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Antioxidant Activity and Oxidative Stress Survival of *Limosilactobacillus reuteri* LR92 in Fermented Milk with Juçara Pulp

Maria Thereza Carlos Fernandes [1,2], Fernanda Silva Farinazzo [1,3], Carolina Saori Ishii Mauro [1,4], Thais de Souza Rocha [1,*], Karla Bigetti Guergoletto [1] and Sandra Garcia [1]

[1] Department of Food Science and Technology, State University of Londrina, Celso Garcia Cid Road, Km 380, Londrina 86051-970, Brazil; thereza.fernandes@hotmail.com (M.T.C.F.); fsfarinazzo@gmail.com (F.S.F.); carol.saori@gmail.com (C.S.I.M.); karla@uel.br (K.B.G.); sgarcia@uel.br (S.G.)
[2] School of Pharmacy, University of Northern Paraná, Campus Arapongas, PR 218 Road, Km 01, Arapongas 86702-670, Brazil
[3] School of Food Engineering, Federal University of Fronteira Sul, Campus Laranjeiras do Sul, BR-158, s/n, Laranjeiras do Sul 85301-970, Brazil
[4] School of Pharmacy, Universidade Positivo Londrina, João Rogério Ribeiro Bonesi St., 150, Londrina 86047-625, Brazil
* Correspondence: tsrocha@uel.br; Tel.: +55-43-3371-4987

Abstract: Fermented milk with probiotic bacteria is a functional food, and adding fruit can enhance its taste. Juçara, the fruit of the *Euterpe edulis* Martius palm tree, is known for its natural antioxidant properties. This study aimed to assess the antioxidant capacity of milk fermented by *Limosilactobacillus reuteri* LR92 with juçara pulp (JFM) over 30 days of storage at 4 °C and its protective effect on probiotic cells against reactive oxygen species (ROS). Phenolic compounds and antioxidant activities were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) assays during storage. The resistance of *L. reuteri* to hydrogen peroxide, superoxide anions, and hydroxyl radicals was also tested. The results indicated that JFM maintained stability in its composition, except for color, which showed reduced brightness by the end of the 30 days. Although antioxidant activity measured by DPPH and FRAP decreased (83.92–67.03 µmol TEAC.g^{-1} and 1185.64–830 g TEAC.100 g.mL^{-1}, respectively), it remained higher than the control (21.90–24.50 µmol TEAC.g^{-1} and 235.77–229.87 g TEAC.100 g.mL^{-1}, respectively). Phenolic content remained consistent. In addition, juçara pulp significantly protected *L. reuteri* cells from ROS. Therefore, juçara-enriched fermented milk not only improved antioxidant properties but also shielded probiotics from oxidative stress, highlighting its potential as a functional food with added health benefits.

Keywords: *Euterpe edulis*; milk-based fruit beverages; ROS; FRAP; phenolics

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1. Introduction

Nowadays, consumers are more concerned about their health and are seeking healthy substitutes for their food consumption habits. This shift has prompted the food industry to develop novel food processing technologies and functional products. Typically, functional foods are dairy-based products, well-known for their positive impacts on consumer health. Dairy-based beverages are particularly suitable for the development of probiotic drinks, which remain the most consumed among functional beverages [1].

Lactic acid bacteria (LAB), used in fermentation and known for their potential probiotic effects, like *Limosillactobacillus reuteri* (*L. reuteri*), are exposed to oxidative stress caused by the accumulation of reactive oxygen species (ROS) present in the food matrix [2]. Oxidative stress is a significant cause of viability loss in lactic acid bacteria used in the food industry [3]. The low survival rate of fermenting bacteria with probiotic potential can limit their ability to exert beneficial effects on human health [4]. Therefore, foods with high antioxidant

capacity can mitigate these effects, ensure greater cell viability, and improve consumer health, as plant extracts, phytocompounds, and LAB and their fermented products have the potential to reduce oxidative stress [5].

The use of *L. reuteri* in dairy products offers both technological advantages and functional benefits. Studies have demonstrated this microorganism's ability to produce reuterin, a compound with antimicrobial properties that can inhibit many pathogens and organisms that spoil dairy products [6–8]. The health benefits of *L. reuteri* include reducing cholesterol levels in the body (TC, TG, and LDL) and improving symptoms in patients with type 2 diabetes mellitus. Additionally, *L. reuteri* is notable for its role in managing intestinal diseases, with mechanisms that include protecting the intestinal barrier and suppressing the immune responses, inflammation, and oxidative stress, thereby regulating the intestinal microbiota and metabolism [9,10].

The addition of fruits in milk bases is used industrially to add to the food better sensory and functional characteristics such as taste, aroma, texture, and color, varying the products available on the market [11]. The use of unconventional fruits, such as juçara (*Euterpe edulis Martius*), is an economical and more sustainable alternative to the extraction of palm hearts, which leads to the death of the palm tree. Native to the Brazilian Atlantic Forest, this fruit is characterized by its rounded shape, fleshy mesocarp, and deep purple color, attributed to its high anthocyanin content [12].

The fruit of the juçara palm is considered a "super fruit" because it contains high nutritional value and bioactive compounds. In its composition, there are fatty acids such as oleic and linoleic, minerals, and proteins [3]. Compared to other berries, such as açaí and blackberry, the bioactives of juçara demonstrate higher values in antioxidant activities [13]. As an energy source, juçara (with an average of 65.5 kcal per 100 g) also stands out compared to fruits such as blackberries (43 kcal), cranberries (46 kcal), raspberries (52 kcal), and blueberries (57 kcal), indicating its potential use as a nutritional supplement for weight gain [14].

Its antioxidant capacity is mainly due to its anthocyanins, phenolics, and other flavonoid contents, which stand out in this fruit. The components found in juçara fruit include quercetin, rutin, aromadendrin, hispidulin, and myricitrin, among others, which confer health benefits when consumed [8–13]. Research indicates that juçara fruit extracts obtained by organic extraction have a high potential in deactivating 2,2-diphenyl-1-picrylhydrazyl (DPPH) and exhibit high electron transfer capacity, demonstrating significant ferric-reducing antioxidant potential (FRAP). This antioxidant capacity is greater than that of fruits like açaí (*Euterpe oleracea*), which are known for their high antioxidant capacity [13]. Due to the health-promoting properties of polyphenols, such as those found in juçara, their incorporation into dairy beverages has aroused significant interest in the food industry. From a technological standpoint, the inclusion of such fruits is also valuable for fermented lactic foods.

However, adding these compounds to dairy matrices can present challenges, such as color fading and loss of bioactivity due to chemical degradation during technological processes, as well as external factors like the food matrix, light, oxygen, temperature, and pH [15]. Therefore, it is crucial to evaluate the stability of the antioxidant activity of products with the ability to supply these bioactive compounds [15]. To ensure their functionality and considering the consumption of antioxidants through the diet, the objective of this study was to evaluate the stability of the antioxidant activity and resistance to oxidative stress of milk fermented by *L. reuteri* LR92 with the addition of juçara pulp during its storage. Given the lack of data in the literature on the behavior of antioxidant activity in the shelf life of dairy products with the addition of juçara pulp, despite its potential as a promising source of functional food, this research addresses an important gap.

2. Materials and Methods

2.1. Microorganisms

The lyophilized *L. reuteri* strain LR92 (Sacco DSM 26866-Cadorago, Italy) was stored at a concentration of 0.1% (*w*/*v*) in reconstituted skimmed milk (RSMP) (Molico®, Nestlé, São Paulo, Brazil) containing 18% (*w*/*v*) solids at −18 °C until use. The inoculum was activated twice in RSMP for 24 h at 37 °C prior to inoculation.

2.2. Juçara Pulp

The fruits of juçara (Sisgen A880DD5) were collected from palm trees at Fazenda Bimini (23°14′0.48″ S; 51°4′0.43″ W) in Rolandia, Brazil. The pulp was obtained and pasteurized, according to Guergoletto, Mauro, and Garcia (2017) [16]. The seeds were separated for planting, and the pulp was frozen in fractions until use. The obtained pulp had 4.30 ± 0.20 °Brix.

2.3. Production of Fermented Milk with the Addition of Juçara Pulp (JFM)

The development of the fermented milk followed the method described by Fernandes et al. (2020) [6]. The extraction of juçara pulp followed the methodology used for the production of pulp from this fruit in industrial demands and used by other authors, ensuring maximum preservation of the characteristics and antioxidant compounds [16,17].

For the production of the fermented milk, 18% (*w*/*v*) of skimmed milk powder (Molico®, -Nestlé, Araras, Brazil) was reconstituted, pasteurized at 95 °C for 5 min, and then cooled in an ice bath to 37 °C. Afterward, 100 mM of food-grade glycerol (Arcolor®, São Paulo, Brazil) and 1% (*v*/*v*) of active *L. reuteri* cells were added. The fermentation was carried out in sterilized glass containers, incubated at 37 °C in an anaerobic jar (Permution, CuritibaBrazil) with an anaerobiosis generator (Probac, São Paulo, Brazil) for 12 h until reaching 7.00 log CFU/mL of *L. reuteri*.

After the fermentation, 10% (*w*/*v*) of sucrose (Camil, São Paulo, Brazil) and 5% (*v*/*v*) of pasteurized juçara pulp were added to the fermented milk (JFM). Fermented milk (FM) with the addition of 10% (*w*/*v*) of sucrose was used for control.

For storage, the FM and the JFM were stored in glass containers at 4 °C for 30 days under anaerobic conditions (adapted from Langa and coauthors, 2014) [18]. Samples were taken for analysis every 15 days.

2.4. Physicochemical Analysis

A colorimeter (Minolta®, model CR400, Osaka, Japan) was used to assess color parameter values. A macronutrient composition analysis was also performed to determine moisture, ash, protein, lipids, and carbohydrate content by difference [19].

2.5. Total Phenolic Compounds and Antioxidant Activity

The methodology described by Hung et al. (2009) [20] was used to extract samples for the analysis of phenolic compounds and antioxidant activity. The total content of phenolic compounds was determined by the spectrophotometric method (at 760 nm) described by Swain and Hills (1959) [21]. Quantification used the standard analytical curve of gallic acid (Sigma-Aldrich, San Luis, MO, USA), and the results were expressed in mg of gallic acid equivalents (GAEs) per 100 g of sample.

The antioxidant capacity was measured by the scavenging-free DPPH• (2,2-Diphenyl-1-picrylhydrazyl), according to the methodology described by Brand-Williams, Cuvelier, & Berset (1995) [22] and Sánchez-González, Jiménez-Escrig, & Saura-Calixto (2005) [23]. The results were expressed as µmol Trolox Equivalent Antioxidant Capacity (238813, Sigma-Aldrich, San Luis, MO, USA) per g of sample (µmol TEAC.g^{-1}).

To determine the antioxidant capacity through iron reduction, the methodology described by Benzie, Strain (1999) [24] was used. The results of the antioxidant activity were expressed in g Trolox equivalent per 100 g sample (g TEAC.100 g.mL^{-1}).

2.6. Evaluation of Resistance to Reactive Oxygen Species (ROS)

Samples of JFM and FM were taken during storage for the analysis of oxidative stress. The analyses were performed according to Sections 2.6.1–2.6.3 described below.

2.6.1. Evaluation of Resistance to Hydrogen Peroxide

Resistance to hydrogen peroxide was measured by suspending the culture in peptone water (0.1% *w*/*v*) and incubating with 1.5 mM hydrogen peroxide (50% *w*/*w* in water, Sigma-Aldrich, San Luis, MO, USA) at 37 °C. Every 2 h for 6 h, aliquots were withdrawn and plated on MRS agar (De Man Rogosa Shape, Himedia, Kelton, PA, USA). The plates were then incubated at 37 °C for 48 h, and the number of viable cells was determined [25].

2.6.2. Evaluation of Resistance to Superoxide Anions

Resistance of *L. reuteri* to superoxide anions induced by paraquat (1,1′-dimethyl-4,4′-bipyridinium, Sigma-Aldrich, San Luis, MO, USA) was tested using the diffusion assay procedure [26]. Overnight JFM and FM cultures were suspended in saline. A total of 0.1 mL of the samples was spread onto MRS agar plates. Ten microliters of a 10 mM paraquat solution in sterile Millipore water was spotted on a paper disk placed in the center of each agar plate. The plates were then incubated for 48 h at 37 °C, and the zone of growth inhibition (mm) was measured.

2.6.3. Evaluation of Resistance to Hydroxyl Radicals

For the resistance to hydroxyl radicals, 1 mL of JFM and FM samples was exposed to hydroxyl radicals generated by the reaction of a 10 mM terephthalic acid solution (Sigma-Aldrich, San Luis, MO USA) in sodium phosphate buffer (pH 7.5) (Sigma-Aldrich, San Luis, MO, USA) and 0.01 mM $CuSO_4 \cdot 5H_2O$ (Synth®, São Paulo, Brazil). The reaction was initiated by adding 1 mM H_2O_2. Every 2 h for 6 h, the number of viable cells was estimated as log CFU/mL on MRS agar plates after incubating at 37 °C for 24 h [25].

2.7. Statistical Analyses

All analyses were performed in genuine triplicate, using variance analysis (ANOVA) and Tukey's test for comparison at a 5% level of significance using Statistica 10 program (StatSoft® Inc., Palo Alto, CA, USA, 2011).

3. Results and Discussion

3.1. Centesimal Composition

The results of the proximate composition for FM and JFM formulations during 1, 15, and 30 days of storage are shown in Table 1. The pH and concentrations of the acids present in the samples are in accordance with Fernandes et al. (2020) [6]. The pH value of 4.5 was established as one of the parameters for the fermentation process, and there were no significant variations during the storage period or between the samples.

The dairy industry faces particular challenges because the addition of fruit and vegetable extracts to fermented products can significantly affect the values of chemical parameters (lipids, proteins, and carbohydrates) and physical parameters (moisture) [26,27]. In our study, we observed (Table 1) that the addition of juçara pulp did not alter the protein and lipid parameters in the product when compared to the control. When comparing moisture and carbohydrates, a significant but small difference was observed between the samples. This difference is due to the addition of solids from the juçara pulp [13], even in low quantities. A study on the centesimal composition of juçara pulp presented values in 100 g of 88.90 g, 4.36 g, 0.09 g, 0.17 g, and 6.27 g in the parameters moisture, lipids, proteins, ashes, and carbohydrates (by difference) [13]. This result coincides with the differences between JFM and FM.

Table 1. Proximate composition and color of fermented milk (FM) and fermented milk with added juçara pulp (JFM) during 30 days of storage (4 °C).

		FM			JFM		
Days		1	15	30	1	15	30
Proteins %		3.05 ± 0.22 [a]	3.47 ± 0.17 [a]	3.20 ± 0.10 [a]	3.47 ± 0.30 [a]	3.48 ± 0.32 [a]	3.56 ± 0.01 [a]
Lipids %		0.30 ± 0.00 [b]	0.30 ± 0.00 [b]	0.31 ± 0.00 [a;b]	0.33 ± 0.01 [a;b]	0.31 ± 0.02 [a;b]	0.34 ± 0.04 [a;b]
Carbohydrates % [Φ]		13.28	14.83	16.73	14.15	15.58	17.61
Ashes %		0.30 ± 0.01 [e]	0.34 ± 0.01 [d]	0.37 ± 0.01 [c]	0.40 ± 0.10 [b]	0.45 ± 0.02 [a]	0.47 ± 0.00 [a]
Moisture %		83.06 ± 0.00 [a]	81.05 ± 0.10 [b]	79.39 ± 0.10 [d]	81.70 ± 0.06 [b]	80.18 ± 0.03 [c]	78.02 ± 0.00 [e]
Color	L*	80.27 ± 0.70 [a]	80.10 ± 1.50 [a]	80.00 ± 2.14 [a]	64.25 ± 0.10 [a]	62.45 ± 0.10 [b]	47.54 ± 0.21 [c]
	a*	−2.78 ± 0.20 [c]	−1.81 ± 0.30 [b]	−1.51 ± 0.80 [a]	5.63 ± 0.30 [a]	3.43 ± 0.10 [b]	1.57 ± 0.00 [c]
	b*	8.73 ± 0.10 [a]	6.93 ± 0.50 [b]	5.70 ± 0.08 [c]	4.91 ± 0.30 [c]	5.81 ± 0.05 [b]	6.05 ± 0.60 [a]

[a–e] Averages followed by the same lowercase letter on the line do not differ at the 5% level by Tukey's test. [Φ] Calculated by difference.

Regarding the stability of the products during storage, the protein and lipid levels remained stable, indicating that the addition of juçara pulp did not cause any interference in these parameters when compared to the control. However, it was observed that there was a slight decrease in the moisture content and an increase in the ash and carbohydrate contents. It was first speculated that there was a possibility of evaporation of water during storage, but this would have caused a concentration of all other components. Thus, it is more likely that other compounds were formed during storage, increasing the solid contents and decreasing moisture. Such compounds could be, for example, reuterin, which is excreted from *L. reuteri* during fermentation under anaerobic conditions [6]. These results also suggest that the anaerobic condition was sustained during the storage period.

3.2. Color

The analysis of the color parameter using a colorimeter revealed different profiles between the FM and JFM samples (Table 1). These differences were due to the addition of juçara pulp. The fruit is rich in anthocyanins, compounds responsible for the coloration that can range from red to purple. As a result, JFM exhibited a purple coloration throughout the 30-day analysis, while FM presented a white coloration typical of dairy products without the addition of fruits or dyes.

During storage, a variation was observed in all three color parameters (L, a*, and b*) analyzed for both FM and JFM. The brightness parameter L* (0 = Black and 100 = White) remained constant in the FM sample, which was lighter compared to the JFM sample. In the sample with juçara pulp, a decrease in brightness parameters was observed, which at first was 64.25 and, at the end of 30 days, reached 47.54, which indicates a darkening of the sample [28]. This reduction in luminosity can be related to the degradation of anthocyanins and cyanidins present in the juçara pulp. Oxidation by the action of polyphenol oxidase enzymes, which are present in lactobacillus and fruit extract, can cause the degradation of these compounds, resulting in brown compounds that directly affect luminosity [29]. Polyphenol oxidase and peroxidase are the main enzymes responsible for anthocyanin degradation, possibly interfering with the antioxidant capacity of the product.

The a* and b* parameters for the JFM sample also showed changes after 30 days, which corroborate the instabilities of the color-related compounds. The stability of these compounds is one of the main challenges in producing foods with their addition [30].

According to Fernandes et al. (2020) [6], when evaluating the sensory attributes (appearance, taste, texture, and odor) of fermented milk with the addition of juçara pulp, color is an important attribute for product acceptance and is considered the attribute with the greatest prominence of the product compared to FM. The variations observed in the a*, b*, and L* parameters of JFM during storage indicate a loss in the quality of this important parameter. These color changes occurred in the JFM due to the unstable nature of their pigment compounds, like anthocyanins and flavonoids. This raises concerns about the

storage conditions of these products upon commercialization. The package must protect the oxygen exchange, avoiding oxidation. Another number of ways to control enzymatic browning could be applied and were explored elsewhere [31].

3.3. Determination of Phenolic Compounds and Antioxidant Activity

Table 2 presents the results of the quantification of phenolics and the antioxidant activity by the methods used, DPPH and FRAP. The study by Cunha de Souza and collaborators [8] indicated that the high levels of anthocyanins (634.26 to 2929 mg of cyanidin-3-glucoside 100 g^{-1}) and total phenolic compounds (415.1 to 9778.20 mg equivalents of gallic acid 100 g^{-1}) were the bioactive compounds most related to the antioxidant scavenging capacity in juçara pulp.

Table 2. Total phenolics and antioxidant activity of fermented milk (FM) and fermented milk with the added juçara pulp (JFM) during 30 days of storage (4 °C).

Samples	Day	Total Phenolic Compounds (mg EAG.100 g^{-1})	DPPH (μmol TEAC.g^{-1})	FRAP (g TEAC.100 g.mL^{-1})
FM	1	1.66 ± 0.17 ^{b}	21.90 ± 5.00 ^{c}	235.77 ± 9.80 ^{d}
	15	1.41 ± 0.24 ^{b}	23.03 ± 0.09 ^{c}	229.87 ± 9.70 ^{d}
	30	1.51 ± 1.26 ^{b}	24.50 ± 1.88 ^{c}	229.87 ^{d} ± 9.10 ^{d}
JFM	1	3.04 ± 0.07 ^{a}	83.92 ± 4.00 ^{a}	1185.64 ± 4.20 ^{a}
	15	3.03 ± 0.08 ^{a}	73.86 ± 4.15 ^{ab}	1062.05 ^{b} ± 4.90 ^{b}
	30	2.81 ± 0.03 ^{a}	67.03 ± 1.67 ^{b}	830.00 ± 9.83 ^{c}

$^{a–d}$ Means with different letters in the columns differ significantly ($p < 0.5$).

During the entire storage period, JFM results showed an average of 44% more phenolic compound content compared to FM samples. Initially, FM showed 1.66 ± 0.17 against 3.04 ± 0.07 in the JFM sample content for phenolic compounds. This variation agrees with Leite (2015) [3], in which yogurt with 5% juçara pulp had a phenolic content 43% higher than the sample without added pulp. Both samples remained stable during storage. Among most fruits and vegetables, anthocyanins are the main phenolic compound present [32]. Despite the darkening observed for the JFM sample during storage being attributed to anthocyanin degradation, this degradation may not have been so intense, preserving this bioactive compound.

In the analysis of the DPPH radical, it was possible to observe that the FM samples did not show variations ($p < 0.05$) during the storage period, averaging 23.12 μmol TEAC.g^{-1}, which were lower than the samples containing juçara pulp (83.92 ± 4.00 to 67.03 ± 1.67 μmol Trolox.g^{-1}). The quantification of antioxidants by the DPPH method is based on the number of molecules of this compound that are reduced in the presence of hydroxyl groups in the food. The degradation of anthocyanins can generate melanin compounds that do not have hydroxyl groups, reducing this reaction and, consequently, its quantification [32]. Thus, based on the results of color, a decrease in DPPH antioxidant activity for JMF samples was already expected during storage. The DPPH results presented in our study are in agreement with those reported by Galani et al. (2017) [33]. The authors analyzed 19 types of vegetables and found reductions in DPPH values in all samples tested during the 15 days at 4 °C, resulting in a significant moderate correlation (ρ = 0.661) with the anthocyanins present in the vegetables. Their findings demonstrate that, despite anthocyanins being the main phenolic compound in fruits and vegetables, they are not the only compound responsible for DPPH antioxidant activity. Even though the degradation of anthocyanins occurred during the storage of JFM samples, darkening the product and decreasing its DPPH antioxidant activity, it is possible to draw two conclusions that are not exclusionary. First, not all anthocyanin was degraded in this process, and second, there are other compounds, probably other phenolics, in the product that are capable of being reduced in the antioxidant reaction. Such phenolic compounds could be rutin, quercitin, and p-coumaric acid [34].

The antioxidant activity measured by ferric-reducing antioxidant power (FRAP) showed the same trend as DPPH. In FM samples, this parameter remained stable during storage, averaging 232 g TEAC.100 g.mL^{-1}. The JFM samples had five times more iron reduction capacity than the FM sample. However, its initial capacity of 1185.64 ± 4.2 g TEAC.100 g.mL^{-1} was reduced to 830 ± 9.83 at the end of 30 days of storage. De Oliveira and collaborators [35], when analyzing the stability of juçara juice, observed that FRAP and TEAC (Trolox-equivalent antioxidant capacity) values showed a gradual decrease during refrigerated storage, with retentions of 52% and 38%, respectively, after 60 days. In the same study, a drastic reduction (98% in 15 days) in anthocyanin levels was also noted at the end of the storage period. In another study, it was shown that there was no significant correlation or negligible correlation between total anthocyanin content and FRAP activity [33] for several fruits and vegetables, implying that anthocyanins are not the main thing responsible for FRAP activity.

This study demonstrated that the storage time influenced the antioxidant activity of the JFM samples, which can be attributed to the degradation of bioactive compounds, mainly anthocyanins. The values obtained in the color and luminosity parameters corroborate these results. Tunin et al. (2023) [36] presented results similar to this study, reporting that the color and the concentration of anthocyanins remained stable in the dairy product from juçara pulp only for the first nine days.

Despite the reduction in antioxidant activity after 30 days of storage, milk with juçara pulp still exhibited significantly higher antioxidant activity compared to FM, indicating that even with the degradation of anthocyanins, JFM presents itself as a source of other antioxidant compounds. There are a number of factors that can affect the stability of the antioxidant compounds present in juçara pulp, such as direct sunlight, oxygen, and high temperatures [36–38], and conditions that were minimized in our storage process and upon commercialization of the product could be improved. In order to increase anthocyanin stability and bioavailability, studies are seeking alternatives such as liposomes, microcapsules, or emulsions for these compounds [39,40].

3.4. Resistance to Reactive Oxygen Species (ROS)

3.4.1. Resistance to Superoxide Anions

The adaptation of *L. reuteri*, regardless of the addition of juçara pulp, in fermented milk involves the optimization of environmental conditions such as temperature, pH, and nutrient availability to increase the survival and activity of the bacteria. In addition, the ability of the strain to produce beneficial compounds such as reuterin and its resilience in the acidic environment of fermented milk make it a promising candidate to increase the nutritional and health benefits of dairy products, improving their texture and flavor while maintaining their probiotic properties. However, the formation of free radicals, such as the superoxide radical, can compromise the survival of *L. reuteri* [41].

The superoxide radical is highly unstable and reacts quickly with other molecules, causing damage to tissues and cells. The JFM sample better protected the *L. reuteri* cells against this radical at all storage times (Table 3) compared to the FM sample. On the first day of analysis, no inhibition zone was detected for the JFM samples. In contrast, the FM sample initially showed an inhibition zone of 8 mm. The results of this analysis showed the same trend as the hydrogen peroxide test regarding storage time, with reduced protection after 15 days of storage. After 15 days, the JFM sample presented an inhibition zone of 11 mm, a result repeated at 30 days. During this same period, there was also an increase in the inhibition zone in the FM sample as well as a lower cell density on the plate.

In addition to protecting the cells in the fermented milk developed in this study, research has shown that anthocyanins present in juçara pulp activate important pathways in combating metabolic radicals. The primary mechanism lies in anthocyanin's ability to neutralize harmful reactive radical species through single electron transfer reactions and by abstracting hydrogen atoms from phenolic groups [42]. This highlights their significance in preventing cellular oxidation and, consequently, in the pathogenesis of

cardiovascular diseases, cancer, liver diseases, and diabetes. Studies have demonstrated the ability of anthocyanins to upregulate antioxidant defenses and modulate signaling pathways involved in reducing oxidative stress [20,43]. Thus, even though it is highly probable that anthocyanin degradation occurred during the storage of JFM samples because of the darkening and decreased antioxidant activity, there may still be a high content of total anthocyanin remaining in the JFM product due to the cell protection observed.

Table 3. Resistance to superoxide anion in fermented milk with the addition of juçara pulp (JFM) and fermented milk (FM) during 30 days of storage at 4 °C.

Day	Sample	
	JFM	FM
	Zone of Inhibition (mm)	
1	-	8
15	11	21
30	11	21

3.4.2. Resistance to Hydrogen Peroxide

The resistance of *L. reuteri* in fermented milk with and without juçara pulp is shown in Figure 1. *L. reuteri* was resistant to hydrogen peroxide for 6 h in both samples. However, the FM and JFM samples showed different behaviors. Up to the 15th day of the storage period, the JFM sample showed a protective effect on *L. reuteri* cells compared to FM samples. Hydrogen peroxide has toxicity related to the production of highly reactive hydroxyl radicals [44]. The antioxidant activity of anthocyanins protects against the formation of free radicals, which, when they reach cells and tissues, cause oxidative damage [45].

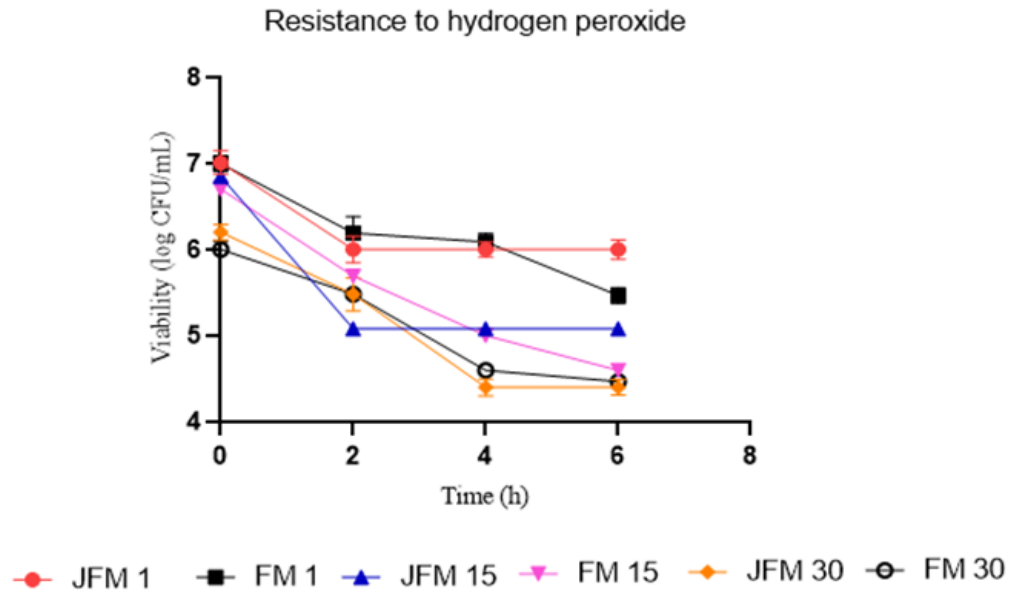

Figure 1. Survival of *L. reuteri* LR92 in fermented milk (FM) and fermented milk with added juçara pulp (JFM) in the presence of 1.5 mM of hydrogen peroxide during 1, 15, and 30 days of storage period (4 °C).

The bioactive compounds present in the pulp possibly exerted a protective effect on the cells against the stress generated by 1.5 mM of hydrogen peroxide. After 15 days, the juçara pulp was no longer protective, coinciding with the decrease in antioxidant activity and the darkening observed for JFM samples.

At the end of 6 h on day one of storage of the JFM, there was a reduction of 7.0 ± 0.01 log CFU/mL for 6.0 ± 0.00 log CFU/mL of *L. reuteri*, equivalent to 1 log CFU/mL.

In 30 days, the reduction was 1.58 log of CFU/mL of the microorganism in JFM, equaling the result of 30 days of the FM sample. The results highlight the beneficial usage of juçara pulp to improve the survival of the probiotic microorganism, favoring the consumer's health.

3.4.3. Resistance to Hydroxyl Radicals

The addition of juçara pulp to fermented milk also showed positive results in relation to resistance to hydroxyl radicals in comparison to fermented milk without pulp, as shown in Figure 2.

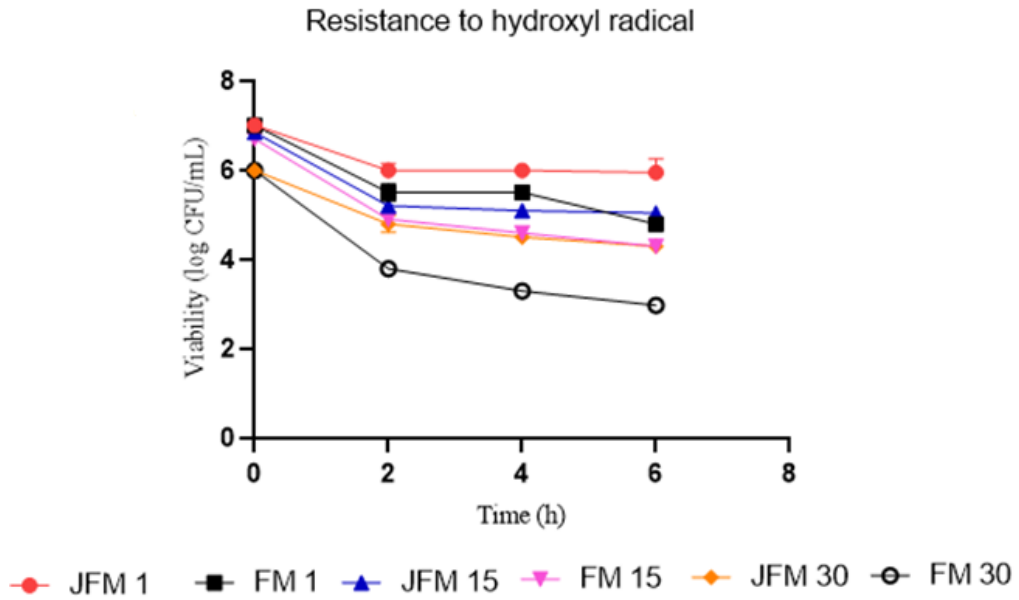

Figure 2. Survival of *L. reuteri* LR92 in fermented milk (FM) and fermented milk with added juçara pulp (JFM) in the presence of hydroxyl radical during 1, 15, and 30 days of storage period (4 °C).

During the storage time, the protection that the juçara pulp had in relation to *L. reuteri* shifted. Initially (one day), the decrease in the number of viable cells after 6 h was 1.06 log CFU/mL (from 7.01 log CFU/mL to 5.95 log CFU/mL); but, after 30 days, the reduction was 1.7 log CFU/mL. Even with this reduction in the survival rate of *L. reuteri* at the end of the 30 days, the resistance of the microorganism was statistically higher in the JFM sample than for the FM sample under the same conditions.

The protective efficiency against oxidative stress agents (such as hydroxyl radical) of anthocyanins depends on their chemical structure, the degree of glycosylation, and the number of hydroxyl groups [14], which can be reduced during their degradation, decreasing their antioxidant activity, as it was observed in the present study. However, juçara fruit has other bioactive compounds, such as quercetin and rutin, that also contribute to its antioxidant capacity [13]. Consequently, even with the degradation of anthocyanins and other antioxidant compounds during storage, the addition of juçara pulp boosts bioactive components that help protect the probiotic microorganism cells against oxidative stress agents.

Thus, the addition of juçara pulp promoted a protective effect for the viability of *L. reuteri* used for the preparation of fermented milk, with significantly higher counts compared to FM throughout the storage period. As it is a potentially probiotic culture, its viability must remain sufficient to provide beneficial health effects to consumers.

4. Conclusions

The addition of juçara pulp to fermented milk increases the phenolic compounds and antioxidant contents to the product. Additionally, juçara pulp also protects *L. reuteri* cells against reactive oxygen species, enhancing their viability when compared to cells in

fermented milk without juçara pulp. Our study demonstrates the potential of juçara pulp in the production of fermented dairy products, improving their functional and technological characteristics. Future studies are needed to ensure greater product stability, as it is a promising source of antioxidants and an excellent vehicle for probiotics.

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