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Histopathological analysis of *Colossoma macropomum* liver, treated with bioproducts used by controlling parasites

Análisis histopatológico del hígado de *Colossoma macropomum*, tratado con bioproductos empleados en el control de parásitos

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Abstract

Possible histological changes in the liver of *Colossoma macropomum* Cuvier, 1816 (tambaqui) generated by the use of products incorporated into the diet as a control method of *Neoechinorhynchus buttnerae* Golvan, 1956 were evaluated. 1036 juveniles tambaqui with 77±3.0g of average weight were used, maintained in 28 polyethylene tanks of 1000L, with constant aeration and water recirculation system, using UV filter. The experimental units (30 fish per experimental unit) were distributed into four treatments, with seven replicates. The products, incorporated in fish oil, were added to the feed, according to the treatments: control group with commercial feed (Nutrizon Karino 36% GW) (A); extract of garlic (B); organic acids (C) and extract of garlic + organic acids (D). A parasitic and histopathological evaluation was performed at time zero (T0). During the experiment, two samples were collected, with an interval of five weeks, evaluating 15 fish per experimental unit. At the end, 63 liver samples for histological analysis were collected. The evaluated animals presented the expected growth for the species. 87.2% of efficacy was obtained with the association extract of garlic + organic acids. Hepatic morphological lesions compatible with toxicological processes were not observed, indicating a low toxicity for tested concentrations.

Resumen

Se realizaron análisis histopatológicos del hígado de *Colossoma macropomum* Cuvier, 1816 (tambaqui), tratado con productos incorporados a la dieta para el control de *Neoechinorhynchus buttnerae* Golvan, 1956. Se mantuvieron 1036 juveniles de tambaquis con un peso promedio de 77±3.0g en 28 tanques de polietileno de 1000L, con aireación constante asociada a la recirculación de agua, utilizando un filtro UV. Estos se dividieron en cuatro tratamientos, con siete repeticiones cada uno, correspondientes a: (A) grupo control (solo alimento comercial Nutrizon Karino 36% GW); (B) alimento comercial adicionado con extracto de ajo; (C) Alimento comercial, adicionado con ácidos orgánicos; y (D) alimento comercial, adicionado con extracto de ajo + ácidos orgánicos. Se realizó una evaluación parasitaria e histopatológica en el tiempo cero (T0). Durante el experimento, se colectaron dos muestras con un intervalo de cinco semanas, evaluándose 15 peces por unidad experimental. Al final, se recolectaron 63 muestras de hígado para análisis histológico. Los animales evaluados mostraron el crecimiento esperado para la especie. Se obtuvo un 87.2% de efectividad con la combinación extracto de ajo + ácidos orgánicos. No se observaron lesiones morfológicas hepáticas compatibles con procesos toxicológicos, lo que indica baja toxicidad a las concentraciones evaluadas.

Introduction

Brazilian fish farming maintained its growth at 4.9% in 2019, with production of around 758000 tons [1]. In this context, the emergence of health problems increases, mainly with the intensification of production systems. Factors such as environmental imbalance (for example, inadequate management practices and changes in abiotic factors) that predispose to the emergence of parasitic infestations, as well as bacterial, fungal and viral infections, since the aquatic environment is highly favorable to spread and disease development [2,3]. The *Colossoma macropomum* Cuvier, 1816 is a *'piracema'* fish native to the Amazonas and Orinoco basins, widely distributed in the tropical part of South America and Central Amazon [4] This is due, in particular, to the fact that parasites act as limiting productivity, causing delay in fish growth and high mortality rates, leading to economic damage [5-7]. The diseases origin that affect cultivated fish are many, but the infestation carried out by *Acanthocephala helminthes (Neoechinorhynchus buttnerae*), has attracted the attention of fish farmers due to the high infestations recorded in the northern region of Brazil. In the state of Rondônia, *N. buttnerae* is also present in tambaqui from fish farms in the Jamari Valley region, encompassing nine municipalities that represent the main productive pole of the state [8].

Products such as formalin, copper sulphate, chloramine T, organophosphorus pesticides and diflubenzuron have been constantly used in the fight against fish parasites, as well as antibiotics, such as oxytetracycline, in the treatment of bacterial infections. These products, in addition to a toxic effect on fish tissues, especially for the gills, integument and liver, may accumulate residues in the musculature [9], and represent risks to the food safety of consumers [10,11]. In addition, they may provide toxic injury to fish and deleterious effects on the aquatic ecosystem [12], therefore, excessive use of chemotherapeutic agents in aquaculture, can negatively affect animal and human health, as well as the aquatic environment, and should be better evaluated and regulated [13].

Another alternative has been the increasing use of alternative products such as plant extracts, which can drastically decrease the use of chemotherapeutic and antimicrobial agents in fish farming [14]; eradicate parasites and minimize negative impacts on the environment, to prevent the emergence of species of parasites and bacteria resistant to the commonly used products. Likewise, the use of organic acids in diets for animals of zootechnical interest, has received special attention in the agricultural sector.

The aim of the present research was to evaluate possible histological changes in the liver of *C. macropomum* generated by the use of products incorporated into the diet as a method of control of *N. buttnerae*, in order to obtain alternatives for the development of an economical and sustainable aquaculture.

Methods

Study area and experimental conditions

The assay has been approved by the Ethics Committee for Animals Use of the Federal University of Rondônia UNIR, Brazil (Protocol number 035/16), and executed at the Experimental Animal Health Center (CESA) and at the Laboratory of Environmental Sciences, belonging to the UNIR, campus Presidente Médici, Brazil (latitude 11° 10' 18" S, longitude 61° 54' 12" W, elevation 191 m.a.s.l). There was used 28 polyethylene tanks with 1000 L capacity each. The source of water for supply comes from an artesian underground reservoir, supplying directly to the piping of the CESA system.

The tanks have independent water inlet, outlet, and constant aeration system. All water drained from these, passes through a water recirculation system, by using an ultra violet (UV) filter. Water quality parameters such as temperature (ºC) and dissolved oxygen (DO), were daily checked in the morning and late afternoon with Yellow Spring Instrument (YSI™), digital multiparameter equipment. Values of pH, alkalinity, hardness and ammonia were weekly measured by using colorimetric kits (Alfakit®, Florianópolis, Brazil).

Experimental design

A total of 1036 tambaqui juveniles with 77±3.0g of average weight, were obtained from a commercial fish farm located at the municipality of Ariquemes, Rondônia State, Brazil; naturally infested with *N. buttnerae*. The presence of parasites was identified by the owner of the fish farm and 100% infestation was confirmed by the evaluation of a 10% sampling (T0) of the purchased animals. These fishs were transported to the CESA in a transport box (Transfish®) with oxygenation system. The acclimatization to the system and feeding regime, occurred in water tanks for 43 days. The fish were fed twice a day with approximately 2.0% of body weight.day^{-1}. During the adaptation period, the amount of feed daily consumed was 60g per experimental unit.day^{-1}. Subsequently, in T0, all fish were weighed (g) on a digital scale and measured for standard length (SL) and total length (TL). Then, they were randomly distributed in a density of 30 fish per box, with four treatments (A, B, C and D) and seven replicates, totaling 840 fish. Fish were submitted to food deprivation for gastrointestinal emptying, 24 hours before the beginning of the experiment, which lasted for 85 days, starting the experimental feed on April 20 and ending on July 13, 2017. The control group (A) received Nutrizon Karino® commercial feed (GW = 36%, grain size 3 to 4 mm, 2x/day), while the other groups received the same commercial feed, plus extract of garlic (B), organic acids (C), extract of garlic + organic acids (D), in the proportion of 200g.ton^{-1}. The products were incorporated into fish oil by using a plastic container. Subsequently, added to commercial feed with a hand sprayer and mixed in a concrete mixer, checking out a good product aggregation. To determine the initial parasitic and histopathology evaluation, 84 fish were analyzed on T0. From these, the liver of seven specimens was removed for histological procedure. The others collections were made in T45 (I-C) an T85 (II-C). At each collection, 15 fish per experimental unit passed through desensitization by using a sharp object, weighing and measurement. Thereafter, a longitudinal incision was made on the median-ventral line of the pectoral fins to the anus for evisceration of the animal. Hence, only the liver of one fish per experimental unit was removed for histopathological analysis, fixed in 10% formalin for 48 hours, and later conserved in alcohol 70%, making up 28 samples per collection.

Statistical analyses

After identification and quantification of parasites, their mean number and prevalence for each treatment were established, according to Bush et al. [14], in addition to determining the percentage of efficacy [15]. The results were submitted to ANOVA. The means of the number of parasites found, were compared by the Tukey test. The statistical program used was SAS® version 2001, with a confidence level of 95%.

Histological procedures

At the end of the experiment, 63 liver samples were collected (seven referring to T0 collection and 56 to I-C and II-C), for histological analysis, at the Veterinary Pathology and Parasitology Laboratory of the Federal University of Goiás, campus Jataí, Goiás State, Brazil. Samples were routinely processed by increasing alcohol dehydration, diaphanization in xylol and paraffin inclusion. 5µm thick sections obtained by microtome, which were stained with HE-hematoxylin-eosin-according to Junqueira and Carneiro [15]; and subsequently, analyzed and photographed in optical microscopy (Nikkon™, E200) coupled with Moticam™ 3.0 MP camera. Two slides of each sample past to analyze, with the aim of identify and characterize possible lesions, with magnifications of 200x and 400x.

Results

The water temperature during the experiment ranged from 22°C to 35°C, dissolved oxygen, from 5 to 8 mg.L^{-1}, pH, between 7 and 8, alkalinity, between 39 and 63 mg.L^{-1}, hardness between 12 and 45 mg.L^{-1} and ammonia did not exceed 0.6 mg.L^{-1}. The weight (W), total length (TL) and standard length (PL) variables, analyzed during the experiment, were not influenced ($P< 0.05$) by the use of the products incorporated into the diet (tables 1, 2). In the parasitic evaluation of the animals, only the *N. buttnerae* was present in almost the entire gastrointestinal tract, strongly fixed in the intestinal mucosa, many of which still alive, even after the use of the experimental ration. Table 1 shows the results obtained after five weeks of treatment (I-C). The animals presented the same corporal development, gaining in the total and standard lengths, and weight ($P<0.05$). Among treatments, the association between vegetable extract of garlic + organic acids (D) was the most effective (53.8%) of the treatments used in the parasites control.

In II-C (table 2), at 85 days of medicated feed supply, an even greater reduction in the number of parasites (1.23) of the fish fed with treatment D (extract of garlic + organic acids), reaching 87.2% of efficacy, when compared to control treatment. Followed by treatment B (extract of garlic), which showed efficacy in the parasites control of 85.7% with respect to the reference values obtained in the T0 collection. Anatomically, the liver presented a brownish red color, consisting of three hepatic lobes, being a right lateral lobe, a left lateral lobe and a ventral lobe. In all specimens, it was located ventral to the swim bladder and cranium-dorsally to the stomach. Microscopically, the hepatic architecture was full of ducts, sinusoids, capillaries and veins, which were

Table 1 Analysis of the *Colossoma macropomum* development, average parasites of *N. buttnerae* count and efficacy test after five weeks of treatment (I-C)

Variable	Time 0	Treatments A	Treatments B	Treatments C	Treatments D
Total length (cm)	16.6±1.1[a]	17.8±1.3[b]	17.9±1.2[b]	18.0±1.4[b]	18.2±1.4[b]
Standard length (cm)	13.2±1.0[a]	16.1±1.1[b]	16.2±1.1[b]	16.2±1.2[b]	16.4±1.3[b]
Weight (g)	77±3.0[a]	110.6±3.2[b]	110.5±2.8[b]	113.6±2.9[b]	118.5±2.6[b]
Parasites (%)	9.6[a]	5.28[b]	5.0[b]	4.8[b]	4.5[c]
Efficacy (%)	-	45.0[a]	48.6[b]	50.1[c]	53.8[d]

Different lowercase letters indicate significant difference at the 0.5% level (*P*<0.05).

Table 2 Analysis of the *Colossoma macropomum* development, mean parasites of *N. buttnerae* count and efficacy test after eighty-five days of treatment (II-C)

Variable	Time 0	Treatments A	Treatments B	Treatments C	Treatments D
Total length (cm)	16.6±1.2[a]	18.3±1.6[b]	18.7±1.5[b]	18.8±1.6[b]	18.5±1.4[b]
Standard length (cm)	13.2±1.0[a]	16.4±1.2[b]	16.8±1.3[b]	16.8±1.4[b]	16.8±1.4[b]
Weight (g)	77±3.0[a]	118.1±2.4[b]	128.7±2.8[b]	131.1±3.0[b]	122.0±3.2[b]
Parasites (%)	9.6[a]	1.96[b]	1.4[b]	2.5[b]	1.2[c]
Efficacy (%)	-	79.63[a]	85.7[a]	74.1[a]	87.2[b]

Different lowercase letters indicate significant difference at the 0.5% level (*P*<0.05).

Interspersed among polyhedral hepatocytes with central nuclei, strongly stained marginally and evident nucleoli. Associated with the hepatic parenchyma, areas of pancreatic tissue were randomly distributed, involving vessels of different calibers. In this context, hepatic morphological lesions compatible with toxicological processes were not observed (figure 1), indicating a low toxicity for tested concentrations.

Discussion

An increased number of studies point to the benefits of the natural products in treatment of animal (including fishes) diseases of different orders. The use of plant extracts for pharmacological purposes, tends to decrease production costs and the side effects of the synthetic substance application to the environment, since natural products tend to be more biodegradable. In addition, the presence of a high diversity of molecules with diverse mechanisms of action in a plant extract and its toxicity degree, may be another positive factor in relation to the use of natural substances [16,17]. Ocampo and Auró [18], reported that the aqueous extract of garlic at concentrations of 200 mg.L^{-1} presented 99% efficacy in the treatment of Acanthocephala in tilapia, while for onion, the concentration was of 2400 mg.L^{-1} during five days against *Pomphorhynchus laveis* and *Acantocephalus anguillae* in Mozambican tilapia. On the other hand, Adineh et al. Ndong and Fall [19], evaluated the effects of garlic incorporated into the diet (0%, 0.5% and 1%) of juveniles of hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) during four weeks. They observed improvement in the immune system, with increase of leukocytes, phagocytic activity, phagocytic index and lysosome activity of fish fed with 0.5% of garlic.

In a preliminary study in *C. macropomum* by our research group, DAAB (Agriculture and Livestock Development of Brazilian Amazon), the use of plant extract for only seven days reduced the average number of parasites, obtaining an efficacy of 37.6%. In the present study, also with tambaqui, we obtained an efficacy of 85.7%, when we using extract of garlic, 85 days after. Other natural products are the organic acids, which correspond to most of the acidifiers tested with commercial interests, because they have low potential for corrosion and toxicity when compared to inorganic acids [20], showing excellent results in several species of animals, mainly young and adults in the state of stress [21]. Some combinations of organic acids show advantages over their isolated use, since they resulted in better control of the pathogenic bacteria proliferation, suggesting a synergistic effect not yet explained, as demonstrated in the present study, when garlic + organic acids extract was associated, obtaining an

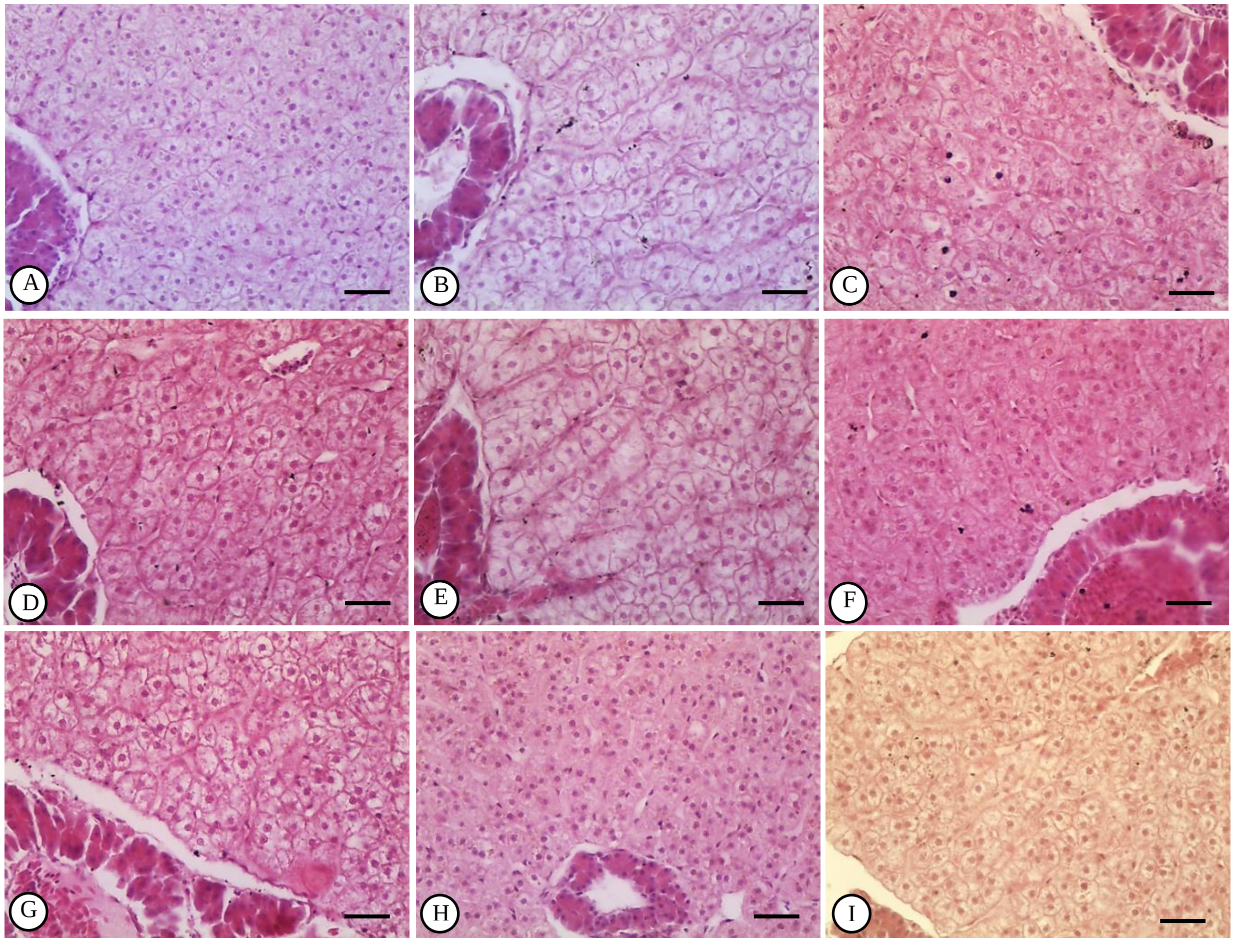

Figure 1 Microscopic images of *Colossoma macropomum* liver submitted to different types of treatment, demonstrating the absence of morphological changes compatible with intoxication process. **A)** Sample of T0; **B)** I-C sample, treatment A; **C)** I-C sample, treatment B; **D)** I-C sample, treatment C; **E)** I-C sample, treatment D; **F)** II-C sample, treatment A; **G)** II-C sample, treatment B; **H)** II-C sample, treatment C; **I)** II-C sample, treatment D. HE, 400x. Bar = 20µm.

efficacy of 85.7%, 85 days after. According to Lu et al. [22], studies with natural products in fish farms are necessary, as the market is increasingly demanding in relation to the quality and sustainability of the product. As a result, fish farmers have to adapt to the trend and new market parameters such as quality, safety, elimination of pollutants, concomitants, antibiotics and carcinogens during aquaculture activities, reducing the use of chemicals and antimicrobials that can harm the environment and compromise food safety and health. Presence of Acanthocephala is a problem in many Brazilian fish farms. It can present a prevalence of up to 100%, as in the case of a growing season in the municipality of Itacoatiara in the state of Amazonas [23], or in the central region of the state of Rondônia, Brazil where a prevalence of 45.7% of parasitism by *N. buttnerae* was found in *C. macropomum* [24]. Probably, the incidence of these parasites is due to problems arising from lack of sanitary management, not control of water quality, unbalanced ration and origin of the fingerlings.

Generally, this infestation is restricted to the portion of the anterior intestine; however, in high infestation rates, both the middle and the posterior intestine, are also affected [25], similarly to the observed in this study. Despite it, the animals evaluated did not suffer interference in their performance with the use of the products incorporated into the feed, maintaining their growth within the expected for the species. However, possibly due to the experimental conditions, they underwent and to the process of parasitic infestation, they were unable to achieve optimal growth for a suitable cropping system. By other side, hepatic tissue plays a vital role in metabolism such as synthesis, secretion, storage, biotransformation and metabolism [26]. Histopathological changes such as vacuolization of hepatocytes, glycogen depletion, and inflammation, alterations in the shape of sinusoid vessels and neoplasms in the liver of fish are efficient biomarkers that indicate the previous exposure of the animal to stressors present in the environment [27,28]. During this experimental development, liver color was also observed, by considering that it is an important growth factor, directly related to the amount of hepatic energy. In this study, hepatic morphological changes suggestive of lesions compatible with toxicological or pathological processes (figure 1) did not occur. This indicating that the tested concentrations of extract of garlic and organic acids, used in the animal feeding, were of low toxicity, but efficient in the purpose of their use, resulting in a high efficacy in controlling *N. buttnerae*.

Conclusion

The absence of hepatic morphological changes, in all treatments, indicates a high efficacy of extract of garlic and organic acids at tested concentrations, by controlling helminthes in *C. macropomum*. The results point to natural products as a sustainable alternative for micro, small and medium fish farmers, due to their low cost, associated to appropriate management practices for production, human health and animal welfare.

Consent for publication

The authors read and approved the final manuscript.

Competing interest

The authors declare no conflict of interest. This document only reflects their point of views and not that of the institution to which they belong.

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