Dietary guarana (Paullinia cupana) powder for tambaqui (Colossoma macropomum): growth, hemato-immunological parameters and bacterial challenge

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Abstract

The present study evaluated the effects of commercial guarana (*Paullinia cupana*) powder as a growth-promoter and natural antioxidant supplement in aquafeeds. In Experiment 1, fish were fed with graded levels of guarana powder (0, 1.25, 2.5, 5 and 10 g/kg) during 60 days. Growth, hemato-immunological and biochemical performance were evaluated in healthy fish and in fish after infection by *Aeromonas jandaei*. In Experiment 2, the supplemented feed was stored for 90 days, in which tests were carried out regarding the quantity of phenolic compounds and the inhibition of lipid peroxidation. As a result of Experiment 1, fish fed guarana-supplemented diets and infected by *A. jandaei* presented better parameters of leukocyte respiratory activity, total proteins, globulin, albumin, alanine aminotransferase (ALT), leukocytes, lymphocytes, neutrophils, monocytes and thrombocytes. As a result of Experiment 2, levels of phenolic compounds were not altered ($p > 0.05$) by the guarana-supplemented diets. An increase in the concentration of malondialdehyde (MDA) and a lower oxygen radical absorbance capacity (ORAC) was observed in the fish fed diets with the highest level of guarana supplementation. As conclusion, guarana did not impair productivity and revealed a possible cytoprotective and hepatoprotective effect in fish infected by *A. jandaei*. Therefore, guarana seems to be an interesting additive to be included at levels of up to 1% in special feeds to be used prior to stressful situations in aquaculture. Additionally, guarana had no protective effect against lipid peroxidation and showed a pro-oxidant effect in diets with higher supplementation levels.

1. Introduction

Nutrition is the largest input in aquaculture and represents between 40 and 75% of the cost of production, and thus becomes one of the main determinants of the economic success of this activity (Ansari et al. 2021). In addition, inadequate nutrition impairs growth, animal health and the quality of the final product. However, the use of natural food components can enhance production and reduce nutrition costs.

Natural inputs of plant origin have been gaining prominence in aquaculture, after showing proven positive effects as growth promoters, immunostimulants, antioxidants as well as providing increased resistance to diseases (Amer et al. 2018; Hassaan et al. 2019; Kong et al. 2020; Mahmound et al. 2021). Some species of plants that are native to South America, such as copaiba (*Copaifera duckei*) (Malheiros et al. 2020; Seixas et al. 2020) and açaí (*Euterpe oleracea*) (Colombo et al. 2020; Schmitz et al. 2020), have already shown potential for use in aquaculture. The diversity of the Brazilian fauna, largely little studied, suggests that there are native species with the potential to boost animal production. Although widely consumed in the Amazon region, the effects of guarana (*Paullinia cupana*) are still unknown in aquaculture.

The guarana is a shrub that belongs to the Saindaceae family, which is found mainly in the Amazon region, and is classified into two geographical varieties – *Paullinia cupana* Kunth var. *typica* (found mainly in Venezuela and Colombia) and *Paullinia cupana* Kunth var. *sorbilis* (from the Brazilian flora) (Correia 1984). The guarana seed has a high content of alkaloids of the type methylxanthines (caffeine,

theophylline and theobromine), and phenolic compounds (catechin, epicatechin and epicatechin gallate) (Yonekura et al. 2016; Marques et al. 2019; Cavalcanti et al. 2020; Santana and Macedo 2021; Torres et al. 2022). Due to these alkaloids, the guarana fruit has a stimulating effect, with a caffeine content 2 to 4 times higher than coffee and up to 30 times higher than cocoa (Santana and Macedo 2018; Konstantinos and Heun 2019). The phenolic compounds of guarana, on the other hand, are mainly responsible for its antioxidant effect (Junior et al. 2020; Pinho et al. 2021 Torres et al. 2022). As well as other natural antioxidants that have already been identified in foods such as grape seeds (*Salvia hispanica* L.), chia (*Vitis vinifera* L.) (Munekata et al. 2020) and gác fruit (*Momordica cochinchinensis*) oil (Chamchong et al. 2021), guarana shows potential to be used as a natural antioxidant, and is able to replace synthetic antioxidants used in feed, such as BHT (butylated hydroxytoluene) and BHA (3-tert-butyl-4-hydroxyanisole), which are classified by the International Agency for Research on Cancer as carcinogens in animals (IARC, 1986).

The potential of guarana to improve growth and health has also been reported in other studies, as it has antioxidant, immunomodulatory, anticancer properties and a low cytotoxic effect (Lima et al., 2018; Marques et al., 2019; Kaushik et al., 2020; Junior et al., 2020). It also has antimicrobial properties against the bacteria *Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*, which were proven in different guarana seed extracts by Carvalho et al. (2016), and by Junior et al. (2020) against *Pseudomonas aeruginosa, E. coli and Candida albicans*. Thus, guarana has been demonstrated to be a potential to boost productive sectors, such as aquaculture, by increasing disease resistance in fish, and by improving the health and productivity of animals such as tambaqui (*Colossoma macropomum*).

The tambaqui is the most produced native species in Brazil, with a production of 101,079 tons in 2019, with the northern region of the country being the largest producer of this fish. However, with the intensification of fish farming, there is an increase in stressful conditions, which may be linked to the remarkable drop in productivity of the species. This went from 29.43% of Brazilian fish production in 2014, to 19.09% of production in 2019 (Hilsdorf et al. 2022). As such, it is important to research products that are capable of favoring the development of the animal and, in turn, boost the production chain of tambaqui. Based on this context, the objective of this study was to evaluate the effects of commercial guarana powder as a growth-promotor and natural antioxidant, and describe its effects on the productive and sanitary parameters of tambaqui, as well as its protective effects against lipid peroxidation in the feed.

2. Materials And Methods

The present study was conducted in two experiments. Experiment 1 aimed to evaluate growth performance and health of tambaqui fed diets with graded levels of guarana powder, and Experiment 2 aimed to evaluate the protective effects of guarana against lipid peroxidation and its potential as a natural antioxidant in the feed.

2.1. GROWTH PERFORMANCE AND HEALTH OF TAMBAQUI FED WITH GRADED LEVELS OF GUARANA POWDER

Preparation of the feed

For the experiment, the feeds (31% crude protein; 5.77% lipids; 1.85% crude fiber; 8.50% ash; 3,370 kcal gross energy/Kg) were formulated with conventional ingredients to meet the nutritional needs of the tambaqui. Guarana seed extract powder replaced a proportional amount of wheat bran in the formulation (Table 2). Commercial guarana seed powder was obtained in the city of Maués, Amazonas, Brazil. Guarana was added to the experimental feeds at concentrations of 1.25, 2.5, 5 and 10 g/kg. A control was formulated without supplementation of guarana powder. The ingredients were ground, mixed and extruded at 100°C in a single screw extruder (INBRAMAQ, MX – 80) to ensure full cooking, starch gelatinization and buoyancy of the pellets. After extrusion, the pellets were dehydrated in a forced ventilation oven at 55 ºC for 24 hours.

Table 1
Composition of experimental feed

Ingredients (%)	Control	1.25 g/kg	2.5 g/kg	5 g/kg	10 g/kg
Commercial guarana	-	0.125	0.25	0.5	1.0
Soy bran	53.50	53.50	53.50	53.50	53.50
Corn meal	31.50	31.50	31.50	31.50	31.50
Wheat bran	6.32	6.20	6.07	5.82	5.32
Bone/meat meal	5.0	5.0	5.0	5.0	5.0
Sybean oil	2.0	2.0	2.0	2.0	2.0
Vitamin and mineral supplement[a]	1.0	1.0	1.0	1.0	1.0
Dicalcium phosphate	0.50	0.50	0.50	0.50	0.50
Common salt	0.10	0.10	0.10	0.10	0.10
DL – Methionine	0.08	0.08	0.08	0.08	0.08

[a]Vitamin and mineral mix (Nutron©) per kg of product: folic acid (250 mg), pantothenic acid (5,000 mg), antioxidant (600 mg), biotin (125 mg), cobalt (25 mg)., copper (2,000 mg), iron (13,820 mg), iodine (100 mg), manganese (3,750 mg), niacin (5,000 mg), selenium (75 mg), vitamin A (1,000,000 IU), vitamin B1 (1,250 mg), vitamin B12 (3,750 mg), vitamin B2 (2,500 mg), vitamin B6 (2,485 mg), vitamin C (28,000 mg), vitamin D3 (500,000 IU), vitamin E (28,000 IU), vitamin K3 (500 mg), zinc (17,500 mg).

Experimental design and conditions of the animal experiment

All experimental procedures were performed at the Experimental Aquaculture Station of the National Institute Amazonian Research – INPA, Manaus, Brazil, in accordance with the Ethics Committee on the Use of Animals – CEUA, under protocol 027/2019. Initially, the fish were kept in circular tanks with constant aeration and water renewal for acclimation for one week. During the acclimation period, the fish were fed commercial feed for omnivorous fish. After this period, the tambaqui, with an average weight of (25.9 ± 2.03 g), were randomly distributed in 20 water tanks (310 L; 15 fish per tank) with water recirculation and constant aeration. In a completely randomized design, the tanks were then divided into five treatments (feeds with guarana powder in four different concentrations and a control group) with four repetitions each. The fish were fed with 4% of their total biomass which was divided into 2 feeds per day (8:00 h and 17:00 h) for 60 days.

The water quality parameters for dissolved oxygen (6.86 ± 0.51 mg L^{-1}), temperature (29.01 ± 0.82°C) and pH (6.53 ± 0.25) were monitored daily using a multiparameter probe (PRO ODO, YSI®). The ammonia (0.23 ± 0.08 mg L^{-1}) and nitrite (0.09 ± 0.01 mg L^{-1}) levels were monitored weekly using colorimetric and titrimetric kits (Alfakit AT 101, Alfakit, Florianópolis, SC, Brazil). All the data collected during experiments showed that the levels were acceptable for tambaqui farming (Lima et al. 2019). After 30 days of the experiment, three tambaqui were randomly selected from each tank to perform hemato-immunological analyses. After 60 days of the experiment, all fish were collected for evaluation of zootechnical parameters, with three fish randomly selected from each tank for hemato-immunological analyses.

Zootechnical parameters

The productive performance of the fish was analyzed according to weight gain (WG), apparent feed conversion (AFC) and relative growth rate (RGR). These variables were calculated using the following formulas:

WG (g) = (final weight – initial weight)

AFC = food consumption/relative weight gain

RGR (%/day) = (eg^1-1)*100

Where “e” is the Neperian number and g=(ln(FW)-ln(IW))/(Ne); where: FW = final weight; IW = initial weight; Ne = number of experimental days

Experimental infection

To analyze the immune response of the fish to the challenge by *Aeromonas jandaei*, 50 tambaqui (118.55 ± 29.21 g) were collected at the end of the experiment and distributed in 10 plastic tanks of 70 L, in duplicate, 10 per treatment, in a closed system, with partial water renewal (50% every 24 h) and with constant aeration.

For the preparation of the inoculum, a strain from the Microbiology Laboratory of Nilton Lins University (Manaus, Amazonas), identified as *A. jandaei* by Maldi-ToF mass spectrometry and sequencing of recA, rpoD and gyrB genes, was reactivated. For reactivation, the isolate was striated (stored in 15% glycerol at -20 ºC) on tryptone soy agar (TSA, Himedia) and incubated at 28 ºC for 24 h. The colonies were dissolved in sterile PBS until reaching an optical density of 0.786 (OD625), with a concentration of $3x10^8$ CFU/mL.

In the challenge, the fish were anesthetized with benzocaine (Sigma-Aldrich) in an immersion bath at a concentration of 0.1 g/L. Subsequently, the bacteria were inoculated via an intraperitoneal injection with a volume of 0.1 mL of inoculum per 10 g of live weight. During 24 h, behavioral changes, manifestations of clinical signs and mortality of the challenged animals were observed. After the challenge period (24 h), as there was no mortality, the fish were euthanized via an immersion bath with benzocaine at a concentration of 0.3 g/L. After euthanasia, blood was collected for hemato-immunological analyses.

Hemato-immunological analyses

Collection of whole blood and serum

On days 30 and 60 of the experiment, and after 24 hours of bacterial challenge, the fish were previously anesthetized in a benzocaine immersion bath (0.1 g/L) and blood samples were collected by caudal puncture. An aliquot of blood was used to perform blood smears on glass slides, part was heparinized (3 µL/sample) and the rest was kept without anticoagulant and centrifuged at 3,000 *x*g/10 minutes (4°C) to obtain the serum. Heparinized whole blood was used at the time of collection for analysis of leukocyte respiratory activity (burst), while serum was stored at -80°C for further analysis of total protein, albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Total and differential leukocyte counts

For the total and differential count of leukocytes, after drying and application of the dye May-Giemsa-Grunwald-Wright (Ranzani-Paiva et al. 2013), the blood components were observed under an optical microscope (Primo Star, Zeiss). The total leukocyte count was performed using the indirect method of Ranzani-Paiva et al. (2013): total leukocytes (µL) = [(number of leukocytes counted in component times the number of erythrocytes in a Neubauer chamber/2000)]. In the differential leukocyte count, 200 cells were counted, thus establishing the percentage and absolute value of each cell component of interest, according to the Ranzani-Paiva et al. (2013) protocol.

Burst analysis

The burst was measured by reactive oxygen species (ROS) and was determined using NBT (nitrotetrazolium blue chloride, Sigma N6876), according to the protocol of Siwicki et al. (1994), with some adaptations. Whole blood with anticoagulant (50 µL) was incubated in 2 mL microtubes containing 50 µL of buffer solution (HSSB, Sigma H6136) and NBT (0.2%) (Sigma, N5514), at room temperature for 30 min and stored in the dark. Then, 50 µL of the mixture was removed and 1 mL of dimethylformamide

(DMF, Sigma D4551) was added. Following this, the samples were centrifuged at 3,000 *x*g for 5 minutes. After centrifugation, the sample was read in microplate reader (Spectramax M5, Molecular Devices Inc., USA) at room temperature, at the optical density (OD) of 540 nm.

ALT and AST analyses

Colorimetric ALT and AST analyses were performed using a commercial kit (Labtest®), in which the enzyme assays were incubated at 37°C for 5 minutes, after which time, the initial absorbance (A0) was read. Another two subsequent readings (A1, A2), one every minute for two minutes, were carried out. The absorbance was read at the wavelength of 340 nm. After reading, the calculation of the mean variation of absorbances was performed by calculating:

$\Delta A/min = (A1-A2)/2$

To calculate the activity of ALT/AST, the following calculation was used:

$ALT/AST\ (U/L) = \Delta A/min \times 1,746$

Total protein

Colorimetric total protein analyses were carried out using a commercial kit (Labtest®). The blank was determined using 0.02 mL of distilled water + 1 mL of biuret reagent, and the standard absorbance (SA) using 0.02 mL of the standard solution (bovine albumin + sodium azide) + 1 mL of biuret reagent, both read at a wavelength of 545 mm. Each serum sample (0.02 mL) was added to 1 mL of biuret reagent, in which they were incubated at 37°C for 10 minutes. After which, the absorbance of the test (A1) was read at a wavelength of 545 Nm. After reading, the calculation of total protein was performed as follows:

$Total\ protein = A1/SA \times 4$

Albumin

The colorimetric albumin analysis was performed using a commercial kit (Labtest®). The standard absorbance (Ap) was determined using 0.01 mL of the standard solution (bovine albumin + sodium azide) + 1 mL of color reagent (nº1), mixed and, after 2 minutes, read at a wavelength of 630 nm. Each serum sample (0.01 mL) was added to 1 mL of color reagent (No.1), mixed and, after 2 minutes, the absorbance of the test (A1) was read at the wavelength of 630 nm. After reading, the following albumin calculation was performed:

$Albumin = A1/SA \times 3.8$

Globulin

Globulin was calculated by subtracting albumin from the total protein values, using the calculation:

Globulin = total protein-albumin

2.2. ANTIOXIDANT POTENTIAL

Experimental design

The feeds produced for Experiment 1 were the same used in this experiment, and the five treatments were randomized in three replications each. The feeds were then packed in airtight bags (500 g per sample) and sent, along with 500 g of the commercial guarana powder, to the Laboratory of Food Components and Health (LACAS) at the School of Public Health – USP, in São Paulo, Brazil. The feeds and commercial powder were stored at room temperature, without light, for 90 days, and 150 g of each feed were crushed and stored in three 50 mL Falcon tubes. The tubes were covered with aluminum foil and protected from light. At 30 and 90 days of storage, 100 grams of each sample were collected for determination of total phenolics and inhibition of lipid peroxidation.

Determination of total phenolics

The determination of total phenolics was performed by the method described by Singleton and Rossi (1965), adapted to microplates and in triplicate. The guarana sample was diluted in deionized water (1:400, v/v), and aliquots of 120 µL of the feeds (1:20, v/v) were mixed with 50 µL of Folin-Ciocalteau reagent (Merck) in a transparent polystyrene 96-well microplate (Greiner, Bio One). The microplate was shaken for five seconds and incubated for three minutes at room temperature. Subsequently, 30 µL of sodium carbonate (200 g/L) was added to the mixture, which was incubated at 37°C for one hour. The blue color produced by the reduction of Folin-Ciocalteau reagent by phenolics was measured in plate reader (Molecular Devices, SPECTRA max) at 765 nm. The quantification of total phenolics was performed using the analytical curve of the gallic acid standard, in concentrations of 0.024 to 0.398 µg/mL and an R^2 = 0.999. The results were expressed in mg of gallic acid equivalents/100 g of feed.

Inhibition of lipid peroxidation

Lipid peroxidation inhibition was evaluated according to thiobarbituric acid reactive substances (TBARS) using the method described in the literature, with modifications (Vyncke 1970; Tamura 1994; Soares et al. 2020). The samples were triturated in turrax (0.5 g) and 5 mL of TCA and centrifuged for 25 minutes at 5,000 *x*g. After centrifugation, the supernatant was deposited in a digester tube, to which 10 mL of HCL (4N) and 3 drops of defoamer were added for distillation. An aliquot of 480 µL of the buffer with linoleic acid and 10 µL of the distilled sample and 10 µL of the aqueous solution of ferrous sulfate (20 mM) were pipetted into a 2 mL microtube. The microtube was vortexed and incubated for 16 hours at 37°C to induce lipid peroxidation in the incubator. After 16 hours, 10 µL of the BHT STOPPER solution (91 mM) was added by pipette, and 150 µL was removed in triplicate, which were placed in a 2 mL microtube with the addition of 150 µL of the TBA solution and 450 µL of HCl 0.05 N. This was vortexed and incubated for 30 minutes at 100°C with sealing lock, cooled in an ice bath. Then, 750 µL of n-butanol was added and the mixture was vortexed vigorously. After stirring, the microtube was centrifuged for 10 minutes at 2,500 rpm, 200 µL of the n-butanol layer (upper layer) was removed, which was then placed in a

transparent 96-well microplate and read in a microplate reader (spectrophotometer) at an absorbance of 535 nm. The standard 1,1,3,3-tetraethoxypropane was used for the standard curve. Lipid peroxidation was expressed in mg MDA/100 g of feed.

Oxygen radical absorbance capacity (ORAC)

This test was performed according to a modified version of the method described by Ou et al. (2001). All solutions were prepared in phosphate buffer at 75 mmol/L (pH 7.4), since the fluorescence of fluorescein (3',6'-dihydroxy-spiro[isobenzofuran-1[3H],9'[9H]-xanten]-3-one) is sensitive to pH; when pH is lower than 7.0, the fluorescence intensity decreases considerably. The analysis was performed on 96-well black microplates (FLUOTRAC™ 200, Greiner Bio One). An aliquot of 50 µL of the sample (1:50000) was pipetted into 75 mmol/L (pH 7.4) of phosphate buffer, 150 µL of fluorescein (93.54 nmol/L) and 50 µL of AAPH (2,2'-azobis(2-aminopropane)) (221 mmol/L) (Sigma-Aldrich, USA). The plate was incubated at 37°C for 15 minutes prior to the addition of AAPH. For a period of 1 hour, the fluorescence decay was measured every minute at 37°C (excitation = 493 nm; emission = 515 nm), in a plate reader (SPECTRAmax, Molecular Devices). 75 mmol/L (pH 7.4) of phosphate buffer were used as the blank. To verify the linearity of the test, a calibration curve was prepared with Trolox at the following concentrations: 6.25: 12.5: 25; 50 and 100 µmol/L). The results were expressed in µmol of Trolox equivalents/100 g of feed. The calculation of the AUC of the sample, the blank and the standard was performed using the following equation:

AUC = (0.5 + f1/f0 + f2/f0 + f3/f0.....+f59/f0 + f60/f0

In which: f0 = fluorescence at time 0 minutes and f60 = fluorescence at 60 min

ORAC = C Trolox (AUCsample – AUCblank)/(AUCTrolox – AUCblank).K

Where: C Trolox = concentration of Trolox (20 µmol/L), AUC = area under the fluorescein reduction curve and K = sample dilution factor.

2.3. STATISTICAL ANALYSIS

The data involved two factors (guarana concentration in the feed and samples before and after challenge) that were evaluated using a two-way ANOVA in SigmaStat software (version 3.5, Systat Software Inc., Point Richmond, USA). The productive performance analyses were submitted to one-way analysis of variance (ANOVA). For normal data, the comparison of the means was performed using the Tukey test. Non-normal data were transformed into natural logarithm (NL) or evaluated via the Kruskal-Wallis test, which was followed by Dunn's test. In all analyses, a level of 0.05 was considered significant.

3. Results

3.1. GROWTH PERFORMANCE AND HEALTH OF TAMBAQUI FED WITH GRADED LEVELS OF GUARANA POWDER

In Experiment 1, it was observed that the fish ate the feed well and without refusal. There were no mortalities during this period. Juvenile tambaqui that received the feed with the guarana powder added showed no differences in the zootechnical performance variables (Table 2).

Table 2
Zootechnical performance of tambaqui provided with feed containing different levels of guarana

Parameter	Concentration of guarana in the feed (g/kg)				
	0	1.25	2.5	5	10
FW	129.21 ± 36.26	129 ± 37.52	101.82 ± 28.08	124.90 ± 37.02	104.91 ± 36.25
WG	88.18 ± 3.35	96.66 ± 0.73	85.16 ± 3.68	93.65 ± 4.82	81. 15 ± 3.92
ACF	1.16 ± 0.09	1.07 ± 0.03	1.10 ± 0.04	1.09 ± 0.03	1.22 ± 0.13
RGR	7.75 ± 0.07	7.92 ± 0.01	7.69 ± 0.08	7.86 ± 0.09	7.60 ± 0.09
FW: final weight (g). WG: weight gain (g). AFC: apparent feed conversion. RGR: relative growth rate.					

Hemato-immunological analyses

The tambaqui that were fed with guarana powder added to the feed showed no differences in hematological parameters after 30 and 60 days of feeding (Table 3).

In biochemical and immunological parameters, no differences were found after 30 days. At 60 days, significant differences ($p < 0.05$) were observed in these parameters only in fish fed feed containing 1.25 and 10 g/kg of guarana, and showed a reduction in burst values (Table 4).

Table 3
Hematological parameters of tambaqui receiving feed containing different levels of guarana

Parameter		Concentration of guarana in the feed (g/kg)				
		0	1.25	2.5	5	10
RBC (x10^6/µL)	30D	1.84 ± 0.12A	1.96 ± 0.11	1.67 ± 0.11A	1.87 ± 0.11A	1.84 ± 0.11A
	60D	2.37 ± 0.11B	2.39 ± 0.11	2.26 ± 0.12BC	2.29 ± 0.11B	2.37 ± 0.11B
	PCC	2.05 ± 0.13AB	1.95 ± 0.19	2.14 ± 0.14C	2.01 ± 0.14AB	2.31 ± 0.17AB
Total leukocytes (x10^3/µL)	30D	110.61 ± 9.49B	120.11 ± 8.66A	97.35 ± 8.66A	110.00 ± 8.66	100.96 ± 8.66A
	60D	143.49 ± 9.04C	151.51 ± 8.66B	150.73 ± 9.05B	137.33 ± 8.66	150.71 ± 8.66B
	PCC	53.10 ± 10.00Ab	124.01 ± 15.00ABa	138.75 ± 10.61Ba	133.08 ± 10.61a	162.19 ±13.42Ba
Lymphocytes (x10^3/µL)	30D	43.89 ± 5.16B	44.71 ± 4.71	35.95 ± 4.71A	40.78 ± 4.71	43.14 ± 4.71A
	60D	46.50 ± 4.71C	46.76 ± 4.71	47.86 ± 4.92B	41.92 ± 4.71	43.88 ± 4.71B
	PCC	16.81 ± 5.441Ab	39.49 ± 8.15a	45.93 ±5.78Ba	38.80 ± 5.77a	62.86 ± 7.29Ba
Neutrophils (x10^3/ µL)	30D	10.04 ± 1.47B	11.64 ± 1.34A	9.46 ± 1.34A	11.10 ± 1.34	11.04 ± 1.34A
	60D	12.03 ± 1.47B	16.80 ± 1.34B	15.77 ± 1.40B	13.76 ± 1.34	14.51 ± 1.34AB
	PCC	3.36 ± 1.55Ab	12.46 ± 2.33ABa	13.86 ± 1.65Ba	14.76 ± 1.65a	16.83 ± 2.08Ba
Monocytes (x10^3/ µL)	30D	9.24 ± 1.33B	9.39 ± 1.22	7.98 ± 1.22	8.55 ± 1.22	9.19 ± 1.22
	60D	13.01 ± 1.22B	12.39 ± 1.22	11.70 ± 1.33	11.68 ± 1.22	12.43 ± 1.22
	PCC	2.87 ± 1.40Ab	9.62 ± 2.11a	10.52 ± 1.49a	11.97 ± 1.49a	12.65 ± 1.88a

Different uppercase letters indicate significant difference ($p < 0.05$) between the collections and different lowercase letters indicate significant difference ($p < 0.05$) between the treatments. PCC: post-challenge collection. SCG: special granulocytic cells.

Parameter		Concentration of guarana in the feed (g/kg)				
		0	1.25	2.5	5	10
SCG (x10^3/µL)	30D	0.80 ± 0.31	0.74 ± 0.28	0.51 ± 0.28	0.81 ± 0.30	1.29 ± 0.28
	60D	0.88 ± 0.28	0.97 ± 0.28	1.48 ± 0.30	0.65 ± 0.28	0.65 ± 20.8
	PCC	0.27 ± 0.33	0.67 ± 0.49	0.90 ± 0.35	0.87 ± 0.35	0.80 ± 0.44
Eosinophils (x10^3/µL)	30D	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.06	0.18 ± 0.06
	60D	0.06 ± 0.06	0.11 ± 0.06	0.06 ± 0.06	0.05 ± 0.06	0.05 ± 0.06
	PCC	0.03 ± 0.07	0.16 ± 0.10	0.18 ± 0.07	0.008 ± 0.07	0.16 ± 0.09
Thrombocytes (x10^3/µL)	30D	43.89 ± 5.16B	44.71 ± 4.71	35.95 ± 4.71	40.78 ± 4.71	43.14 ± 4.71
	60D	46.50 ± 4.71B	46.76 ± 4.71	47.86 ± 4.92	41.92 ± 4.71	43.88 ± 4.71
	PCC	16.81 ± 5.44Ab	39.49 ± 8.15ab	45.93 ± 5.77a	38.80 ± 5.77ab	62.86 ± 7.33a

Different uppercase letters indicate significant difference ($p < 0.05$) between the collections and different lowercase letters indicate significant difference ($p < 0.05$) between the treatments. PCC: post-challenge collection. SCG: special granulocytic cells.

Table 4
Biochemical and immunological parameters of tambaqui receiving feed containing different levels of guarana.

Parameter		Concentration of guarana in the feed (g/kg)				
		0	1.25	2.5	5	10
Burst	30D	0.35 ± 0.01A	0.33 ± 0.01A	0.33 ± 0.01A	0.36 ± 0.01A	0.35 ± 0.01A
	60D	0.55 ± 0.01Ba	0.48 ± 0.01Bbc	0.52 ± 0.01Bab	0.54 ± 0.01Ba	0.44 ± 0.01Bc
	PCC	0.34 ± 0.02Ab	0.31 ± 0.02Ab	0.34 ± 0.02Ab	0.37 ± 0.02Aab	0.41 ± 0.02Ba
Total protein	30D	3.31 ± 0.10A	3.51 ± 0.11A	3.47 ± 0.26A	3.35 ± 0.11A	3.23 ± 0.11A
	60D	3.81 ± 0.10B	3.51 ± 0.11A	3.85 ± 0.47B	3.78 ± 0.11B	3.68 ± 0.11B
	PCC	4.08 ± 0.12Ba	4.14 ± 0.15Ba	3.71 ± 0.38ABa	2.90 ± 0.12Cb	3.08 ± 0.12Ab
Albumin	30D	0.57 ± 0.03	0.58 ± 0.03	0.59 ± 0.03	0.59 ± 0.03B	0.58 ± 0.03B
	60D	0.60 ± 0.03	0.60 ± 0.03	0.62 ± 0.03	0.60 ± 0.03B	0.60 ± 0.04B
	PCC	0.58 ± 0.03a	0.55 ± 0.04a	0.57 ± 0.03a	0.47 ± 0.03Aa	0.30 ± 0.03Ab
Globulin	30D	2.73 ± 0.11A	2.94 ± 0.11A	2.89 ± 0.11	2.76 ± 0.11A	2.64 ± 0.11A
	60D	3.18 ± 0.11B	2.94 ± 0.11A	3.22 ± 0.11	3.20 ± 0.12B	3.07 ± 0.14B
	PCC	3.50 ± 0.12Ba	3.60 ± 0.16Bab	3.14 ± 0.12a	2.43 ± 0.12Ac	2.78 ± 0.12ABbc
ALT	30D	6.46 ± 3.60A	7.70 ± 3.43	5.76 ± 5.09	7.25 ± 3.60A	6.19 ± 3.43
	60D	6.27 ± 3.43A	6.98 ± 3.80	6.98 ± 3.43	6.51 ± 3.43A	7.70 ± 3.43
	PCC	19.08 ± 3.80B	17.17 ± 4.65	15.89 ± 3.60	36.76 ± 3.80B	15.32. ± 3.80
AST	30D	17.38 ± 9.88A	19.68 ± 9.88A	18.16 ± 10.36A	21.65 ± 10.36A	17.20 ± 10.36A
	60D	26.35 ± 9.88A	26.12 ± 9.46A	26.27 ± 9.88A	27.06 ± 10.36A	30.36 ± 10.92A
	PCC	132.35 ± 10.36B	97.92 ± 13.37B	95.94 ± 10.36B	87.56 ± 10.36B	98.30 ± 10.36B

Different uppercase letters indicate significant difference ($p < 0.05$) between the collections and different lowercase letters indicate significant difference ($p < 0.05$) between the treatments. PCC: post-challenge collection.

In the results of the post-challenge fish (PC), the groups fed with guarana presented higher values of total leukocytes, lymphocytes, neutrophils and monocytes in relation to the fish of the control group ($p<0.05$) (Table 3). The post-challenge fish fed with 2.5 and 10 g/kg of guarana showed increased thrombocyte values in relation to the fish of the control group ($p<0.05$) (Table 3). In the biochemical and immunological analyses, an increase in burst was observed in fish fed with the highest level of guarana ($p<0.05$) (Table 4). The animals fed with 5 and 10 g/kg of guarana presented the highest levels of total proteins and globulin ($p<0.05$) (Table 4), while the highest levels of albumin were observed in the tambaqui fed with 10 g/kg of guarana ($p<0.05$) (Table 4). No significant differences ($p>0.05$) were observed in the ALT and AST levels of fish fed with guarana in relation to fish in the control group (Table 4).

Comparing the results of the fish at 60 days and post-challenge, it is noted that the tambaqui of the control group showed a significant reduction ($p<0.05$) in the levels of total leukocytes, lymphocytes, neutrophils, monocytes and thrombocytes (Table 3). Fish fed with 1.25 g/kg of guarana showed an increase in total protein and globulin levels ($p<0.05$) (Table 4), while tambaqui fed with 5 g/kg showed a significant reduction ($p<0.05$) of total protein, albumin and globulin (Table 4). However, animals fed with 10 g/kg of guarana showed a reduction in total protein and albumin levels ($p<0.05$) (Table 4). Post-challenge fish showed a significant increase ($p<0.05$) of ALT in relation to day 60 fish, in the control group and in the group fed with 5 g/kg of guarana (Table 4). All post-challenge fish showed an increase in AST levels compared to day 60 fish ($p<0.05$) (Table 4).

As a general aspect of the infection, all fish evaluated presented ventral bulging, hemorrhage at the base of the fins and on the back, anal prolapse and fluid accumulation in the peritoneal cavity (Figure 1).

3.2. *ANTIOXIDANT POTENTIAL*

Antioxidant evaluation of feed

In the analysis of phenolic compounds, it was observed that the commercial guarana powder has 546.75 mg/100 g of phenolic compounds per sample. The feeds of the control group and those with the supplementation with guarana showed no significant difference ($p > 0.05$) between the levels of phenolic compounds (Fig. 2).

In the ORAC (Fig. 3), it was observed that the feed with 1.25 g/kg of guarana presented higher oxygen radical absorbance capacity in relation to diets with higher levels of supplementation with guarana.

The concentration of MDA (Fig. 4) increased in all groups in the comparison between 30 and 90 days and, between treatments, the group with 10 g/kg of supplementation had the highest levels of MDA at 30 days and 90 days.

4. Discussion

To our knowledge, there are currently no published studies that investigate the use of guarana powder in aquafeeds. In this study, it was shown that guarana positively affected cellular parameters related to fish health when infected by aeromonosis.

Regarding the comparison of the results before the challenge and after infection by *A. jandaei*, the tambaqui of the control group showed a significant reduction in total leukocytes, which was due to a reduction in lymphocytes, monocytes and neutrophils, as well as a reduction in thrombocytes and from the burst. The infection also caused a significant increase in ALT and AST enzymes in the fish in the control group. The observed responses were similar to those recently described during infection by another species of *Aeromonas* (*A. hydrophila*) in tambaqui (Gallani et al. 2021), which suggests that the host immune system reacts to both bacteria of the same genus in a similar way.

Leukocytes are important defense cells since they act on immune responses and are able to eliminate pathogens and maintain animal homeostasis. Among them, monocytes, lymphocytes and neutrophils are one of the first lines of defense within the body (Guilliams et al. 2018; Paz et al. 2019; Libanori et al. 2021). The reduction of leukocytes, observed only in the post-challenge control group, may be related to possible oxidative damage caused in cells due to increased ROS activity during the challenge with *A. jandaei*. It can also be justified by the occurrence of a mechanism of programmed cell death called ETosis, which is a process that has already been described in tambaqui, and is classed as a last defense resource. It causes a programmed cell explosion for the release of granular proteins and chromatin, which form a network of extracellular fibers, bind to bacteria, degrade their virulence factors and kill these pathogens (Gallani et al. 2021).

The fact that all groups supplemented with guarana maintain post-infection cellular levels similar to pre-infection levels is one of the most relevant results of the study, and this may be a reflection of cellular protection against oxidative damage (Yilmaz 2019; Yonar et al. 2019). Studies show that guarana protects the cell membrane, since it has antioxidant substances that are capable of binding to free radicals, such as reactive oxygen and nitrogen species, which are generated during oxidative processes and inflammation. These free radicals, when they are not inhibited by antioxidants, react with the animal's cells and cause oxidative damage (Aldhahrani 2021). In this way, protection against cell death allowed cell levels to remain intact. Similar results were observed in rainbow trout fed with increased curcumin in the feed and challenged with the bacterium *A. salmonicida* subsp. *Achromogenes*; the fish in the control group had the lowest leukocyte values (Yonar et al. 2019).

In bony fish, thrombocytes have a function that is similar to platelets in mammals. In addition to hemostatic processes, these cells also have an immunological action in animals, and participate in phagocytosis and the destruction of pathogens, thus demonstrating a probable role as an antigen-presenting cell (APC) (Stosik et al. 2019). The reduction in thrombocytes, observed only in the post-challenge control group, may be related to oxidative damage caused by increased ROS activity, as already discussed above, which would justify the reduction in the number of thrombocytes, along with the

reduction in leukocytes, in the challenged fish. Therefore, the cellular protection of guarana would also extend to this group of cells.

The respiratory activity of leukocytes (burst) has been used as an indicator of the innate immune system of animals. It measures the formation of ROS, which is produced through NADPH oxidase, present in phagocytes, which are one of the main defense mechanisms of the body. These ROS are synthesized in hydrogen peroxide by the action of superoxide dismutase (SOD), and the myeloperoxidase then converts the H_2O_2 into acids, such as hypochlorous acid (HOCl), which have a great capacity to fight pathogens (Nguyen et al. 2017; Biller and Takahashi 2018). Post-challenge fish fed a diet containing 10 g/kg of commercial guarana powder showed maintenance of high levels of burst while a significant reduction was observed in the control, which is possibly related to the immunostimulating effect of guarana, and may be a consequence of the maintenance of high levels of leukocytes found in these fish. Tambaqui fed with pepper-rosemarin essential oil (*Lippia sidoides*) and with macroalgae extract (Ashour et al. 2020) also showed higher levels of burst when challenged with the bacterium *A. hydrophila*, (Monteiro et al. 2021). This demonstrates that plant products with immunostimulating properties, when added to the tambaqui diet, are able to increase the immune response of animals.

Serum proteins (albumin and globulin) are indicators of animal health, and may alter in situations of stress, kidney and liver diseases and in situations of immunostimulation (such as infections) (Hoseini et al. 2018). During the experiment, the levels of total proteins, albumin and globulin did not show significant differences; however, after the bacterial challenge, a significant reduction of total proteins and globulin was observed in the groups with 5 and 10 g/kg of commercial guarana powder supplementation in the feed, as was albumin reduction in the group with 10 g/kg of supplementation. The reduction of these proteins after bacterial challenge was also observed in tambaqui (Gallani et al. 2021) and pacu (Garcia and Moraes 2009) when challenged with the bacterium *A. hydrophila*. This reduction may be related to the demand for these proteins in damaged tissues and/or loss of these proteins in hemorrhagic processes (Jain 1986).

The enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes that are commonly used as biomarkers of liver lesions in several species of animals. In addition to being present in the liver, these enzymes can be found in other organs, such as the kidneys, brain, and in the heart and skeletal muscles, which may indicate that changes have occurred in them (Hoffman and Solter 2008; Amin et al. 2021; Silva et al. 2021). The fish showed no significant differences in the enzymes AST and ALT during the experiment, thus indicating that the commercial guarana powder as a supplement did not affect the liver and other organs in a negative way. After the bacterial challenge, the tambaqui did not present any significant difference between the treatments; however, when comparing the pre- and post-challenge results, the fish of the control groups and with 5 g/kg of supplementation showed significant elevation of levels of ALT. The increase in these biochemical indicators is commonly observed after bacterial challenge, and this is due to the lesions that bacteria cause in the organs of animals, including the kidneys and liver (Mbokane and Moyo 2018). Post-challenge fish with a higher level of supplementation with guarana did not show a significant increase in ALT levels when compared to fish at

60 days, and this is possibly due to the hepatoprotective effect of guarana. This effect has previously been observed in rats subjected to liver damage induced by carbon tetrachloride (CCl_4) (Kober et al. 2015).

Regarding the evaluation of the antioxidant potential of guarana, it is known that it presents phenolic compounds that are natural bioactive molecules, with the capacity to donate hydrogen atoms and/or electrons to free radicals, which are considered antioxidants (Albuquerque et al. 2021). However, there was no significant difference in the levels of phenolic compounds between the feeds, and this is probably due to the low amount of guarana added to the feed (up to the limit of 1% as a natural additive/ingredient), which does not significantly affect its final content. The phenolic compounds present in guarana amounted to 546 mg per 100 g of guarana; thus, in the diet with the highest amount of the supplement (10 g/kg), phenolic compounds represented approximately only 0.005% of its composition.

Oxygen radical absorbance capacity (ORAC) is a method that assesses the antioxidant capacity in substances. The method is based on the inhibition of peroxyl radical-induced oxidation, which is initiated by the thermal decomposition of azo compounds, such as AAPH. In the 30- and 90-day analyses, it was observed that the feeds with higher levels of supplementation with guarana presented lower oxygen radical absorbance capacity, which demonstrates a lower antioxidant effect. These results are probably linked to the alkaloids, such as caffeine, present in guarana. The pro-oxidant effects of methylxanthines, such as caffeine, have already been described in the literature. Li et al. (2019) observed increased ROS production in *Caenorhabditis elegans* when exposed to higher levels of caffeine. Therefore, higher levels of guarana in the feed may result in a greater oxidizing effect, and lead to faster lipid peroxidation.

The commercial guarana powder, as well as the feed with and without supplementation, showed an increase in MDA values between days 30 and 90. This increase occurred due to lipid peroxidation that occurs in food, even in antioxidant products such as guarana. Malondialdehyde (MDA) is a by-product derived from oxidative processes, such as lipid peroxidation, and is able to be used as a biomarker of rancidity and food spoilage (Wang et al. 2019). In the analyses of 30 and 90 days, it was observed that the feed with the highest level of guarana supplementation presented the highest levels of MDA ($p < 0.05$), even in relation to the control group, which does not contain any antioxidant product. This increase in MDA may be related to the pro-oxidant effects of alkaloids present in guarana, as already described above.

5. Conclusion

As a growth promoter, the commercial guarana powder did not affect the zootechnical performance of the animals; however, it positively affected the cellular parameters related to the health of the fish, thus demonstrating a possible cytoprotective and hepatoprotective effect in the tambaqui when challenged with the bacterium *A. jandaei*. As a natural antioxidant, guarana did not present a protective effect

against lipid peroxidation in the feed and, at higher levels of supplementation, it demonstrated a pro-oxidant effect, which is possibly related to the alkaloids present in its composition.

Declarations

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Ethical Approval

All the procedures performed were in accordance with the Brazilian National Council for Control of Animal Experimentation and the experimental protocol was approved by the Ethics Committee on the Use of Animals of the National Institute Amazonian Research (INPA), under protocol 027/2019.

Competing interests

The authors have no competing interests.

Authors' contributions

Conceived and designed the analysis: LFS MOREIRA, GN SAMPAIO, EAFS TORRES, LU GONÇALVES, GMR VALLADÃO.

Collected the data: LFS MOREIRA, IEC FERNANDES, IMC FERNANDES, MJ SOARES, IMS RIBEIRO, FM DANTAS.

Contributed data or analysis tools: LFS MOREIRA, IEC FERNANDES, IMC FERNANDES, MJ SOARES, FM DANTAS, FGN SAMPAIO, GMR VALLADÃO.

Performed the analysis: LFS MOREIRA, IEC FERNANDES, IMC FERNANDES, MJ SOARES,IMS RIBEIRO.

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Review the paper: all authors.

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Availability of data and materials

Derived data supporting the findings of this study are available from the corresponding author (VALLADÃO, G.M.R.) on request.

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Figures

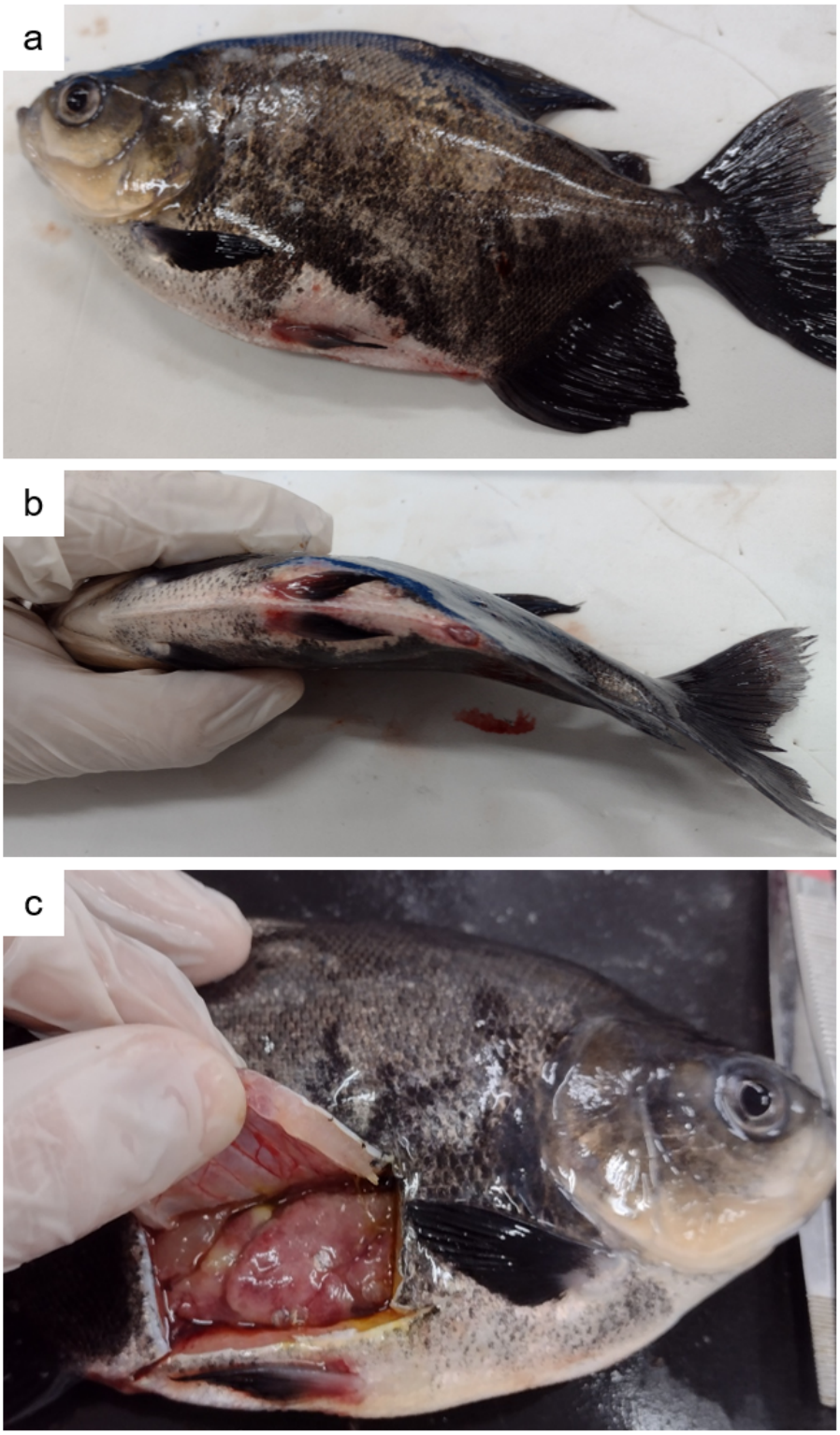

Figure 1

Ventral bulging (A), hemorrhage at the base of the fins and abdomen (B), and fluid accumulation in the peritoneal cavity (C).

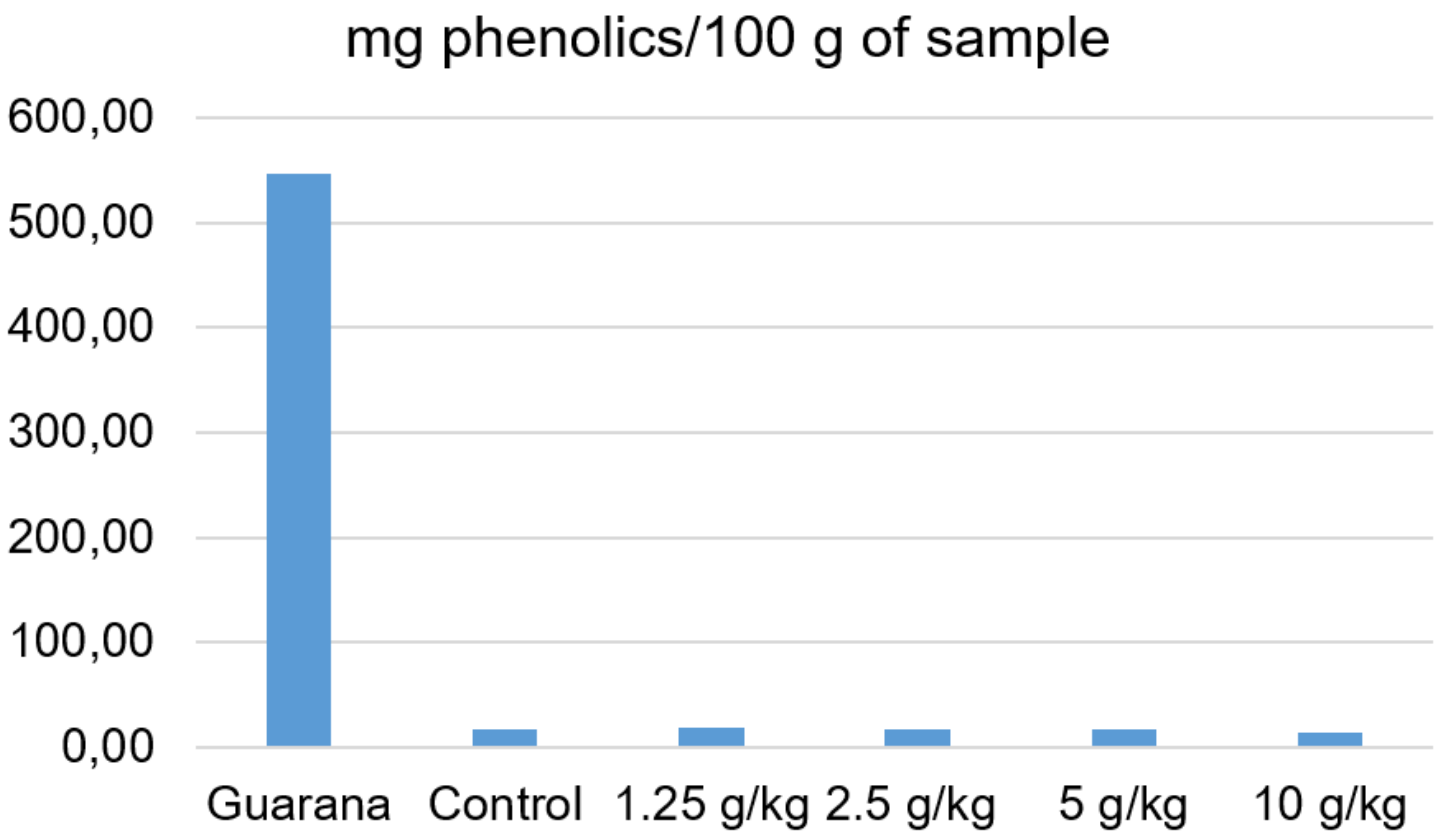

Figure 2

Concentration of guarana in the control feed and in feeds with different levels of supplementation with the commercial guarana powder.

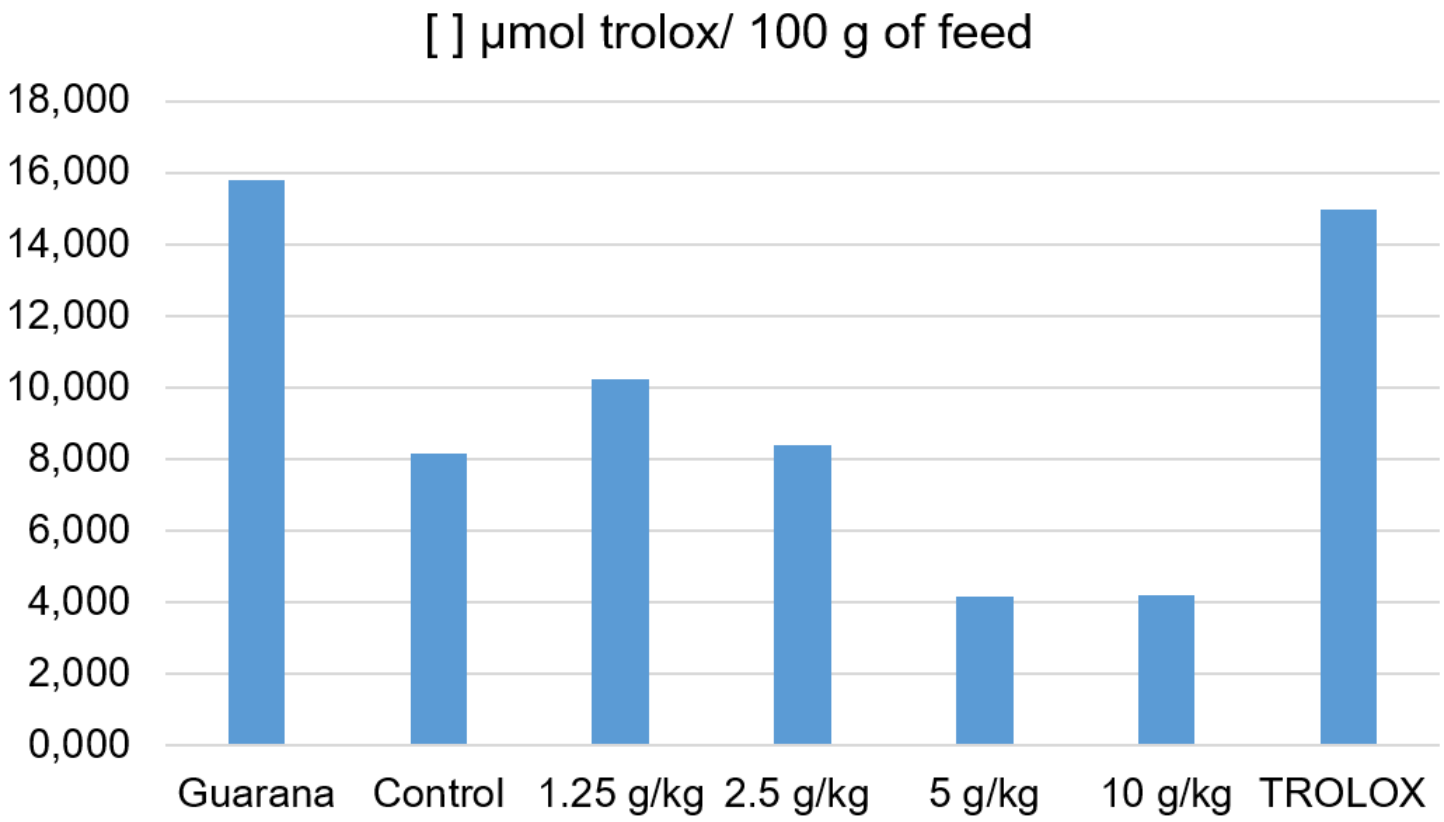

Figure 3

Evaluation of oxygen radical absorbance capacity (ORAC) in the guarana, in the control feed, in treatments with different levels of supplementation with the commercial guarana powder, and in Trolox. Different lowercase letters indicate significant difference ($p<0.05$) between feeds.

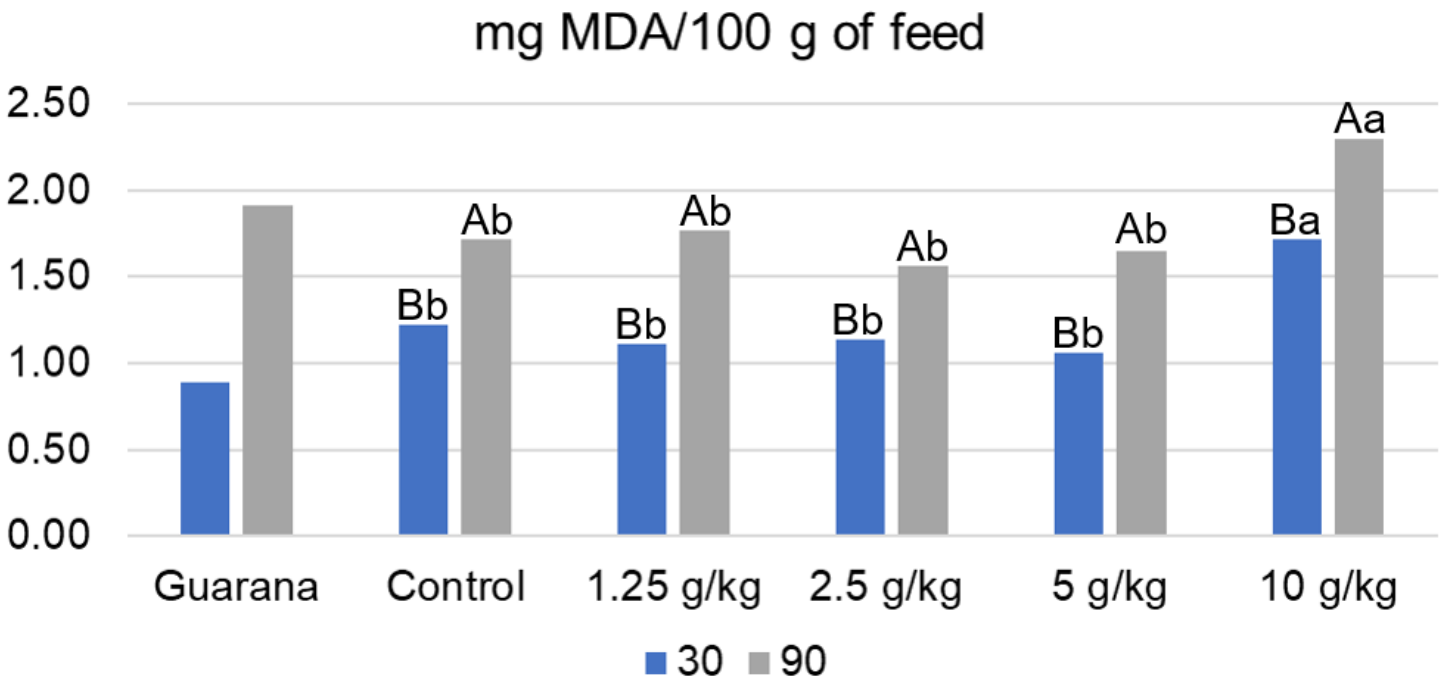

Figure 4

Concentration of MDA in guarana, in the control feed and in treatments with different levels of supplementation with the commercial guarana powder. Different uppercase letters indicate significant difference ($p<0.05$) between storage periods, and different lowercase letters indicate significant difference ($p<0.05$) between feeds.