

Hematological responses of tambaqui *Colossoma macropomum* (Serrassalmidae) fed with diets supplemented with essential oil from *Mentha piperita* (Lamiaceae) and challenged with *Aeromonas hydrophila*

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ABSTRACT

In fish farmings, diseases can be reduced by using immunostimulants. The aim of this study was to evaluate the immunostimulant potential of *Mentha piperita* in tambaqui fed with 0, 0.5, 1.0 and 1.5% of oil per kg of commercial fish feed. The fish were inoculated with *Aeromonas hydrophila* to challenge them. Hematological and biochemical parameters were determined after 30 days of feeding and seven days after the challenge. There was no mortality and *M. piperita* oil did not influence fish production parameters. However, blood hemoglobin concentration (Hb) increased in the fish fed with 0.5 and 1.5% of oil per kg of diet; albumin increased in those fed with 1.0%; cholesterol increased in all groups with oil; and triglycerides increased in those fed with 0.5%. After the bacterial challenge, the fish showed decreases in Hb when fed with diet enriched with 1.5% oil per kg of diet, in mean corpuscular volume with 1.0% and in mean corpuscular hemoglobin concentration with 0 and 1.5%. Protein levels increased in groups with 0 and 1.5% of oil and albumin when fed with 0 and 1.0%; cholesterol levels increased in the control group; and high levels of triglycerides were observed in the groups with 0, 0.5 and 1.5%. Thus, *M. piperita* essential oil promoted hematological alterations in tambaqui and can be recommended in diets containing up to 1.0% per kg, because of the minimal physiological modifications caused. However, additional studies are necessary to obtain more information regarding to the physiological effects of this immunostimulant.

KEYWORDS: Immune system; natural product; bacterial challenge; biochemistry.

Respostas hematológicas de tambaqui *Colossoma macropomum* (Serrassalmidae) alimentado com dietas suplementadas com óleo essencial de *Mentha piperita* (Lamiaceae) e desafiados com *Aeromonas hydrophila*

RESUMO

Em pisciculturas, doenças podem ser minimizadas com o uso de imunoestimulantes. O objetivo deste estudo foi avaliar o potencial imunoestimulante do óleo essencial de *Mentha piperita* na dieta de tambaqui com dietas contendo 0; 0,5; 1,0; 1,5% de inclusão do óleo por kg de ração comercial para peixes. Os tambaquês foram desafiados com a bactéria *Aeromonas hydrophila*. Hematologia e bioquímica dos tambaquês foram determinados após 30 dias de alimentação e sete dias do desafio. Não houve mortalidade dos peixes durante o experimento, sendo que o óleo não influenciou os parâmetros produtivos dos peixes. Entretanto, a concentração de hemoglobina (Hb) aumentou em peixes alimentados com 0,5 e 1,5% de óleo por kg de dieta; a albumina aumentou em 1,0%; o colesterol aumentou em todos os grupos com óleo; e triglicérides em 0,5% por kg de dieta. Após desafio bacteriano ocorreram diminuições da Hb nos peixes alimentados com 1,5% de óleo; do volume corpuscular médio com 1,0%; e da concentração de hemoglobina corpuscular média com 0 e 1,5%. Níveis aumentados de proteína nos grupos 0 e 1,5%; da albumina em 0 e 1,0%; do colesterol no controle; e dos triglicérides em 0, 0,5 e 1,5% de óleo na dieta. Assim o óleo essencial de *M. piperita* na dieta promoveu alterações hematológicas e pode ser recomendado seu uso em inclusões de até 1,0% por kg da dieta de tambaqui, devido às mínimas alterações fisiológicas causadas. Entretanto, estudos adicionais são necessários, de modo a obter informações sobre os efeitos fisiológicos deste imunoestimulante.

PALAVRAS-CHAVE: Sistema imune; produto natural; desafio bacteriano; bioquímica.

INTRODUCTION

Use of antibiotics and chemotherapeutics for treatment and prophylaxis in aquaculture can have many negative impacts, and this has led to a search for alternative products, such as probiotics and immunostimulants from plants (Logambal *et al.* 2000; Biller-Takahashi and Urbinati 2014; Mehana *et al.* 2015). Evaluation of the use of phytochemicals in aquaculture is a relatively new research area, but this has been generating promising results (Anderson 1992; Citarasu 2010; Biller-Takahashi and Urbinati 2014).

In *Lates calcarifer*, *Allium sativum* causes hematological changes and improves both oxygen transport and the immune response (Talpur and Ikhwanuddin 2012); *Ocimum basilicum* (basil) increases the resistance of *Cyprinus carpio* against infection by *Aeromonas hydrophila* (Amirkhani and Firouzbaksh 2015); and diets containing *Mangifera indica* (mango) kernel increase the resistance of *L. rohita* (Sahu *et al.* 2007). Astaxanthin, a carotenoid pigment produced by some algae, and bacteria, was used at concentrations of 50 and 100 mg kg⁻¹ in the diet of common carp *C. carpio* and, was found to stimulate growth, hematological alterations, immune response and specific bacterial disease protection (Jagruthi *et al.* 2014).

Aeromonas hydrophila is among the pathogenic bacteria that have been correlated with high levels of fish mortality, on fish farms throughout the world over the last decade, thus resulting in huge economic losses (Monette *et al.* 2006). This bacterium is frequently associated with hemorrhagic septicemia, fin erosion and abdominal cavity distension, among other pathological conditions (Paniagua *et al.* 1990; Joseph and Carnahan 1994; Silva *et al.* 2012). The use of antibiotics as a preventive measure has been questioned (Kashhedikar and Chhabra 2010), because this alters the intestinal microbiota and induces bacterial resistance through nonspecific defense mechanisms (Sakai 1999). In this manner, immunostimulants have shown beneficial effects for fish farms, not only as an alternative for disease management, but also for promoting growth and achieving improvements of both specific and the nonspecific the immune system (Harikrishnan and Balasundaram 2008; Harikrishnan *et al.* 2009 a,b; Mehana *et al.* 2015).

The tambaqui is a native fish species of the Amazon region and is farmed in many regions of Brazil. It successfully adapts to captive farming, because of good feed conversion, good growth, easy handling and good availability of juveniles (Val *et al.* 2000; Izel and Melo 2004; Tavares-Dias 2011).

Thus, the present study aimed to evaluate the hematological responses of tambaqui after being fed with a diet that included essential oil from *M. piperita*, as an immunostimulant, and after application of a bacterial challenge protocol using *A. hydrophila*, in order to evaluate the immunocompetence and

disease resistance of this fish species, consequent to the use of this immunostimulant in its diet.

MATERIALS AND METHODS

This experiment was authorized by Embrapa (proc. no. 02001.002877/2014-64) and IBAMA (no. 002/2008), in accordance with current Brazilian legislation. Tambaqui specimens were acquired from local fish farms at an average weight of 36.0 ± 7.7 g and were transported to Embrapa Amapá, in Macapá (AP). They were randomly distributed to 12 water tanks (20 fish per tank) with a capacity of 500 L. These tanks were connected to a biological filter, with continuous water flow and constant aeration, so as to maintain constancy of physicochemical variables: dissolved oxygen (DO) 7.32 ± 0.58 mg L⁻¹ (HI 9146, HANNA, Woonsocket, RI, USA); pH 7.46 ± 0.46 (Ecosense pH 100, YSI, China); temperature 27.78 ± 0.52 °C (Ecosense pH 100, YSI, China); ammonia 0.4 ± 0.1 mg L⁻¹ (HI 96715C, HANNA, Woonsocket, USA, RI.); alkalinity 26.43 ± 14.3 mg CaCO₃ L⁻¹ (Acqua Supre, Acqua Análises, Jundiaí, SP, Brazil); and hardness 16.67 ± 8.56 mg CaCO₃ L⁻¹ (Acqua Supre, Acqua Análises, Jundiaí, SP, Brazil). The fish were kept under laboratory conditions for 20 days and were fed with commercial fish food containing 32% crude protein (CP). Tank cleaning was performed every three days to remove excess food and feces.

Four diets were tested: 0% or control, without inclusion of the oil; 0.5%: inclusion of 0.5%; 1.0%: inclusion of 1.0%; and 1.5%: inclusion of 1.5% of the essential oil from *M. piperita* per kg of commercial fish food. The treatments were performed in triplicate, such that the fish were fed until apparent satiety, twice daily (8:00 and 16:00 hours). The *M. piperita* essential oil samples were obtained from the Medicinal Plant Laboratory, Embrapa Western Amazon (Manaus, AM). The *M. piperita* essential oil presented the following chemical components: alpha-pinene (1.1%), beta-pinene (1.9%), myrcene (1.1%), *p*-cymene (4.6%), limonene (3.1%), menthone (18.2%), iso-menthone (13.4%), menthol (30.8%), 4-terpineol (1.5%), pulegone (13.7%) and menthyl acetate (9.7%). To incorporate the essential oil into the diet, solutions for each level of inclusion were prepared using cereal alcohol (Dairiki *et al.* 2013), adding *M. piperita* essential oil at the necessary quantity. These solutions were sprayed manually onto commercial fish food (32% CP and 4 mm ration size) to be incorporated into it. The food was then dried at room temperature for 24 hours before its use in feeding the fish.

After the 30-day feeding period, blood collection was performed by caudal vein puncture in fifteen fish from each treatment, using sodium heparin anticoagulant, after they had previously been anesthetized using 0.1% benzocaine (Sigma-Aldrich, St. Louis, MO, USA). The blood samples collected were kept on ice to obtain the hematocrit (Ht),

hemoglobin concentration (Hb) and erythrocytes count (Er); and to calculate Wintrobe's hematimetric index: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), in accordance with Ranzani-Paiva *et al.* (2013). The remaining blood was centrifuged at 13.709 g for 5 min (Centrifuge 5424, Eppendorf, Hamburg, Germany). The plasma samples thus obtained were used to determine the concentrations of glucose, protein, albumin, cholesterol and triglycerides using colorimetric kits (Bio Tecnica®, Varginha, MG, Brazil), and absorbance readings were performed using a spectrophotometer (Biospectro SP-220; Curitiba, PR, Brazil).

After blood sample collection, the remaining tambaqui specimens were infected with *A. hydrophila* (ATCC 7966) (Seshadri *et al.* 2006). For this, the fish were intraperitoneally inoculated with *A. hydrophila* at a concentration of 1.5×10^8 UFC mL⁻¹. For 15 days, these fish were observed with regard to mortality and clinical signs of occurrences of aeromonosis, such as hemorrhagic petechiae, abdominal cavity distension, ulcerative injuries on the body surface, fin hemorrhage, fish isolated from the shoal and blackish coloring, among others. Seven days after inoculation of the bacterium, blood collection was performed on nine fish per treatment.

The results obtained from the hematological and biochemical evaluations on the fish, after they had been fed with diets containing different levels of *M. piperita* essential oil (0, 0.5, 1.0 and 1.5%), and after bacterial challenge, were statistically compared using parametric and nonparametric tests to find out which groups were different between each other ($P < 0.05$), after applying the normality test (Zar 2010).

RESULTS

The bacterial challenge with *A. hydrophila* at a concentration of 1.5×10^8 UFC mL⁻¹, performed on tambaqui of the present study, did not produce any clinical signs of aeromonosis (from viewing the external appearance of the fish) and there were no deaths.

The concentrations of essential oil from *M. piperita* that were tested in the diets, in the present study, did not influence the fish production performance parameters, since there were no significant differences in weight gain, although there was a tendency towards reduction in the group fed with 1.0% of oil in the diet (Table 1).

The hemoglobin concentration in tambaquis fed with diets containing 0.5 and 1.5% of *M. piperita* essential oil increased significantly in relation to the other diets. After experimental bacterial infection with *A. hydrophila*, a decrease in Hb was observed among the fish fed with 1.5% of oil in the diet ($P < 0.05$). The inclusion of the essential oil did not change the MCV of the tambaquis, but the MCV decreased after the bacterial challenge

in the group fed with the diet that included 1.0% of *M. piperita* oil. Feeding with *M. piperita* oil did not alter the MCHC values, but after the bacterial challenge with *A. hydrophila*, these values decreased in the control fish group (0%) and the group with 1.5% of oil in the diet (Table 2).

The plasma glucose concentrations in the tambaquis fed with diets containing *M. piperita* oil did not show any alterations, even after the bacterial challenge. The plasma protein concentration in the tambaquis only showed an alteration after the bacterial challenge, with increased values in the groups with 0 and 1.5% of the essential oil in the diet. Increased levels of albumin were found in the fish fed with diets containing 0 and 1.0% of the oil after the bacterial challenge.

The plasma cholesterol levels increased in all the fish fed with diets containing essential oil (from 0.5 to 1.5% inclusion per kg of diet). After the bacterial challenge, only the control group presented a significant increase in cholesterol levels. The plasma triglyceride values were lowest in the fish fed with diets enriched with 0.5% of essential oil. However, after the challenge with *A. hydrophila*, the fish fed with 0, 0.5 and 1.5% of oil in the diet presented significantly increased values.

DISCUSSION

The inclusion levels of 0.5 to 1.5% of *M. piperita* essential oil used in the diet for the tambaquis did not promote weight gain. This was contrary to the study by Talpur (2014), who reported body weight gain in *L. calcarifer*, with levels of 2 to 5 g of *M. piperita* leaves per kg of food. Moreover, this author reported that there was a good feed conversion rate with higher amounts of *M. piperita* in the diet, coming from this plant's characteristic of stimulating fish appetite. According to Sharafi *et al.* (2010), *M. piperita* essential oil presents very important antioxidant properties and is appropriate for use in food, cosmetics and medicine. Thus, it has been found that the form of use of the plant, i.e. its leaves (Talpur 2014) or its essential oil (Sharafi *et al.* 2010; present study), can influence the acceptance of the diet by fish and the action of its bioactive components.

The coefficient of variation among the tambaquis after feeding on *M. piperita* essential oil was higher (57%) when fed with diets containing 0.5% of the essential oil, in relation to the other groups. The coefficient of variation was 29% for fish fed without essential oil; 39% for fish fed with 1.0% of oil in the diet; and 40% in fish fed with 1.5% oil in the diet. This may indicate that the acceptance of diets with inclusion of this oil was not good among the fish, especially among those fed with diets containing 0.5% of oil in the diet. The fish that may have accepted the diet best were possibly those fed with 1.0% of the oil, because the FCR was 1.66 (Table 1). However, in terms of performance, the fish with the best FCR were those that received the diet without essential oil.

Table 1. Initial and final weight, weight gain (mean ± standard deviation) and feed conversion rate (FCR) of tambaqui groups fed with diets containing 0, 0.5, 1.0 and 1.5% of *Mentha piperita* essential oil per kg of diet for 30 days.

	Inclusion levels of <i>M. piperita</i> essential oil			
	0%	0.5%	1.0%	1.5%
Initial weight (g)	20.35 ± 4.84	20.74 ± 4.64	20.19 ± 4.74	20.10 ± 3.33
Final weight (g)	75.81 ± 22.39	63.56 ± 36.09	59.13 ± 23.03	68.74 ± 27.74
Weight gain (g)	58.15 ± 19.21	44.20 ± 34.54	38.94 ± 22.69	48.64 ± 28.36
FCR	1.07	1.42	1.66	1.28

There were no statistical differences ($P > 0.05$).

Table 2. Hematocrit (Ht), hemoglobin concentration (Hb), erythrocytes count (Er) and hematocrit indices (mean corpuscular volume, MCV; mean corpuscular hemoglobin, MCH and mean corpuscular hemoglobin concentration, MCHC) (mean ± standard deviation) of tambaqui groups fed with diets containing 0, 0.5, 1.0 and 1.5% of *Mentha piperita* essential oil per kg of diet for 30 days, before *Aeromonas hydrophila* challenge and seven days afterwards.

	Inclusion levels of <i>M. piperita</i> essential oil			
	0%	0.5%	1.0%	1.5%
Before bacterial challenge				
Ht (%)	17.54 ± 3.73a	19.75 ± 3.36a	18.20 ± 4.16a	19.65 ± 2.22a
Hb (g dL ⁻¹)	5.71 ± 1.27b	6.83 ± 0.98a	6.30 ± 1.32b	7.76 ± 0.62a
Er (x10 ⁶ μL ⁻¹)	1.04 ± 0.34a	0.93 ± 0.25a	0.94 ± 0.35a	1.04 ± 0.34a
MCV (fL)	181.09 ± 62.41a	192.20 ± 58.43a	169.25 ± 41.52a	181.09 ± 62.41a
MCH (g dL ⁻¹)	70.33 ± 14.37a	65.97 ± 15.98a	67.24 ± 10.74a	65.63 ± 10.29a
MCHC (g dL ⁻¹)	42.46 ± 16.76a	34.33 ± 8.33a	39.29 ± 12.16a	42.93 ± 13.68a
After bacterial challenge				
Ht (%)	20.05 ± 2.97a	19.94 ± 3.08a	16.28 ± 4.53a	18.50 ± 3.31a
Hb (g dL ⁻¹)	5.29 ± 2.26a	5.94 ± 1.64a	4.60 ± 2.21a	4.29 ± 1.79a*
Er (x10 ⁶ μL ⁻¹)	0.66 ± 0.18a	0.71 ± 0.22a	0.89 ± 0.37a	1.21 ± 0.24a
MCV (fL)	271.90 ± 176.32a	248.37 ± 139.73a	107.85 ± 38.73a*	187.59 ± 106.33a
MCH (g dL ⁻¹)	95.82 ± 27.79a	75.59 ± 22.23ab	51.26 ± 10.65b	60.71 ± 14.19b
MCHC (g dL ⁻¹)	35.72 ± 16.37a*	35.95 ± 18.66a	46.70 ± 18.03a	36.32 ± 18.80a*

Small different letters in the same row indicate statistically significant differences ($P < 0.05$) among essential oil inclusion levels; asterisk (*) indicates statistically significant differences ($P < 0.05$) between values before and after bacterial challenge. fL means femtoliter, i.e. a volume measurement unit equivalent to 10⁻¹⁵ liter or to 1 μm³.

Table 3. Glucose, protein, albumin, cholesterol and triglycerides plasma levels (mean ± standard deviation) of tambaqui groups fed with diets containing 0, 0.5, 1.0 and 1.5% of *Mentha piperita* essential oil per kg of diet for 30 days, before *Aeromonas hydrophila* challenge and seven days afterwards.

	Inclusion levels of <i>M. piperita</i> essential oil			
	0%	0.5%	1.0%	1.5%
Before bacterial challenge				
Glucose (mg dL ⁻¹)	79.25 ± 22.50a	87.85 ± 24.93a	85.48 ± 20.80a	76.49 ± 18.02a
Protein (g dL ⁻¹)	3.07 ± 0.59a	2.81 ± 0.81a	2.68 ± 0.54a	2.50 ± 0.45a
Albumin (g dL ⁻¹)	0.94 ± 0.23a	0.89 ± 0.17a	0.95 ± 0.17a	1.00 ± 0.23a
Cholesterol (mg dL ⁻¹)	62.85 ± 9.83b	83.78 ± 16.71a	86.64 ± 39.92a	98.93 ± 18.61a
Triglycerides (mg dL ⁻¹)	272.96 ± 29.93a	224.76 ± 54.30b	271.49 ± 60.49a	286.17 ± 45.21a
After bacterial challenge				
Glucose (mg dL ⁻¹)	79.50 ± 7.21a	76.81 ± 20.55a	74.61 ± 16.74a	81.89 ± 10.92a
Protein (g dL ⁻¹)	3.44 ± 0.73a*	3.14 ± 0.42b	3.30 ± 0.25a	3.23 ± 0.36a*
Albumin (g dL ⁻¹)	1.09 ± 0.10b*	0.98 ± 0.20b	1.48 ± 0.14a*	0.97 ± 0.12b
Cholesterol (mg dL ⁻¹)	137.37 ± 11.80a*	127.58 ± 48.54a	114.65 ± 9.28a	118.18 ± 19.39a
Triglycerides (mg dL ⁻¹)	472.57 ± 32.78a*	290.12 ± 32.60b*	306.28 ± 63.60b	422.15 ± 38.47a*

Small different letters in the same row indicate statistically significant differences ($P < 0.05$) among essential oil inclusion levels; asterisk (*) indicates statistically significant differences ($P < 0.05$) between values before and after bacterial challenge.

The tambaquis of the present study did not die during the experimental period and did not show any clinical signs of disease. According to Amirkhani and Firouzbaksh (2015), diets supplemented with ethanol extract of *O. basilicum* increased the resistance of *C. carpio* against infection by *A. hydrophila*, due to improvement of the nonspecific immune system. This was also observed in *L. rohita* fed with diets containing *M. indica* and challenged with *A. hydrophila* (Sahu *et al.* 2007). Ingestion of essential oil, such as from *M. piperita*, through diets, can be used as an immunostimulant, as reported for several species of plants and animals (Bairwa *et al.* 2012; Biller-Takahashi and Urbinati 2014; Mehana *et al.* 2015).

The increased hemoglobin concentration in the tambaquis fed with diets containing 0.5 and 1.5% of *M. piperita* essential oil could be related to an increase in oxygen supply, which promotes fish welfare. Similar results were found for *L. calcarifer* fed with diets containing *A. sativum* (Talpur and Ikhwanuddin 2012) and leaves of *M. piperita* (Talpur 2014). With the experimental bacterial infection, there was a decrease in Hb in the tambaqui fed with 1.5% of oil in the diet, due to the stress induced by the pathogen, as reported by Talpur and Ikhwanuddin (2012) in *L. calcarifer*. The Hb and MCHC of the tambaquis presented significant reductions after the challenge with *A. hydrophila*, consequently decreasing the O₂ carrying capacity of the fish (Vijayan *et al.* 1997). In this study, it was also possible to observe a decrease in MCV after the bacterial challenge, among the fish fed with diets enriched with 1.0% of *M. piperita* essential oil, probably due to the tendency towards increased number of erythrocytes in the blood with the diet included 1.0% of the essential oil from *M. piperita*.

Feeding tambaquis with diets containing *M. piperita* did not cause stress in the fish, as indicated by the maintenance of plasma glucose at levels similar to those of the control group, even after application of the bacterial challenge with *A. hydrophila*. According to Talpur (2014), the inclusion of *M. piperita* stimulated insulin action, and therefore reduced glucose levels were also observed in *L. calcarifer* after feeding. Moreover, after the bacterial challenge, the glucose reduction could be attributed to fasting, consequent to the presence of the pathogen. Only after the bacterial challenge did the plasma protein concentration increase in the fish of the control group and in the fish fed with the diet containing 1.5% of *M. piperita* essential oil. Feeding fish with garlic (*A. sativum*), ginger (*Zingiber officinale*) and neem (*Azadirachta indica*) (Talpur and Ikhwanuddin 2012; 2013 a,b) increased protein concentration in *L. calcarifer*; and this was also reported to *C. carpio* fed with *M. piperita* extract (Abasali and Mohamad 2010). However, in tambaquis fed with *M. piperita* oil, plasma protein levels remained constant. Thus, exposure of the fish to stressful situations, such as the presence of pathogens,

can result in a series of adjustments through homeostasis mechanisms (Almeida-Val *et al.* 2006).

Increased albumin levels in tambaqui plasma were observed only after the bacterial challenge in the fish fed with 0 and 1.0% inclusion of oil in the diet. Since plasma proteins include several humoral elements of the nonspecific immune system, this increase in albumin at the inclusion level of 1.0% could reflect the increase in the nonspecific immune response of tambaqui. Sahu *et al.* (2007) reported similar results, since the protein levels (albumin and globulin levels) in young *L. rohita* fed with *M. indica* were greater than the levels of the control group.

The plasma cholesterol levels increased in all the tambaquis fed with *M. piperita* essential oil. After the bacterial challenge with *A. hydrophila*, there was only an increase in the control fish, which could indicate that the immunostimulant used may have provided a protective effect for the hepatic tissue of these fish, thus not causing serious dyslipidemia. The plasma triglyceride level decreased significantly in the tambaqui fed with 0.5% of *M. piperita* oil in the diet. After the bacterial challenge, the highest triglycerides levels were found in the fish fed with 0.5 and 1.5% of *M. piperita* oil. Unlike the present study, *L. calcarifer* fed with diets containing *M. piperita* showed reductions of total lipids, triglycerides and cholesterol (Talpur 2014).

CONCLUSIONS

The hematological and biochemical responses of tambaqui fed with diets supplemented with essential oil from *M. piperita* indicated that the oil had positive effects, due to plasma protein increases. The use of this essential oil can be recommended in diets for tambaquis at levels of up to 1.0% per kg of food for 30 days, because of the minimal physiological modifications that are caused. Nonetheless, additional studies using essential oil from *M. piperita* in diets for tambaquis over different feeding periods are necessary, in order to obtain information regarding to the physiological effects of this immunostimulant on these fish.

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