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Dietary supplementation with Melaleuca alternifolia essential oil improves survival and resistance to Saprolegnia in tambatinga

Suplementação alimentar com óleo essencial de Melaleuca alternifolia melhora a sobrevivência e a resistência à Saprolegnia em tambatinga

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Highlights

Tea tree oil improves tambatinga survival.
Protective effect of tea tree essential oil.
Indication of antifungal action of essential oil.

Abstract

Fish diets should not only be formulated to meet nutritional needs (e.g., protein and energy), but also to prevent disease and promote health. In this regard, essential oils addition to diets of fish of commercial interest has been intensifying in recent years. This 45-day study assessed the efficacy of dietary supplementation with the essential oil of *Melaleuca alternifolia* (EOMA) in improving growth and survival of tambatinga (*Colossoma macropomum* x *Piaractus brachypomus*) naturally infected with *Saprolegnia* sp. The juveniles (10.60 ± 2.18 g; 8.46 ± 0.61 cm) were distributed into 20 150-L polyethylene tanks operated as a recirculating system under constant aeration (n=160; 8 fish/tank). The fish received diets containing one of the following EOMA concentrations: 0.0, 1.0, 1.5, 2.0, and 2.5 mL/kg feed. At the

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end of the trial, no statistical difference was seen between treatments regarding weight gain, specific growth rate, and condition factor. On the other hand, dietary inclusion of EOMA showed a positive linear relationship with survival rate. EOMA demonstrated antifungal efficacy, since fish mortality decreased as its concentration was raised. Incorporation of EOMA into the diet of tambatinga juveniles enhanced resistance to the pathogenic oomycete *Saprolegnia* sp., resulting in increased final survival rates at concentrations exceeding 1.5 mL/kg feed.
Key words: Alternative supplement. Nutrition. Health. Aquaculture. Tea tree.

Resumo

As dietas para peixes não devem ser formuladas somente para suprir necessidades nutricionais (por exemplo, proteína e energia), mas também para prevenir doenças e promover saúde. Nesse sentido, o uso de óleos essenciais em dietas de peixes de interesse comercial tem-se intensificado nos últimos anos. Este estudo de 45 dias avaliou a eficácia da suplementação alimentar com óleo essencial de *Melaleuca alternifolia* (OEMA) em promover o crescimento e a sobrevivência de tambatingas (*Colossoma macropomum* x *Piaractus brachypomus*) naturalmente infectados com *Saprolegnia* sp. Os juvenis (10,60 ± 2,18 g; 8,46 ± 0,61 cm) foram distribuídos em 20 caixas de polietileno de 150L em sistema de recirculação de água e aeração constante (n=160; 8 peixes/caixa). Os peixes receberam dietas contendo OEMA em uma das seguintes concentrações: 0,0, 1,0, 1,5, 2,0 e 2,5 mL/kg ração. Ao final do experimento, não houve diferença estatística entre os tratamentos em relação ao ganho de peso, taxa de crescimento específica e fator de condição. No entanto, a inclusão dietética de OEMA apresentou uma relação linear positiva com a taxa de sobrevivência. O OEMA demonstrou eficácia antifúngica, uma vez que a mortalidade dos peixes diminuiu conforme sua concentração foi elevada. A incorporação de OEMA à dieta de juvenis de tambatinga aumentou a resistência ao oomiceto patogênico *Saprolegnia* sp., resultando em maiores taxas de sobrevivência final em concentrações superiores a 1,5 mL/kg de ração.
Palavras-chave: Suplemento alternativo. Nutrição. Saúde. Aquicultura. Árvore do chá.

Introduction

Despite the vast diversity of Brazilian native fish species, local farmers tend to opt for hybrids such as tambatinga (*Colossoma macropomum* x *Piaractus brachypomus*), which is of substantial importance in some regions of Brazil. Tambatinga is economically relevant in the North and Northeast of the country (Instituto Brasileiro de Geografia e Estatística [IBGE], 2020), emerging as the third most cultured species in the latter, where Maranhão State is the major producer. This species shows excellent zootechnical performance and is able to adapt to climate variability (M. C. S. Silva et al., 2021). Moreover, it reaches commercial weight within a short period, and can be fed with low levels of crude protein, thus reducing production costs and contributing to diversify Brazilian aquaculture (Carvalho et al., 2020).

The search for growth promoters as a means to control disease has given rise to new alternatives such as plant extracts and essential oils. These products have been increasingly used in fish farming to optimize not only basic nutrition but also feed efficiency and health (Souza et al., 2017, 2019; Lopes et al., 2020), and antioxidant activity (Lopes et al., 2019), for they contain a great quantity and variety of secondary bioactive metabolites (phytochemicals) (Costa et al., 2022).

Enrichment of diets with essential oils has been reported to improve growth, oxidative status, and immune responses (Dawood et al., 2022) as well as to enhance survival in fish infected with bacteria (Souza et al., 2019; Soltani et al., 2021). These oils, which may be obtained from all parts of aromatic plants, are also important antifungal agents (Sinkar et al., 2021). Some of them, mainly those of Lamiaceae plants, have been successfully tested as antifungals both *in vitro* and *in vivo* (Hoskonen et al., 2015).

Melaleuca alternifolia, commonly known as the tea tree, is endemic to Australia and belongs to the Myrtaceae family. The essential oil of *M. alternifolia* (EOMA) contains terpinen-4-ol and γ-terpinene as major components, and is widely explored for showing antifungal, antibiotic, and anti-inflammatory characteristics (Souza et al., 2019; L. L. Silva et al., 2019; Roana et al., 2021).

The rapid development of intensive aquaculture systems in Brazil has triggered the emergence of previously unknown fungal diseases, among which are those caused by the oomycete *Saprolegnia* sp. Water molds may significantly affect fish health, being able to colonize eggs as well as immature and mature stages (Nardoni et al., 2019).

There is a need to acquire further knowledge on the health of tambatinga, considering its commercial value for the eastern mesoregion of Maranhão State as well as the lack of management strategies to efficiently tackle pathogenic oomycetes in aquaculture. Thus, this study was aimed at evaluating the efficacy of dietary supplementation with EOMA in improving growth and survival of juvenile tambatinga naturally infected with *Saprolegnia* sp. during cultivation.

Material and Methods

Extraction and characterization of essential oils

EOMA was purchased from Importadora Química Delaware Ltda® (Porto Alegre, Brazil). Its composition was determined using a gas chromatograph connected to a flame ionization detector as described in Souza et al. (2020); terpinen-4-ol (45.85%), γ-terpinene (20.01%), and α-terpinene (10.26%) were the main components identified (Table 1).

Table 1
Composition of *Melaleuca alternifolia* essential oil used in tambatinga diets for 45 days

Compound	(%) relative*
α- Felandrene	0.73
α- Pinene	2.15
β-pinene	0.58
β-Mircene	0.77
α- Tujene	0.48
α- Terpinene	**10.26**
Cimene	3.13
Limonene	1.75
Eucaliptol	4.62
γ-Terpinene	**20.01**
Terpinolene	5.82
Terpinen-4- ol	**45.85**
α- Terpineol	3.85
Total	**100**

*The calculation of the relative percentage of each substance identified in the sample was carried out based on the area of each peak in the chromatogram, with the sum of the areas considered 100%.

Animals and culture conditions

This study was conducted in the Fish Farming Laboratory at the Universidade Federal do Maranhão (UFMA), located in the eastern mesoregion of Maranhão State. A completely randomized design with five treatments and four replications (n=8/experimental unit) was applied; 160 tambatinga juveniles (initial weight: 10.60 ± 2.18 g; initial length: 8.46 ± 0.61 cm) were distributed into 20 150-L polyethylene tanks (operated as a closed recirculating system under constant aeration) and maintained for 45 days. The water quality parameters remained stable throughout the experiment (Table 2). The pH was monitored using an AT-315 digital pH meter (Alphakit, Florianópolis, Brazil). Dissolved oxygen and temperature were verified with a Y5512 digital oximeter (YSI, Yellow Springs, USA). Total ammonia levels were measured using a commercial kit (Labcom, Camboriú, Brazil). All experimental work was approved by the Ethics Committee on the Use of Animals of the Universidade Federal do Maranhão - CEUA/UFMA (protocol nº. 23115.028167/2019-61).

Table 2
Water quality parameters during supplementation of tambatinga diet with Melaleuca alternifolia essential oil (EOMA)

Treatment	Dietary levels of EOMA (mL/kg)				
	0.0	1.0	1.5	2.0	2.5
T (°C)	23.6 ± 0.73	23.8 ± 0.67	23.8 ± 0.91	23.6 ± 0.95	23.2 ± 0.67
O_2D (mg/L)	7.2 ± 1.1	6.8 ± 0.5	6.8 ± 0.4	7.0 ± 0.4	6.7 ± 0.8
pH	6.5 ± 0.4	6.4 ± 0.4	6.4 ± 0.5	6.3 ± 0.4	6.4 ± 0.4
NH_3 (mg/L)	0.005 ± 0.03	0.006 ± 0.05	0.009 ± 0.01	0.008 ± 0.02	0.011 ± 0.01

T - Temperature; O_2D - Dissolved oxygen; pH - Hydrogen potential; NH_3 - Non-ionized ammonia. Values are expressed as mean ± standard deviation.

Experimental diets

The diets were formulated as detailed in Dairiki et al. (2013), using commercial feed containing 35% crude protein and 3.000 kcal/kg, and offered during the 10-day adaptation period. No signs of fungal contamination were detected within this time length.

For each kilogram of feed, 0.0, 1.0, 1.5, 2.0, and 2.5 mL of EOMA were diluted in 100 mL of rice alcohol and then sprayed onto the feed. Rice alcohol alone at 100 mL/kg was added to the feed to prepare the control diet. The fish were fed to apparent satiety twice a day (9 a.m. and 5 p.m.) throughout the trial.

Biometric measurements were taken at days 0, 30 and 45; the juveniles were anesthetized with eugenol at 40 mg/L for 2 minutes (Cunha et al., 2010), weighed and returned to their original tanks.

The following performance parameters were evaluated:

- Specific Growth Rate (SGR): {[ln (Final Weight) - ln (Initial Weight)] / (time in days)} x 100;

- Condition Factor (CF): (Final Weight / Final Total Length 3) x 100;

- Weight Gain (WG): Final Weight - Initial Weight;

- Survival Rate (SR): Final number of fish / Initial number of fish x 100

Identification and isolation of fungi

Animals that died during the experiment were collected daily and refrigerated for subsequent assessment of injuries. Small portions of the fungal structures located on the body surface of infected fish were removed by directly scraping the lesions with a scalpel, tweezers, and nickel-chromium pliers, which were duly sterilized for isolation and preparation of slides. Optical microscopy allowed to examine the fungal structures of the colonies and identify the pathogen, following the technique described in Alfenas et al. (2007).

The fungal colonies isolated from infected tissues were cultivated on cornmeal agar as proposed by Dotta and Piazza (2012).

The colonies were maintained in pure culture and incubated in a BOD culture chamber throughout the research period. Pure colonies of the fungus were obtained using the direct isolation technique, facilitated by the presence of cotton tufts (cotton mycelium) developed on the surface of infected tissues.

Direct isolation involved transferring the infected tissue with fungal structures onto Petri dishes containing cornmeal medium to stimulate fungal growth and sporulation. The isolated colonies were exposed to continuous light to promote sporulation. Microscopic observations were conducted using the methods outlined in Barnett and Hunter (1972) and Singh (1991). Identification was performed using a gender-specific identification key.

Statistical analysis

Data were subjected to Levene's test to verify homogeneity of variances, and expressed as the mean ± standard deviation. Comparisons between treatments were performed via one-way ANOVA followed by Tukey's post-hoc test. Survival was assessed by linear regression using the SAS statistical package.

Results and Discussion

Oomycete infection

The inclusion of plant-derived essential oils as feed additives for fish has shown great promise, contributing to improved disease resistance, growth, and animal welfare. In this study, the essential oil of Melaleuca alternifolia (EOMA) was evaluated in tambatingas, focusing on zootechnical performance and disease resistance. Mortality was observed in all experimental units in the first week of assessment, along with external lesions primarily on the fins (Figures 1A and 1B) resulting from infection with Saprolegnia sp. (Figures 1C, 1D, 2A and 2B), which was present in the water throughout the experimental period.

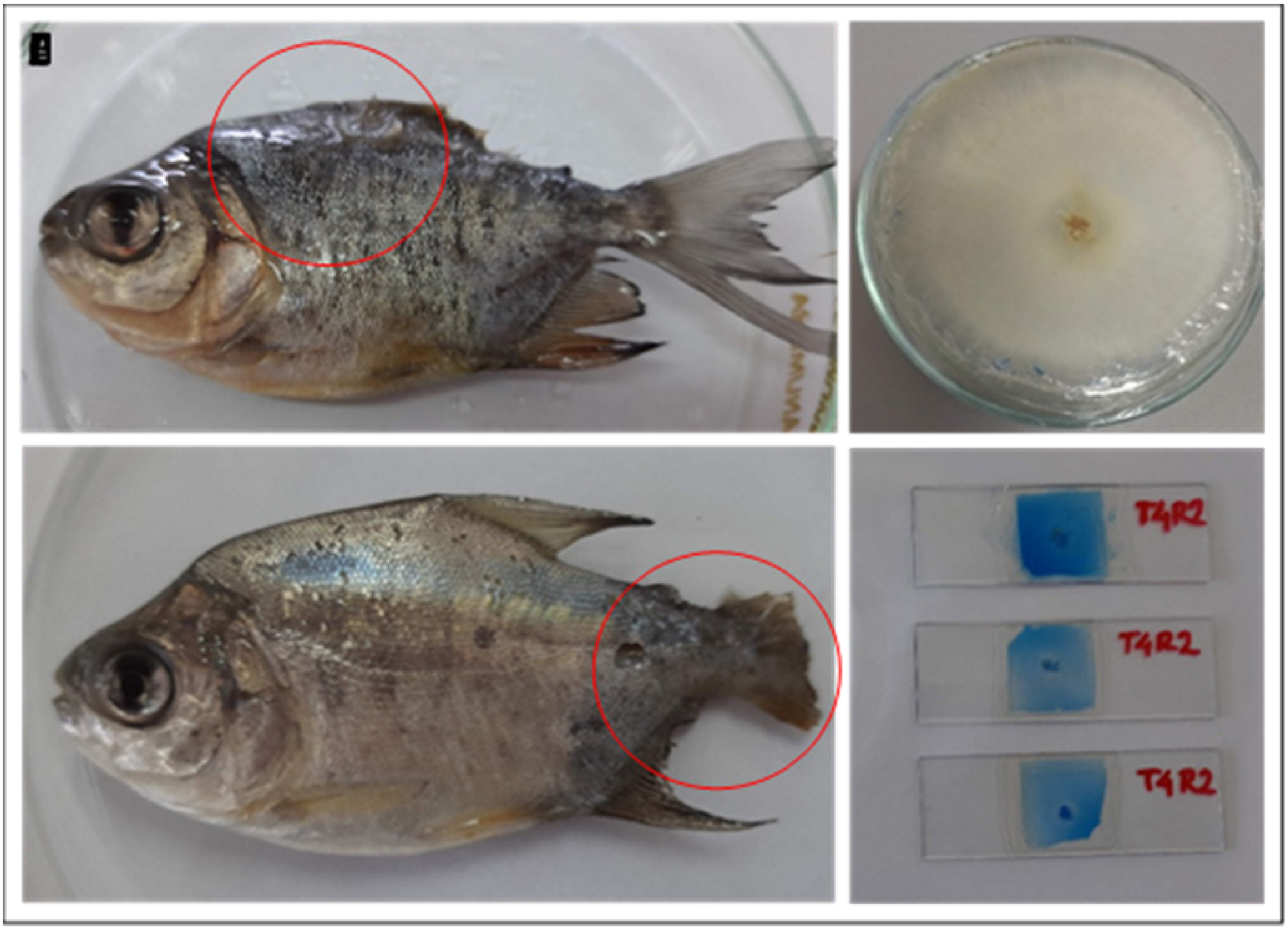

Figure 1. Development of fungal colonies of *Saprolegnia* sp. in infected superficial tissues of tambatinga juveniles that died during the experiment with *Melaleuca alternifolia* essential oil.
A: Tambatinga infected by *Saprolegnia* sp. in the dorsal region; **B:** Tambatinga infected by *Saprolegnia* sp. in the tail region; **C:** Pure isolate of *Saprolegnia* sp. grown on cornmeal medium; and **D:** Blades with fungal structures of *Saprolegnia* sp. obtained from pure colonies isolated from scrapings of infected superficial tissues.

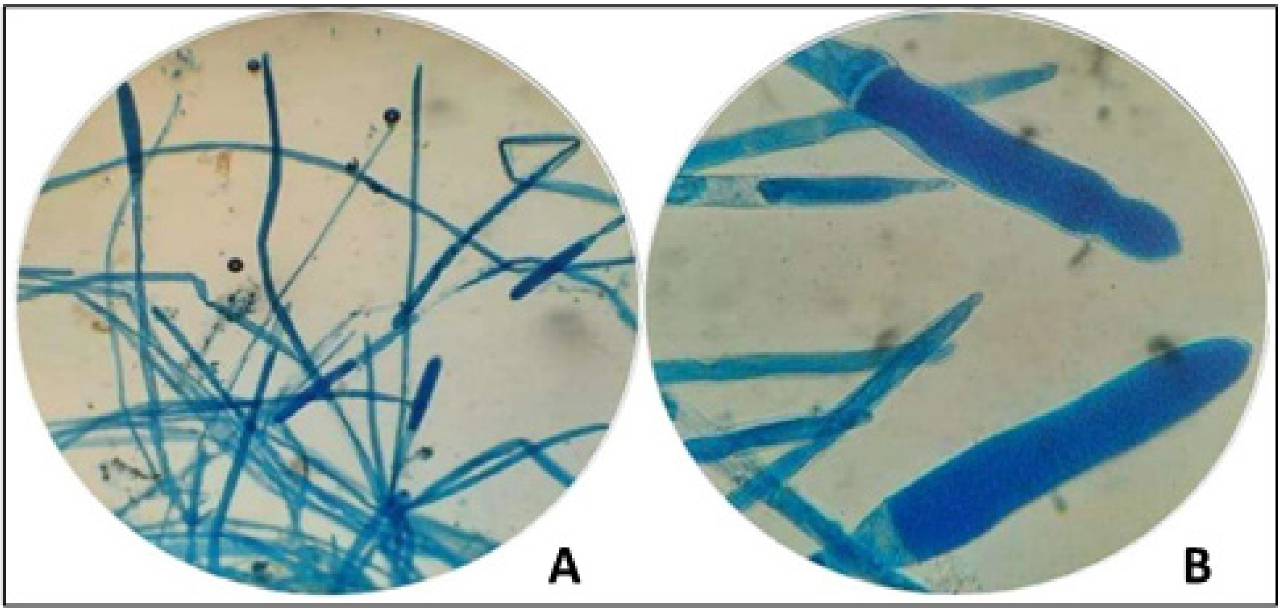

Figure 2. Photomicroscopy of fungal structures of *Saprolegnia* sp. from infected superficial tissues of tambatinga juveniles that died during the experiment with *Melaleuca alternifolia* essential oil. **A:** Hyphae developed with the formation of reproductive structures; and **B:** Detail of the reproductive structure of the fungus. Photomicrographs were obtained by optical microscopy at 40x magnification.

The low water temperature registered in the present investigation resulted in mortality in all treatments, which was attributed to infection by the aquatic fungus belonging to the genus Saprolegnia within the class of Oomycetes. Saprolegniosis manifests itself in the epidermis and dermis as white or grayish spots, characterizing the mycelial growth that is visible to the naked eye (Barbosa, 2018).

Fungus-like aquatic molds (Oomycota, Saprolegniales) are common in freshwater habitats and include Saprolegnia species, secondary pathogens that cause significant economic losses, amounting to millions of dollars in global aquaculture (Eszterbauer et al., 2024; Magray et al., 2019). This opportunistic saprophytic oomycete proliferates in stressed and physically injured fish; it thrives on decomposing organic matter, and infections can occur when fish have macroscopic lesions or reduced mucus due to handling (Ali et al., 2020).

Mortality was effectively controlled after 30 days, indicating that EOMA had a positive impact against Saprolegnia sp. The levels of EOMA tested in this experiment did not cause toxicity, as mortality was also observed in control fish as well as in those subjected to 1.0 mL/kg EOMA. The fish exposed to 1.5, 2.0 and 2.5 mL/kg EOMA presented higher survival rates, 40%-70%, than those receiving 0.0 and 1.0 mL/kg EOMA. Progressive increases in EOMA concentration raised survival rates by approximately 10.30%. The model explains 67% of the relationship between EOMA concentration and survival (Figure 3)

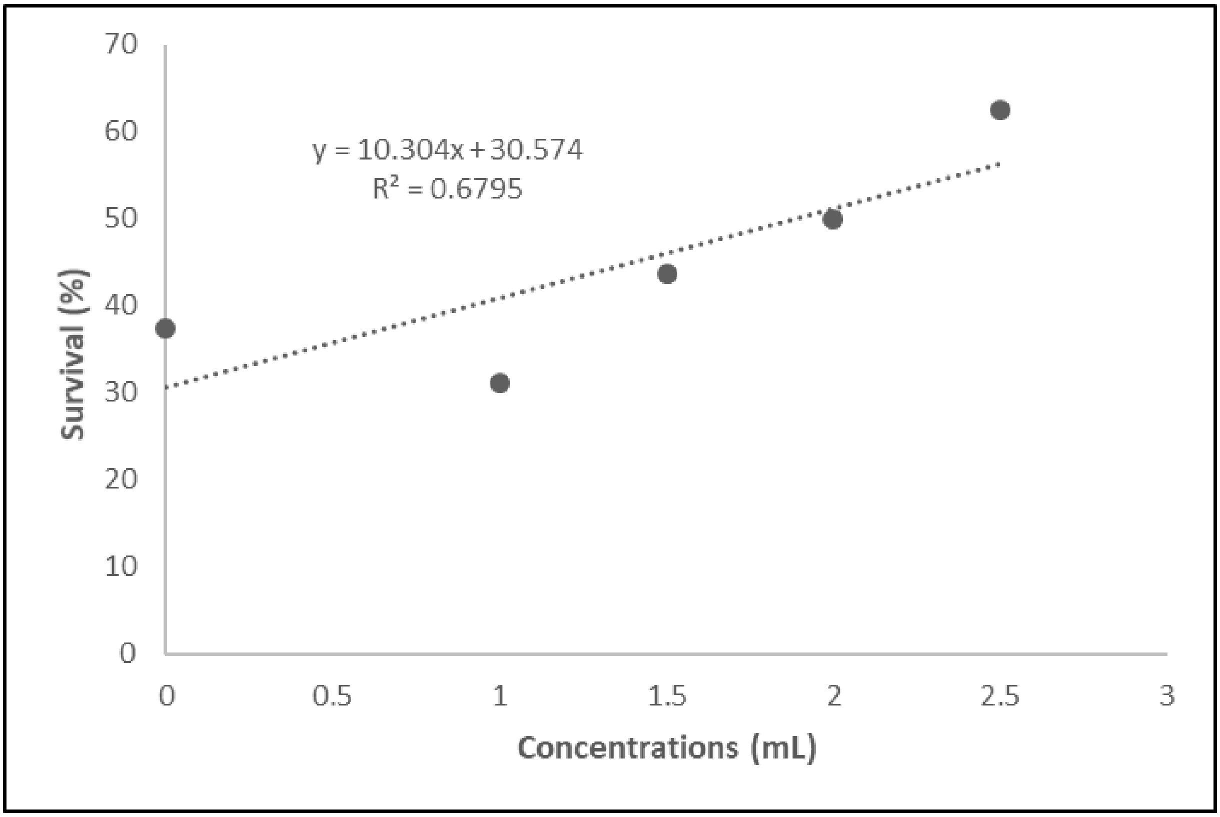

Figure 3. Survival rate of tambatinga juveniles fed diets containing different levels of *Melaleuca alternifolia* essential oil for 45 days.

The reduced mortality seen with the addition of EOMA may be attributed to its medicinal properties, which have been proven beneficial in combating fish pathogens (Souza et al., 2017; Baldissera et al., 2018). Mertas et al. (2015) achieved promising results when using EOMA against strains of Candida albicans, suggesting a potential in targeting fungi that are resistant to conventional antifungal medicines.

Zootechnical performance

Inclusion of EOMA in tambatinga diets had no effect upon the evaluated zootechnical variables within the first 30 days of testing (P>0.05). After 45 days, feed intake and survival increased linearly with the EOMA concentration (P<0.05), and feed conversion started to improve from 1.5 mL/kg EOMA upwards (P<0.05) (Table 3). This may be related to an initial adaptation period to the diet, or to latency of the fish's response to the bioactive effects of EOMA upon metabolism and appetite. On the other hand, addition of EOMA to tambatinga diets elicited improvements in feed conversion ratio, a linear increase in feed intake, and greater survival rates, although it did not promote growth at the end of the 45-day trial.

The absence of influence on fish growth may be explained by the low water temperature recorded during the study, 23.6 ± 1.4 °C, which is below the recommended range of 25-32 °C for tropical species (Pinto et al., 2019). When kept at temperatures from 24 to 28 ºC, tropical fish have an ideal consumption rate to maximize growth; however, food consumption is reduced below 24 ºC, leading to decreased growth and increased mortality (M. C. S. Silva et al., 2021). Besides, the optimal temperature for ideal growth of tambaqui and pirapitinga, which are the parent species of the hybrid tambatinga, is in the range of 28-31 ºC (Gomes et al., 2021). Tambatinga is more susceptible to disease and mortality when cultivated in regions of Brazil where temperature variations occur (Fernandes et al., 2018); in order to avoid such an outcome, management adaptations and supplementation with dietary substances that are able to boost the immunity are required.

Table 2
Zootechnical performance of tambatinga juveniles fed diets containing different levels of *Melaleuca alternifolia* essential oil (EOMA) for 30 and 45 days

	Dietary levels of EOMA (mL/kg)					
	0.0	**1.0**	**1.5**	**2.0**	**2.5**	**CV (%)**
IW	10.5±1.4	11.0±1.1	10.0±0.9	10.4±1.0	10.9±1.0	
IL	8.3±0.4	8.6±0.3	8.3±0.1	8.6±0.3	8.3±0.4	
			30 days			
WG	8.0±2.1	12.8±2.5	12.4±5.5	12.2±4.2	11.1±3.2	32.93
SGR	1.9±0.4	2.5±0.2	2.6±0.8	2.5±0.6	2.3±0.6	24.56
CF	1.9±0.2	1.8±0.1	2.0±0.1	1.9±0.07	2.0±0.1	9.33
SR	43.7±7.2	43.7±23.9	46.8±21.3	53.1±15.7	65.6±18.7	
			45 days			
WG	21.2±3.2	22.6±5.3	24.3±4.4	23.0±8.3	24.6±6.4	27.69
SGR	2.4±0.3	2.7±0.1	2.6±0.6	2.5±0.5	2.6±0.4	16.20
CF	1.9±0.1	2.0±0.3	1.8±0.1	1.7±0.3	1.91±0.1	11.83
FI1	35.85±3.54b	47.04±2.09a	38.75±3.59b	47.06±5.01a	50.08±3.71a	8.43
FC2	1.15±0.11a	1.10±0.134a	1.00±0.17ab	0.98±0.12ab	0.91±0.06b	11.95
SR*	37.5±17.6	31.2±16.1	40.6±16.1*	50.0±20.4*	62.5±22.8*	

IW - Initial Weight (g); IL - Initial Length (cm); WG - Weight Gain (g); SGR - Specific Growth Rate (%); CF - Condition Factor; SR - Survival Rate (%); FI - Feed intake (g); FC - Feed conversion; CV (%) - Coefficient of Variation. Values are expressed as mean ± SD. (n=8)
*Statistical difference between treatments. ***y = 10.304x + 30.574, R^2 = 0.6795**
[1]Statistical difference between treatments. **[1]y = 4.1585x + 38.342, R^2 = 0.353**
[2]Statistical difference between treatments. **[2]y = - 0.0983x + 1.1652, R^2 = 0.3466**

When used as feed additive, EOMA may boost growth, antioxidant capacity, and innate immunity. For instance, supplementation of *Macrobrachium rosenbergii* diet with 100 mg/kg EOMA for eight weeks produced positive outcomes on growth, antioxidant capacity, and intestinal immunity, thus contributing to the animals' overall health (Liu et al., 2022). Similarly, *Rhamdia quelen* receiving 1.0 mL/kg EOMA and kept at 21°C for 21 days exhibited enhanced zootechnical performance and reduced hepatic oxidative damage (Reis et al., 2021).

Prieto et al. (2005) and Schalch et al. (2009) have reported that extracts and essential oils, such as those from garlic (*Allium sativum*), crabgrass (*Polygonum hydropiper*), donkey grass (*Sapium sebiferum*), acalypha (*Acalypha australis*), shrub cairatia (*Cayratia japonica*), mugwort (*Artemisia argyi*), wild strawberry (*Duchesnea indica*), helenium (*Helenium quadridentatum*), and carnation (*Syzygium romanticum*), may be used to treat fungal diseases in fish farming. Additionally, EOMA may be considered an alternative product for controlling *Saprolegnia* sp. It may be included in fish diet at concentrations exceeding 1.5 mL/kg feed as a prophylactic measure.

The current findings demonstrate the antifungal efficacy of EOMA, which is likely attributed to its main component, terpinen-4-ol (45.85%), since it is said to possess antifungal activity (Felipe et al., 2018). Terpinen-4-ol inhibits cellular respiration and alters the structure and permeability of fungal and bacterial cell membranes, thus impeding the action of these pathogens, as observed in this investigation. EOMA may be incorporated into tambatinga diet as an antimicrobial and antifungal agent. Nevertheless, the current results indicate the requirement for further studies in order to better understand its effects on growth and biochemical parameters, and to determine its optimal concentrations and treatment time in different farming conditions (e.g., more suitable temperatures). Its mechanism of action in fish must also be elucidated.

Conclusion

Incorporation of EOMA into the diet of tambatinga juveniles enhanced resistance to Saprolegnia sp., resulting in increased final survival rates at concentrations exceeding 1.5 mL/kg.

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Conflicts of Interest

The authors declare no conflict of interest.

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