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Effects of thymol: carvacrol association on health and zootechnical performance of tambaqui *Colossoma macropomum*

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ABSTRACT

Research in aquaculture shows that thymol and carvacrol are promising additives in fish diets. In this study, the effects of the thymol:carvacrol combination on health parameters and the zootechnical performance of tambaqui (*Colossoma macropomum*) were evaluated. The compounds were incorporated into five concentrations of feed. At intervals of 30 and 60 days, fish were sampled to evaluate haematological, biochemical, and immunological parameters, and after 60 days, zootechnical performance, parasitic load, and resistance to bacteriosis were evaluated. After 30 days, fish that received the highest concentrations of thymol:carvacrol showed a reduction in the number of thrombocytes and lymphocytes and an increase in eosinophils ($p \leq 0.05$). After 60 days of feeding, haematological parameters were similar between all groups. Regarding the antiparasitic effect of the compounds, all groups had a high parasitic load (monogenean infestation). Regarding the prevention of bacteriosis, high mortality was detected in all groups after the experimental challenge with *Aeromonas jandaei*, with no greater protection for fish fed thymol:carvacrol. In conclusion, it was observed that haematological results corroborate with research on mammals, in which the anti-inflammatory/immunosuppressive effects of thymol and carvacrol are highlighted. However, the use of additives for aquaculture should be discussed carefully, as the cost-benefit of supplementation is not a consensus.

Keywords: Anti-inflammatory, essential oil, isolate compound, nutrition, thyme.

Efeitos da associação timol:carvacrol na saúde e desempenho zootécnico do tambaqui *Colossoma macropomum*

RESUMO

Pesquisas em aquicultura mostram que timol e carvacrol são aditivos promissores na dieta de peixes. Neste estudo, foram avaliados os efeitos da combinação timol:carvacrol sobre parâmetros de saúde e o desempenho zootécnico de tambaqui (*Colossoma macropomum*). Os compostos foram incorporados em cinco concentrações na dieta. Em intervalos de 30 e 60 dias, peixes foram amostrados para avaliação hematológica, bioquímica e imunológica e, após 60 dias, para desempenho zootécnico, carga parasitária e resistência à bacteriose. Após 30 dias, peixes que receberam maiores concentrações de timol:carvacrol apresentaram redução de trombócitos e linfócitos e aumento dos eosinófilos ($p \leq 0,05$). Após 60 dias de alimentação, os parâmetros hematológicos foram semelhantes entre todos os grupos. Com relação ao efeito antiparasitário dos compostos, todos os grupos apresentaram alta carga parasitária (infestação monogênica). Quanto à prevenção da bacteriose, foi detectada alta mortalidade em todos os grupos após desafio com *Aeromonas jandaei*, não havendo maior proteção para os peixes alimentados com timol:carvacrol. Em conclusão, observou-se que os resultados hematológicos corroboram pesquisas em mamíferos, que destacam a ação anti-inflamatória/imunossupressora do timol e do carvacrol. No entanto, o uso desses aditivos para a aquicultura merece ser discutido cautelosamente, pois o custo-benefício dessa suplementação para o produtor não é um consenso.

Palavras-chave: Anti-inflamatório, composto isolado, nutrição, óleo essencial, tomilho.

INTRODUCTION

Additives regularly used for animal feed are substances, microorganisms, or products formulated to be intentionally included in the diet that are not normally an ingredient but can improve the characteristics of products intended for animals, benefiting the performance of healthy individuals and meeting the nutritional demand needed by them (Brasil, 2015). To combine the increase in productivity with the improvement of the health of fish from aquaculture, studies have been developed for food supplementation with varied additives and distributed in different classes, such as immunostimulants

(Huttenhuis et al., 2006; Dawood et al., 2018), probiotics (Burr et al., 2005; Balcázar et al., 2006; Hai, 2015; Tachibana et al., 2019), and prebiotics (Li and Gatlin, 2004; Ringø et al., 2010). Additives include plant products that are biodegradable and range from herbs and spices to commercial products (Dawood et al., 2018). There are plant by-products that are known for their chemical classes, such as phenolics, polyphenols, alkaloids, quinones, terpenoids, lectins, and polypeptides (Rodríguez-Estrada and Ranzani-Paiva, 2019), which are largely responsible for their biological effects.

Compounds such as thymol and carvacrol, which are monoterpene phenols isolated from plants, have medicinal properties and have been highlighted in aquaculture (Alagawany et al., 2015; 2021; Dhama et al., 2015). Various plants have these compounds in abundance in their composition; for example, the different species of thyme (*Thymus vulgaris* and *Thymus riatarum*) (Silva, 2018; Yousefi et al., 2018) are rich in thymol, in addition to black cumin (*Nigella sativa*), marjoram (*Origanum majorana*), and oregano (*Origanum vulgare*), which are rich in carvacrol (Silva, 2018). In aquaculture studies, products based on thymol and carvacrol are described as an anaesthetic for silver catfish (*Rhamdia quelen*) (Bianchini et al., 2017) and carp (*Cyprinus carpio*) (Yousefi et al., 2018), growth promoters for different species of fish (Amer et al., 2018; Morselli et al., 2020; Kong et al., 2021; Silva et al., 2021), and antimicrobial for silver catfish infected with *Aeromonas hydrophila* (Cunha et al., 2019). These characteristics can be attributed to the ability of these compounds to suppress free radicals and harmful constituents by interacting with cellular biological components and their ability to manipulate the balance of the intestinal microbiota, increasing digestion, metabolism, and nutrient absorption (Alagawany et al., 2015; 2021).

Tambaqui (*Colossoma macropomum*) is currently the main native species farmed in aquaculture in South America (Brazilian Institute of Geography and Statistics Foundation — IBGE, 2021). These fish are valuable species farmed for human consumption in Bolivia, Brazil, Colombia, Ecuador, Peru, and Venezuela. However, there is still a lack of inputs, such as additives that improve the zootechnical performance or health of tambaqui. Recently, Valladão et al. (2018) described how the integration of producers, researchers, and industry stands out as an issue of extreme importance, and that these types of inputs should become available as soon as possible. Knowing that zootechnical, immunological, and sanitary performances are parameters easily compromised when animals are subjected to confinement, the use of phytogenics can cooperate with the restoration of physiological, metabolic, and health damage that the production environment imposes on fish.

This study investigated the effects of dietary supplementation with different levels of the association of thymol:carvacrol on health parameters that include biochemical and haemato-immunological analyses, parasitism, and resistance to infection as well as zootechnical parameters.

MATERIALS AND METHODS

Tambaqui juveniles were obtained from commercial fish farming and acclimated in a 500-L tank with constant water flow and aeration, in addition to providing commercial feed for juveniles once a day for two months. During the acclimatisation period, the animals were healthy without expressing any clinical signs of disease before being part of the experimental groups of the nutritional trial. All adopted procedures with fish followed the ethical principles adopted by the Brazilian College of Animal Experimentation in accordance with the principles of ethics in animal experimentation. This study was approved by the Ethics Committee on Animal Use (CEUA) of Universidade Nilton Lins (protocol 002/2021).

Experimental design

A total of 180 juvenile tambaqui (32.17 ± 5.76 g; 12.21 ± 0.73 cm) were randomly distributed at a density of 9 fish/tank in 20 tanks with a capacity of 300 L each (system of recirculation, maintained with biofilter and partial water changes weekly), comprising five treatments (control, 500, 1,000, 1,500, and 2,000 mg/kg) of the combination of thymol and carvacrol (1:1 p/p), with four replicates. The experiment with supplementation lasted for 60 days. The combination was chosen because it showed excellent results against tambaqui parasites in in vitro studies performed by the current research group (unpublished data).

The physical and chemical parameters of the water were monitored throughout the experimental period and were found to be within the conditions suitable for the species: temperature (29 ± 0.62°C), dissolved oxygen concentration (3.47 ± 0.94 mg/L), and potential of hydrogen-pH (5.92 ±0.12).

Preparation of experimental diet and bromatology

A basal diet was formulated to meet the tambaqui's nutritional needs (Table 1). First, the basal feed was hot extruded in a single screw extruder (INBRAMAQ, Model MX-80) to ensure starch cooking and the improvement of nutrient digestibility. The basal feed pellets were dried in an oven with forced air circulation at 55°C for 24 h and then ground (VIEIRA®, model MCS 280). The bran feed was divided into five portions, which were supplemented with increasing levels of the mixture of thymol and carvacrol (1:1 w/w, Sigma-Aldrich, ≥ 98% purity): 0 (control), 500, 1,000, 1,500, and 2,000 mg/kg. The isolated compounds were solubilised in a hydroalcoholic solution (1:1 ratio) and gradually mixed into the bran diets in the respective concentrations. It was then cold extruded to form the pellets at 20°C (ITALVISA, model P55), guaranteeing the stability of the thymol:carvacrol mix. The pellets were dried in an oven with forced air circulation at 55°C until the moisture content was below 10%, and moisture was quantified by means of the automatic moisture analyser (Denver Instrument, Model IR 35). All experimental diets were packed in plastic bags, labelled, and

Table 1. Basal diet formulation and proximate composition.

Ingredients	%
Soybean meal	53.50
Corn grain	31.50
Wheat bran	6.30
Meat and bone meal	5.00
Soy oil	2.00
Premix*	1.00
Dicalcium phosphate	0.50
Common salt	0.10
DL-methionine	0.08
Butylated hydroxytoluene	0.02
Nutrients and Energy	
Crude protein (%)	30.01
Fat (%)	4.91
Crude fibre (%)	4.05
Starch (%)	25.86
Ashes (%)	5.72
Gross energy (kcal/kg) **	3,601.10

*Premix: vitamin and mineral supplement (g/kg); potassium chloride 2; magnesium oxide 0.60; iron sulphate 7.50; copper sulphate 1; manganese oxide 2; sodium selenite 0.07; calcium iodate 0.25; choline chloride 80; vitamin K3 0.70; nicotinic acid 10; pantothenic acid 5; folic acid 0.10; biotin 0.05; vitamin A 2,000,000 IU; vitamin D3 600,000 IU; vitamin E; vitamin B1 2; victim B2 4; vitamin B6 5; vitamin B12 0.01; vitamin C 80; inositol 4; ethoxyquin 1; BHT 5; **Value calculated according to the formula EB (kcal/g) = (5.7 × g PB) + (9.4 × g EE) + [4.1 × (g ENN + g FB)].

stored at -4°C until use. A portion of the basal diet was analysed bromatologically according to the analysis methodology developed by the Brazilian Compendium of Animal Feeding (2017) (Table 1).

Evaluation of zootechnical parameters

The food provided for each experimental unit was previously weighed, and after each feeding, the leftover diet was collected in individual bags for drying in an oven (55°C for 24 h), followed by weighing. Biometrics for weighing fish were established on days 0 and 60. Based on the data obtained, final weight, weight gain (final weight — initial weight), total consumption/tank, feed conversion (g dry weight fed/g live weight gain), and protein efficiency ratio (weight gain/protein intake) were calculated according to the methodology of Fracalossi et al. (2013).

Fish health assessment

Samples were collected on days 30 and 60 after initiating supplementation. For procedures, fish were anesthetised by immersion in benzocaine (100 mg/L) diluted in water. Eight fish per treatment (two fish/replicate) were randomly sampled to collect blood via puncture of the caudal vessel. The whole blood of each animal was used to prepare smears and obtain serum. An aliquot of the blood was heparinised to assess the leukocyte respiratory burst activity and red blood cell (RBC) count.

At the end of the experimental period, eight fish per treatment were also collected for parasitological analysis, and the remaining fish were used for the experimental challenge with the aim of evaluating the prevention of motile aeromonas septicemia.

Haematological, immunological, and biochemical parameters

The RBC count was calculated to be 10^6 cells/mm^3 using heparinised whole blood diluted 1:200 in formalin citrate solution. Counting was performed according to Hesser (1960) in a Neubauer chamber.

The blood extensions were stained with May Grünwald-Giemsa-Wright. For complete blood cell data, 2,000 erythrocytes were counted for the quantification of white blood cells (WBCs) and thrombocytes. For differential blood cell counts, 200 WBCs were examined and counted as lymphocytes, monocytes, neutrophils, eosinophils, or special granulocytic cells.

BURST was evaluated by reducing nitroblue tetrazolium (Sigma-Aldrich, CA, USA, ref. N6876) to formazan granules with readings performed on a spectrophotometer (BIOCHROM, model Libra S32) at 540 nm optical density, according to the methodology described by Biller-Takahashi et al. (2013).

Biochemical analyses of alanine aminotransferase (ALT), aspartate aminotransferase (AST), protein, and albumin were measured from the serum in a spectrophotometer (BIOCHROM, model Libra S32), following the manufacturer's instructions. ALT and AST were performed with a commercial kit (VIDA Biotecnologia, ref. 80785070010 and 80785070006, respectively), and the values were reported in IU/L. The concentrations of total protein and albumin were determined in the serum with a commercial kit (Labtest Diagnóstica SA, ref. 99/250 and 19/250, respectively). Globulin was calculated by subtracting the albumin value from the protein. Values were expressed in g/dL.

Parasitological analysis

To analyse the influence of supplementation with additives on ectoparasites of the Monogenea class, after 60 days of feeding, eight fish from each group were analysed. The fish were euthanised by spinal cord section, and after the procedure, mucus was obtained from the integument and gills according to the methodology of Pala et al. (2018). The mucus of the integument was scraped with a glass slide and deposited in a collecting pot with 20 mL of 5% formaldehyde. As for the collection of mucus from the gills, all branchial arches were deposited in a universal collector containing 20 mL of formaldehyde (1:4,000) for 2 h. After this period, the mucus was scraped with scalpel blades in the same collecting pot, which was added with the same volume

of 10% formaldehyde. The prevalence and mean intensity of the parasites of the integument and gills were determined by analysis in a Sedgewick-Rafter chamber (optical microscopy).

Bacterial challenge

At the end of the supplementation period, the remaining fish from the respective groups were distributed at a density of 8 fish/tank, in a total of 10 tanks with a capacity of 70 L (closed system with continuous aeration), maintaining the five groups (0, 500, 1,000, 1,500, and 2,000 mg/kg) with two replicates. All groups were inoculated intraperitoneally with a strain of *A. jandaei* (AM-71), identified by matrix-assisted laser desorption-ionization time of flight (MALDI-ToF) mass spectrometry (Bruker Daltonics, Germany). This strain was chosen because it was recently confirmed as pathogenic for tambaqui by Koch's postulate (Mielke et al., 2022).

The concentration used to infect the animals was defined through previous experiments (lethal dose), where mortality of more than 50% of the fish was selected to compare the protective effects of the groups that received supplementation. To prepare the inoculum, the strain (stored at -20°C in tryptone soy broth, enriched with 15% glycerol) was streaked on tryptone soy agar (TSA, Himedia, India) and incubated at 28°C for 24 h. After growth, the colonies were homogenised in sterile phosphate buffer saline to an optical density of 0.8 in a spectrophotometer (600 nm wavelength), corresponding to approximately 10^8 CFU/mL. The inoculum was administered at a dose of 0.1 mL/10 g of body weight.

The fish were fed the control diet for 7 days of the experimental period until apparent satiety. Daily, the animals were analysed for the appearance of clinical signs, behavioural changes, and mortality. The newly killed fish were sterilised (peeled and washed with neutral detergent, 0.1% iodised alcohol, and 70% alcohol), and fragments of the brain and kidney were assessed in a sterile microbiological flow for re-isolation and confirmation of the infectious agent. The contents of each organ were striated in TSA (Himedia) and incubated at 28°C for 24 h. All isolates were again identified for ribosomal proteins by MALDI-ToF mass spectrometry, following the methodology described by Assis et al. (2017).

Statistical analysis

The normality of the data and the homogeneity of the variances were assessed by the Shapiro-Wilk and Levene tests, respectively. The data were expressed as the mean ± standard deviation. Values referring to productive performance and all health parameters were submitted for analysis of variance (one-way ANOVA) using the statistical software Sigma Stat version 3.5. When statistical differences were observed, the means were compared using the Tukey's test at a 95% confidence level. Based on the experimental design of increasing levels of inclusion of the additive, the zootechnical parameters were also subjected to polynomial regression analysis.

RESULTS

Zootechnical performance

For all parameters of zootechnical performance, no statistical differences were observed between treatments ($p \geq 0.05$) (Table 2). However, the polynomial regression analysis showed that the final weight of the fish increased according to the concentration of thymol:carvacrol (Table 2; Figure 1). Therefore, there was a directly proportional and positive relationship between the level of inclusion of thymol:carvacrol and the final weight of the fish.

Health parameters

Haematological parameters

Thirty days after the start of supplementation, fish fed the three highest concentrations of the thymol:carvacrol association showed a decrease in thrombocytes ($p \leq 0.05$) when compared to fish in the

Table 2. Effect of supplementation with different concentrations of thymol:carvacrol, after 60 days, under the zootechnical parameters of tambaqui *Colossoma macropomum*.

Zootechnical parameters	Control	500 mg/kg	1,000 mg/kg	1,500 mg/kg	2,000 mg/kg	p-value	
						One-way ANOVA	Regression
Final weight (g)	68.57 ± 18.84	76.71 ± 14.87	79.95 ± 20.18	83.33 ± 16.75	91.72 ± 12.75	0.431	0.0445
Weight gain (g)	36.56 ± 18.36	47.60 ± 14.03	47.53 ± 19.73	49.98 ± 17.44	60.17 ± 12.16	0.432	0.0582
Total feed consumption/tank (g)	582.22 ± 98.15	614.55 ± 106.18	636.25 ± 165.38	637.32 ± 88.50	689.25 ± 73.40	0.739	0.158
Feed conversion	1.08 ± 0.64	1.04 ± 0.31	0.97 ± 0.31	1.01 ± 0.35	0.87 ± 0.12	0.946	0.243
Protein efficiency ratio	4.04 ± 2.72	4.15 ± 1.65	3.98 ± 0.47	4.40 ± 1.86	4.38 ± 1.01	0.972	0.717

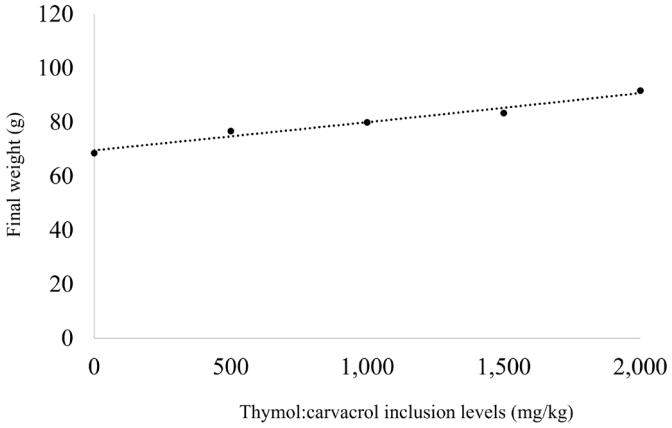

Figure 1. Polynomial regression ($y = 2E\text{-}07x^2 + 0.0102x + 69.565$; $R^2 = 0.9661$) on the effect of inclusion levels of thymol:carvacrol on the final weight of tambaqui *Colossoma macropomum*.

control group. In the same period, fish fed the highest concentration of thymol:carvacrol had lower amounts of lymphocytes ($p \leq 0.05$) when compared to the other groups. For the counting of eosinophils, the group fed the highest level of thymol:carvacrol supplementation showed a significant increase when compared to the control group ($p \leq 0.05$) after 30 days of experimentation.

After 60 days of the experiment, fish supplemented with 1,000 mg/kg phytogenics had higher number of eosinophils than the control group ($p \leq 0.05$). The quantification of the other cells did not vary according to the level of supplementation with the additives in the diet (Table 3).

Biochemical parameters

After 30 days of supplementation, the enzymatic activity of ALT increased ($p \leq 0.05$) in fish fed the lowest level of the additive in the diet (500 mg/kg) when compared to the other groups. The values of albumin, total protein, globulin, and AST did not

Table 3. Effect of supplementation with different levels of the association of thymol: carvacrol under the haematological parameters of tambaqui *Colossoma macropomum*.

Haematological parameters	30 days					One-way ANOVA
	Control	500 mg/kg	1,000 mg/kg	1,500 mg/kg	2,000 mg/kg	
RBC ($\times 10^6$/µL)	1.74 ± 0.17	2.19 ± 0.39	1.17 ± 0.24	2.26 ± 0.53	1.80 ± 0.62	0.244
Total leukocytes ($\times 10^3$/µL)	25.35 ± 9.88	19.41 ± 3.40	15.83 ± 2.63	18.73 ± 5.91	15.33 ± 8.02	0.090
Thrombocytes (x10^3/µL)	39.38 ± 4.56a	29.85 ± 10.29a	17.73 ± 7.03b	16.36 ± 15.23b	15.05 ± 7.53b	0.008
Neutrophils ($\times 10^3$/µL)	3.83 ± 3.04	4.39 ± 0.84	4.06 ± 1.60	2.59 ± 1.52	3.50 ± 2.22	0.747
Lymphocytes ($\times 10^3$/µL)	14.70 ± 5.91a	8.52 ± 1.48a	6.88 ± 1.29a	6.91 ± 3.27a	5.79 ± 3.96b	0.027
Monocytes (x10^3/µL)	6.60 ± 3.85	5.61 ± 1.23	4.32 ± 1.37	4.13 ± 4.46	5.30 ± 2.79	0.781
Special granulocytic cells ($\times 10^3$/µL)	0.40 ± 0.20	0.47 ± 0.38	0.20 ± 0.13	0.11 ± 0.07	0.06 ± 0.05	0.095
Eosinophils ($\times 10^3$/µL)	0.31 ± 0.06a	0.56 ± 0.19ab	0.38 ± 0.15a	0.36 ± 0.05a	1.02 ± 0.53b	0.009
Haematological parameters	**60 days**					**One-way ANOVA**
	Control	**500 mg/kg**	**1,000 mg/kg**	**1,500 mg/kg**	**2,000 mg/kg**	
RBC ($\times 10^6$/µL)	2.32 ± 0.30	2.36 ± 0.24	2.49 ± 0.64	2.66 ± 0.51	2.28 ± 0.25	0.714
Total leukocytes ($\times 10^3$/µL)	24.08 ± 4.93	21.82 ± 6.39	24.23 ± 7.21	26.88 ± 6.76	23.54 ± 6.16	0.871
Thrombocytes ($\times 10^3$/µL)	35.37 ± 5.13	35.32 ± 5.92	38.09 ± 9.73	41.13 ± 6.77	37.65 ± 9.41	0.501
Neutrophils ($\times 10^3$/µL)	6.46 ± 3.33	5.68 ± 2.22	5.85 ± 3.29	6.91 ± 3.47	7.61 ± 2.14	0.882
Lymphocytes ($\times 10^3$/µL)	11.31 ± 2.92	11.86 ± 4.36	12.05 ± 6.04	14.61 ± 6.31	10.63 ± 2.89	0.8
Monocytes ($\times 10^3$/µL)	5.88 ± 2.22	3.51 ± 0.86	4.69 ± 0.63	6.05 ± 2.79	4.81 ± 1.28	0.293
Special granulocytic cells ($\times 10^3$/µL)	0.10 ± 0.08	0.16 ± 0.11	0.07 ± 0.08	0.09 ± 0.11	0.05 ± 0.06	0.527
Eosinophils ($\times 10^3$/µL)	0.51 ± 0.27a	0.71 ± 0.38a	1.73 ± 0.90b	0.66 ± 0.37a	0.74 ± 0.63a	0.049

All values were expressed as mean ± standard deviation. Different letters express significant differences ($p \leq 0.05$, one-way ANOVA followed by the Tukey's test); RBC: erythrocytes.

change significantly during this period (Table 4). After 60 days of feeding, all biochemical parameters maintained similar results to those of the control group ($p \geq 0.05$), as described in Table 4.

Immunological parameters

The use of diets with different concentrations of additives for periods of 30 and 60 days did not stimulate the leukocyte respiratory activity ($p \geq 0.05$) between the groups (Figure 2).

Antiparasitic potential

Parasitological analysis, performed at the end of the experiment, revealed a high level of Monogenean infestation in all groups, without differences in prevalence and parasitic intensity ($p \geq 0.05$) when compared to supplemented groups with the control group (Table 5).

Protective effect against haemorrhagic septicaemia by motile Aeromonas

After 24 h of the experimental bacterial challenge, the fish began to show clinical signs suggestive of haemorrhagic septicaemia, such as lethargy, loss of scales, melanosis, pale gills, bulging of the celomatic cavity suggestive of fluid accumulation, diffuse

Table 4. Effect of supplementation with different levels of the association of thymol: carvacrol on the biochemical parameters (serum protein profile and liver functions) of tambaqui *Colossoma macropomum*.

Biochemical parameters	30 days					One-way ANOVA
	Control	500 mg/kg	1,000 mg/kg	1,500 mg/kg	2,000 mg/kg	
Protein (g/dL)	3.04 ± 0.54	3.00 ± 0.36	2.70 ± 0.14	3.05 ± 0.51	2.76 ± 0.20	0.589
Albumin (g/dL)	0.66 ± 0.11	0.68 ± 0.05	0.64 ± 0.11	0.71 ± 0.14	0.67 ± 0.09	0.882
Globulin (g/dL)	2.38 ± 0.43	2.30 ± 0.33	2.06 ± 0.24	2.33 ± 0.44	2.10 ± 0.17	0.594
ALT (U/L)	9.28 ± 3.66a	19.97 ± 4.94b	16.37 ± 2.53a	12.00 ± 4.13a	14.51 ± 2.00a	0.008
AST (U/L)	70.93 ± 17.00	95.00 ± 30.63	86.86 ± 12.65	137.82 ± 65.37	103.23 ± 41.00	0.205
Biochemical parameters	**60 days**					**One-way ANOVA**
	Control	**500 mg/kg**	**1,000 mg/kg**	**1,500 mg/kg**	**2,000 mg/kg**	
Protein (g/dL)	3.35 ± 0.60	3.50 ± 0.70	3.96 ± 0.63	3.00 ± 1.02	3.20 ± 0.40	0.403
Albumin (g/dL)	0.46 ± 0.05	0.51 ± 0.12	0.50 ± 0.13	0.50 ± 0.06	0.48 ± 0.15	0.963
Globulin (g/dL)	1.42 ± 0.32	1.44 ± 0.08	1.63 ± 0.63	1.40 ± 0.40	1.30 ± 0.24	0.805
ALT (U/L)	5.56 ± 1.65	5.68 ± 1.92	5.90 ± 1.97	6.33 ± 2.09	4.70 ± 1.30	0.777
AST (U/L)	91.12 ± 33.73	87.08 ± 21.15	83.49 ± 31.70	84.80 ± 16.97	95.70 ± 18.76	0.185

All values were expressed as mean ± standard deviation. Different letters express significant differences ($p \leq 0.05$, one-way ANOVA followed by the Tukey's test); ALT: alanine aminotransferase; ALT: aspartate aminotransferase.

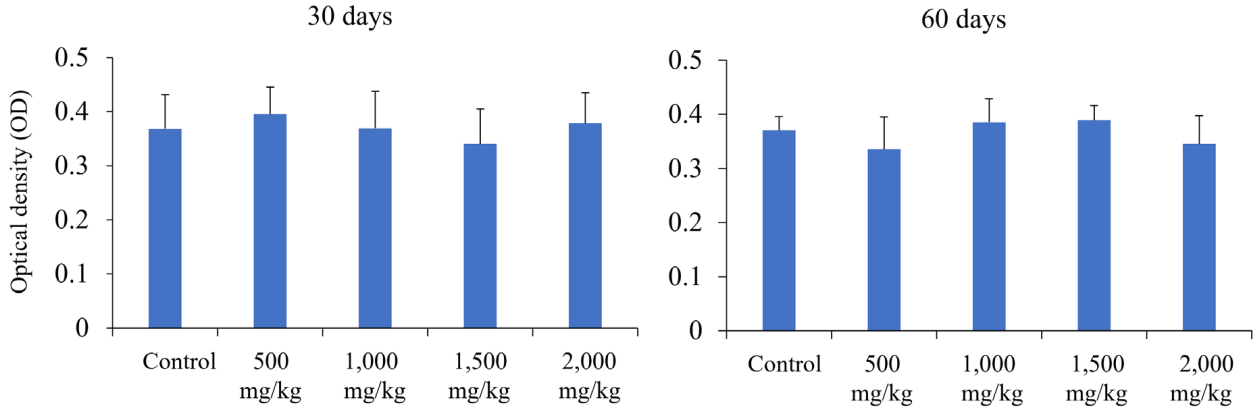

Figure 2. Oxidative burst (optical density [OD]) of tambaqui *Colossoma macropomum* fed diet containing different levels of the association of thymol:carvacrol.

Table 5. Parasitological indexes of Monogenean infestation in juveniles of tambaqui *Colossoma macropomum* after 60 days of experiment.

Indexes	Gills					One-way ANOVA
	Control	500 mg/kg	1,000 mg/kg	1,500 mg/kg	2,000 mg/kg	
Prevalence (%)	100	100	100	100	100	–
Parasitism intensity	11,175 ± 3,297.89	9,210 ± 1,929.25	9,080 ± 2,68.81	9,785 ± 2,281.48	9,500 ± 3,148.63	0.797
Indexes	**Skin**					**One-way ANOVA**
	Control	**500 mg/kg**	**1000 mg/kg**	**1500 mg/kg**	**2000 mg/kg**	
Prevalence (%)	100	100	100	100	100	–
Parasitism intensity	300.00 ± 100.37	533.75 ± 74.54	517.50 ± 213.48	521.25 ± 279.86	508.75 ± 117.07	0.457

haemorrhage, and rectal prolapse. A high cumulative mortality was observed in all challenged groups, with no statistical difference ($p \geq 0.05$) (Figure 3). The experiment resulted in a cumulative mortality rate of 87.5, 100, 93.75, 100, and 100% in the control group and 500, 1,000, 1,500, and 2,000 mg/kg treatment groups, respectively. Through the re-isolation of the bacteria and analysis of MALDI-ToF mass spectrometry identification, the cause of mortality was confirmed by the presence of *A. jandaei* bacteria.

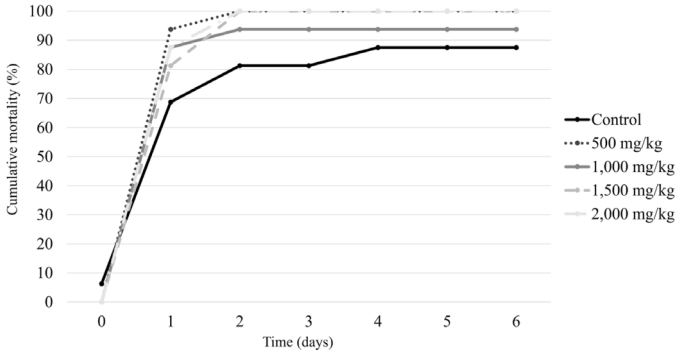

Figure 3. Cumulative mortality (%) of tambaqui *Colossoma macropomum* fed diet containing increasing levels of the thymol:carvacrol association and challenged experimentally with *Aeromonas jandaei*.

DISCUSSION

Products derived from medicinal plants correspond to one of the most researched additives in aquaculture today, being responsible for beneficial effects on fish, including promoting growth, increasing disease resistance, and improving meat quality (Pu et al., 2017). Considering the multiple benefits described in the literature for isolated compounds, there is a potential to improve the productive (Morselli et al., 2020) and reproductive (Alagawany et al. 2021) performances, increase the bioavailability of nutrients (Aanyu et al., 2018), improve immunity and general health, and reduce disease outbreaks (Morselli et al., 2020). These properties have been attributed, for example, to antioxidant, antimicrobial, and immunomodulating agents (Abd El-Hack et al., 2016). On the mechanism of action of carvacrol and thymol, they can prevent free radicals and hazardous compounds from interacting with cellular DNA and the ability to change the gut microflora, improving the digestion coefficient and absorption of nutrient compounds (Alagawany et al., 2015; 2021). The synergistic effect of thymol and carvacrol has already been proven by some authors who have noted better efficacy of the combination as larvicidal (Youssefi et al., 2019), antioxidant (Rathod et al., 2021), and antimicrobial (Heckler et al., 2021). However, this was not evident for tambaqui at the tested concentrations, showing that the compounds can interact in specific ways between different species of fish, and the responses can also be strongly influenced by the time of exposure and concentration of supplementation.

The effect of thymol and carvacrol isomers on fish performance is controversial. Aanyu et al. (2018) showed that thymol supplemented up to 500 ppm (500 mg/kg) did not improve the growth of tilapia. Mahboub and Tartor (2020) concluded that the use of carvacrol as a dietary additive significantly enhanced the growth performance and immunological parameters of tilapia. Morselli et al. (2020) revealed an increase in carp weight gain using thymol at concentrations of only 100 mg/kg. Kong et al. (2021) defined a thymol concentration of 356 mg/kg for the best performance of snakehead fish, and Silva et al. (2021) observed a better zootechnical performance of tambaqui fed 10 g/kg of carvacrol. The results of zootechnical performance in this study did not indicate differences between treatments, but the regression analysis revealed that there was a significant increase in the final weight of the fish as the level of supplementation with thymol:carvacrol increased. In our results, the zootechnical parameters tended to improve as supplementation increased, meaning there was a tendency to increase weight gain with feed consumption; however, only the use of higher concentrations of supplementation can provide more significant results. Recent research involving the use of carvacrol for tambaqui

showed that concentrations above 10 g/kg promoted benefits in the growth of the animal (Silva et al., 2021), which represents concentrations five times higher than those used in this study. However, the cost-benefit of high supplementation with isolated plant compounds deserves to be discussed in new studies, as it is known that the cost of feed is a key point for fish farmers, and supplementation with these additives may become impractical.

This study showed that a diet containing thymol:carvacrol interfered with the health of the host, altering cellular haematological parameters, since the levels of thrombocytes and lymphocytes were significantly lower after 30 days of the experiment. This indicates mild immunosuppression caused by the compound thymol:carvacrol. This can be explained by the fact that both compounds are recognised to cause decreased expression of essential stimulatory molecules (e.g., CD40 and CD86) and inhibition of dendritic cell maturation in mammalian studies (Amirghofran et al., 2016). In fact, several mammalian studies have revealed the anti-inflammatory effects of thymol and carvacrol (Pivetta et al., 2018; Chamanara et al., 2019; Sheorain et al., 2019). To the best of our knowledge, this is the first study in fish to demonstrate immunosuppression caused by thymol and carvacrol, and these compounds enter the list of potential anti-inflammatory drugs for aquatic animals, which still has an unusual applicability compared to medicine in mammals. In addition, the association tested showed an influence on the increase in the number of eosinophils. Cases of increased eosinophilia are associated with sensitivities to drugs or medications in general (Chauffaille, 2010), which may be an explanation for tambaqui. Studies on the sensitivity of aquatic animals to drugs are scarce and deserve more detailed investigation.

In this study, the inclusion of concentrations up to 2,000 mg/kg of the compounds in the diet could not exert significant changes in biochemical parameters, such as total proteins, albumin, and globulin, in the period of 30 and 60 days. The only change observed was a point increase in the ALT enzyme after 30 days in the group supplemented with the lowest concentration of thymol:carvacrol. The activity of enzymes, such as ALT and AST, is used to ascertain the metabolic response of the liver when the animal is exposed to pollutants or chemical molecules, such as drugs, and can indicate negative effects on liver tissue (Zadmajid and Mohammadi, 2017). When using additives in the diet, the values expressed by the activity of liver enzymes can be compromised; however, this study showed that the change was punctual for tambaqui, as the levels of the enzymes did not increase with the highest concentrations or with the time of greater exposure (60 days). Therefore, the tested concentrations did not reveal the potential for significant hepatotoxicity of the molecules.

Regarding the strong antiparasitic and antibacterial effects of thymol and carvacrol, which have been evidenced in numerous in vitro studies (Xu et al., 2008; Marchese et al., 2016; Kachur and Suntres, 2020), this study showed that administration as a feed supplement did not reproduce the same efficacy for tambaqui. However, the medicinal potential of these molecules cannot be ruled out when supplied and added to the water, where they can have a direct action on pathogens, which has already been proven against parasites (Hashimoto et al., 2016; Costa et al., 2017) and bacteria (Baldissera et al., 2017; Cunha et al., 2018).

In conclusion, the results of this study were consistent and similar to the responses of thymol and carvacrol already described some years ago for mammals and show the anti-inflammatory potential of the compounds, which deserves to be investigated in depth from a medical point of view. In addition, it differs from most recent studies in aquaculture that describe these compounds as potent growth promoters and immunostimulants that increase fish resistance to disease. Additionally, the use of such compounds in aquaculture deserves reservation due to the increase in the cost of feed.

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CONFLICT OF INTERESTS

Nothing to declare.

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AUTHORS' CONTRIBUTION

Frota, R.M.: Conceptualization, Data curation, Formal Analysis, Investigation, Writing – original draft, Writing – review & editing. Gallani, S.G.: Conceptualization, Data curation, Investigation, Methodology. Santos, P.D.P.: Conceptualization, Data curation, Formal Analysis, Investigation, Writing – review & editing. Pereira, C.S.: Data curation, Investigation, Methodology, Writing – review & editing. Oishi, C.A.: Conceptualization, Formal Analysis, Writing – review & editing. Gonçalves, L.U.: Conceptualization, Formal Analysis, Writing – review & editing. Valladão, G.M.R.: Conceptualization, Data curation, Formal Analysis, Investigation, Writing – original draft, Writing – review & editing.

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