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Citric acid minimizes oxidative stress in Amazonian fish (*Colossoma macropomum*) when fed plant protein-based diets

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ABSTRACT - This study aimed to evaluate the antioxidant potential of citric acid in plant protein based-diets offered to juvenile tambaqui, *Colossoma macropomum*. Four isonitrogenous and isoenergetic diets containing different levels of citric acid (0, 10, 20, and 30 g.kg^{-1}) were formulated. Fish (n = 160; 27.56±1.03 g) were randomly distributed in 310-L water tanks (n = 16), with four replicates per treatment. After the experimental period (80 days), animals were anesthetized, and blood was collected for cell count. They were euthanized and dissected to remove livers for subsequent oxidative stress analyses. No significant differences were observed in the levels of thrombocytes, total leukocytes, lymphocytes, positive granular leukocytes, and immature leukocytes among the experimental treatments. However, there were fewer monocytes and neutrophils in fish fed the diet containing 30 g.kg^{-1} of citric acid. All citric acid diets increased glutathione concentration and reduced lipid peroxidation levels in livers. Therefore, citric acid acts as a potent antioxidant in juvenile tambaqui fed plant protein-based diets and, thus, can improve their maintenance conditions in production systems.

Keywords: feed additive, fish, nutrition, organic acid

1. Introduction

Soybean meal corresponds to one of the best amino acid profiles compared with other plant sources (Hertrampf and Piedad-Pascual, 2000). However, the antinutritional factors of this ingredient may limit its use as a source of integral protein for fish (Francis et al., 2001; Antonopoulou et al., 2017). For example, high proportions of soybean meal in fish diets can inhibit growth, increase production of reactive oxygen species (ROS), and, consequently, affect oxidative homeostasis of fish (Chen et al., 2018; Zhang et al., 2016).

The imbalance between the generation and elimination of ROS induces oxidative stress, which damages important biomolecules (Nimse and Pal, 2015). Free radicals generated in fish tissues are effectively eliminated by the antioxidant system, which consists of enzymes and nonenzymatic agents (Dong et al., 2013). In addition, other substances such as glutathione and malondialdehyde are important biomarkers for assessing oxidative stress (Rosmini et al., 1996; Dong et al., 2013; Lin and Cheng, 2017). Although the production of reactive species is potentially toxic for the metabolism, it is important during the maximum activation of neutrophils and monocytes in the inflammatory phase (Ferreira and Matsubara, 1997; Rahal et al., 2014). Therefore, changes in antioxidant and defense capacity can be considered important indicators of the nutritional value of plant protein sources.

Some studies have reported how organic acids can mitigate oxidative stress in fish (Li et al., 2017; Zhang et al., 2016; Chen et al., 2018). Citric acid, for example, is widely used in the food and drug

industry for its buffering, anticoagulant, and antioxidant abilities (Su et al., 2014; Li et al., 2017). However, very little information is available about the use and action of citric acid in the antioxidant system of fish (Chen et al., 2018). This organic acid can act as a substrate in the intermediate metabolism and, when included in diets, can be directed to the Krebs cycle for emergency ATP synthesis under stressful conditions to thus improve the body's nonspecific immunity (Lehninger et al., 1993; Li et al., 2017). Therefore, including acid citric in diet can increase the activation of the defense system and, consequently, contribute to lower oxidative stress levels for farmed fish.

Tambaqui (*Colossoma macropomum*) is an omnivorous fish found in the Amazon and Orinoco River Basins and is one of the most farmed native freshwater species in Brazil (Gomes et al., 2010; Woynárovich and Van Anrooy, 2019). Although citric acid does not positively affect the performance of juvenile tambaqui, Nascimento et al. (2020) suggested that the use of this organic acid increases nutrient digestibility. However, other effects of citric acid on the health and welfare of fish need to be analyzed. Thus, the present study evaluated the antioxidant potential of citric acid when added to plant protein-based diets offered to juvenile tambaqui.

2. Material and Methods

The experimental trial was conducted in Manaus, AM, Brazil (3°06'26" S and 60°01'34" W), in accordance with the ethical principles in animal experimentation adopted by the National Council for Animal Experimentation Control (CONCEA) and was approved by the Animal Use Ethics Commission (CEUA), case no 018/2016.

Experimental diets (Table 1) were formulated to be isonitrogenous (330 g.kg^{-1} of crude protein), based on soybean and wheat bran plant protein, and isoenergetic (14.65 MJ.kg^{-1} of crude energy).

Table 1 - Ingredients and proximate chemical composition of experimental diets

Item	Experimental diets (citric acid; g.kg^{-1} of diet)			
	0	10	20	30
Ingredient (g.kg^{-1})				
Soybean meal	510.000	510.000	510.000	510.000
Corn gluten meal	221.400	221.400	221.400	221.400
Wheat middlings	180.000	180.000	180.000	180.000
Soybean oil	25.600	25.600	25.600	25.600
Dicalcium phosphate	13.900	13.900	13.900	13.900
Calcium	5.200	5.200	5.200	5.200
Cellulose	30.000	20.000	10.000	0.000
DL-methionine (99%)	7.700	7.700	7.700	7.700
Sodium chloride	1.000	1.000	1.000	1.000
Vitamin-mineral premix[1]	5.000	5.000	5.000	5.000
Citric acid[2]	0.000	10.000	20.000	30.000
Butyl hydroxy toluene (BHT)	0.200	0.200	0.200	0.200
Proximate analysis				
Dry matter (g.kg^{-1})[3]	896.300	893.000	896.600	893.700
Crude energy (MJ.kg^{-1})[3]	14.650	14.650	14.650	14.650
Crude protein (g.kg^{-1})[3]	335.100	336.100	334.500	338.500
Crude fat (g.kg^{-1})[3]	40.300	42.600	39.500	40.400
Ash (g.kg^{-1})[3]	73.400	69.400	75.300	70.200

[1] Composition of vitamin-mineral premix (Vaccinar, Belo Horizonte, MG, Brazil) in mg/kg of diet: vitamin A, 500,000 IU; vitamin D3, 250,000 IU; vitamin E, 5,000 mg; vitamin K3, 500 mg; vitamin B1, 1,000 mg; vitamin B2, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 2,000 mg; niacin, 2,500 mg; folic acid, 500 mg; biotin, 10 mg; vitamin C, 10,000 mg; choline, 100,000 mg; inositol, 1,000 mg; selenium, 30 mg; iron, 5,000 mg; copper, 1,000 mg; manganese, 5,000 mg; zinc, 9,000 mg; cobalt, 50 mg; iodine, 200 mg.
[2] Citric acid (Synth, Diadema, SP, Brazil).
[3] Analyzed values according to AOAC (2012).

Ingredients were ground in a knife mill (Wiley Mill, TE-650/1) with a 0.5-mm sieve, homogenized in an automatic "Y" mixer, moistened (20% water), and processed in a single-thread extruder (2.5 mm). Anhydrous citric acid (Synth, Diadema, SP, Brazil) was included as 0, 10, 20, and 30 $g.kg^{-1}$ fractions to formulate four experimental diets, proportionally at the expense of cellulose. After that, pellets were dried in a forced-ventilation oven at 55 °C for 24 h to be stored at −18 °C. Proximate composition of ingredients and diets was determined by standard methods of the Association of Official Analytical Chemists (AOAC, 2012); the following were determined: content moisture by drying for 24 h at 110 °C to constant weight, protein by the Kjeldahl method, crude fat by diethyl ether extraction, and ash by heating at 450 °C for 24 h.

A completely randomized design consisting of four treatments was used: 0, 10, 20, and 30 $g.kg^{-1}$ of citric acid added to otherwise identical plant protein-based diets. One hundred and sixty juvenile tambaqui were used, whose average initial weight was 27.56±1.03 g. They were randomly distributed in 16 polyethylene tanks (310 L; 10 fish/tank) with a static water system and 20% water renewal every two days.

Animals were fed daily at 08.00, 11.00, 13.00, and 16.00 h until apparent satiation for 80 days, according to the treatment. During this experimental period, water quality parameters were measured: temperature (27.74±0.28 °C), which was controlled by a digital thermostat (500 W); dissolved oxygen (6.18±0.76 $mg.L^{-1}$), which was maintained with an air compressor; and pH (7.56±0.34). These parameters were measured daily in the morning by a multiparameter meter (G-50, Horiba, Minami-ku, Kyoto, Japan). Total ammonia (0.04±0.01 $mg.L^{-1}$) and nitrite (0.01±0.00 $mg.L^{-1}$) concentrations were monitored in the morning in weekly intervals using colorimetric kits (Alfakit®). These parameters remained at suitable levels for this studied species, as described by Araújo-Lima and Goulding (1997).

After the experimental 80-day period and 24 h of fasting, 12 fish per treatment (i.e., three fish from each tank) were anesthetized with 100 $mg.L^{-1}$ of benzocaine (Sanchez et al., 2016) for blood collection and euthanized by severing the spinal cord behind the head. After that, fish are dissected, and the livers were removed and washed in cold saline solution (0.9% NaCl). Tissue samples were stored in a freezer (−80 °C).

Blood samples were collected by caudal puncture by syringes with 3% EDTA. Three slides were used per fish, which were stained by May Grünwald-Giemsa-Wright following the technique described by Tavares-Dias and Moraes (2004). Total number of thrombocytes and leukocytes was calculated by the indirect method (Ranzani-Paiva et al., 2013).

Hepatic biomarkers of the antioxidant system were also analyzed. Tissue samples were homogenized using a Potter Elvehjem homogenizer (Teflon pestle) in 0.1 M sodium phosphate buffer, pH 7.0. To generate the homogenate, the liver was weighed, and 20X liver weight of 0.1 M sodium phosphate buffer, pH 7, was added. After homogenization, the material was used to quantify total protein for glutathione (GSH) concentration and lipid peroxidation analyses.

The GSH concentration was determined according to Ellman (1959). This method consists in analyzing GSH reactivity with dinitrobenzoic acid (DTNB), which forms a yellowish-colored thiol called trinitrobenzene (TNB) that can be measured in a spectrophotometer at 412 nm. Lipid peroxidation was estimated by the method described by Buege and Aust (1978). It was determined by the formation of lipid peroxidation, a malondialdehyde (MDA) byproduct, which is a reactive substance to the heating of thiobarbituric acid (TBA) and is formed during lipid peroxidation. The 1,1,3,3-tetraethoxypropane was used in the preparation of a standard curve of MDA, with a concentration range of 0.5-100 nmol/L. Total protein concentration of liver homogenates was determined by the Bradford method (Bradford, 1976) for the later quantification of MDA per mmol of MDA.mg of $protein^{-1}$.

Our results are expressed as mean±standard error (SE). Normality of the data was previously assessed using a Shapiro-Wilk test, and homogeneity of variance was also verified using the Levene test. Effects of experimental diets on blood cell count were analyzed using One-Way ANOVA. The following mathematical model was adopted:

$$Y_{ij} = \mu + \tau_i + e_{ij},$$

in which Y_{ij} is the quantitative response variable, μ is the general mean, τ_i is the effect of treatment i, and e_{ij} is the standard error. When significant differences were detected, data were compared with Tukey's HSD for multiple comparisons.

Nonparametric data (immature leukocyte, GSH, and MDA) were assessed by the Kruskal-Wallis test, followed by the LSD Fisher test for multiple comparisons. Statistical analyses were conducted using SPSS software (version 20.0, IBM, Armonk, NY), and differences were considered statistically significant when $P<0.05$.

3. Results

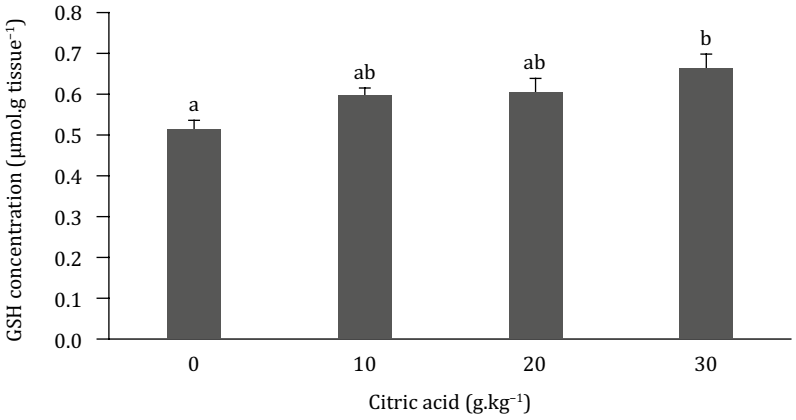
No significant differences were observed for thrombocyte, total leukocyte, lymphocyte, and positive granular leukocytes (LG-PAS) counts (one-way ANOVA, $F\leq2.31$; $P>0.09$; Table 2) and immature leukocytes (Kruskal-Wallis, $X^2 = 9$, $P = 0.51$; Table 2) in animals fed the experimental diets. However, there were fewer monocytes and neutrophils in fish fed diets containing 30 $g.kg^{-1}$ of citric acid ($P<0.05$; Table 2).

The GSH concentration in livers showed significant differences (one-way ANOVA, $F\geq4,92$; $P<0.001$; Figure 1) as a higher GSH concentration was observed in animals treated by adding 30 $g.kg^{-1}$ of citric acid to diet compared with the control group (Tukey, $P<0.05$). However, this concentration was not

Table 2 - Thrombogram and leucogram of juvenile tambaquis (*Colossoma macropomum*) fed diets containing different citric acid levels (0, 10, 20, and 30 $g.kg^{-1}$)

Parameter	Citric acid ($g.kg^{-1}$)				P-value
	0	10	20	30	
Thrombocyte ($\times10^3.\mu L^{-1}$)	57.622±8.618	54.807±3.324	46.150±2.083	54.355±3.461	0.443
Leukocyte ($\times10^3.\mu L^{-1}$)	52.087±9.584	41.162±5.738	30.265±3.940	47.162±5.399	0.156
Lymphocyte ($\times10^3.\mu L^{-1}$)	18.582±3.283	30.397±3.856	25.960±6.559	16.280±1.694	0.120
Monocyte ($\times10^3.\mu L^{-1}$)[1]	9.507±1.241a	4.515±0.304ab	3.700±0.429b	3.270±0.265b	0.018
Neutrophil ($\times10^3.\mu L^{-1}$)	5.257±0.721a	3.205±0.221ab	4.889±0.895a	2.125±0.350b	0.003
LG-PAS ($\times10^3.\mu L^{-1}$)	0.370±0.121	0.897±0.048	0.617±0.280	0.350±0.041	0.093
Immature leukocyte ($\times10^3.\mu L^{-1}$)[1]	0.142±0.042	0.369±0.185	0.323±0.190	0.110±0.066	0.518

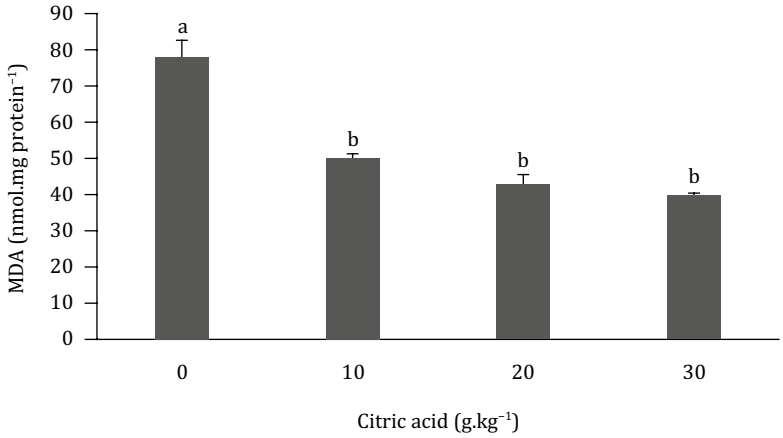
LG-PAS - positive granular leukocytes.
Values are presented as mean±SE, n = 12.
[1] Data were analyzed by a nonparametric Kruskal-Wallis test ($P<0.05$).
Different letters indicate significant differences between treatments (LSD and Tukey, $P<0.05$).



Values are presented as mean±SE, n = 12.
Different letters indicate significant differences between treatments (Tukey, $P<0.05$).

Figure 1 - Glutathione (GSH) concentration in the liver of juvenile tambaquis (*Colossoma macropomum*) fed diets containing different citric acid levels (0, 10, 20, and 30 $g.kg^{-1}$).

statistically different in treatments to which 10 and 20 g.kg^{-1} of citric acid were added (Tukey, P>0.05; Figure 1). Differences were observed in lipid peroxidation levels found in livers (Kruskal-Wallis, X^2 = 11.33; P = 0.01), with a higher MDA concentration for the control treatment (LSD, P<0.05). No significant differences were observed among treatments supplemented with 10, 20, and 30 g.kg^{-1} of citric acid in diet (LSD, P>0.05; Figure 2).



Values are presented as mean±SE, n = 12.
Different letters indicate significant differences between treatments (LSD, P<0.05).

Figure 2 - Malondialdehyde (MDA) concentration in the liver of juvenile tambaquis (*Colossoma macropomum*) fed diets containing different citric acid levels (0, 10, 20, and 30 g.kg^{-1}).

4. Discussion

The use of plant protein sources in diets can affect the immune and antioxidant defense systems of aquatic organisms (Dong et al., 2013; Lin and Mui, 2017). According to Tavares-Dias and Sandrim (1998), blood cells are very important for assessing immune responses in fish. These groups consist of lymphocytes, neutrophils, monocytes, eosinophils, and basophils, and use the bloodstream to monitor possible tissue damage or injury in fish, which thus increases their defense mechanism (Iwama, 1998; Tavares-Dias and Moraes, 2004). These cells are also sensitive to the effects of feeding fish, and their values may vary according to diet formulation (Flajnik and Du Pasquier, 2004). Thus, it is important to evaluate these parameters when using alternative ingredients in nutritional diet compositions.

Although no significant differences were observed in the number of thrombocytes, total leukocytes, lymphocytes, LG-PAS, and immature leukocytes among the experimental treatments, a higher level of citric acid inclusion in diet lowered the number of monocytes and neutrophils in juvenile tambaquis. This decrease is probably not harmful to the immune defense of fish, since the supplementation of citric acid reduced monocytes and neutrophils to values that are close to or above baseline levels (control group) of *C. macropomum* (e.g., Tavares-Dias et al., 2001; Inoue et al., 2016; Paz et al., 2019), indicating that the organism remains with functional cellular defense system. Moreover, neutrophils and monocytes involved in inflammation participate in initiation and propagation of ROS (Rahal et al., 2014). Thus, the reduction in number of these cell groups may suggest lower ROS production via leukocyte respiratory activity for tambaqui fed a diet supplemented with citric acid.

Free radicals generated in fish tissue are eliminated by the antioxidant system of enzymatic defense (e.g., glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase) and nonenzymatic defense (e.g., vitamin E, vitamin C, reduced glutathione, and carotenoids) (Dong et al., 2013; Hong et al., 2015). When the antioxidant activity of defense system decreases, ROS production increases and causes oxidative stress (Martínez-Álvarez et al., 2005; Packer, 1995). Therefore, fish health is

linked to ROS production and to the defense mechanisms of the antioxidant system, responsible for eliminating and protecting cell membranes against these reactive species (Dong et al., 2013).

The concentration of compounds and substances, such as reduced GSH and MDA, are used as biomarkers to measure the level of damage caused by oxidative stress (Martínez-Álvarez et al., 2005; Srikanth et al., 2013; Wang and Powell, 2010). Glutathione is an important low-molecular-weight thiol in cellular defense against ROS and other peroxides and also serves as a substrate for other detoxifying enzymes (Srikanth et al., 2013). Malondialdehyde is the lipid peroxidation product that provides direct evidence for toxic stress caused by free radicals and is also one of the most popular and commonly used methods to assess tissue peroxidation (Rosmini et al., 1996). Thus, the analysis of these compounds and substances is deemed suitable for assessing the oxidative state of fish fed diets based on soy protein (Dong et al., 2013; Lin and Cheng, 2017).

According to Jiang et al. (2018) and Zhang et al. (2016), high soy protein inclusion rates in fish diets can cause serious damage to animals' organisms. Increased ROS production can cause apoptosis of the intestinal epithelium and reduce mucin synthesis, which would expose intestinal tissue to intestinal content, which could lead to organ inflammation (Matés et al., 2008; Yan et al., 2008).

Chen et al. (2018) suggested supplementing diets with 15 and 30 $g.kg^{-1}$ of citric acid containing soy protein to enhance antioxidant capacity and reduce MDA content in the intestine of *Scophthalmus maximus*. Lin and Cheng (2017) also found that the hepatic TBARS value was higher in fish fed the control diet than the diets with organic acids (butyrate and lactate). A similar response was found by Zhang et al. (2016), who demonstrated that citric acid reduces intestinal oxidative damage (lipid peroxidation and carbonyl protein) by increasing total antioxidant capacity and activities of copper zinc superoxide dismutase and superoxide dismutase in *Larimichthys crocea* when fed plant protein at high doses. Zhu et al. (2014) and Li et al. (2017) also observed a reduction in ROS levels in *Pelteobagrus fulvidraco* and MDA in *Oncorhynchus mykiss* when fed diets to which organic acids had been added. These responses are also corroborated in the present study as the GSH concentration in juvenile tambaqui was significantly higher in the treatment to which 30 $g.kg^{-1}$ of citric acid were added. The MDA concentration was higher in fish fed the control diet and lower in those fed the diet with added citric acid. Hence, these results indicate that citric acid is a mitigating agent for oxidative stress in plant protein-based diets administered to the studied species.

Supplementation of citric acid generally stimulated the activation of the antioxidant defense system of juvenile tambaqui. This is evident because citric acid acts by protecting from damage caused by oxidative stress and chelate mineral elements, and thus reduces the pro-oxidation effect (Su et al., 2014). This organic acid also acts as a substrate in the intermediate metabolism of the Krebs cycle and provides more energy than glucose for emergency ATP synthesis under stressful conditions to improve the body's nonspecific immunity and resistance to stress (Lehninger et al., 1993; Su et al., 2014; Li et al., 2017). In addition, the supplementation of this organic acid in diets does not negatively affect fish nutritional status, health, or welfare and increases nutrient and mineral availability (Nascimento et al., 2020). Considering that, citric acid can be useful when added as a feed ingredient to the diet of juvenile *C. macropomum*.

5. Conclusions

The supplementation of citric acid to diets formulated with plant protein provides an antioxidant effect for juvenile tambaqui, *C. macropomum*.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: M.S. Nascimento, B.O. Mattos and T.B. Carvalho. Data curation: B.O. Mattos and T.B. Carvalho. Formal analysis: M.S. Nascimento, A.P. Amaral, B.O. Mattos and T.B. Carvalho. Methodology: M.S. Nascimento, A.P. Amaral, B.O. Mattos and T.B. Carvalho. Supervision: B.O. Mattos and T.B. Carvalho. Validation: B.O. Mattos and T.B. Carvalho. Writing-original draft: M.S. Nascimento, A.P. Amaral, B.O. Mattos and T.B. Carvalho. Writing-review & editing: M.S. Nascimento, A.P. Amaral, B.O. Mattos and T.B. Carvalho.

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