890 system coupled with an Agilent 5973N mass selective detector, operating in electron ionization mode (70 eV).

According to methodology described by Chagas *et al.* (2020), for component separation, an HP5-MS capillary column (30 m × 0.25 mm × 0.25 µm) was used with a temperature program from 60 to 240 °C, increasing at 3°C/min. Samples (1.0 µL) were injected in split mode (1:100) into an injector maintained at 250 °C. Helium was used as the carrier gas (1.0 mL·min⁻¹). For relative quantification (area %) of the EO components, a gas chromatograph Agilent 6890N equipped with a flame ionization detector (FID) set at 280 °C was used, along with an HP5 capillary column (30 m × 0.32 mm × 0.25 µm),

and hydrogen as the carrier gas ($1.5 \text{ mL} \cdot \text{min}^{-1}$). The remaining analytical parameters were the same as described above.

Constituent identification for each oil was carried out by comparing the obtained mass spectra with those in the Wiley mass spectral library (6th edition), as well as by comparing the calculated retention indices with data from the literature (VAN DEN DOOL; KRATZ, 1963; ADAMS, 2007). Quantitative data were obtained from peak areas using the FID, normalized with an internal standard (ethyl octanoate). All EO samples were analyzed in triplicate.

10.3. Antimicrobial activity of essential oils against *Aeromonas* strains

Antimicrobial activity of the essential oils of *C. sacaquinha*, *C. nardus*, *C. longa*, *L. origanoides*, *M. arvensis*, *O. basilicum*, *O. micranthum*, *P. hispidum*, and *P. marginatum* was evaluated by determining the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). One isolate of *Aeromonas jandaei* (A-08) and five isolates of *Aeromonas hydrophila* (A-30, A-34, A-37, A-38, and A-44) were used in this study. These isolates were obtained from juvenile tambaqui farmed in small-scale fish farms in the Metropolitan Region of Manaus (Amazonas, Brazil). For the assays, the strains were cultured in Trypticase Soy Broth (TSB, Difco) for 18 hours at 29 °C.

The Minimum Inhibitory Concentration (MIC) was determined using the broth microdilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2003). A quantity of 3.2 g of each of nine EO was weighed, based on density calculation, and diluted in 50% v/v DMSO (dimethyl sulfoxide). The mixture was then filtered, and 1 mL of the filtrate was diluted in 4 mL of Mueller-Hinton Broth (Difco), yielding a stock solution of $640,000 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$. Briefly, 100 μL of Mueller-Hinton Broth (MHB) was dispensed into the wells of a 96-well microplate. Then, 100 μL of the EO stock solution was added to the first well, homogenized, and serially transferred to obtain final concentrations ranging from $320,000$ to $5,000 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ for each EO. The initial bacterial inoculum was prepared with turbidity equivalent to 0.5 on the McFarland scale and diluted (1:1000) in Mueller-Hinton Broth. Then, 100 μL of the bacterial suspension was added to each well containing the EO or diluent. Plates were incubated at 29 °C for 24 hours under aerobic conditions. A negative control, consisting of culture medium only, and a positive control, consisting of culture medium with inoculum, were also included in the assays. In addition, an antibiotic (oxytetracycline) was used as a

positive control against the bacteria. All assays were performed in triplicate.

For the determination of the Minimum Bactericidal Concentration (MBC), a 10 μ L aliquot from each well was plated on Mueller–Hinton agar (Difco). After 24 h of incubation at 29 °C, the MBC was defined as the lowest concentration of EO or antibiotic required to kill the bacterial inoculum.

11. RESULTS

Using chemical analyses, 94.5% of the chemical components of the EO from *C. sacaquinha*, 100% from *C. nardus*, 99.9% from *C. longa*, 99.8% from *L. origanoides*, 100% from *Mentha arvensis*, 99.3% from *O. basilicum*, 89.6% from *Ocimum micranthum*, 94.9% from *Piper hispidum*, and 98.0% from *Piper marginatum* were identified. Table 4 presents the main components identified in each of the EO evaluated in this study, along with their Relative Area and Linear Retention Index.

Table 4. Major components of the essential oils evaluated in the present study.

Botanical name	Common name	Compound identification	Relative area (%)	Linear retention index
<i>Croton sacaquinha</i>	Sacaquinha	β -selinene	14.5	1477
		α -cedrene	12.8	1402
		allo-aromadendrene	6.5	1416
		α -muurolene	6.0	1493
		elemol	6.0	1542
		linalool	4.7	1098
		β -copaene	4.4	1420
<i>Cymbopogon nardus</i>	Citronela	geraniol	53.3	1256
		geranial	18.0	1269
		neral	13.4	1238
		citronellol	4.7	1226
<i>Curcuma longa</i>	Açafrão	ar-tumerone	30.2	1666
		α -turmerone	13.7	1663
		curlone	13.7	1696
		α -phellandrene	10.2	1003
		1,8-cineole	7.0	1027
		α -zingiberene	3.1	1489
		terpinolene	3.0	1084
		β -sesquiphellandrene	3.0	1517
<i>Lippia origanoides</i>	Sálvia marajó	carvacrol	43.3	1303
		<i>p</i> -cymene	12.0	1021
		γ -terpinene	9.5	1054
		thymol methyl ether	5.3	1231
		(<i>E</i>)-caryophyllene	4.9	1410
		thymol	4.8	1291
		bicyclogermacrene	3.4	1487
<i>Mentha arvensis</i>	Hortelã japonesa	menthol	88.5	1174
		isomenthone	3.6	1160

		menthone	2.9	1149
<i>Ocimum basilicum</i>	Manjeriç�o	methyl-chavicol	33.9	1197
		linalool	21.4	1099
		1,8-cineole	12.7	1027
		limonene	9.7	1025
		carvone	3.2	1239
		terpeninen-4-ol	3.0	1172
<i>Ocimum micranthum</i>	Alfavaca	piperitenone oxide	36.2	1364
		limonene	18.8	1025
		menthol	8.4	1169
		1,8-cineole	3.8	1027
		terpeninen-4-ol	3.1	1137
<i>Piper hispidum</i>	Jaborandi	γ -terpinene	34.1	1058
		α -terpinene	17.0	1016
		terpinolene	8.0	1088
		α -selinene	6.3	1491
		β -selinene	5.6	1482
		<i>p</i> -cymene	5.3	1023
		(<i>E</i>)- β -caryophyllene	3.9	1416
		selin-11-en-4-ol	3.2	1650
<i>Piper marginatum</i>	Capeba	δ -3-carene	13.1	1009
		propiopiperone	8.0	1530
		(<i>E</i>)-caryophyllene	6.8	1414
		α -copaene	6.5	1371
		(<i>E</i>)- β -ocimene	5.1	1045
		spathulenol	4.8	1572
		bicyclogermacrene	4.1	1492
		methyl-eugenol	4.0	1403
		germacrene D	3.1	1476

The main components of the EO from *C. sacaquinha* were β -selinene (14.5%), α -cedrene (12.8%) and allo-aromadendrene (6.5%) among 59 identified compounds. For *C. nardus*, the main constituents were geraniol (53.3%) geranial (18.0%) and neral (13.4%) among 20 identified compounds. Ar-turmerone (30.2%), α -turmerone and curlone (13.7% each) were the main components in *C. longa*, which had 35 identified compounds. For *L. organoides*, carvacrol (43.3%) *p*-cymene (12.0%) and γ -terpinene (9.5%) were the main components among 38 identified compounds. Menthol (88.5%), isomenthone (3.6%) and menthone (2.9%) were the main components in *M. arvensis* among 20 identified compounds. For *O. basilicum*, the main constituents were methyl chavicol (33.9%), linalool (21.4%) and 1,8-cineole (12.7%) among 33 compounds. In *O. micranthum*, the predominant components were piperitone oxide (36.2%), limonene (18.8%) and menthol (8.4%) among 50 compounds. In *P. hispidum*, γ -terpinene (37.1%), α -terpinene (17.0%) and terpinolene (8.0%) were the main constituents among 24 compounds. For *P. marginatum*, the major components were δ -3-carene (13.1%), propiopiperone (8.0%) and (*E*)-caryophyllene (6.8%), among 47 compounds.

Regarding the chemical classes, oxygenated monoterpenes predominated among the identified components in the EO from *C. nardus* (96.4%), *M. arvensis* (96.8%), *P. hispidum* (59.1%), *L. organoides* (58.2%), *O. micranthum* (55.7%) and *O. basilicum* (46.4%). In *C. longa*, oxygenated sesquiterpenes were the most prevalent (63.6%), while in *C. sacaquinha* and *P. marginatum*, sesquiterpenes dominated.

The EO showed MIC values ranging from 320,000 to 10,000 $\mu\text{g}\cdot\text{mL}^{-1}$ against *A. jandaei* and 320,000 to 9,200 $\mu\text{g}\cdot\text{mL}^{-1}$ against *A. hydrophila* (Table 5).

Table 5. Minimum Inhibitory Concentration (MIC) in $\mu\text{g}\cdot\text{mL}^{-1}$ of the essential oils from *Croton sacaquinha*, *Ocimum basilicum*, *Ocimum micranthum*, *Curcuma longa*, *Cymbopogon nardus*, *Lippia organoides*, *Piper hispidum*, *Piper marginatum*, *Mentha arvensis*, and the antimicrobial agent oxytetracycline against *Aeromonas jandaei* and *Aeromonas hydrophila* strains.

Treatments	Strains					
	<i>A. jandaei</i>			<i>A. hydrophila</i>		
	A-08	A-30	A-34	A-37	A-38	A-44
<i>C. sacaquinha</i>	80,000	320,000	320,000	320,000	320,000	320,000
<i>C. nardus</i>	10,000	10,000	10,000	10,000	10,000	10,000
<i>C. longa</i>	80,000	160,000	160,000	160,000	160,000	160,000
<i>L. organoides</i>	10,000	9,200	10,000	10,000	9,200	10,000
<i>M. arvensis</i>	15,000	15,000	15,000	15,000	15,000	15,000
<i>O. basilicum</i>	80,000	160,000	160,000	320,000	320,000	320,000
<i>O. micranthum</i>	80,000	320,000	160,000	320,000	320,000	320,000

<i>P. hispidum</i>	320,000	160,000	160,000	320,000	320,000	320,000
<i>P. marginatum</i>	15,000	15,000	15,000	15,000	15,000	15,000
Oxytetracycline	0.23	30	0.94	1.88	0.23	0.23

MBC values ranged from 320,000 to 10,000 $\mu\text{g}\cdot\text{mL}^{-1}$ against *A. jandaei* and *A. hydrophila* (Table 6) for the nine EO evaluated. Tables 2 and 3 also present the results for oxytetracycline, which showed MIC values ranging from 0.23 to 30 $\mu\text{g}\cdot\text{mL}^{-1}$, and MBC values ranging from 0.47 to 30 $\mu\text{g}\cdot\text{mL}^{-1}$.

Table 6. Minimum Bactericidal Concentration (MBC) in $\mu\text{g}\cdot\text{mL}^{-1}$ of the essential oils from *Croton sacaquinha*, *Cymbopogon nardus*, *Curcuma longa*, *Lippia organoides*, *Mentha arvensis*, *Ocimum basilicum*, *Ocimum micranthum*, *Piper hispidum* e *Piper marginatum*, and the antimicrobial agent oxytetracycline against *Aeromonas jandaei* and *Aeromonas hydrophila* strains.

Treatments	Strains					
	<i>A.jandaei</i>			<i>A. hydrophila</i>		
	A-08	A-30	A-34	A-37	A-38	A-44
<i>C. sacaquinha</i>	160,000	320,000	320,000	320,000	320,000	320,000
<i>C. nardus</i>	10,000	10,000	10,000	10,000	10,000	10,000
<i>C. longa</i>	160,000	160,000	160,000	160,000	160,000	160,000
<i>L. organoides</i>	20,000	20,000	20,000	20,000	20,000	20,000
<i>M. arvensis</i>	30,000	30,000	30,000	30,000	30,000	30,000
<i>O. basilicum</i>	160,000	160,000	160,000	320,000	320,000	320,000
<i>O. micranthum</i>	160,000	320,000	160,000	320,000	320,000	320,000
<i>P. hispidum</i>	320,000	160,000	160,000	320,000	320,000	320,000
<i>P. marginatum</i>	30,000	30,000	30,000	30,000	30,000	30,000
Oxytetracycline	0.47	30	3.75	1.88	0.47	0.47

12. DISCUSSION

Essential oils can be defined as mixtures of various low molecular weight, lipophilic, aromatic, and volatile compounds, usually consisting of monoterpenes, sesquiterpenes, and phenylpropenes, as well as their oxygenated derivatives: alcohols, aldehydes, esters, ketones, phenols, and oxides (REICHLING, 2020). They have the ability to inhibit the growth of a wide range of pathogens due to the presence of these natural substances produced by plant organs. Their unique aroma and bioactive properties depend essentially on their constituents (SWAMY *et al.*, 2016).

The chemical composition of EO is primarily determined by genetic factors; however, certain biotic and abiotic environmental factors may lead to significant changes in the production and accumulation of these constituents, as they redirect metabolic pathways and promote the biosynthesis of different compounds (SOARES *et al.*, 2019). According to Shankar *et al.* (2021), the mechanism of action of each EO is directly linked to its chemical composition.

Croton sacaquinha, also known as sacaquinha or saca-rabo, is a Brazilian native plant found mainly in the Cerrado and Northeastern regions. Adaptable to various soil types, it is valued in folk medicine for its anti-inflammatory and wound-healing properties. As an indigenous species, few studies have explored the chemical profile of its EO. Chagas *et al.* (2016) analyzed the essential oil obtained from this species, using plants from the same region as the present study, and identified sesquiterpene germacrene (12.0%) as the major compound, while our study found β -selinene (14.5%), also a sesquiterpene, as the most abundant constituent. Similarly, Lopes *et al.* (2003) found β -elemene as the main component in the same region.

Cymbopogon nardus EO in the present study had geraniol as its main monoterpene (53.3%), a result consistent with Santos *et al.* (2017), who found it at 33.7% in EO of plants grown in Lavras (Minas Gerais, Brazil). However, studies in other countries show different profiles; for instance, Bayala *et al.* (2020) reported citronellal (33.06%) as the main component in oil extracted in Ouagadougou (Burkina Faso), while Das *et al.* (2021), using a similar method in Bucaramanga (Colombia), found citronellal (25.3%), citronellol (17.9%), and geraniol (11.6%) as the main components.

Curcuma longa (turmeric) is a perennial rhizomatous herb native to Southeast Asia, widely cultivated in tropical and subtropical regions (DOSOKY; SELTZER, 2018). Unlike the other species, *C. longa* EO in this study showed high chemical consistency with reports from other Brazilian regions, with ar-turmerone, α -turmerone, and curlone

(β -turmerone) as the major compounds, in similar proportions as reported by Guimarães *et al.* (2020) in Jaborandi (Bahia). These authors emphasized the EO's chemical stability as reported in the literature. Similar results were reported by Camilo *et al.* (2020) in Ceará and Teles *et al.* (2019) in Maranhão, both in Northeastern Brazil.

The major components found in *Lippia origanoides* EO in the present study were the same as those reported by Majolo *et al.* (2022) for the essential oil of *L. origanoides* A, with only minor variations observed in the concentrations of the three major components, carvacrol, *p*-cymene, and γ -terpinene. In contrast, Brasil *et al.* (2019), who analyzed *L. origanoides* oil from plants cultivated in Manaus and identified thymol and *p*-cymene as predominant. This differentiation suggests that the authors investigated the genotype *L. origanoides* B (syn. *Lippia sidoides*), which may account for the variation in essential oil composition observed for the same species cultivated within the same region. In contrast, Sousa *et al.* (2020) identified camphor, camphene, and β -bisabolene as the main constituents of the same essential oil, compounds that differ from those reported for both subtypes of *L. origanoides* in the Amazon region, in plants cultivated in Montes Altos, Maranhão, Brazil.

Despite chemical variations, the *Mentha arvensis* EO described here had menthol, isomenthone, and menthone as its main constituents, similar to the findings of Chagas *et al.* (2020) with plants from the same region. Interestingly, these components have also been reported as major in various studies across different regions (KHAN *et al.*, 2019; ISEPPI *et al.*, 2020; SHARMA *et al.*, 2023).

Ocimum basilicum (basil) is one of the most important medicinal plants of the family Lamiaceae and, in addition to its culinary use, exhibits anti-inflammatory, antifungal, and antibacterial properties. Minor variations in relative percentages have been reported; however, a consistent pattern of major constituents—particularly methyl chavicol and linalool—has been observed in the essential oil of this species across different geographic regions (MONFORT *et al.*, 2018; AGHAMIRZAEI *et al.*, 2024), in agreement with the findings of the present study. In contrast, Alimi *et al.* (2022) identified estragole (80.87%), followed by linalool (16.12%), as the predominant compound from plants cultivated in northeastern Tunisia.

Differently from the *Ocimum micranthum* EO evaluated here, Sousa *et al.* (2023) reported eugenol (42.7%) and elemicin (13.2%) as the main constituents in plants from Fortaleza (Ceará, Brazil). Silva *et al.* (2004) previously reported eugenol as the major compound in oils from the same region, supporting the claim that EO composition tends

to be more similar within the same geographical area. Zeppenfeld *et al.* (2019) also found a different profile for *O. micranthum* EO in Santa Maria (Rio Grande do Sul, Brazil), with methyl chavicol and linalool as the major compounds, distinct from both the current study in Amazonas and the study in Fortaleza.

Similarly, when comparing the composition of *Piper hispidum* oil in the present study with that reported by Majolo *et al.* (2019), both from the Amazon region and obtained using the same methodology, there was similarity in the main component γ -terpinene (34.1% and 27.3%, respectively), but differences in the second and third most abundant components: α -terpinene (17.0%) and terpinolene (8.0%) in this study; *p*-cymene (14.0%) and α -terpinene (12.0%) in Majolo *et al.* (2019). Those authors also evaluated the composition of *Piper marginatum* EO, describing the same major components in similar amounts as reported here. Ayres *et al.* (2023), when investigating the chemical composition of *P. marginatum* EO from the same region, also reported δ -3-carene as the major constituent, followed by (*E*)- β -ocimene, which in the present study accounted for only 5.1% of the total composition.

Santana *et al.* (2016) and Jaramillo-Colorado *et al.* (2019) studied *P. hispidum* EO composition in Panama and Colombia, respectively, and found distinct major components, dillapiole (57.7%) and limonene (17.2%), using the same extraction method as the present study. The chemical composition of *P. marginatum* EO obtained here differed from that reported by Santana *et al.* (2015), who used plants from different locations in Rondônia (Brazil) and found (*E*)-methyl isoeugenol (27.08%), (*E*)-anethole (23.98%), and (*Z*)-methyl isoeugenol (12.01%) as major constituents. These results reinforce the idea that plant geographical origin directly affects EO composition, influenced by soil type, environmental conditions, plant age, and developmental stage (SANTIAGO *et al.*, 2016; EL YAAGOUBI *et al.*, 2021). Therefore, approaches such as the selection of stable chemotypes and genotypes (MUNDA *et al.*, 2020; SAKAR *et al.*, 2023), the control of cultivation and seasonal harvesting conditions (CHENG *et al.*, 2024), and the systematic chemical characterization of each batch (KLŪGA *et al.*, 2021) have been highlighted as promising alternatives to ensure greater consistency and effectiveness of these essential oils. Additionally, the application of nanotechnologies, including the use of nanoparticles and nanoemulsions, may enhance the stability and bioavailability of bioactive compounds, thereby contributing to increased efficacy of essential oils in the treatment of bacterial diseases in tambaqui (MATHEWS *et al.*, 2023).

In the present study, all essential oils tested demonstrated in vitro antimicrobial

activity against *Aeromonas* strains. However, *C. nardus*, *L. origanoides*, *M. arvensis* and *P. marginatum* EO showed the most pronounced effects against the *Aeromonas* strains. The Minimum Inhibitory Concentration (MIC) values did not differ between *A. jandaei* and *A. hydrophila* strains for these essential oils.

L. origanoides EO showed the best result, with MIC of 9,200 $\mu\text{g}\cdot\text{mL}^{-1}$ against *A. hydrophila* strains A-30 and A-38, and 10,000 $\mu\text{g}\cdot\text{mL}^{-1}$ against the *A. jandaei* strain (A-08) and *A. hydrophila* strains A-34, A-37, and A-44. Recent studies have shown strong antimicrobial activity of *L. origanoides* EO against various bacterial strains, contrasting with our findings. These differences may also be attributed to variations in major components, namely thymol and carvacrol (CÁCERES *et al.*, 2020; GÓMEZ-SEQUEDA *et al.*, 2020; MARTÍNEZ *et al.*, 2023).

Furlani *et al.* (2021) reported a similar chemical composition for *L. origanoides* EO from the Brazilian Northeast, yet found moderate antimicrobial activity, with MIC of 560 $\mu\text{g}\cdot\text{mL}^{-1}$ against *Staphylococcus aureus*. The efficacy of EO may rely on individual mechanisms of its compounds or synergistic effects among them, making it difficult to determine exact modes of action (MATTE *et al.*, 2023). Bacterial species and environmental origin of strains also play a role. The strains used here were isolated from farmed tambaqui, and water may act as a vector for spreading resistance and virulence genes (PÉREZ-ETAYO *et al.*, 2020).

Majolo *et al.* (2022), studying *Lippia* species in the same region, described a similar composition for *L. origanoides* (type A: carvacrol 35.6%, *p*-cymene 15.7%, γ -terpinene 9.1%) and found stronger activity against *Aeromonas* strains from tambaqui (MIC: 260.4–833.3 $\mu\text{g}\cdot\text{mL}^{-1}$). This may suggest that *Aeromonas* strains are becoming more resistant to natural antimicrobials due to selective pressures (from medical, agricultural, and livestock practices), promoting the transfer of virulence factors that hinder the mechanisms of action of essential oils (GOMES *et al.*, 2021).

Natural aquatic ecosystems, often the endpoint of terrestrial runoff, are recognized reservoirs for antimicrobial resistance and bacterial virulence genes (CHEN *et al.*, 2018). The resident microbiota, even without clinical relevance, can serve as a genetic pool for terrestrial microbes or enter anthropogenic cycles (PETERSON; KAUR, 2018). *Aeromonas* spp. can acquire resistance mechanisms and spread them via horizontal gene transfer, making them suitable indicators for monitoring antimicrobial resistance in water (CANELLAS *et al.*, 2021).

Another factor possibly associated with the lower antimicrobial effectiveness of

L. origanoides EO in this study compared to Majolo *et al.* (2022) is temperature variation. Abiotic factors can modulate gene expression of bacterial virulence factors (STEINMANN; DERSCH, 2013; YI *et al.*, 2022; ZHANG *et al.*, 2023). The recent record-high temperatures in the Amazon may have altered regulatory systems in *Aeromonas*, increasing resistance to EO components.

C. nardus EO showed MIC of 10,000 $\mu\text{g}\cdot\text{mL}^{-1}$ against *A. jandaei* and *A. hydrophila*. Wei and Wee (2013) reported strong antimicrobial activity of the essential oil of this species against bacterial strains isolated from different aquatic organisms in Malaysia, with MIC values ranging from 0.244 to 0.977 $\mu\text{g}\cdot\text{mL}^{-1}$. The substantial variation in the antimicrobial effectiveness of the same essential oil can be explained by differences in its chemical profile, as the study conducted in Malaysia identified citronellal as the major constituent (29.2%), whereas geraniol was the predominant component in the present study.

Geraniol is an acyclic monoterpenoid alcohol belonging to the class of terpenoids and occurs naturally in the essential oils of several plant species, such as citronella, lemongrass, lavender, and geranium, among others (FAJDEK-BIEDA *et al.*, 2024). In addition to its widespread use in the perfumery and cosmetic industries, this compound exhibits antimicrobial and anti-inflammatory activities and has been shown to reduce biofilm formation (LIRA *et al.*, 2020). Guo *et al.* (2023) investigated the activity of geraniol in comparison with commonly used antimicrobials for the treatment of mastitis in dairy cows caused by bacterial infections. The authors reported that geraniol exhibited efficacy comparable to that of cephalexin and kanamycin, significantly inhibiting pathogenic bacteria without disrupting intestinal microbial communities. Moreover, no geraniol residues were detected in milk four days after treatment withdrawal, whereas antibiotic residues were still detected seven days after drug suspension.

Several studies have demonstrated the synergistic effect of geraniol in enhancing the activity of antimicrobials against resistant bacterial strains, mainly through the inhibition of bacterial efflux pumps, highlighting its potential as a promising antimicrobial adjuvant (KIM *et al.*, 2022). Araújo *et al.* (2025) reported that the use of geraniol in combination with antibiotics was effective in reducing the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* strains. According to these authors, the absence or low intrinsic antibacterial activity may be considered a favorable characteristic for substances with synergistic antimicrobial effects, since ideal efflux pump inhibitors do not exhibit direct antibacterial activity. Therefore, the essential oil of

C. nardus obtained in the present study demonstrates potential for use in association with antibiotics.

Kačániová *et al.* (2021) also reported weak antimicrobial activity of *C. nardus* essential oil against bacterial strains of different species isolated from fish, corroborating the findings of the present study. Despite the extensive characterization of its chemical composition, few studies have focused on evaluating the antimicrobial activity of *C. nardus* essential oil against bacterial strains, particularly *Aeromonas* spp., highlighting the need for further investigations involving this plant species.

The essential oil of *P. marginatum* exhibited an MIC of 15,000 $\mu\text{g}\cdot\text{mL}^{-1}$ against *A. jandaei* and *A. hydrophila* strains, with δ -3-carene identified as the major constituent. Majolo *et al.* (2019) evaluated the antimicrobial activity of essential oils from different *Piper* species against *A. hydrophila* strains also isolated from farmed tambaqui. In contrast to the present study, the authors reported strong to moderate antimicrobial activity of *P. marginatum* essential oil, with MIC values ranging from 234 to 2,500 $\mu\text{g}\cdot\text{mL}^{-1}$. Although both studies described a similar chemical composition for the essential oil of this species, its antimicrobial effectiveness against the *A. hydrophila* and *A. jandaei* strains tested in the present study was lower.

The essential oil of *M. arvensis* also exhibited an MIC of 15,000 $\mu\text{g}\cdot\text{mL}^{-1}$ against *A. jandaei* and *A. hydrophila* strains, with menthol as the major component. Chagas *et al.* (2020), when evaluating the antimicrobial activity of essential oils from several *Mentha* species cultivated in the Amazon region, reported the presence of the same compounds in proportions similar to those observed in the present study, with MIC values ranging from 1,250 to 10,000 $\mu\text{g}\cdot\text{mL}^{-1}$. Although these values were lower than those obtained in the present work, the authors also classified the antimicrobial activity of this essential oil as moderate to weak against *Aeromonas* strains isolated from tambaqui.

The essential oils of *C. sacaquinha*, *C. longa*, *O. basilicum*, *O. micranthum*, and *P. hispidum* evaluated in the present study exhibited weaker antimicrobial activity, with MIC values of 80,000 $\mu\text{g}\cdot\text{mL}^{-1}$ for *A. jandaei* and 160,000 to 320,000 $\mu\text{g}\cdot\text{mL}^{-1}$ for *A. hydrophila*. However, when considering the MBC/MIC ratio, the essential oils of these species demonstrated effective bactericidal activity, highlighting their potential for use in synergy with antimicrobials and their possible role in reducing the amount of drugs required to control pathogenic bacterial strains.

13. CONCLUSION

It can be concluded that, due to the wide diversity of metabolites present and their synergistic modes of action, essential oils represent a promising alternative for the treatment and control of bacterial diseases in aquaculture. The results obtained in this study demonstrated antibacterial activity of the essential oils of *C. sacaquinha*, *C. nardus*, *C. longa*, *L. origanoides*, *M. arvensis*, *O. basilicum*, *O. micranthum*, *P. hispidum*, and *P. marginatum* against *A. jandaei* and *A. hydrophila* strains, with bactericidal effects, indicating their potential use in combination with antimicrobials. This strategy may enhance therapeutic efficacy, reduce the use of synthetic drugs in aquatic environments, and consequently minimize selective pressure for the emergence of resistant bacterial strains in aquaculture systems. Among the essential oils evaluated, *Lippia origanoides* exhibited the lowest MIC values, followed by *Cymbopogon nardus*, *Mentha arvensis*, and *Piper marginatum*. However, despite these promising findings, potential risks related to the toxicity of essential oils must be carefully considered prior to their recommendation for use in aquaculture. Future *in vivo* studies are required to assess the safety of the concentrations proposed herein, including their effects on zootechnical, physiological and immunological parameters of tambaqui, as well as potential impacts on non-target organisms, in order to validate both the efficacy and safety of these compounds under experimental conditions closer to field reality.

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CHAPTER IV – Dietary supplementation with essential oils of *Cymbopogon nardus*, *Lippia origanoides* and *Mentha arvensis* on the growth and hemato-immunological parameters of *Colossoma macropomum* challenged with *Aeromonas hydrophila*

15. MATERIAL AND METHODS

15.1.Fish

Juvenile tambaqui ($112,37 \pm 23,59$ g; $18,65 \pm 1,37$ cm), were obtained from a commercial fish farm located in the municipality of Rio Preto da Eva (Amazonas, Brazil). The fish were acclimated for two weeks in 1000-L tanks under a recirculating aquaculture system with water heating and continuous aeration at the aquaculture sector of Embrapa Amazônia Ocidental (Manaus, Amazonas, Brazil). During this period, the fish were fed twice daily with a commercial diet containing 32% crude protein until apparent satiation.

Water quality parameters were monitored daily. Mean water temperature was 31.23 ± 0.85 °C and dissolved oxygen concentration was 4.97 ± 0.16 mg L⁻¹, measured using a dissolved oxygen meter (YSI Pro20, YSI Inc., USA), while pH was 5.46 ± 0.28 , measured using a digital pH meter (YSI F-1100, YSI Inc., USA). Water samples were collected weekly from the tanks for the determination of alkalinity (23.05 ± 4.71 mg L⁻¹) and total ammonia (0.017 ± 0.013 mg L⁻¹), according to the methodology described by APHA (1998).

All experimental procedures were conducted under a protocol approved by the Animal Ethics Committee of the Federal Institute of Education, Science and Technology of Amazonas (IFAM) (Protocol nº 1741.0810/2020).

15.2.Essential oils

EOs of *C. nardus*, *L. origanoides* and *M. arvensis* were obtained through hydrodistillation using a Clevenger-type apparatus. The plant material used for extraction consisted of leaves, inflorescences and/or rhizomes collected from mature plants cultivated in the Medicinal Plants sector of Embrapa Amazônia Ocidental (Manaus, Amazonas, Brazil). Samples of these EOs were used for the characterization of their chemical composition by gas chromatography and mass spectrometry. The major compounds identified were geraniol (53.3%), geranial (18.0%), and neral (13.4%) in the EO of *C. nardus*; carvacrol (43.3%), *p*-cymene (12.0%), and γ -terpinene (9.5%) in the EO of *L. origanoides*; and menthol (88.5%), isomenthone (3.6%), and menthone (2.9%)

in the EO of *M. arvensis*.

15.3. Experimental diets

During the experimental period, fish were fed the experimental diets, which consisted of a commercial feed (32% crude protein) supplemented with essential oils (EOs) incorporated through an ethylcellulose coating process to prevent leaching of the essential oils into the water (CORDEIRO *et al.*, 2022). Briefly, for each kilogram of feed, the EOs and ethylcellulose (0.75% concentration) were diluted in 100 mL of alcohol. Subsequently, the solutions were sprayed onto the diet. In the control treatment, only the ethylcellulose solution was applied. The experimental diets were dried at 25 °C for 24 h, packaged, and stored under refrigeration (4 °C). Thereafter, the diets were provided twice daily based on the biomass of stocked fish (3% of body weight/day) in the experimental units for a period of 30 days.

15.4. Experimental design

Juvenile tambaqui ($n = 273$; 112.37 ± 23.59 g; 18.65 ± 1.37 cm) were randomly distributed into 21 tanks of 1000-L ($n = 13$ per tank), comprising seven treatments in triplicate. The treatments consisted of: T1) 0 g kg⁻¹ (control); T2) 9.2 g kg⁻¹ of *L. origanoides* EO; T3) 18.4 g kg⁻¹ of *L. origanoides* EO; T4) 10 g kg⁻¹ of *C. nardus* EO; T5) 20 g kg⁻¹ of *C. nardus* EO; T6) 15 g kg⁻¹ of *M. arvensis* EO; and T7) 30 g kg⁻¹ of *M. arvensis* EO.

Fish were fed based on stocked biomass (3% of body weight/day), twice daily (8:00 a.m. and 4:00 p.m.), for 30 days. After this period, the fish underwent biometric assessment and blood sampling for the determination of zootechnical, hematological, biochemical, and immunological parameters. Following the 30-day feeding period, the fish were challenged with *A. hydrophila* (10⁸ CFU mL⁻¹), and mortality was monitored for 15 days.

15.5. Growth performance

After 30 days of feeding, data on initial weight (IW, g), final weight (FW, g), final length (FL, cm), and feed intake were used to determine the zootechnical performance parameters of the fish, as follows: weight gain (WG, g) = FW – IW; feed conversion ratio (FCR) = feed intake/WG; condition factor (CF) = $100 \times (\text{final weight})/(\text{final length})^3$; specific growth rate (SGR, %) = $[(\ln \text{ final weight} - \ln \text{ initial weight})/\text{feeding days}] \times 100$;

and survival (S, %) = (final number of fish / initial number of fish) $\times$ 100.

15.6. Blood and collection analyses

Blood sampling was performed at the end of the 30-day feeding period. Fish (n = 12 per treatment) were anesthetized with benzocaine (100 mg L⁻¹). Blood samples (approximately 1.5 mL) were collected by caudal vein puncture using sterile syringes. Half of the collected blood was stored in 2-mL microtubes containing heparin as an anticoagulant for hematological analyses. The remaining blood was transferred to microtubes without heparin to obtain serum for biochemical and enzymatic analyses.

The following hematological analyses were performed: hematocrit (Hct, %), determined by blood centrifugation in capillary tubes followed by reading on a standardized scale; hemoglobin (Hb, g dL⁻¹), determined by the cyanmethemoglobin method according to Collier (1944); and erythrocyte count (RBC, 10⁶ cells μ L⁻¹), performed using a Neubauer chamber after blood dilution in formol-citrate solution (NATT; HERRICK, 1952). The erythrocyte indices were determined: mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), and mean corpuscular hemoglobin concentration (MCHC, %), according to (RANZANI-PAIVA *et al.*, 2013). In addition, blood smears were prepared, fixed with methanol, and stained using Rosenfeld stain. Differential leukocyte and thrombocyte counts were performed under light microscopy by examining 200 cells. Total leukocyte, thrombocyte, and erythrocyte counts were estimated indirectly from the same blood smears by counting 2,000 cells.

Finally, blood samples were centrifuged (10,000 $\times$ g for 5 min), and the resulting serum aliquots were collected and stored at -80 °C for subsequent biochemical analyses. Total protein levels (g dL⁻¹) were determined using the biuret method, and albumin levels (g dL⁻¹) were determined using the bromocresol green method, using commercial kits (Labtest®) in both cases. Globulin concentration was calculated using the following equation: globulin = total proteins - albumin. Enzymatic analyses of aspartate aminotransferase (AST, U L⁻¹) and alanine aminotransferase (ALT, U L⁻¹) were performed in kinetic mode using commercial kits (Labtest®), following the manufacturer's recommendations.

15.7. Challenge with *Aeromonas hydrophila*

The *A. hydrophila* strain A-30 was cultured on Tryptic Soy Agar (TSA – Kasvi, Spain) at 29 °C for 24 h in the microbiology laboratory of Embrapa Amazônia Ocidental

(Manaus, Brazil). Subsequently, the bacterial colonies were diluted in sterile saline solution (0.85%) to achieve a concentration equivalent to 0.5 on the McFarland scale.

For the challenge assay, six fish per tank were collected and anesthetized with benzocaine (100 mg L⁻¹). The fish were intraperitoneally inoculated with 0.1 mL per 10 g of body weight of *A. hydrophila* suspension (1.6×10^8 CFU mL⁻¹) using 1-mL syringes. After inoculation, the fish were returned to their respective tanks and observed daily for a period of 10 days to record the occurrence of clinical signs, behavioral alterations, and mortality.

15.8. Statical analysis

All data were analyzed by one-way ANOVA using the R software (2021), following the guidelines outlined by Logan (2010). When significant effects were detected, Tukey's honestly significant difference (HSD) test was applied for multiple comparisons among means. The results are presented as means, and statistical significance was declared at $p \leq 0.05$.

16. RESULTS

Dietary supplementation with the essential oils of *C. nardus*, *L. origanoides*, and *M. arvensis* significantly influenced final weight (FW), feed intake (FI), and feed conversion ratio (FCR) of juvenile tambaqui ($p \leq 0.05$, Table 7). Final weight was higher in the treatments supplemented with *C. nardus* (18.4 g kg⁻¹) and *M. arvensis* (15 g kg⁻¹), differing significantly from the control group, whereas the remaining treatments showed intermediate values. It should be noted that fish in the *M. arvensis* (30 g kg⁻¹) treatment presented a lower mean initial weight than those in the *C. nardus* (9.2 and 18.4 g kg⁻¹), *L. origanoides* (10 and 20 g kg⁻¹), and *M. arvensis* (15 g kg⁻¹) treatments, while the control group exhibited intermediate values. Regarding feed intake, the *C. nardus* (18.4 g kg⁻¹) treatment showed higher feed consumption than both the control group and the *M. arvensis* (30 g kg⁻¹) treatment, whereas the remaining groups did not differ significantly from each other. A significant difference was also observed for feed conversion ratio (FCR), with the *C. nardus* (18.4 g kg⁻¹) treatment exhibiting a lower FCR than the control group, while the other treatments showed intermediate values. Final length (FL), weight gain (WG), specific growth rate (SGR), condition factor (CF), and survival (SUR) did not differ significantly among treatments ($p > 0.05$).

Table 7. Growth performance (mean $\pm$ standard deviation) of juvenile tambaqui (*Colossoma macropomum*) fed diets supplemented with different concentrations of the essential oils of *C. nardus*, *L. origanoides*, and *M. arvensis*.

Variables ¹	Control	<i>Cymbopogon nardus</i>		<i>Lippia origanoides</i>		<i>Mentha arvensis</i>		p-value ²	CV ³ , %
		9,2 g.kg ⁻¹	18,4 g.kg ⁻¹	10 g.kg ⁻¹	20 g.kg ⁻¹	15 g.kg ⁻¹	30 g.kg ⁻¹		
IW	103.92 $\pm$ 25.96 ^b	113.14 $\pm$ 20.98 ^a	116.14 $\pm$ 24.98 ^a	114.65 $\pm$ 22.75 ^a	115.59 $\pm$ 20.21 ^a	116.83 $\pm$ 25.60 ^a	107.49 $\pm$ 24.35 ^b	0.05	21.16
FW	167.29 $\pm$ 48.78 ^c	175.55 $\pm$ 42.80 ^b	191.56 $\pm$ 45.84 ^a	178.59 $\pm$ 45.49 ^b	182.05 $\pm$ 42.78 ^{ab}	188.33 $\pm$ 40.06 ^a	175.46 $\pm$ 46.04 ^b	0.05	14.92
FL	21.80 $\pm$ 2.09	22.08 $\pm$ 1.72	22.69 $\pm$ 1.67	22.11 $\pm$ 1.53	22.26 $\pm$ 1.54	22.63 $\pm$ 1.48	21.84 $\pm$ 1.91	0.16	7.82
FI	112.92 $\pm$ 43.90 ^c	118.49 $\pm$ 38.52 ^b	129.30 $\pm$ 41.26 ^a	120.54 $\pm$ 40.94 ^{ab}	122.88 $\pm$ 38.50 ^{ab}	127.11 $\pm$ 36.05 ^a	118.44 $\pm$ 41.44 ^b	0.02	14.92
WG	63.36 $\pm$ 52.58	62.41 $\pm$ 43.72	75.41 $\pm$ 56.55	63.94 $\pm$ 48.45	66.45 $\pm$ 49.36	71.49 $\pm$ 42.54	67.96 $\pm$ 46.47	0.91	17.89
FCR	1.78 $\pm$ 0.83 ^b	1.89 $\pm$ 0.77 ^a	1.71 $\pm$ 0.35 ^c	1.88 $\pm$ 0.59 ^a	1.84 $\pm$ 0.81 ^{ab}	1.77 $\pm$ 0.69 ^b	1.74 $\pm$ 0.58 ^b	0.05	17.19
SGR	1.56 $\pm$ 0.82	1.42 $\pm$ 0.93	1.64 $\pm$ 1.18	1.44 $\pm$ 0.97	1.47 $\pm$ 0.99	1.59 $\pm$ 0.99	1.60 $\pm$ 0.84	0.94	16.44
CF	1.57 $\pm$ 0.10	1.61 $\pm$ 0.67	1.64 $\pm$ 0.85	1.44 $\pm$ 0.97	1.47 $\pm$ 0.99	1.59 $\pm$ 0.99	1.60 $\pm$ 0.84	0.95	16.05
SUR	100.00	100.00	100.00	100.00	100.00	100.00	100.00	-	-

¹ IW: Initial Weight (g). FW: Final Weight (g). FL: Final Length (cm). FI: Feed intake (g). WG: Weight Gain (g). FCR: Feed Conversion Ratio (g.g⁻¹). SGR: Specific Growth Rate (%). CF: Condition Factor. SUR: Survival.

² Means followed by different letters within the same row present significant differences (p $\leq$ 0.05).

CV: Coefficient of variation.

Among the hematological and biochemical parameters evaluated (Table 8), significant differences were observed for hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) ($p \leq 0.05$). For Hb, the control group showed the highest mean value, differing from the group supplemented with *Cymbopogon nardus* (9.2 g kg^{-1}), whereas the remaining treatments exhibited intermediate values. MCH values were higher in the control group and in the group supplemented with *Mentha arvensis* (30 g kg^{-1}), differing from the groups supplemented with *C. nardus* (18.4 g kg^{-1}), *Lippia origanoides* (20 g kg^{-1}), and *M. arvensis* (15 g kg^{-1}), while the other treatments presented intermediate values. For MCHC, the highest values were recorded in the control group and in the *M. arvensis* (30 g kg^{-1}) treatment, whereas the lowest values were observed in the groups supplemented with *C. nardus* (9.2 and 18.4 g kg^{-1}) and *L. origanoides* (10 and 20 g kg^{-1}); the remaining treatments showed intermediate values. Regarding the hepatic enzymes, ALT activity was highest in the group supplemented with *L. origanoides* (20 g kg^{-1}), differing from the groups supplemented with *M. arvensis* (15 g kg^{-1}) and *C. nardus* (9.2 g kg^{-1}), whereas the other treatments exhibited intermediate values. For AST, the group supplemented with *M. arvensis* (30 g kg^{-1}) showed the highest enzymatic activity, differing from the groups supplemented with *C. nardus* (9.2 and 18.4 g kg^{-1}) and *L. origanoides* (10 g kg^{-1}), while the control group, *L. origanoides* (20 g kg^{-1}), and *M. arvensis* (15 g kg^{-1}) presented intermediate values. Hematocrit (Hct), red blood cell count (RBC), mean corpuscular volume (MCV), glucose (Glu), total protein (TP), albumin (Alb), and globulin (Glob) did not differ significantly among treatments ($p > 0.05$).

Table 8. Hematological and biochemical parameters (mean $\pm$ standard deviation) of juvenile tambaqui (*Colossoma macropomum*) fed diets supplemented with different concentrations of the essential oils of *C. nardus*, *L. origanoides*, and *M. arvensis*.

Variables ¹	Control	<i>Cymbopogon nardus</i>		<i>Lippia origanoides</i>		<i>Mentha arvensis</i>		p-value	CV, %
		9,2 g.kg ⁻¹	18,4 g.kg ⁻¹	10 g.kg ⁻¹	20 g.kg ⁻¹	15 g.kg ⁻¹	30 g.kg ⁻¹		
Hct	27.41 $\pm$ 3.47	25.58 $\pm$ 4.25	27.33 $\pm$ 3.52	28.00 $\pm$ 3.07	26.33 $\pm$ 3.60	27.08 $\pm$ 6.22	25.91 $\pm$ 4.05	0.77	15.20
Hb	9.90 $\pm$ 2.55 ^a	7.90 $\pm$ 1.55 ^c	8.66 $\pm$ 3.05 ^b	8.87 $\pm$ 2.04 ^b	8.14 $\pm$ 1.97 ^c	9.29 $\pm$ 2.29 ^{ab}	9.46 $\pm$ 2.49 ^a	0.05	16.26
RBC	2.57 $\pm$ 0.83	2.45 $\pm$ 0.82	2.77 $\pm$ 0.80	2.65 $\pm$ 0.84	2.59 $\pm$ 0.81	2.91 $\pm$ 0.81	2.57 $\pm$ 0.77	0.86	19.74
MCV	119.26 $\pm$ 46.88	113.43 $\pm$ 35.85	108.07 $\pm$ 44.23	113.83 $\pm$ 32.27	110.61 $\pm$ 34.38	97.84 $\pm$ 29.34	110.97 $\pm$ 46.02	0.91	14.42
MCH	42.96 $\pm$ 19.14 ^a	34.84 $\pm$ 10.77 ^b	32.21 $\pm$ 8.53 ^b	35.54 $\pm$ 10.99 ^b	33.26 $\pm$ 9.30 ^b	33.46 $\pm$ 9.71 ^b	41.95 $\pm$ 26.72 ^a	0.05	14.26
MCHC	36.72 $\pm$ 11.31 ^a	31.75 $\pm$ 8.29 ^b	31.62 $\pm$ 9.36 ^b	31.94 $\pm$ 7.59 ^b	31.98 $\pm$ 9.40 ^b	35.40 $\pm$ 9.40 ^a	36.60 $\pm$ 7.98 ^a	0.05	17.02
Glu	43.28 $\pm$ 4.18	45.43 $\pm$ 8.68	43.17 $\pm$ 4.63	41.62 $\pm$ 3.58	43.51 $\pm$ 4.20	45.81 $\pm$ 8.00	43.36 $\pm$ 4.96	0.61	13.08
TP	5.95 $\pm$ 0.26	6.00 $\pm$ 0.23	5.95 $\pm$ 0.30	5.81 $\pm$ 0.17	5.98 $\pm$ 0.27	5.96 $\pm$ 0.41	5.94 $\pm$ 0.29	0.78	4.80
Alb	4.00 $\pm$ 0.18	4.06 $\pm$ 0.16	4.02 $\pm$ 0.19	3.92 $\pm$ 0.13	4.01 $\pm$ 0.20	4.00 $\pm$ 0.29	4.00 $\pm$ 0.20	0.78	4.96
Glob	1.95 $\pm$ 0.10	1.94 $\pm$ 0.07	1.93 $\pm$ 0.11	1.89 $\pm$ 0.04	1.97 $\pm$ 0.09	1.96 $\pm$ 0.13	1.94 $\pm$ 0.10	0.65	5.12
ALT	77.59 $\pm$ 22.46 ^b	63.61 $\pm$ 13.30 ^b	77.82 $\pm$ 57.51 ^b	81.81 $\pm$ 21.91 ^b	134.93 $\pm$ 121.49 ^a	68.52 $\pm$ 26.56 ^c	74.66 $\pm$ 16.17 ^b	0.03	6.08
AST	17.51 $\pm$ 6.13 ^c	16.76 $\pm$ 6.78 ^c	16.55 $\pm$ 5.98 ^c	15.88 $\pm$ 5.59 ^c	24.32 $\pm$ 5.66 ^a	20.91 $\pm$ 5.25 ^b	25.81 $\pm$ 3.27 ^a	0.05	6.82

¹ Hct: Hematocrit (%). Hb: Hemoglobin (g dL⁻¹). RBC: Red Blood Cell Count ($\times 10^6$ μ L⁻¹). MCV: Mean Corpuscular Volume (fL). MCH: Mean Corpuscular Hemoglobin (pg). MCHC: Mean Corpuscular Hemoglobin Concentration (g dL⁻¹). Glu: Glucose (mg dL⁻¹). TP: Total Protein (g dL⁻¹). Alb: Albumin (g dL⁻¹). Glob: Globulin (g dL⁻¹). ALT: Alanine Aminotransferase (U L⁻¹). AST: Aspartate Aminotransferase (U L⁻¹).

² Means followed by different letters within the same row present significant differences ($p \leq 0.05$).

CV: Coefficient of variation.

Dietary supplementation with the essential oils did not significantly influence total leukocyte and thrombocyte counts or the differential leukocyte profile of juvenile tambaqui ($p > 0.05$; Table 9). The relative proportions of lymphocytes, monocytes, neutrophils, eosinophils, and special granulocytic cells (Figure 3) were comparable among treatments, demonstrating that supplementation with the tested concentrations of essential oils did not alter the circulating leukocyte profile of the fish.

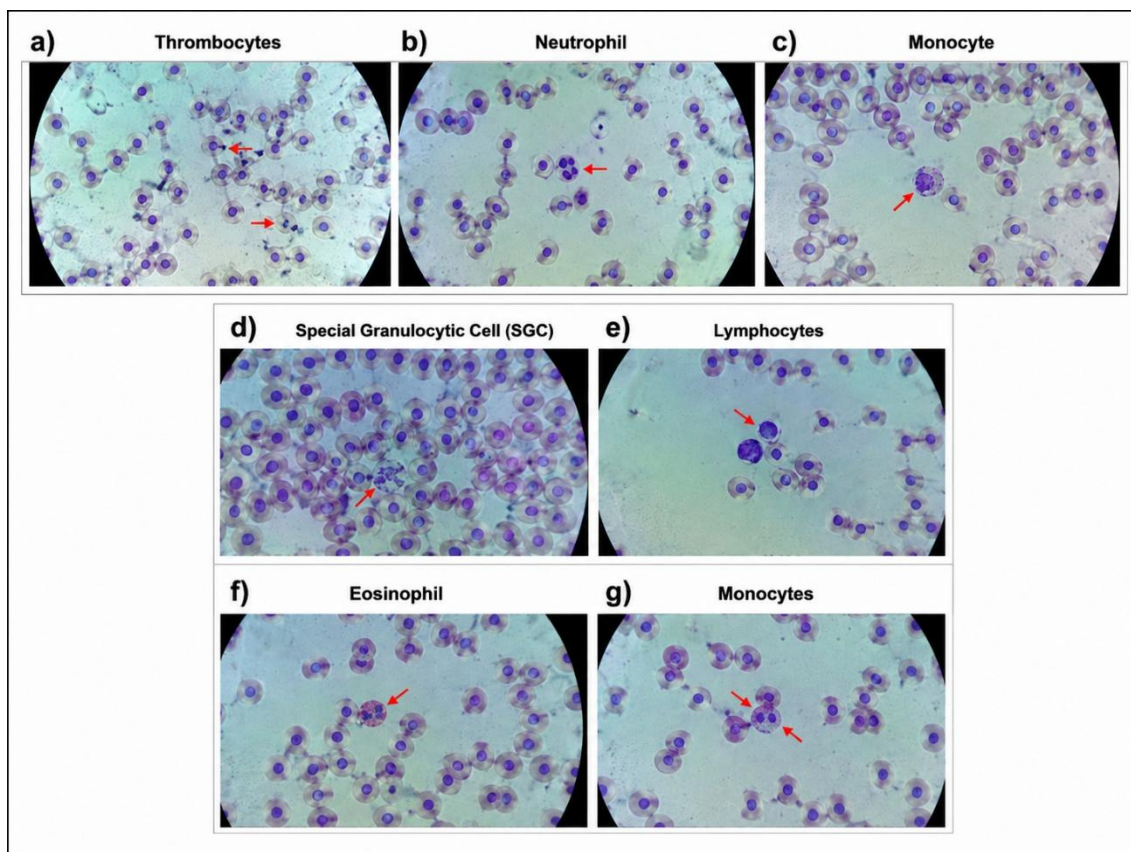


Figure 3. Representative photomicrographs of blood smears from tambaqui (*Colossoma macropomum*) fed diets supplemented with different concentrations of the essential oils of *C. nardus*, *L. origanoides* and *M. arvensis*, stained with the Giemsa–Wright method, showing the main blood cell types: (a) thrombocytes; (b) neutrophil; (c) monocyte; (d) special granulocytic cell (SGC); (e) lymphocyte; (f) eosinophil; and (g) monocytes. Red arrows indicate the identified cells.

Table 9. Total leukocyte and thrombocyte, and differential leukocyte count (mean $\pm$ standard deviation) of juvenile tambaqui (*Colossoma macropomum*) fed diets supplemented with different concentrations of the essential oils of *C. nardus*, *L. origanoides*, and *M. arvensis*.

Cells	Controle	<i>Cymbopogon nardus</i>		<i>Lippia origanoides</i>		<i>Mentha arvensis</i>	
		9,2 g.kg ⁻¹	18,4 g.kg ⁻¹	10 g.kg ⁻¹	20 g.kg ⁻¹	15 g.kg ⁻¹	30 g.kg ⁻¹
Leucócitos totais (μL ⁻¹)	23871.4 $\pm$ 7240.8	22158.4 $\pm$ 9461.9	26322.5 $\pm$ 9432.4	26997.6 $\pm$ 9640.6	25047.8 $\pm$ 8408.9	29422.2 $\pm$ 8047.6	26042.6 $\pm$ 7568.9
Trombócitos totais (μL ⁻¹)	9378.9 $\pm$ 3728.9	6434.6 $\pm$ 3750.2	7367.5 $\pm$ 4208.6	9227.3 $\pm$ 6463.6	7003.5 $\pm$ 2468.9	9425.2 $\pm$ 5604.2	7054.0 $\pm$ 3797.3
Linfócitos (%)	63.00 $\pm$ 12.71	64.18 $\pm$ 21.24	69.66 $\pm$ 5.55	68.37 $\pm$ 8.69	67.94 $\pm$ 11.70	70.67 $\pm$ 10.92	64.27 $\pm$ 12.49
Monócitos (%)	16.00 $\pm$ 5.43	16.85 $\pm$ 11.75	15.14 $\pm$ 5.39	13.39 $\pm$ 6.80	12.70 $\pm$ 6.93	10.72 $\pm$ 4.87	13.65 $\pm$ 4.77
Neutrófilos (%)	17.64 $\pm$ 6.76	15.52 $\pm$ 7.95	14.13 $\pm$ 5.12	16.63 $\pm$ 5.50	18.50 $\pm$ 6.19	16.22 $\pm$ 7.58	20.2 $\pm$ 9.68
Eosinófilos (%)	0.21 $\pm$ 0.57	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.09 $\pm$ 0.20	0.06 $\pm$ 0.17	0.00 $\pm$ 0.00
CGE (%)	3.36 $\pm$ 2.48	3.45 $\pm$ 3.84	1.07 $\pm$ 1.26	1.6 $\pm$ 1.90	0.86 $\pm$ 1.05	2.39 $\pm$ 2.45	1.88 $\pm$ 1.48

* Célula Granulocítica Especial

Relative percent survival of juvenile tambaqui following the experimental challenge with *Aeromonas hydrophila* differed significantly among treatments (Figure 4). Fish fed diets supplemented with *Lippia origanoides* (10 and 20 g kg⁻¹) and *Mentha arvensis* (15 g kg⁻¹) exhibited the highest survival rate (88.89%), differing significantly from the control group (33.33%). The treatments supplemented with *Cymbopogon nardus* (9.2 and 18.4 g kg⁻¹) and *Mentha arvensis* (30 g kg⁻¹) showed intermediate survival rates (66.67%, 66.67%, and 77.78%, respectively), with no significant differences from either the control group or the treatments with the highest survival rates ($p > 0.05$).

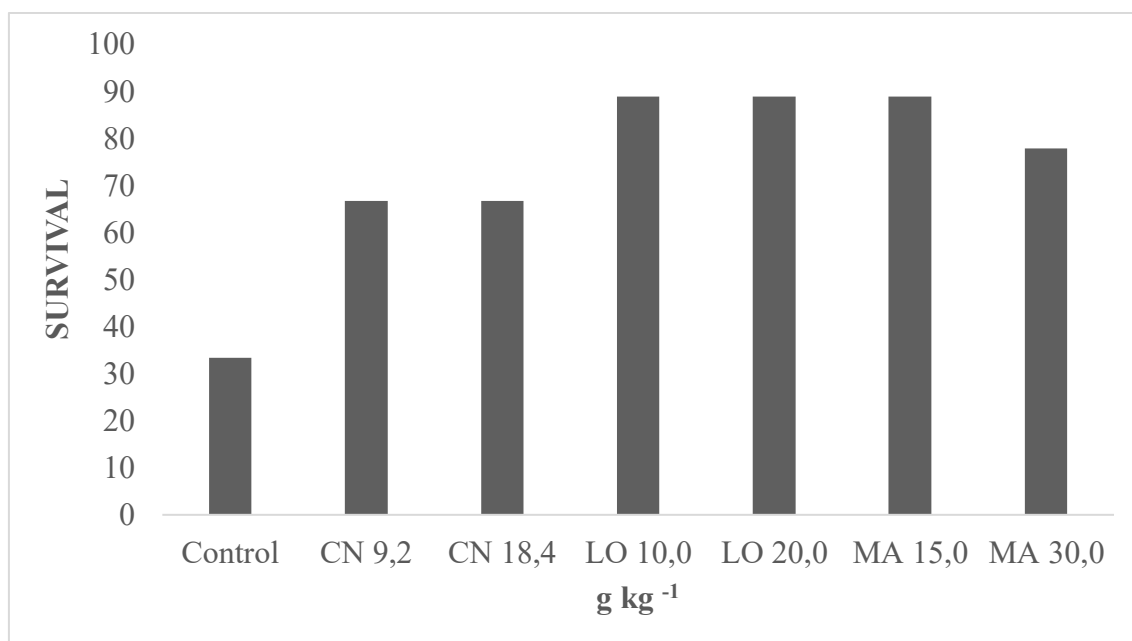


Figure 4. Survival of juvenile tambaqui (*Colossoma macropomum*) fed diets supplemented with different concentrations of the essential oils of *C. nardus*, *L. origanoides* and *M. arvensis*. CN: *Cymbopogon nardus*; LO: *Lippia origanoides*; MA: *Mentha arvensis*.

17. DISCUSSION

Dietary supplementation with the essential oils elicited distinct responses in the zootechnical performance of juvenile tambaqui, significantly affecting final weight, feed intake, and feed conversion ratio, whereas final length, weight gain, specific growth rate, condition factor, and survival did not differ among treatments. This pattern indicates that the essential oils exerted specific effects on selected aspects of productive performance without promoting generalized changes in fish growth.

In aquaculture, essential oils do not always result in consistent improvements in growth performance, with their effects being more commonly associated with enhanced nutrient digestibility and utilization, modulation of the intestinal microbiota, increased antioxidant capacity, and improved immune responses (ORZUNA-ORZUNA; GRANADOS-RIVERA, 2024). However, the magnitude of these responses depends on the cultured species, the chemical composition of the essential oil, the inclusion level, the supplementation period, and the experimental conditions (DAWOOD *et al.*, 2022; KOLYGAS *et al.*, 2025). In addition, factors such as botanical origin, edaphoclimatic conditions, plant developmental stage, and extraction method may alter the chemical profile of essential oils and, consequently, their biological activity, contributing to the variability observed among studies (MIURA *et al.*, 2021).

Fish fed diets supplemented with *Cymbopogon nardus* (18.4 g kg⁻¹) and *Mentha arvensis* (15 g kg⁻¹) exhibited higher final weight than the control group, indicating a positive effect of these inclusion levels on productive performance. However, the absence of significant differences in weight gain warrants caution when interpreting this result, as slight differences in initial weight were observed among some treatments. Fish supplemented with *M. arvensis* (30 g kg⁻¹) had a lower initial weight than those in other groups, which may have reduced the sensitivity to detect differences in growth-related variables. Nevertheless, the association between higher final weight and improved feed conversion ratio in the *C. nardus* (18.4 g kg⁻¹) treatment suggests a biological effect of supplementation rather than merely an influence of initial differences among groups.

The improvement in feed conversion ratio observed in the treatment containing *C. nardus* (18.4 g kg⁻¹) represents one of the main findings of this study. Feed conversion ratio is an important indicator of nutritional efficiency in aquaculture systems, as it reflects the animal's ability to convert ingested feed into biomass. A reduction in this index suggests greater efficiency in nutrient utilization, possibly resulting from improvements in digestion, intestinal absorption, and the metabolic utilization of dietary

nutrients (ELVY *et al.*, 2023). The chemical characterization of the essential oil from this species identified geraniol as the major constituent, followed by geranial and neral. These monoterpenes possess well-documented antimicrobial, antioxidant, and anti-inflammatory activities, which may promote intestinal homeostasis, modulate the gut microbiota, and reduce oxidative stress, thereby contributing to improved digestive efficiency and nutrient utilization (GUTIÉRREZ-PACHECO *et al.*, 2023; LI *et al.*, 2025).

Another relevant finding was the higher feed intake observed in the treatment supplemented with *C. nardus* (18.4 g kg⁻¹). This effect may be associated with both enhanced diet palatability and physiological changes induced by the bioactive compounds on metabolism and gastrointestinal function. Since the increase in feed intake was accompanied by an improvement in feed conversion ratio, the results indicate that the additional feed consumed was converted more efficiently into biomass, reinforcing the potential of this essential oil to optimize nutrient utilization.

Similar findings were reported by Al-Sagheer *et al.* (2018) who evaluated dietary supplementation with *Cymbopogon citratus* essential oil in juvenile Nile tilapia (*Oreochromis niloticus*). The authors observed that supplementation at 200 and 400 mg kg⁻¹ increased final weight, daily weight gain, specific growth rate, and protein efficiency, while reducing feed conversion ratio, whereas feed intake increased only at the 200 mg kg⁻¹ inclusion level. Similarly, supplementation with *C. nardus* at 18.4 g kg⁻¹ in the present study resulted in higher final weight, greater feed intake, and improved feed conversion ratio in juvenile tambaqui. Although the studies evaluated different species within the *Cymbopogon* genus, both demonstrate that these essential oils can improve feed efficiency and productive performance without compromising survival.

In contrast, García-Márquez *et al.* (2021) did not observe changes in final weight or weight gain in juvenile gilthead seabream (*Sparus aurata*) fed diets supplemented with *C. nardus* essential oil. In the present study, however, supplementation with 18.4 g kg⁻¹ increased final weight and improved feed conversion ratio, suggesting that the response to this essential oil depends on fish species, inclusion level, oil chemical composition, and experimental conditions. Thus, the present findings indicate that, in juvenile tambaqui, this essential oil may act as a feed efficiency promoter by enhancing nutrient utilization.

Although both studies used *C. nardus* essential oil, the differences observed in zootechnical performance may be related to differences in the chemical profile of the oils. In the present study, the essential oil was characterized by a high concentration of geraniol

(53.3%), followed by geranial (18.0%) and neral (13.4%), whereas García-Márquez *et al.* reported an essential oil composed mainly of citronellal, citronellol, and geraniol.

The results obtained with *Mentha arvensis* also suggest a dose-dependent response. While supplementation at 15 g kg⁻¹ increased final weight, inclusion at 30 g kg⁻¹ provided no additional benefits. This pattern has frequently been reported for essential oils used in fish nutrition, as moderate inclusion levels tend to optimize physiological responses, whereas higher doses may reduce diet palatability, increase the metabolic costs associated with the biotransformation of bioactive compounds, or negatively interfere with nutrient digestion and absorption (KHALAFALAA *et al.*, 2025). Therefore, intermediate inclusion levels appear to represent the most suitable supplementation rate for this essential oil in juvenile tambaqui.

The response observed in the present study is partially supported by Aguiar *et al.* (2023), who evaluated dietary supplementation with *Mentha piperita* essential oil in juvenile Nile tilapia. The authors reported that dietary inclusion levels ranging from 0.2 to 0.8 g kg⁻¹ increased weight gain and improved feed conversion ratio, with the best performance estimated at approximately 0.6 g kg⁻¹, a concentration that also enhanced digestive enzyme activity and feed efficiency. In addition, survival was not affected by the treatments. Although weight gain, specific growth rate, and feed conversion ratio did not differ among the treatments containing *M. arvensis*, the increase in final weight at the intermediate inclusion level reinforces the potential of *Mentha* essential oils as phytogenic feed additives capable of improving productive performance, possibly through enhanced digestion, nutrient utilization, and intestinal health.

Despite the similarities observed, differences in the magnitude of the responses may be attributed to the physiological characteristics of the species studied and to differences in the essential oils used. The *M. piperita* essential oil evaluated by Aguiar *et al.* (2023) consisted primarily of menthol (46.5%), menthone (26.2%), and neomenthol (11.2%), whereas the *M. arvensis* essential oil used in the present study also contained menthol as the major constituent, but at a substantially higher concentration (88.5%).

The stability of most hematological and biochemical parameters observed in the present study is consistent with part of the literature regarding the use of essential oils in tambaqui. Monteiro *et al.* (2021) reported that diets supplemented with essential oils of *Lippia sidoides*, *Ocimum gratissimum*, and *Zingiber officinale* did not affect growth or total protein levels, although they promoted specific immune responses following challenge with *Aeromonas hydrophila*. Similarly, in the present study, total protein,

albumin, globulins, glucose, hematocrit, erythrocyte count, and MCV remained unchanged, indicating that the essential oils did not compromise the physiological homeostasis of juvenile tambaqui.

The changes observed in hemoglobin, MCH, and MCHC are also supported by previous studies in tambaqui. Ribeiro *et al.* (2016), when evaluating dietary supplementation with *Mentha piperita* essential oil, reported changes in hemoglobin concentration and erythrocyte indices without impairing productive performance. In the present study, although Hb, MCH, and MCHC differed among treatments, the absence of changes in hematocrit, erythrocyte count, and MCV suggests that these variations were isolated and do not indicate impairment of erythropoiesis or of the overall oxygen transport capacity.

The differences observed in ALT and AST activities should be interpreted with caution, as studies evaluating essential oils in tambaqui have reported variable responses depending on the type of oil and the inclusion level. Chung *et al.* (2021) reported that dietary supplementation with ginger (*Zingiber officinale*) essential oil reduced ALT and AST activities at an inclusion level of 0.5 mL kg⁻¹, whereas higher levels were associated with hepatic histopathological alterations. In contrast, Copatti *et al.* (2022) observed increased ALT activity in tambaqui fed higher inclusion levels (1.0 and 1.5 mL kg⁻¹) of *Lippia sidoides* essential oil. In the present study, the highest ALT activity was observed in the *Lippia organoides* (20 g kg⁻¹) treatment, whereas the highest AST activity occurred in the *Mentha arvensis* (30 g kg⁻¹) treatment, without concomitant changes in glucose, total protein, albumin, or globulin levels. Therefore, these responses appear to reflect treatment-specific metabolic adjustments rather than generalized hepatotoxicity.

Taken together, these findings reinforce that the effects of essential oils in tambaqui are dependent on the plant species, chemical composition, and inclusion level. While some studies have reported positive effects on growth, metabolism, immunity, and resistance to *A. hydrophila*, others have demonstrated only minor physiological changes. Accordingly, the results of the present study indicate that supplementation with the evaluated essential oils was generally well tolerated by juvenile tambaqui, promoting isolated changes in hematological and enzymatic indicators without consistent evidence of metabolic imbalance or physiological impairment.

The differences observed in ALT and AST activities should also be interpreted in light of the well-documented antioxidant, anti-inflammatory, and hepatoprotective properties of the major constituents of the essential oils evaluated. Lei *et al.* (2019)

reported that geraniol exerts its biological effects primarily through modulation of cellular redox homeostasis by increasing the activity of antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR), reducing lipid peroxidation, and modulating inflammatory signaling pathways, including NF- κ B, p38 MAPK, COX-2, and iNOS. In addition, geraniol has demonstrated hepatoprotective activity in different experimental models by reducing ALT and AST activities, preserving mitochondrial function, decreasing the production of reactive oxygen species (ROS), and restoring normal liver histological architecture. These mechanisms provide a consistent biological basis for the protective effects frequently attributed to geraniol-rich essential oils.

Among the compounds evaluated in the present study, evidence in fish is more robust for carvacrol and menthol, whereas evidence for geraniol remains indirect. Carvacrol, the major constituent of *Lippia origanoides*, has demonstrated marked hepatoprotective potential in Nile tilapia exposed to thallium nitrate. Abdel-Moneam *et al.* (2025) reported that dietary supplementation almost completely restored serum ALT and AST activities, reduced lipid peroxidation (MDA), restored reduced glutathione (GSH) concentrations, normalized the expression of antioxidant genes, including *glutathione peroxidase* (GPX) and *glutathione S-transferase* (GST), and minimized histopathological alterations, including vascular congestion and hepatic vacuolar degeneration. These findings demonstrate that carvacrol preserves redox homeostasis and liver functional integrity under conditions of oxidative stress.

For menthol, the major constituent of *Mentha arvensis*, Dawood *et al.* (2020) reported similar effects in Nile tilapia subjected to chlorpyrifos intoxication, an insecticide used in that study as a toxic stress-inducing agent to evaluate whether a menthol-rich essential oil could protect fish against such damage. Dietary supplementation significantly reduced ALT and AST activities, increased serum total protein, albumin, and globulin levels, decreased urea, creatinine, and bilirubin concentrations, improved the expression of the antioxidant genes CAT and GPX, and reduced inflammatory lesions in the liver, gills, and intestine, demonstrating marked antioxidant and anti-inflammatory activity.

Regarding geraniol, Abdel Rahman *et al.* (2020) demonstrated that dietary supplementation with *Pelargonium graveolens* essential oil, which is rich in this monoterpene, reduced malondialdehyde (MDA) levels, a marker of lipid peroxidation, restored reduced glutathione (GSH) concentrations, one of the major cellular

antioxidants, increased the activity of hepatic antioxidant enzymes, and restored ALT and AST activities. In addition, it almost completely restored the histological architecture of the liver and kidneys of common carp exposed to profenofos, an organophosphate insecticide commonly used in experimental studies to induce oxidative stress, while also reducing tissue apoptosis.

In the present study, although significant differences in ALT and AST activities were observed among some treatments, these changes were not accompanied by consistent alterations in glucose, total protein, albumin, globulin levels, or hematological parameters. Furthermore, the treatments containing *Lippia organoides* and *Mentha arvensis* exhibited the highest survival rates following challenge with *Aeromonas hydrophila*, indicating that the enzymatic changes most likely reflect physiological adaptations to the metabolism of bioactive compounds rather than hepatotoxicity. This interpretation is consistent with the mechanisms described for carvacrol, menthol, and geraniol, which primarily act by reducing oxidative stress, preserving cellular integrity, and limiting hepatic damage under challenge conditions. Although the present study did not directly assess oxidative stress biomarkers or perform histopathological analyses, the stability of most biochemical and hematological parameters, together with the high survival of the supplemented fish following bacterial challenge, suggests that these essential oils contributed to maintaining physiological homeostasis during the experimental infection.

Dietary supplementation with the essential oils of *Cymbopogon nardus*, *Lippia organoides*, and *Mentha arvensis* did not significantly affect total leukocyte and thrombocyte counts or the differential leukocyte count of juvenile tambaqui. The values observed remained within the expected range for healthy fish, characterized by a predominance of lymphocytes, followed by neutrophils and monocytes, whereas eosinophils and special granulocytic cells occurred at low proportions, as described for teleosts (RANZANI-PAIVA *et al.*, 2013; TAVARES-DIAS; MORAES, 2004). These findings suggest that the evaluated essential oils did not induce a systemic inflammatory response or hematological alterations indicative of physiological stress.

Similar results were reported by Rodrigues-Soares *et al.* (2018), who evaluated dietary supplementation with *Lippia alba* essential oil in Nile tilapia (*Oreochromis niloticus*) for 45 days. The authors found that supplementation did not alter total leukocyte, circulating lymphocyte, or monocyte counts, even after the experimental induction of carrageenan-induced aerocystitis. However, supplemented fish exhibited an

increased number of circulating neutrophils following the inflammatory stimulus, indicating that the essential oil modulated the immune response only when the fish were subjected to an inflammatory challenge, without affecting the basal hematological profile.

In the present study, the fish were not subjected to a sanitary challenge at the time of hematological evaluation, which may explain the absence of changes in the neutrophil population. Taken together, these findings suggest that dietary essential oils tend to preserve hematological homeostasis under physiological conditions, whereas their immunomodulatory effects become more evident following activation of the immune system.

In contrast, some studies have reported an increase in the leukocyte population following dietary supplementation with essential oils. Vazirzadeh *et al.* (2017) found that juvenile rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with *Ducrosia anethifolia* essential oil for eight weeks exhibited a significant increase in mean total leukocyte counts at dietary inclusion levels of 0.01 and 0.1%, whereas erythrocyte counts remained unchanged.

The differences between these studies and the results observed in the present work may be related to the fish species, the chemical profile of the essential oils, the inclusion levels, the duration of supplementation, and the mechanisms of action of the different bioactive compounds. Furthermore, many immunomodulatory effects of essential oils become more evident in functional immune parameters or following sanitary challenges, rather than being accompanied by quantitative changes in circulating leukocyte counts. Therefore, the maintenance of hematological parameters within the normal range observed in the present study suggests that supplementation with *C. nardus*, *L. origanoides*, and *M. arvensis* is hematologically safe and preserves the physiological balance of juvenile tambaqui.

Dietary supplementation with the essential oils significantly increased the resistance of juvenile tambaqui to experimental infection with *Aeromonas hydrophila*, as evidenced by the higher survival rates observed in the treatments containing *Lippia origanoides* (10 and 20 g kg⁻¹) and *Mentha arvensis* (15 g kg⁻¹). While the control group exhibited a survival rate of only 33.33%, fish supplemented with these essential oils achieved a survival rate of 88.89%, representing a substantial increase in their ability to withstand the bacterial challenge. These findings demonstrate the prophylactic potential of essential oils as functional feed additives in aquaculture, reducing mortality caused by one of the major bacterial diseases affecting cultured fish.

However, the response was not uniform among treatments, indicating that the protective effect depends on both the plant species and the inclusion level. Although the treatments containing *Cymbopogon nardus* (9.2 and 18.4 g kg⁻¹) and *Mentha arvensis* (30 g kg⁻¹) showed intermediate survival rates, these did not differ statistically from either the control group or the treatments providing greater protection. Furthermore, the absence of differences between the two inclusion levels of *L. origanoides* suggests that the lower dose was sufficient to achieve the maximum effect under the conditions evaluated, indicating a possible plateau effect. A similar pattern was observed for *M. arvensis*, in which the intermediate inclusion level produced better results than the higher dose, reinforcing that the benefits of essential oils do not always increase proportionally with the administered dose.

The results obtained following the experimental challenge are also consistent with the antimicrobial activity assays previously conducted in this study. Among the evaluated oils, *L. origanoides* exhibited the lowest minimum inhibitory and minimum bactericidal concentrations against *A. hydrophila* isolates, demonstrating high in vitro antibacterial activity, which was accompanied by the highest in vivo survival rates. Although the protection observed following bacterial challenge cannot be attributed exclusively to direct antimicrobial activity, the agreement between the in vitro and in vivo assays suggests that this essential oil possesses high prophylactic potential against aeromoniasis.

The findings of the present study are consistent with the trend reported in the literature, according to which essential oils from the genera *Lippia*, *Mentha*, and *Cymbopogon* frequently enhance fish resistance to *A. hydrophila* infection, although the magnitude of this effect varies according to fish species, the chemical composition of the essential oil, the inclusion level, and the duration of supplementation. In juvenile Nile tilapia, dietary supplementation with *Lippia alba* essential oil at 2.0 mL kg⁻¹ significantly increased survival following *Aeromonas* spp. infection, accompanied by enhanced lysozyme activity and improved feed conversion ratio, without relevant metabolic alterations (DE SOUZA *et al.*, 2019). Likewise, supplementation with *Cymbopogon flexuosus* essential oil (2.0 mL kg⁻¹) simultaneously improved growth, physiological responses, and survival of Nile tilapia following challenge with *A. hydrophila* (SOUZA *et al.*, 2020). Abou Zaid *et al.* (2025) demonstrated that diets containing 0.6% *Mentha piperita* promoted the highest survival rates in Nile tilapia infected with *A. hydrophila*, an effect accompanied by increased phagocytic activity, immunoglobulin M levels, antioxidant enzyme activities, and preservation of intestinal integrity.

Among studies conducted in species more closely related to the present work, those involving *Lippia sidoides*, a species chemically similar to *L. organoides* and likewise rich in carvacrol, deserve particular attention. In koi carp, Medeiros *et al.* (2023) reported that dietary inclusion of 0.125% essential oil resulted in the highest survival following challenge with *A. hydrophila*, although without significant hematological alterations. In tambaqui, Monteiro *et al.* (2021) observed that supplementation with 0.625 g kg⁻¹ of *L. sidoides* essential oil increased leukocyte respiratory burst activity and resulted in the highest survival following challenge with *A. hydrophila*, whereas the essential oils of *Ocimum gratissimum* and *Zingiber officinale* did not significantly affect this parameter. These findings reinforce the hypothesis that essential oils rich in phenolic compounds, particularly carvacrol and thymol, possess greater immunoprotective potential against aeromoniasis.

However, the literature also indicates that this effect is not universal. Copatti *et al.* (2022) reported that dietary supplementation with *Lippia sidoides* essential oil in juvenile tambaqui improved growth performance, hematological parameters, lysozyme activity, and digestive enzyme activity, but resistance following bacterial challenge was not evaluated. In Nile tilapia, Andrade *et al.* (2026) observed improvements in feed conversion ratio and changes in hematological parameters following supplementation with *L. sidoides*; however, the essential oil was not able to mitigate the hematological alterations caused by air exposure stress and experimental infection with *A. hydrophila*. Similarly, Misriani *et al.* (2026) reported only a tendency toward increased survival in Nile tilapia fed citronella (*Cymbopogon nardus*) powder combined with probiotics, without statistically significant differences among treatments, although improvements in hematological parameters were observed. These findings demonstrate that the success of dietary supplementation depends on the formulation used, the chemotype of the essential oil, the cultured species, and the experimental challenge protocol.

The chemical composition of the essential oils likely played a decisive role in the differences observed among treatments in the present study. The essential oil of *L. organoides*, characterized by a high concentration of carvacrol, exhibited the greatest protective effect following bacterial challenge, a result consistent with the well-established antimicrobial, antioxidant, and immunomodulatory properties of this monoterpene. Likewise, the high menthol content of *M. arvensis* may have contributed to the increased survival observed at the 15 g kg⁻¹ inclusion level, considering that this compound possesses antioxidant and anti-inflammatory properties and the ability to

preserve the integrity of different tissues in fish exposed to stressors. In contrast, although *C. nardus*, rich in geraniol, improved zootechnical performance, its effect on survival was less pronounced, suggesting that different chemical profiles may elicit distinct physiological responses.

This pattern indicates that improvements in productive performance are not necessarily associated with greater prophylactic efficacy. In the present study, *C. nardus* stood out primarily for its effects on final weight, feed intake, and feed conversion ratio, whereas *L. origanoides* exhibited a greater capacity to increase survival following infection with *A. hydrophila*. These findings suggest that different essential oils preferentially influence distinct physiological processes, with some primarily enhancing zootechnical performance and others exerting greater effects on host defense mechanisms.

This interpretation is consistent with the recent reviews by Ghosh *et al.* (2025) e Rajendran *et al.* (2026), which summarize evidence from different fish species demonstrating that essential oils can enhance resistance to *A. hydrophila* through a combination of direct antimicrobial activity, modulation of the innate immune response, stimulation of phagocytic activity, enhancement of lysozyme activity, strengthening of antioxidant capacity, and maintenance of physiological homeostasis. However, both reviews emphasize that the efficacy of these compounds depends strongly on fish species, the chemical profile of the essential oil, the inclusion level, the duration of supplementation, and the experimental challenge protocol, factors that explain the variability observed among different studies.

Taken together, the results obtained demonstrate that dietary supplementation with essential oils represents a promising strategy for the prophylaxis of aeromoniasis in tambaqui. Among the evaluated essential oils, *L. origanoides* exhibited the greatest prophylactic potential, whereas *C. nardus* contributed most substantially to productive performance. The integration of the *in vitro* antimicrobial activity, zootechnical performance, physiological parameters, and post-challenge survival results provides consistent evidence that essential oils may represent sustainable alternatives to conventional antimicrobials, contributing simultaneously to the productivity and health of aquaculture production systems.

18. CONCLUSION

Dietary supplementation with the essential oils of *Cymbopogon nardus*, *Lippia origanoides*, and *Mentha arvensis* elicited distinct biological responses in juvenile tambaqui, demonstrating that their effects depend on the plant species and the inclusion level. *Cymbopogon nardus* essential oil was distinguished by its positive effects on zootechnical performance, resulting in higher final weight, greater feed intake, and improved feed conversion ratio, whereas *L. origanoides* exhibited the greatest prophylactic potential against aeromoniasis, as evidenced by its high in vitro antibacterial activity and the highest survival rates following experimental challenge with *Aeromonas hydrophila*. *Mentha arvensis*, at an inclusion level of 15 g kg⁻¹, also significantly increased fish survival, reinforcing its potential as a functional feed additive. Overall, dietary supplementation did not induce consistent hematological or biochemical alterations indicative of physiological impairment, suggesting that the essential oils were well tolerated under the evaluated conditions. Collectively, these findings demonstrate that these compounds represent promising natural alternatives for aquaculture, with the potential to reduce reliance on conventional antimicrobials while simultaneously improving productive performance and the health status of juvenile tambaqui.

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20.FINAL CONSIDERATIONS

This thesis achieved its proposed objective by identifying *Aeromonas* species associated with semi-intensive tambaqui farming systems in the state of Amazonas and evaluating the prophylactic potential of essential oils against experimental aeromoniasis. The findings generated throughout the study provide new insights into the epidemiology and pathogenicity of *Aeromonas* spp. affecting tambaqui, while demonstrating the feasibility of using plant-derived compounds as sustainable alternatives for disease prevention in aquaculture.

The molecular characterization of bacterial isolates revealed the occurrence of *Aeromonas hydrophila* and *Aeromonas jandaei* in apparently healthy tambaqui, together with the presence of multiple virulence genes and high pathogenic potential. These findings demonstrate that clinically healthy fish may serve as reservoirs of virulent *Aeromonas* strains, reinforcing the importance of continuous health surveillance and appropriate management practices to reduce the risk of disease outbreaks in tambaqui farming.

The *in vitro* evaluation demonstrated that all nine essential oils exhibited antibacterial activity against *Aeromonas* isolates, although with marked differences in efficacy. Among the oils evaluated, *Lippia origanoides* showed the greatest antibacterial activity, followed by *Cymbopogon nardus*, *Mentha arvensis*, and *Piper marginatum*. These findings confirm the antimicrobial potential of essential oils against important fish pathogens and provide scientific support for selecting promising candidates for subsequent *vivo* evaluation.

The *in vivo* study further demonstrated that dietary supplementation with essential oils elicited distinct biological responses depending on the botanical species and inclusion level. *Cymbopogon nardus* improved zootechnical performance by increasing final weight, feed intake, and feed efficiency, whereas *Lippia origanoides* exhibited the greatest prophylactic efficacy against aeromoniasis, as evidenced by the highest survival rates following bacterial challenge. *Mentha arvensis* also significantly increased fish survival at the intermediate inclusion level. Moreover, the absence of consistent hematological and biochemical alterations indicative of physiological impairment suggests that these essential oils were well tolerated under the experimental conditions employed.

An important contribution of this thesis lies in the integration of the results obtained throughout its three experimental stages. The identification and characterization

of pathogenic *Aeromonas* strains circulating in tambaqui farms provided the biological basis for selecting the target pathogen. Subsequently, the *in vitro* screening of nine essential oils enabled the identification of the most promising candidates based on their antibacterial activity. Finally, the *in vivo* evaluation demonstrated that the essential oil exhibiting the highest antibacterial activity (*Lippia origanoides*) also provided the greatest protection against experimental infection with *Aeromonas hydrophila*. This sequential approach established a direct relationship between the *in vitro* and *in vivo* findings, demonstrating that antibacterial screening can be successfully used to identify phytogetic compounds with prophylactic potential for aquaculture.

Taken together, these findings indicate that the prophylactic effects of essential oils cannot be attributed solely to their direct antibacterial activity. Instead, their benefits appear to result from the combination of antimicrobial properties with improvements in physiological homeostasis and host resistance, highlighting the multifactorial mechanisms through which phytogetic compounds may contribute to fish health.

From an applied perspective, this thesis provides robust scientific evidence supporting the incorporation of essential oils into health management strategies for tambaqui farming. Their use has the potential to reduce dependence on conventional antimicrobials, minimize the selective pressure associated with antimicrobial resistance, and contribute to the development of more sustainable aquaculture production systems.

Although the results obtained are promising, additional studies are still required before large-scale application. Future research should investigate the long-term effects of dietary supplementation, the mechanisms underlying the immunomodulatory activity of these compounds, their influence on intestinal microbiota and oxidative status, optimal inclusion levels under commercial farming conditions, and their economic feasibility. Furthermore, studies involving nanoencapsulation, combinations of essential oils, and their association with probiotics or other functional feed additives may further enhance their prophylactic efficacy.

Overall, this thesis advances the understanding of host–pathogen interactions involving *Aeromonas* spp. in tambaqui and provides robust evidence that essential oils, particularly *Lippia origanoides*, represent promising natural alternatives for the prevention of bacterial diseases in aquaculture. More importantly, this work establishes a rational framework for the selection of phytogetic compounds by integrating pathogen characterization, *in vitro* antimicrobial screening, and *in vivo* validation within a single experimental approach. This integrated strategy provides a solid scientific basis for the

development of innovative, sustainable, and antibiotic-sparing disease management strategies for tambaqui farming and may serve as a model for future studies involving other fish species and bacterial pathogens.