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ORIGINAL ARTICLE

Bromatological Analysis and Evaluation of Antioxidant Properties of Pasteurized Cupuaçu (*Theobroma grandiflorum*) Juice Fermented by *Lacticaseibacillus rhamnosus* ATCC 9595

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Highlights

(1) Fermentation by L. rhamnosus ATCC 9595 increased the antioxidant capacity of cupuaçu juice.
(2) The juice met microbiological standards, with L. rhamnosus remaining stable for 28 days.
(3) Fermented juice maintained antioxidant activity during storage for 28 days.

ABSTRACT

This study conducted a bromatological analysis and evaluated the antioxidant properties of pasteurized cupuaçu (*Theobroma grandiflorum*) juice fermented by *Lacticaseibacillus rhamnosus* ATCC 9595. The juices were pasteurized (80º C/10 minutes) for subsequent fermentation with *L. rhamnosus* ATCC 9595 (inoculum of 108 CFU/mL). After 48 hours, the viability of *L. rhamnosus*, production of organic acids, and resistance to lysozyme and bile salts were analyzed. The samples were refrigerated for 28 days for bromatological analyses. The juices were extracted with ethyl acetate to evaluate antioxidant activity, phenolic compounds, and flavonoids. *L. rhamnosus* ATCC 9595 grew in pasteurized cupuaçu juice (~9 Log CFU/mL) and remained stable over 28 days ($p > 0.05$). All samples met the microbiological standards established by Brazilian guidelines for juices. Similarly, no significant changes were detected in the levels of ashes, moisture, or Brix degrees during the analyzed periods ($p > 0.05$). *L. rhamnosus* ATCC 9595 cultivated in the juice or MRS medium resisted the action of lysozyme (100 mg/L) and bile salts (0.5% and 1.0%). The antioxidant capacity of cupuaçu juice significantly increased after fermentation by *L. rhamnosus* ATCC 9595, as did the levels of phenolic compounds. The fermented juice samples showed similar IC50 values during storage, while the non-fermented extract showed variation from the 21st day onwards. The results indicate that fermentation by *L. rhamnosus* ATCC 9595 is an efficient strategy to enhance the antioxidant characteristics of cupuaçu juice.

Keywords: fermentation; fruit juice; probiotics; functional food; free radicals.

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INTRODUCTION

Free radicals and reactive oxygen species (ROS) play important roles in the etiology of degenerative pathologies (Parkinson's disease, Alzheimer's disease), cancer, diabetes, and inflammatory disorders[1]. In this sense, organisms have developed various strategies to control these agents, constituting the antioxidant defense system formed by enzymes (such as superoxide dismutase and catalase) and proteins (glutathione and thioredoxin)[2]. However, in many situations, this system cannot cope with the overproduction of reactive species, leading to a state called oxidative stress related to the clinical manifestations described above[3,4].

An alternative way to delay the damage caused by free radicals is by using foods with antioxidant properties that act through different mechanisms[1,5]. Fruits are considered excellent sources of bioactive compounds (such as proteins, vitamins, soluble carbohydrates, flavonoids, and other phenolic compounds)[6]. For example, cupuaçu juice and pulp (*Theobroma grandiflorum*) possess compounds with antioxidant, anti-inflammatory, and hypoglycemic properties[7–9].

Recently, the development of a potentially probiotic cupuaçu juice was reported based on the incorporation of *Lacticaseibacillus rhamnosus* ATCC 9595 (= *Lactobacillus rhamnosus*)[9]. This strain was selected due to its antimicrobial and immunomodulatory properties[10,11]. Fermentation of cupuaçu juice with *L. rhamnosus* ATCC 9595 caused alterations in the metabolite profile of cupuaçu juice due to biotransformation and an increased concentration of some bioactive compounds[9].

Additionally, fermentation by *L. rhamnosus* ATCC 9595 increased the anti-inflammatory properties observed in murine models of lipopolysaccharide-induced endotoxemia[9]. Fermentation with *L. rhamnosus* ATCC 9595 was also employed to improve the functional characteristics of bacuri juice. The fermented bacuri (*Platonia insignis*) juice showed superior anti-infectious effects compared to the non-fermented juice, prolonging the survival of *Tenebrio molitor* larvae infected with an enteroaggregative *Escherichia coli* strain[12].

Given the biotechnological potential of *L. rhamnosus* ATCC 9595 for the production of potentially probiotic beverages, this study conducted a bromatological analysis and evaluated the antioxidant properties of pasteurized cupuaçu juice fermented by *L. rhamnosus* ATCC 9595 stored for 28 days.

MATERIALS AND METHODS

Preparation of pasteurized juices and fermentation with *Lacticaseibacillus rhamnosus* ATCC 9595

Cupuaçu fruits were obtained in São Luís (Maranhão, Brazil). The fruit pulp was manually removed and stored at -20 °C until preparation. In each experiment, samples (30 g) of pulp were dissolved in 250 mL of distilled water (120 mg/mL), and the pH was adjusted to 6.0. Then, each juice was subjected to pasteurization (80 °C for 10 min). The juice was transferred to a container with ice until it reached room temperature, and then 1 mL of *L. rhamnosus* ATCC 9595 suspension (10^8 CFU/mL) was added. The cultures were incubated under shaking (120 rpm). After 48 h, the samples were serially diluted using phosphate-buffered saline (PBS) and plated on MRS agar. The plates were then incubated at 37 °C for 48 hours, and the colony-forming units (CFU) were expressed as CFU/mL[9].

Evaluation of juice stability during storage

Viability of Lacticaseibacillus rhamnosus ATCC 9595 in pasteurized Cupuaçu juice

Samples of fermented and non-fermented juices were stored at 8 °C. After each determined period (7, 14, 21, and 28 days of refrigeration), the viability of *L. rhamnosus* ATCC 9595 was analyzed as described above.

Microbiological and physicochemical analysis

The search for pathogenic microorganisms in the stored juices was carried out according to Normative Instruction No. 60 of 2019[13]. During the storage period, possible variations in color, total ash, moisture, and sugar contents were analyzed[14]. Five samples per batch were produced for each analysis.

In vitro simulation of the gastrointestinal tract of Lacticaseibacillus rhamnosus ATCC 9595 in Cupuaçu juice

Lysozyme resistance

Initially, the microorganisms grown in 10 mL of MRS broth at 37 °C were centrifuged, washed twice, and suspended in 2 mL with phosphate buffer (0.1 M, pH 7.0). Then, 10% of the bacterial suspension was inoculated into a sterile electrolytic solution (SEE) (0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$) in the presence of 100 mg/L of lysozyme (Sigma-Aldrich, St. Louis, USA). For control, the bacterial suspension was also inoculated in SEE without lysozyme. The survival rate was expressed as a percentage of CFU/mL after 30 min and 120 min compared to the count determined at time zero.

Bile salt resistance

The bile salt tolerance test was performed using a previously described method, with modifications[15]. MRS medium and sterile non-fermented juice solutions were prepared containing bile salt (Oxgall Sigma, USA) at 0.5% or 1.0% and pH 7.3. Samples (100 µL) of each solution and 10 µL of the fermented juice were added to the wells of a 96-well plate. The plate was incubated for 3 hours at 37 °C, and the results were read at 630 nm.

In vitro antioxidant assays of cupuaçu juice

Samples (100 mL) of fermented and non-fermented juices were subjected to liquid-liquid extraction using ethyl acetate (1:1; v/v). After separation, the solvent was removed by evaporation, and the extracts were stored at -20 °C.

DPPH and ABTS assays

The free radical scavenging properties of the extracts were measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich) radical and the ABTS (2,2'-azino-bis-(3-ethylbenzothia-zoline-6-sulfonic acid) radical, according to previously described methods[16].

Determination of phenolic compounds

The phenolic compounds were determined in the extracts using the Folin-Ciocalteu reagent. Samples (200 µL at 1000 µg/mL) were added to 1.0 mL of Folin-Ciocalteu reagent (1:1 v/v). After 3 minutes, 800 µL of sodium carbonate (20%) was added. The mixture was incubated at room temperature, protected from light, and left to stand for 2 hours. The absorbance of the mixture was measured at 765 nm. The total phenol content was expressed in µg/mL of gallic acid equivalents (GAE) using a calibration curve obtained with the standard gallic acid solution[16].

Determination of flavonoids

Aliquots of the samples (100 µL at different concentrations) were mixed with 100 µL of the reagent solution (2 g of aluminum chloridediluted in a 2% ethanol solution). The mixture was incubated at room temperature and protected from light, and after 60 minutes, the absorbance was measured at 420 nm. The flavonoid amount was calculated in µg/mL of quercetin equivalent (QE), using a calibration curve constructed with the standard quercetin solution[16].

Data analysis

The experiments were performed in triplicate and in three independent assays. Excel® was used for data tabulation, and GraphPad Prism version 8.1 was used for data analysis using Student's t-tests or One-way Anova or Two-way Anova, depending on the type of experiment. All results are expressed as the mean values of the groups and analyzed, considering a p-value < 0.05 as statistically significant.

RESULTS AND DISCUSSION

Viability of Lacticaseibacillus rhamnosus ATCC 9595 in pasteurized cupuaçu juice during storage

Initially, the growth of *L. rhamnosus* ATCC 9595 in pasteurized cupuaçu juice was analyzed, resulting in a population of approximately 9 Log CFU/mL, which remained stable during the 28 days of storage (p > 0.05) (Figure 1). The juice pH after fermentation was 4.2 ± 0.03, indicating the production of organic acids. These data are consistent with those obtained in the fermentation of cupuaçu juice sterilized by autoclaving[9].

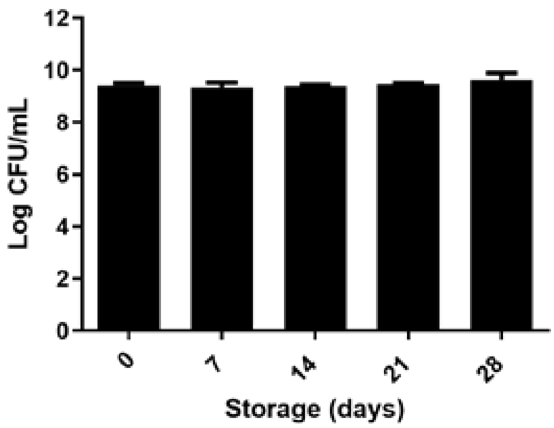

Figure 1 – Storage for 28 days of Lacticaseibacillus rhamnosus ATCC 9595 in pasteurized Theobroma grandiflorum juice. p>0.05. Values were analyzed by One-way Anova test.

The use of probiotics in food products in Brazil requires a prior analysis by the National Health Surveillance Agency (Anvisa), which recommends that the probiotic population should be in the range of 8 to 9 Log CFU/mL in the product ready for consumption[17–19]. In this context, the product formulated in this research meets the probiotic concentration required by Anvisa. In addition, it was confirmed that cupuaçu juice is a suitable matrix for the propagation of probiotic bacteria, as previously demonstrated[9].

Although more commonly associated with milk, lactic fermentation can be carried out using plant matrices, such as fruit pulps and milks, as long as they contain fermentable sugars in their compositions[20]. The incorporation of probiotics into plant-based beverages enhances their nutritional value, intensifies or adds functional characteristics, and extends the shelf life of the beverages[9,21].

Furthermore, developing fruit juices and other plant products with probiotics allows people with milk restrictions (vegans, lactose intolerant, and those with cow's milk protein allergy) to access the multiple benefits of consuming these microorganisms[21,22]. Recent studies demonstrate that consuming probiotics is crucial for balancing the intestinal microbiota and improving digestive and immune function[23]. Additionally, probiotic supplementation has beneficial effects on mental health and in treating infectious and chronic diseases[24, 25].

Microbiological analyses

Microbiological analyses revealed that the samples of pasteurized cupuaçu juice, whether fermented or not, met the standards established by Brazilian guidelines for juices[13], with none of the following pathogens being detectable throughout the 28 days of storage (Table 1).

Table 1 – Microbiological analysis of pasteurized cupuaçu juice (Theobroma grandiflorum)

Pathogen (/mL)	T0	T7	T14	T21	T28
	C/FC	C/FC	C/FC	C/FC	C/FC
Molds and Yeasts	0/0	0/0	0/0	0/0	0/0
Enterobacteriaceae	0/0	0/0	0/0	0/0	0/0
Salmonella	0/0	0/0	0/0	0/0	0/0
Escherichia coli	0/0	0/0	0/0	0/0	0/0

C = Cupuaçu juice; FC = Fermented cupuaçu juice

Physicochemical analysis

During the storage period, the possible physicochemical changes of the products were also analyzed. No significant changes were detected in the ash content, moisture, or Brix ($p > 0.05$) in the analyzed periods. Regarding the pH, the non-fermented juice showed a significant increase at the end of the storage period ($p<0.05$) (Table 2).

Table 2 – Physicochemical analysis of pasteurized cupuaçu juices (Theobroma grandiflorum)

	Ash (%)		**Moisture (%)**		**Brix (*B*°)**		**pH**	
	C	FC	C	FC	C	FC	C	FC
T0	$0.81 \pm 0.04^{a,1}$	$0.86 \pm 0.06^{a,1}$	$96.92 \pm 0.20^{a,1}$	$96.83 \pm 0.20^{a,1}$	$3^{a,1}$	$3^{a,1}$	$5.16 \pm 0.05^{a,}1$	$4.2 \pm 0.03^{a,2}$
T7	$0.81 \pm 0.04^{a,1}$	$0.72 \pm 0.10^{a,1}$	$96.66 \pm 0.03^{a,1}$	$96.61 \pm 0.001^{a,1}$	$3^{a,1}$	$3^{a,1}$	$5.26 \pm 0.05^{b,1}$	$4.4 \pm 0.03^{b,2}$
T14	$0.78 \pm 0.20^{a,1}$	$0.84 \pm 0.10^{a,1}$	$96.78 \pm 0.002^{a,1}$	$96.75 \pm 0.03^{a,1}$	$3^{a,1}$	$3^{a,1}$	$5.12 \pm 0.04^{a,1}$	$4.32 \pm 0.00^{c,2}$
T21	$0.83 \pm 0.20^{a,1}$	$0.83 \pm 0.08^{a,1}$	$96.56 \pm 0.1^{a,1}$	$96.55 \pm 0.05^{a,1}$	$3^{a,1}$	$3^{a,1}$	$5.00 \pm 0.01^{c,1}$	$4.27 \pm 0.01^{c,2}$
T28	$0.81 \pm 0.50^{a,1}$	$0.56 \pm 0.20^{a,1}$	$96.71 \pm 0.01^{a,1}$	$96.60 \pm 0.00^{a,1}$	$3^{a,1}$	$3^{a,1}$	$5.95 \pm 0.01^{d,1}$	$4.34 \pm 0.02^{c,2}$

C = Cupuaçu juice; FC = Fermented cupuaçu juice. In each column, values with statistically significant differences are indicated by the superscript letters ($^{a,\ b,\ c}$) different. For each parameter analyzed at a specific time (rows), values with statistically significant differences are indicated by superscript numbers ($^{1,\ 2}$) different. Values were analyzed by Two-way Anova test.

In vitro simulation of the gastrointestinal tract of Lacticaseibacillus rhamnosus ATCC 9595 in cupuaçu juice

Next, the survival of *L. rhamnosus* ATCC 9595 in cupuaçu juice under various conditions found in the gastrointestinal tract was evaluated (Figure 2). *L. rhamnosus* ATCC 9595 resisted the action of lysozyme (100 mg/L) when cultured in cupuaçu juice and MRS broth (Figure 2A). However, a greater resistance was observed in cupuaçu juice, at the two analyzed times ($p<0.05$). These data were similar to those reported for *L. rhamnosus* ATCC 9595 grown in bacuri juice[12]. Similarly, *L. rhamnosus* ATCC 9595 resisted exposure to bile salts (0.5% to 1.0%), obtaining similar variations when cultured in MRS or cupuaçu juice (Figure 2B).

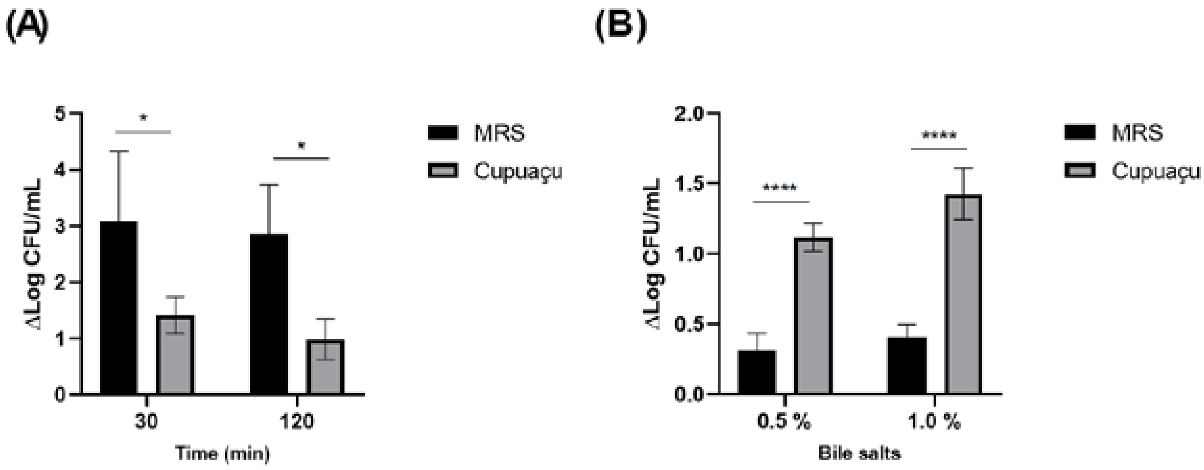

Figure 2 – Evaluation of the tolerance to simulated gastrointestinal conditions.

(A) Lysozyme tolerance test. (B) Bile salts tolerance test. *p<0.05; ****p<0.0001. Values were analyzed by Two-way ANOVA test.

Effect of fermentation on the antioxidant properties and levels of phenolic compounds and flavonoids in cupuaçu juice

The antioxidant capacity of cupuaçu juice, fermented or not by *L. rhamnosus* ATCC 9595, was evaluated by DPPH and ABTS assays (Figure 3). It was observed that the antioxidant properties of cupuaçu juice significantly increased due to fermentation by *L. rhamnosus* ATCC 9595. In the comparative analysis, significant differences were observed at all concentrations in the DPPH assay and at concentrations ≤ 250 µg/mL in the ABTS assay. The fermented juice samples subjected to storage showed IC50 values similar to those of day 0, while the non-fermented extract showed variation from day 21 onwards (Figure 3C).

The results obtained with the fermentation of pasteurized cupuaçu juice are consistent with several studies demonstrating the efficacy of lactic fermentation by *L. rhamnosus* strains (isolated or in co-culture) in increasing the antioxidant properties of fruit juices, such as nettle-fortified orange juice[26] and mixed juçara and mango juice[27].

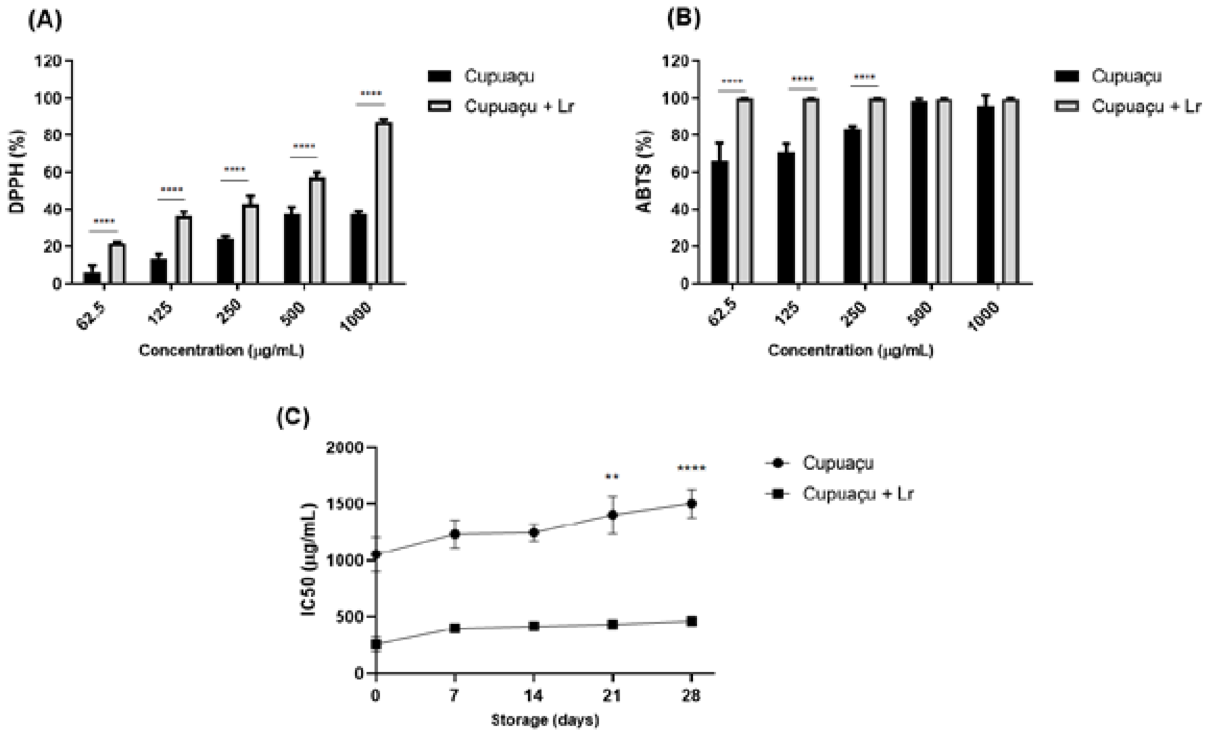

Figure 3 – Evaluation of the antioxidant properties of pasteurized Theobroma grandiflorum juices.

(A) Evaluation of antioxidant activity by the DPPH method. (B) Evaluation of antioxidant activity by the ABTS method; (C) Evaluation of antioxidant activity by the DPPH method of samples obtained during storage. IC50 = concentration that inhibits 50% of the radical; **** p < 0.0001. Values were analyzed by Two-way Anova test.

Figure 4 presents the total phenolic compounds and flavonoids levels in fermented or non-fermented cupuaçu juice samples by *L. rhamnosus*. It was observed that fermentation significantly increased the levels of total phenolic compounds (p<0.01); however, no changes were detected in the flavonoid content.

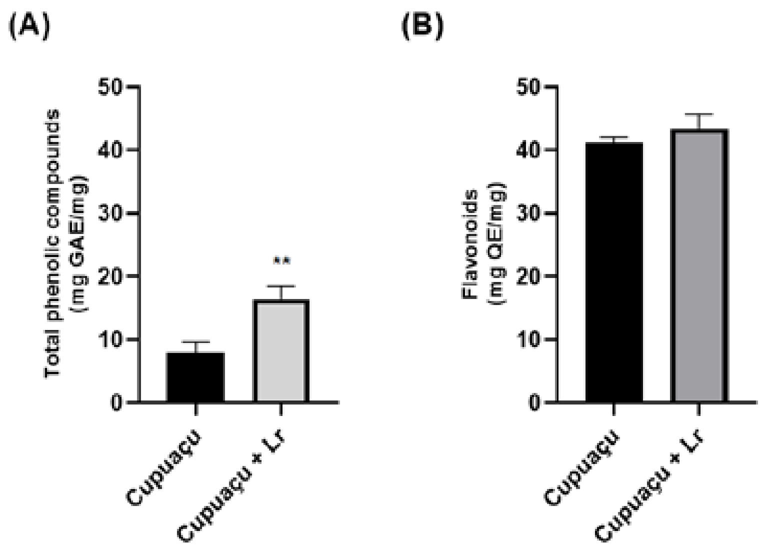

Figure 4 – Measurement of total phenolic compounds (A) and flavonoids (B) in fermented and non-fermented cupuaçu juices.

GAE: gallic acid equivalent; QE: quercetin equivalent. ** p < 0.01. Values were analyzed by the Student's t-test.

A diet rich in phenolic compounds is associated with a decreased risk of myocardial infarction, stroke, and diabetes, as it improves lipid profile, blood pressure, insulin resistance, and systemic inflammation[28]. Particularly, flavonoids are abundant in fruits and responsible for their color, flavor, and aroma. Due to their antioxidant and anti-inflammatory characteristics, they are important in preventing and treating cardiovascular diseases, diabetes, neurological disorders, and cancer[29].

CONCLUSION

The results obtained in this study reaffirm the potential of cupuaçu juice in the development of probiotic beverages. The juice samples (fermented or non-fermented by *L. rhamnosus* ATCC 9595) remained in accordance with Brazilian guidelines even after 28 days of refrigeration. The stored samples of both juices showed no significant changes in ash content, moisture, or Brix values during the analyzed periods; however, an increase in pH values was detected only for the non-fermented juice. Importantly, the population of *L. rhamnosus* ATCC 9595 remained stable during storage. *L. rhamnosus* ATCC 9595 cultured in cupuaçu juice showed resistance to the adverse conditions simulated by the gastrointestinal tract. Additionally, fermentation with *L. rhamnosus* ATCC 9595 significantly increased the antioxidant characteristics of cupuaçu juice, a finding related to the increase in total phenolic compounds.

In this context, it is suggested that the fermentation of cupuaçu juice by *L. rhamnosus* ATCC 9595 is an efficient strategy for enhancing antioxidant properties. The beverage produced is a lactose-free probiotic product, appealing to the vegan market and individuals with intolerances and allergies. It is also a functional food that can potentially alleviate pathological damage induced by free radicals.

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Kátia Sayuri Aragão Aguiar – Methodology, Validation.

Alexia Figueiredo Ferreira – Methodology, Validation.

Marcos Andrade Silva – Methodology

Luís Cláudio Nascimento da Silva – Conceptualization, Formal analysis, Obtaining funding, project administration, supervision and writing of the original manuscript

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