

REGULAR ARTICLE

# Juçara (*Euterpe edulis*) pulp as a substrate for probiotic bacteria fermentation: Optimisation process and antioxidant activity

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## ABSTRACT

Juçara fruit (*Euterpe edulis*) has received considerable attention because of its high content of phenolic compounds. However, its suitability as a matrix for probiotic bacteria is not known yet. The objective of this study was to evaluate the pH and temperature conditions for the fermentation in juçara pulp by *Lactobacillus reuteri* and *Lactobacillus plantarum*. The optimised conditions were determined by central composite rotational design, and used for the analysis of cellular viability, sugar content, pH, lactic acid, total phenolics and antioxidant activity during 80 h of fermentation. The conditions for probiotic growth were 32 °C with a pH of 5.7 for *L. reuteri* and 25 °C with a pH of 5.0 for *L. plantarum*, and 30 h of fermentation time for both bacteria. The viabilities remained above  $8.6 \pm 0.2$  log colony forming units (cfu)/mL, and at 536.7 mg gallic acid equivalent (GAE)/100 mL of total phenolic reached. *L. reuteri* consumed all of the glucose and fructose present in juçara, whereas *L. plantarum* used only glucose. After 28 days of refrigerated storage, the cell viability of probiotics remained at 8.7 log CFU/mL with an increase in the antioxidant activity. Juçara pulp can be effective for a probiotic growth substrate and the production of new functional beverage.

**Keywords:** Probiotic; Experimental design; Juçara palm fruit; Total phenolics

## INTRODUCTION

Juçara (*Euterpe edulis*) is one among the palms belongs to the family Arecaceae. Seven species are distributed in the tropics. In Brazil juçara is found mainly in the Atlantic forests (Borges et al., 2011).

Juçara is known for producing an excellent quality of palm heart with a high economic value. However, the plant has no regrowth mechanism, and palm heart extraction causes death of the plant. This palm tree also produces a round fruit with a glossy black thin pulp covering the seed, similar to the well-known açai berry (*Euterpe oleracea*) (De Brito et al., 2007). Moreover, because of its thin pulp, juçara fruit is often consumed in the form of juice, pulp and as an ingredient in many foods (Felzenszwalb et al., 2013).

Although the juçara tree is in danger of extinction due to illegal palm extraction, the use of its fruit in

foods presents a potential economic, environmental, and nutritional alternative that could help preserve the species. The juçara fruit is rich in bioactive compounds with high antioxidant activity and has mineral elements, such as potassium, iron and zinc in amounts close to or higher than those found in açai (Borges et al., 2011). Recent studies have demonstrated that juçara pulp may exert high antioxidant effects *in vitro* (Borges et al., 2011; Vieira et al., 2013; Schulz et al., 2015), and its consumption can cause a positive effect on the antioxidant status and oxidative damage of healthy individuals (Cardoso et al., 2015). It is well-known that fruits and vegetables are important food sources due to their macro nutrients, vitamins, minerals, dietary fibre and antioxidants. However, they are a perishable commodity and not easily obtained depending on the season and region. For this reason, many technological resources can be employed to maintain or improve the safety, sensory properties and storage of fruits and vegetables.

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In the food industry, fermentation is the main way improve the sensory characteristics and to eliminate undesirable constituents and thereby enriching the nutritional properties (Jaiswal and Abu-Ghannam, 2013). Besides that, fermentation by probiotic bacteria could provide additional health benefits, since the regular consumption of probiotic microorganisms provides benefits, such as the stimulation of the immune system, inhibition of pathogens, regulation of intestinal function, reduction in serum cholesterol levels, improvement in inflammatory bowel disease, and suppression of *Helicobacter pylori* infection in the stomach (Nurmi et al., 1992, Hörmannspurger and Haller 2010, Remus et al., 2011).

Even though most probiotics are found in dairy-based products, new scientific data has addressed the effects of fermented vegetable products on health. This is due to the high levels of beneficial substances in fruits which, allied with the benefits of probiotics, can provide additional contributions to consumer health (Peres et al., 2012).

Although there are several studies showing the antioxidant potential of juçara fruit, there are no studies yet indicating its use as a suitable medium for probiotic bacteria fermentation. This way, this study aimed to optimise the parameters of pH and temperature during the fermentation process by *L. reuteri* LR92 and *L. plantarum* BG 112 in juçara pulp, using a central composite rotational design (CCRD), and bacterial growth as the response function. Under optimised conditions, the fermentation parameters were determined over the course of 80 h. In addition, we also investigated the influence of the fermentation process on the antioxidant activity and the stability during a refrigerated (4 °C) storage period of 28 days, to evaluate the potential use of juçara pulp as a cultivation media for probiotics strains of *Lactobacillus* for further use as a new non-dairy probiotic product.

## MATERIALS AND METHODS

### Juçara pulp

Juçara fruits were kindly provided by Bimini Farm (Rolândia, Paraná, Brazil) and was from the 2012 harvest of *Enterpe edulis* palm trees.

The product used in the present study was the juçara pulp, which was obtained by the following procedure: the fruits were washed with clean water and sanitised for 30 minutes in water containing chlorine at a concentration of 200 mg/kg. The fruits were then rinsed with clean water, and pulp was prepared by adding water to the fruit in a ratio of 2:1 (w/v) and pulped using a homogeniser for 1 minute at low frequency to preserve seed viability. The

seeds were separated for planting and the pulp was frozen in 250 mL portions.

Immediately prior to use, the frozen pulps were thawed under refrigeration for 18 hours and filtered, first through fabric to remove the sludge, and then with a qualitative filter paper (0.5 mm, Synth, Diadema, Brazil) using a Büchner funnel under vacuum. Thereafter, the pH of the pulp was adjusted using 3 N NaOH (Sigma-Aldrich, São Paulo, Brazil) or 10% acetic acid (Sigma-Aldrich, São Paulo, Brazil) according to the central composite rotational experimental design, followed by pasteurization at 80 °C in a water bath for 1 minute, and then cooled in an ice bath.

### Juçara pulp chemical composition

The chemical composition of the resultant juçara pulp was characterised by determining the following: moisture content was measured in a laboratory oven at 105°C, protein by the Kjeldahl method, ash in a muffle at 550°C, and total fibre by the Enzymatic-Gravimetric Method (AOAC, 2005). The pH was measured using a pH meter (potentiometer - Ion PH300), and the soluble solids with a portable refractometer (Atago refractometer pocket, Japan). Total sugar content was analysed by phenol sulfuric acid method (Dubois, 1956) using as glucose as standard, and lipid according to the method of Bligh and Dyer (1959). In determining the mineral content, the pulp samples were first digested with a mixture of nitric and perchloric acids in the ratio 2:1 (v/v), then diluted with 50 mL deionised water and the elements determined according to Silva (2009).

### Microorganisms and pre inoculum preparation

The probiotic strains of *Lactobacillus reuteri* LR92 (DSM 26866) and *L. plantarum* BG112 (LMG 23520) were provided by Clerici Sacco (Italy). Each cultures powders were diluted at concentration of 0.01% (w/v) in pasteurised juçara pulp and the mixture was kept frozen with 20% (v/v) sterile glycerol (Synth, Diadema, Brazil). Prior to testing, pre-inoculums were obtained from two activations of 1% (v/v) on juçara pulp and incubation at 37 °C for 18 hours.

### Optimisation of *L. reuteri* or *L. plantarum* growth in juçara pulp

The optimum fermentation conditions (initial pH and incubation temperature) for both microorganisms were determined using the response surface methodology in a central composite rotational experimental design (CCRD) consisting of a 2<sup>2</sup> factorial [with two levels (-1 and 1) and two factors, x1 (initial pH) and x2 (incubation temperature, °C)], including four axial and three central points, totalling eleven randomly performed trials.

The fermentation parameters applied to the CCRD were established by preliminary tests and based on the range that

the *Lactobacillus* spp. can grow (Pereira and others 2011). In this manner, the initial pH for *L. reuteri* was from 3.6 to 6.4, and for *L. plantarum* from 5.0 to 7.9. The temperature ranged from  $10.8 \pm 1$  °C to  $39 \pm 1$  °C for both bacteria.

For each experimental run with each microorganism, one percent of a pre-inoculum (containing approximately  $10^8$  CFU/mL) was added to screw-cap glass vials containing 100 mL of pasteurised juçara pulp to an initial count of  $10^6$  CFU/mL. This concentration was chosen based on the preliminary tests, in which concentrations of 1.0, 5.5 and 10.0 % did not presented differences on cell viability for both lactobacilli. Moreover, using the lowest pre-inoculum concentration allows economising in product development. Besides, this concentration meets the recommendation for probiotic foods: minimal counts of 6.00 log CFU/mL for better efficacy in regulating beneficial effects. This mixture was incubated for 48 hours at different temperatures, according to the experimental design. The number of viable cells was determined at the end of the incubation period using Man-Rogosa-Sharp (MRS) agar (Himedia, Mumbai, India) and response variables were recorded as functions of log CFU/mL.

A second order response surface model was used to fit the experimental data using equation 1:

$$Y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{12}x_1x_2 \quad (1)$$

Where Y = response function,  $x_1$  and  $x_2$  = coded independent variables, and  $\beta$  = estimated regression coefficients.

The regression and variance analysis (ANOVA) was performed using the software Statistica 7.0 (StatSoft Inc., Tulsa, USA) to estimate and test the significance ( $p \leq 0.05$ ) of the parameters for the mathematical models. The response surface plots were constructed from the adjusted models, and the desirability of the predicted values was evaluated.

### Lactobacilli growth and fermentation process

A lactobacilli growth study was conducted under optimal fermentation conditions, designed using the response surface methodology (pH 5.7 and 32 °C for *L. reuteri*, and pH 5.0 and 25 °C for *L. plantarum*) with the aim of eliciting the appropriate fermentation time and the sugar consumption for the juçara pulp matrix. In addition, analysis for the total phenolic content and antioxidant activity were also conducted to verify the influence of fermentation parameters on juçara antioxidant activity.

### Storage stability

The juçara pulp (150 mL in glass bottles) was fermented for 30 hours under the optimised conditions as determined

by the growth study. After fermentation, the samples were stored in a cold room at 4 °C for 28 days. Analysis of viable cell counts, total phenolic and antioxidant activity during storage were determined every 7 days in triplicate to calculate the average between 3 repetitions.

### Viable cell count determination

The cellular viability was determined using the pour plate method. Fermented juçara pulp dilutions (1:9) were made with sterile peptone-water 0.1% (w/v), followed by plating on MRS agar (Himedia - Mumbai, India) and incubation at 37 °C in anaerobic jars for 48 hours.

### pH, sugars and lactic acid determinations

The pH values of the samples during the growth study were determined through direct measurements in a Ion PH300 potentiometer at 25 °C.

For the determination of sugars and lactic acid, the cultures were first centrifuged at  $10,000 \times g$  for 10 minutes and the supernatant filtered using a Millex-GV, PVDF hydrophilic membrane (0.22  $\mu$ m pore size; Millipore, Billerica, MA, USA).

The sugars (glucose, fructose, galactose and rhamnose) were analysed using high performance anion exchange chromatography (HPAEC). This method has been used for a mixture of sugars, because of benefits like high sensitivity, simplicity, organic solvent freedom and no need for derivatisation (Cai et al., 2005, Guan et al., 2012). Aliquots (10  $\mu$ L) of the filtrate (PVDF membrane, 0.22  $\mu$ m, Millipore, Billerica, MA, USA) were automatically injected into the Ion Chromatography System (ICS5000, Dionex Canada Ltd., Oakville, Canada), coupled with pulsed amperometric detection (PAD, cell with disposable working gold electrode and pH-Ag/AgCl reference electrode, Dionex/Thermo Scientific) and equipped with a gradient pump (Model SP-5). The carbohydrates were separated using a CarboPac<sup>®</sup> PA1 analytical column [250 mm  $\times$  4 mm, 10  $\mu$ m particle size, polystyrene/divinylbenzene (2% cross-linked) substrate agglomerated with quaternary ammonium functionalised latex (5% cross-linked), DionexCarboPac<sup>®</sup>], but preceded by passage through a CarboPac<sup>®</sup> PA1 guard column (50 mm  $\times$  4 mm). The chromatographic runs were performed at a flow rate of 1.0 mL/min at 30 °C with gradient elution using ultra-pure water (resistivity of 18.2 M $\Omega$  cm, solvent A) and 0.25 mol/L NaOH (solvent B). Furthermore, the column was washed for 10 min after each injection using 0.2 mol/L NaOH, and the eluent ratio was returned to the initial conditions after 33 minutes, with a total run time of 47 minutes. The polypropylene bottles containing mobile phases were continuously pressurised with nitrogen gas to minimise contamination by carbonate. A quadruple potential waveform was applied ( $E$  = potential,

$t$  = duration) using the following settings:  $E_1 = +0.1$  V,  $t_1 = 400$  ms;  $E_2 = -2.0$  V,  $t_2 = 20$  ms;  $E_3 = +0.6$ ,  $t_3 = 10$  ms; and  $E_4 = -0.10$ ,  $t_4 = 70$  ms. Integration occurred from 200 ms to 400 ms during the  $E_1$  application.

Carbohydrate standards of glucose, fructose, rhamnose and galactose (all provided from Sigma, Brazil) were used to identify the different sugars on the basis of their retention times and for external calibration solutions. Chromatogram analysis was carried out using Chromeleon software version 6.8 (Dionex Corporation). The carbohydrate concentrations were expressed as the mean  $\pm$  standard deviation in grams of glucose, fructose, galactose or rhamnose per litre of sample.

Lactic acid production was monitored via High Performance Liquid Chromatography (HPLC-Shimadzu) equipped with a C18 column 5  $\mu$ m ODS 1 (4.6 mm x 250 mm) (Waters Spherisorb, Ireland) and a UV detector operating at 210 nm. Elution was conducted at 20 °C with a flow rate of 0.2 mL/min using  $H_2SO_4$  (2 mM) as the mobile phase.

#### Total Phenolics analysis

The total phenolic content was analysed according to the Folin-Ciocalteu method (Fang and Bhandari, 2011) using gallic acid as the standard.

#### Determination of antioxidant activity

The free radical 2,2 Diphenyl-1-picrylhydrazyl (DPPH) and the cation radical [2,2' azinobis (3-ethylbenzthiazoline sulfonic acid-6)] (ABTS<sup>+</sup>) methods were used to determine free radical scavenging potential of each sample.

The DPPH was performed according to Brand-Williams and others (1995) with modifications. Aliquots of 0.05 mL of each juçara pulp were added to 1 mL of acetate buffer (pH 5.5), 1 mL of absolute ethanol and 0.5 mL of DPPH (Sigma-Aldrich, Brazil) solution (0.25 mM). The reaction mixture was left in the dark for 30 minutes 25°C followed by measuring the absorbance at 517 nm. The quantification was calculated using a Trolox (6-hydroxy-2,5,7-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, Brazil) calibration curve ( $0.5 \cdot 10^{-4}$  to 0.02 mM) and the results expressed in micromole Trolox per millilitre of sample.

The cation radical, ABTS<sup>+</sup>, was prepared from ABTS [2,2' azinobis (3-ethylbenzthiazoline sulfonic acid-6)] (Sigma-Aldrich, Brazil), according to Sanches-Gonzales et al., (2005) with modifications. Before analysis, the ABTS<sup>+</sup> (7 mM to 2.45 mM potassium persulfate) solution was prepared in the dark for 16 hours. Upon analysis, the solution was diluted with sodium phosphate buffer (pH 7.4) to provide a solution of absorbance of 0.7  $\pm$

0.02 at 730 nm. The analysis took place by adding 30  $\mu$ L of sample and 6 mL of the diluted solution of ABTS<sup>+</sup>, and after 6 minutes of reaction, the absorbance was read at 730 nm. The quantification was based on a standard curve of Trolox (2.5 to 20 mM) and the results were expressed in micromole Trolox per millilitre of sample.

## RESULTS AND DISCUSSION

#### Chemical and mineral composition of the juçara pulp

The chemical composition of the juçara pulp used as fermentation substrate by probiotic lactobacilli is shown in Table 1. The protein and lipid contents of the extract were lower than those obtained by Borges et al. (2011), who observed a variation of 5.13 to 8.21% dry basis (db) protein and 18.45 to 44.05% (db) lipids in different cultivars of juçara analysed. Regarding the mineral composition, higher amounts of potassium were found in the extract in relation to the other minerals, followed by calcium, magnesium and phosphorus. The levels of iron in the present study were similar to those reported by Garbin (2011), (1.39 mg/100 g). However, the results of our study were higher for the other minerals (Ca: 16.88 mg/100 g, Mg: 5.25 mg/100 g, Mn: 0:26 mg/100 g, Zn: 0:25 mg/100 g and K: 5.51 mg/100 g).

Previous studies demonstrated that similarly to other types of fruits, factors such as geographical location of growth and harvest can significantly affect the chemical composition of juçara fruits (Borges et al., 2011) which justify the differences between the studies. Besides, the chemical composition showing high content of nutrients such as sugars and minerals, juçara pulp can become a promising nutrient for probiotic bacteria growth making a healthy product for human consumption (Ding, Shah, 2008).

#### Optimisation of *L. reuteri* and *L. plantarum* growth in juçara pulp

Since probiotic bacteria are found mainly in dairy products, it is important to study and determine the best conditions for fermentation in fruits and vegetables. For this reason, an experimental design was carried out to evaluate the effect of initial pH and fermentation temperature on *L. reuteri* and *L. plantarum* growth on juçara pulp. The results are presented in Table 2. According to the results, there are different behaviours between the two microorganisms under the different conditions tested. Temperatures below 15 °C were not favourable for the growth of *L. reuteri*, and a small increase in growth was observed for *L. plantarum* when extreme temperatures (10.8 and 39 °C) were used. This behaviour demonstrated the mesophilic characteristics of the studied strains as low and high temperatures were not favourable for growth of both bacterial species.

**Table 1: Chemical and mineral (db) composition of juçara pulp used for fermentation**

Chemical	Mean value±Standard deviation	Mineral*	Mean value±Standard deviation
Moisture (%)	97.11. (±0.10)	P	11.2 (±1.10)
Protein (%)	0.089 .(±0.04)	K	191.3 (±14.0)
Lipids (%)	0.08 .(±0.02)	Mg	13.0 (±0.30)
Soluble solids ( <sup>o</sup> Brix)	4.30 .(±0.20)	Ca	35.4 (±7.00)
Ash (%)	0.002. (±0.00)	Mn	0.9 (±0.07)
pH	4.90. (±0.10)	Fe	1.2 (±0.60)
Sugars (%)	2.80 .(±0.25)	Zn	0.45 (±0.01)
Total fibres (%)	ND		

ND: Not detected, \* Results expressed as mg/100 g, db = dry basis

**Table 2: Experimental design with real and coded values (in parentheses) for variables and responses on *Lactobacillus reuteri* (LR) and *L. plantarum* (LP) grown in juçara pulp after 48 hours of fermentation**

Assay	Initial pH LR	Initial pH LP	Fermentation temperature (°C)	log CFU/mL LR	log CFU/mL LP
1	4.0 (-1)	5.5 (-1)	15±0.5 (-1)	7.20±0.12	8.45±0.02
2	4.0 (-1)	5.5 (-1)	35±0.5 (+1)	8.54±0.06	8.63±0.18
3	6.0 (+1)	7.5 (+1)	15±0.5 (-1)	8.69±0.36	8.59±0.33
4	6.0 (+1)	7.5 (+1)	35±0.5 (+1)	8.70±0.50	8.10±0.02
5	3.6 (-1.41)	5.0 (-1.41)	25±0.5 (0)	7.49±0.09	8.81±0.10
6	6.4 (+1.41)	7.9 (+1.41)	25±0.5 (0)	8.83±0.23	9.04±0.12
7	5.0 (0)	6.5 (0)	10.8±0.5 (-1.41)	6.78±0.02	7.26±0.04
8	5.0 (0)	6.5 (0)	39.0±0.5 (+1.41)	8.78±0.10	7.72±0.05
9	5.0 (0)	6.5 (0)	25±0.5 (0)	8.93±0.05	8.81±0.04
10	5.0 (0)	6.5 (0)	25±0.5 (0)	8.90±0.10	8.83±0.03
11	5.0 (0)	6.5 (0)	25±0.5 (0)	8.91±0.07	8.83±0.05

Estimated effects of the independent variables ( $x_1 = \text{pH}$  and  $x_2 = \text{incubation temperature}$ ) on the response ( $Y = \log \text{CFU/mL}$ ) were also analysed, and according to these results, growth of *L. reuteri* was significantly influenced ( $p < 0.05$ ) by the initial pH and incubation temperature. Both quadratic effects were negative, indicating the presence of maximum bacterial growth. For *L. plantarum*, only the quadratic term of incubation temperature ( $x_2$  variable) had a significant effect ( $p = 0.002$ ) on cell viability, the region of greatest response.

The results shown in Table 2 were subjected to analysis of variance (ANOVA) and adjusted to a quadratic model, from which the equations 2 and 3 were obtained for the cell growth of *L. reuteri* and *L. plantarum*, respectively.

$$\text{Viability (log CFU/mL)} = 8.91 + 0.44\text{pH} - 0.30\text{pH}^2 + 0.52\text{T} - 0.49\text{T}^2 - 0.33\text{pH}\times\text{T} \quad (2)$$

$$\text{Viability (log CFU/mL)} = 8.82 + 0.109\text{pH}^2 - 0.61\text{T}^2 - 0.17\text{pH}\times\text{T} \quad (3)$$

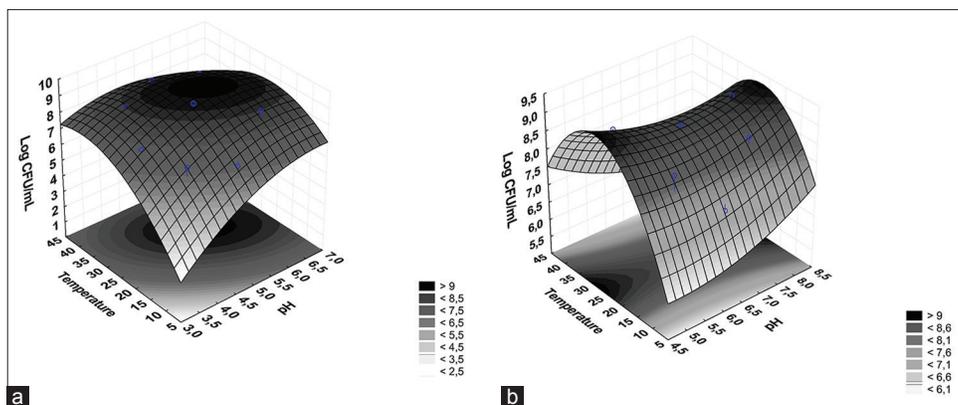
Where, T = temperature (°C) and pH = pH value.

The model obtained for *L. reuteri* growth was significant at the 95% confidence level, as the calculated value F (12.68) was higher than the tabulated value F (5:05). The determination coefficient for the regression was 0.92, indicating that the model could be used as a predictor and

was therefore used to generate the response surface curve (Fig. 1a). Similarly, the model obtained for *L. plantarum* was statistically significant at the 95% confidence level, and again, the calculated value of F (11:56) was higher than the tabulated value F (4:53). The percentage of variance explained by this model was 89%. The response surface was plotted and is shown in Fig. 1b.

It was verified that an increase in *L. reuteri* growth occurred at a temperature of 15 °C when the pH value ranged from 4.0 to 6.0 (Fig. 1a). Similarly, increasing the temperature increased the cell viability, reaching a maximum value in the range of pH 5.0 to 6.4 and temperatures between 25 and 35 °C. The optimal conditions for *L. reuteri* growth in juçara pulp were calculated based on the binomial pH x T, resulting in an initial pH of 5.7 and a fermentation temperature of 32 °C.

For the growth of *L. plantarum* (Fig. 1b), the pH ( $x_1$ ) variation in the range studied exerted little influence on viability. However, the variable  $x_2$  (incubation temperature) resulted in lower probiotic growth at higher (above 35 °C) or lower (below 15 °C) values than within the range of 20 °C and 30 °C. Best growth was observed at 25 °C and at pH values of 5.0 and 7.9. As the pH of the juçara pulp *in natura* was approximately 4.9, choosing an initial pH of 5.0 is more desirable than 7.9 because it may result in reduced costs associated with pulp neutralisation, as well as preventing operational risks with material contamination.



**Fig 1.** 3-D surface plots of (a) *L. reuteri*, and (b) *L. plantarum*. Cell viability (log CFU/mL) in juçara pulp as a function of initial pH and temperature (°C).

The results observed for the growth of *L. reuteri* and *L. plantarum* in juçara pulp were similar from those found in studies with other vegetables (Jaiswal et al., 2012). Yoon et al., (2006), while studying the fermentation of sterilised cabbage juice by *L. plantarum*, *L. casei* and *L. delbrueckii*, reported a growth of  $\sim 8.00$  log CFU/mL within 48 hours of fermentation at 30 °C. Pereira et al., (2011), while fermenting cashew apple juice with *L. casei*, found optimised values of pH 6.4 and 30 °C fermentation temperature. Costa et al., (2013) obtained optimised fermentation conditions with *L. casei* for sonicated pineapple juice with a pH of 5.8 and an incubation temperature of 31 °C. In the latter two studies, a slight variation was observed in conditions that resulted in higher biomass and viability. According to Pereira et al., (2011), the effect of initial pH and growth temperature depends on the substrate and the lactobacilli strains studied. Therefore, the use of an optimisation procedure such as the response surface methodology is an important tool when studying new plant matrices for the growth of probiotic bacteria.

### Fermentation process

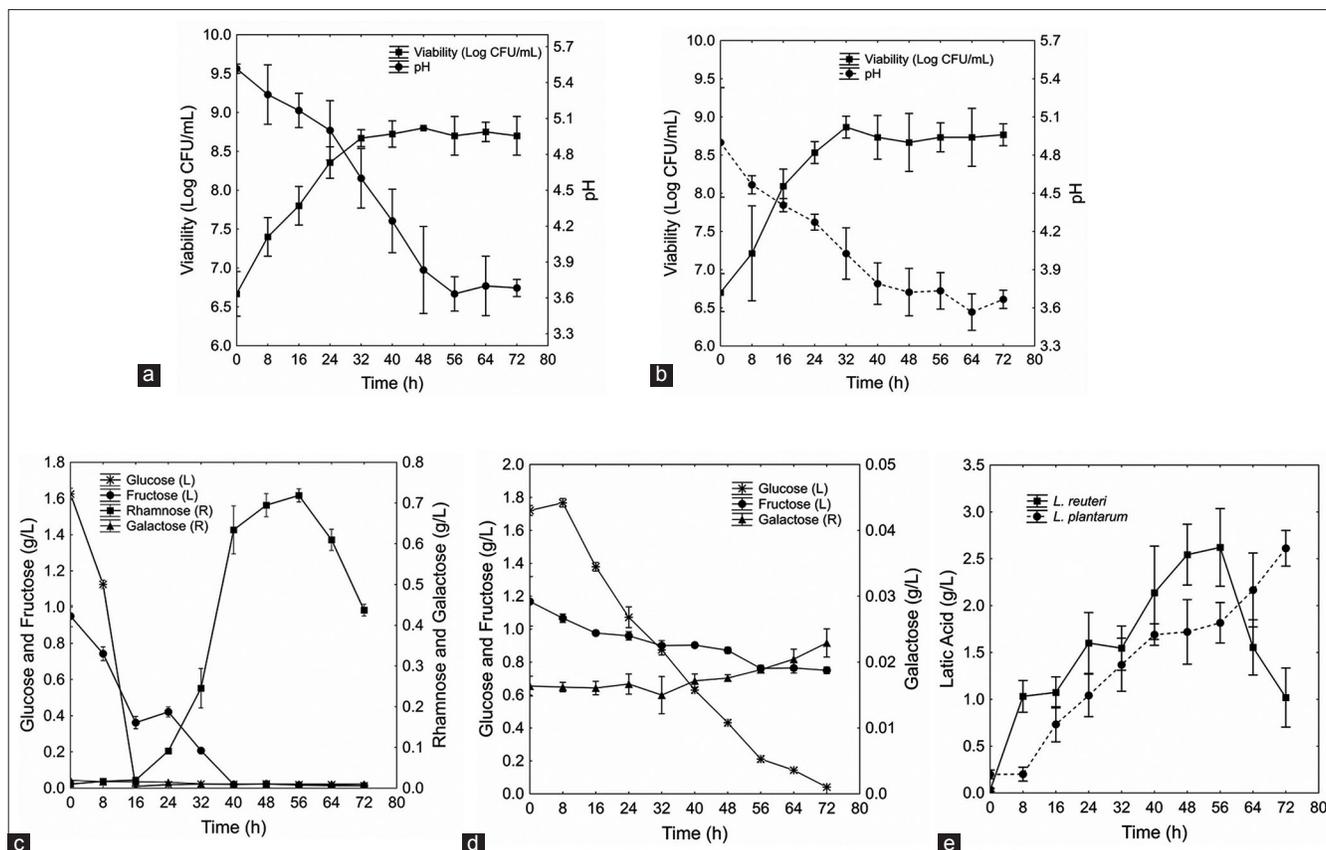
The optimum conditions for the growth of *L. reuteri* (pH 5.7 and incubation temperature at 32 °C) and *L. plantarum* (pH 5.0 and incubation temperature at 25 °C) in juçara pulp obtained from the optimisation experiments were used to evaluate the growth profile and fermentation time. Fig. 2 represents bacterial viability, pH and sugar consumption during fermentation of juçara pulp for 72 hours. It was observed that both *L. reuteri* (Fig. 2a) and *L. plantarum* (Fig. 2b) required a similar fermentation time to achieve the same cell count numbers, between 24 and 32 hours, reaching a maximum growth in the juçara pulp, with a viable count number of  $8.8 \pm 0.2$  log CFU/mL and  $8.6 \pm 0.2$  log CFU/mL, respectively. Similar cell counts were observed by Moreira et al. (2017), which obtained a survival of 8.33 log CFU/mL when evaluating a beverage containing a mixture of mango and juçara pulp with added *Lactobacillus rhamnosus* GG.

The present results are also interesting for commercial scale production, since in market products it is possible to find viable counts starting from 7 log CFU/mL depending on the product matrix and probiotic strain. For example, the American Company, Tropicana, produces beverages containing a mixture of natural juices with the addition of *Bifidobacterium lactis* presenting a count of 9 log CFU/portion, while the Perkii Company produces flavoured waters with *L. casei* (Lc431) at a concentration of 7 log CFU/portion (Wyvill, 2012).

Since cell viability is the main factor to be considered in functional probiotic products when stopping the fermentation at 30 hours. Apart from obtaining the highest number of viable cells, lower pH values are achieved from the formation of acids during fermentation, which increases safety during storage and prevents the development and colonising of pathogenic bacteria (Costa et al., 2013).

The pH decrease caused by lactic acid production occurred from the consumption of sugars present in the juçara pulp during *Lactobacillus* growth (Fig. 2c, d and e). These results are in agreement with other studies (Yoon et al., 2006, Di Cagno et al., 2011, Costa et al., 2013), suggesting that different vegetables such as tomato, cashew, cabbage, and banana can serve as adequate growth substrates for lactic acid bacteria, resulting in higher numbers during lactic acid production.

The major sugars present in the unfermented pulp were glucose ( $1.7 \pm 0.1$  g/L), fructose ( $1.1 \pm 0.1$  g/L) and trace amounts of galactose ( $0.015 \pm 0.001$  g/L). Both microorganisms preferentially used glucose for growth and lactic acid production. However, rapid consumption of glucose by *L. reuteri* occurred, and after 16 hours only trace amounts of glucose were detectable. After 40 hours of fermentation nearly all fructose present in the nutrient medium was consumed by *L. reuteri*. By contrast, the pulp fermented by *L. plantarum* showed a gradual decrease in glucose consumption rate, with total consumption achieved



**Fig 2.** pH values and cell viability (log CFU/mL) of *L. reuteri* (a) and *L. plantarum*; individual sugar consumption by *L. reuteri* (c) and *L. plantarum* (d); and lactic acid production (e) during 72 h fermentation of juçara pulp.

after 72 hours of fermentation. A small amount of fructose was fermented by *L. plantarum* during the study period.

The differences in sugar consumption with different fermentation times may also have caused differences in lactic acid production; *L. reuteri* reached the highest concentration after 48 hours of fermentation, while *L. plantarum* took 72 hours to achieve the same lactic acid concentration (Fig. 2e).

After 16 hours of fermentation with *L. reuteri*, the appearance of rhamnose was observed in the growth medium, which increased during the fermentation period, peaking at 56 hours (Fig. 2a). From 64 hours, a decrease in rhamnose concentration was observed, which may have been caused by the consumption of this sugar by the bacteria, since the other carbohydrate sources had already become exhausted during this period. The rhamnose present after 16 hours of fermentation may have originated from the hydrolysis of cyanidin-3-rhamnoside. According to De Brito et al. (2007), this anthocyanin is found at a concentration of 7 mg/100 g (db) in juçara (*E. edulis*), and some types of Lactic Acid Bacteria, such as *Lactobacillus* (including *L. reuteri*), *Streptococcus*, *Enterococcus*, and *Weissella*; which possessed  $\beta$ -glucosidase activity (Otieno et al., 2005,

Hayek, 2013) directly acting on the anthocyanin, forming aglycones with higher antioxidant capacities compared to the glycosylated forms (Spagna et al., 2000).

Carbohydrate and organic acid metabolism by *Lactobacillus* sp. also varies with species and depends on the substrate and fermentation time (Hou et al., 2000, Wang et al., 2003). Mousavi et al. (2011), evaluating the fermentation of pomegranate using the probiotics, *L. plantarum*, *L. delbrueckii* and *L. paracasei*, found that all of these species were able to grow and metabolise glucose and fructose, however, the consumption rate was different between the species.

### Total phenolic and antioxidant activity during fermentation

In the present study, methods measuring radical scavenging ability of DPPH<sup>-</sup> and ABTS<sup>+</sup> were used to estimate the antioxidant activity of fermented juçara pulp stored for various periods and the results are shown in Table 3. According to Roginski and Lissi (2005), the use of more than one assay is often appropriate to estimate antioxidant activity as each method has its limitations.

Based on the results, fermentation with both microorganisms showed a significant increase in total

**Table 3: Total phenolic compounds and antioxidant activity of juçara pulp fermented by *Lactobacillus plantarum* and *Lactobacillus reuteri* for 72 hours**

Time (hours)	<i>L. plantarum</i>			<i>L. reuteri</i>		
	Total phenolic compounds (mg GAE/100mL)	DPPH. (µg Trolox/100mL)	ABTS.+ (µg Trolox/100mL)	Total phenolic compounds (mg GAE/100mL)	DPPH. (µg Trolox/100mL)	ABTS.+ (µg Trolox/100mL)
0	447.3±2.00 <sup>a</sup>	136.4±10.61 <sup>a</sup>	54.4±3.53 <sup>a</sup>	473.7±0.67 <sup>a</sup>	138.3±5.43 <sup>a</sup>	61.9±10.84 <sup>a</sup>
16	454.6±2.71 <sup>a</sup>	133.6±5.74 <sup>a</sup>	59.9±1.88 <sup>a</sup>	489.3±5.35 <sup>a</sup>	160.2±0.88 <sup>b</sup>	63.6±5.43 <sup>a</sup>
24	514.43±7.36 <sup>b</sup>	156.4±7.98 <sup>a</sup>	60.1±0.23 <sup>a</sup>	482.7±16.06 <sup>a</sup>	165.2±10.61 <sup>b</sup>	67.4±2.12 <sup>a</sup>
32	536.7±4.01 <sup>bc</sup>	133.3±2.56 <sup>a</sup>	59.2±0.47 <sup>a</sup>	537.14±5.35 <sup>b</sup>	160.8±3.68 <sup>b</sup>	76.9±0.47 <sup>a</sup>
48	554.64±5.35 <sup>c</sup>	134.9±2.21 <sup>a</sup>	60.7±0.23 <sup>a</sup>	538±6.02 <sup>b</sup>	161.4±1.77 <sup>b</sup>	119.1±5.89 <sup>b</sup>
64	565.52±16.7 <sup>c</sup>	142.1±13.25 <sup>b</sup>	60.1±0.54 <sup>a</sup>	531.9±13.50 <sup>b</sup>	156.8±11.05 <sup>b</sup>	120.7±2.12 <sup>b</sup>
72	560.32±12.1 <sup>c</sup>	147.1±10.89 <sup>b</sup>	58.9±8.32 <sup>a</sup>	534.1±20.84 <sup>b</sup>	155.7±9.12 <sup>b</sup>	118.2±4.01 <sup>b</sup>

Different letters (a,b,c,d) in the same column are statistically different according to Tukey test carried out at 95 % of confidence level

phenolic content and in antioxidant activity measured by the DPPH method. Moreover, for the ABTS<sup>+</sup> method, an increase of 50% in antioxidant activity was observed for samples fermented with *L. reuteri*. Juçara pulp is a highly pigmented presenting an intense purple colour. According to some studies, the differences between antioxidant activities determined by the above two methods are higher in pigmented foods than in non-pigmented foods (Samaniego Sanchez et al., 2007; Dudonné et al., 2009; Floegel et al., 2011). Besides, the ABTS<sup>+</sup> method presents a stronger correlation with total phenolic than the DPPH assay (Chun et al., 2003; Floegel et al., 2011). These observations can explain the differences obtained in both methods observed in the present study, and a combination of both assays is therefore necessary to better elucidate the antioxidant activity in fermented juçara pulp, however according to Floegel et al., (2011), they are limited as they use non-physiological radicals. both methods are useful and popular in their application.

Although there were no results for antioxidant activity for juçara pulp in the literature, we found that the antioxidant activity was higher than that reported for cranberry, apple and grape juices commercially available in United States, which were  $99.7 \pm 3.1$ ;  $22.8 \pm 2.2$  and  $120.7 \pm 7.0$  (mg vitamin C equivalent (VCE). 100<sup>-1</sup>g) respectively, for the ABTS method, and  $67.2 \pm 6.2$ ;  $18.9 \pm 1.1$  and  $102.6 \pm 1.5$  (mg VCE. 100<sup>-1</sup>g) for the DPPH method (Floegel et al., 2011).

The increased antioxidant activity may be hypothesised as occurring as follows. Firstly, during fermentation of the juçara pulp, the composition of bioactive compounds could be modified by LAB, since fermentation may induce structural breakdown of plant cell wall materials in the pulp through a decrease of pH that creates the condition for bound phenolic constituents to be released through enzymatic processes, leading to the liberation and/or synthesis of various bioactive compounds. Secondly, as discussed above, lactic acid bacteria can possess the

enzyme  $\beta$ -glucosidase, which could modify the contents and structure of anthocyanins, changing from glycosides to the aglycon forms that result in an increased antioxidant activity (Dordevic et al., 2010, Hunaefi et al., 2013).

Some studies demonstrated that malvidin-3-glycoside could be converted to syringic and gallic acids (Fleschhut et al., 2006, Hidalgo et al., 2012), and ferulic and protocatechuic acids are mainly converted from cyanidin biotransformation (Kepler et al., 2005, Flores et al., 2015). Finally, the lower pH during fermentation process could have affected the extraction yield for phenolic content and antioxidant analysis, since the anthocyanins are relatively more stable at lower pH values (Ruenroengklin et al., 2008).

Besides this, the increase in antioxidant capacity of fermented foods with probiotic bacteria may be influenced by factors such as microorganism species, pH, temperature, water content, solvent, fermentation time, kind of food, and aerobic conditions (Hur et al., 2014). Additionally, studies suggest that the antioxidant activity may also be related to proteins present in the cell wall, or cell surface compounds such as exopolysaccharides (Kullisaar et al., 2002, Pan and Mei 2010, Li et al., 2012).

In relation to *L. reuteri*, Juarez et al. (2013) demonstrated that this strain had the highest capacity to reduce inflammatory and oxidative damages during endotoxin shock between others strains of *Lactobacillus*. This could be assigned to the capacity of its cell structure and their secreted molecules, which can explain the highest activity for *L. reuteri* in relation to *L. plantarum* found in the present study.

Although our study shows the influence of LAB fermentation in the juçara pulp for the first time, more detailed studies of the activities of relevant enzymes during fermentation are required to determine the exact mechanisms causing improvement of the nutritional effects (Dordevic et al., 2010).

### Storage stability

The juçara pulp fermented for 30 hours with *L. reuteri* (pH 5.7 and temperature at 32 °C), or *L. plantarum* (pH 5.0 and temperature at 25 °C), were maintained in glass vials at 4 °C for 28 days, and cell viability and antioxidant analysis were conducted every 7 days. During the 28 days of storage, the number of viable cells of *L. reuteri* and *L. plantarum* remained almost constant, maintaining numbers greater than  $8.7 \pm 0.3$  log CFU/mL until the end of the storage period (Table 4). The cell viability during storage is dependent on factors such as oxygen levels within products, oxygen permeability in packaging, fermentation time and storage temperature, among others (Shah 2000, Pereira et al., 2014). In the present study, was used glass bottles, which presents a very low oxygen permeability, and a low storage temperature (4 °C), which could explain the maintenance of cell viability in the fermented juçara pulp. Additionally, according to Shah (2007), in order to produce beneficial effects, the probiotic bacteria must be available and viable at concentrations of at least  $10^6$  CFU/g, and our product presented higher numbers than this right through until the end of storage period assessed.

Pereira et al. (2011), while studying *L. casei* in cashew apple juice fermentation (30 °C for 16 hours) with 42 days of refrigerated storage, reported an increased survival of the probiotic bacteria during the first 21 days of storage. By the 35<sup>th</sup> day, there was a small reduction from 8.72 log CFU/mL to 8.62 log CFU/mL in viable cell numbers.

The antioxidant activity of fermented juçara pulp for either *L. reuteri* or *L. plantarum* during refrigerated storage showed a significant increase as determined by both radical sequestration methodologies (Table 4). As discussed above, this observation may be explained by the increase in the low molecular compounds and by the conversion of existing anthocyanins by fermentation into the aglycone forms (Hussain et al., 2016). Thus demonstrating that higher antioxidant potential can occur even at low temperatures (4°C) (Otieno et al., 2005, Moraes Filho et al., 2014).

Additionally, Hornedo-Ortega et al. (2016) detected that the concentrations of the main anthocyanin compounds were preserved until 60 days of storage at low temperature (4 °C). This trend was also found by Di Cagno et al. (2011) evaluating mashed fermented cherries with addition of lactic starter cultures, and resulted in an increase in antioxidant activity until the end of storage period. Therefore, the fermentation process could be considered an ideal method for preservation and improvement of nutritional properties for juçara pulp even during storage, thereby providing additional health benefits for its consumption by humans.

### CONCLUSION

The maximal response conditions for the growth of each microorganism in juçara pulp was at 32 °C with a pH of 5.7 for *L. reuteri* and 25 °C with a pH of 5.0 for *L. plantarum*, and the proposed models presented a coefficient of determination of above 89% and can be used for predictive purposes. After 30 h of fermentation, the *L. reuteri* and *L. plantarum* showed counts above  $8.6 \pm 0.2$  log CFU/mL, with averages of 536.7 mg GAE/100mL of total phenolic compounds and an increase in antioxidant activity was also observed. During fermentation, *L. reuteri* consumed all glucose and fructose present in juçara pulp, and the appearance of rhamnose was observed, whereas *L. plantarum* consumed only glucose. After 28 days of refrigerated storage, the cell viability of probiotics remained at 8.7 log CFU/mL for both microorganisms with an increase in the antioxidant activity. This study showed for the first time that pure juçara pulp can be used as a carrier of probiotic bacteria, resulting in large numbers of viable cells under optimised conditions, and can be kept viable during storage under refrigeration.

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**Table 4: Viability of *Lactobacillus plantarum* and *Lactobacillus reuteri* (log CFU/mL), total phenolics and antioxidant activity of the fermented juçara pulp after storage for 28 days at 4 °C.**

Sample		log CFU/mL	DPPH (µmol Trolox/mL)	ABTS.* (µmol Trolox/mL)	Total phenolic (GAE/100mL)
<i>L. plantarum</i>	0 day	8.89±0.05 <sup>a</sup>	148.3±12.21 <sup>a</sup>	58.3±3.53 <sup>a</sup>	546.1±5.35 <sup>a</sup>
	7 days	8.81±0.02 <sup>a</sup>	158.5±3.53 <sup>b</sup>	63.9±2.14 <sup>b</sup>	631.7±5.67 <sup>b</sup>
	14 days	8.73±0.06 <sup>b</sup>	160.5±3.76 <sup>b</sup>	74.4±2.36 <sup>b</sup>	705.1±9.37 <sup>c</sup>
	21 days	8.71±0.13 <sup>ab</sup>	163.0±6.70 <sup>bc</sup>	76.5±1.50 <sup>b</sup>	717.4±4.01 <sup>c</sup>
	28 days	8.73±0.05 <sup>b</sup>	170.7±6.88 <sup>c</sup>	81.8±1.41 <sup>b</sup>	635.3±1.33 <sup>b</sup>
<i>L. reuteri</i>	0 days	8.92±0.05 <sup>a</sup>	161.8±5.63 <sup>a</sup>	55.8±2.40 <sup>a</sup>	531.1±3.73 <sup>a</sup>
	7 days	8.85±0.03 <sup>a</sup>	167.6±1.32 <sup>b</sup>	71.3±1.26 <sup>b</sup>	706.4±2.77 <sup>b</sup>
	14 days	8.80±0.10 <sup>ab</sup>	172.4±1.30 <sup>b</sup>	74.1±0.47 <sup>b</sup>	726.3±6.12 <sup>c</sup>
	21 days	8.72±0.07 <sup>b</sup>	174.3±4.43 <sup>bc</sup>	75.3±2.36 <sup>b</sup>	674.9±5.83 <sup>d</sup>
	28 days	8.71±0.05 <sup>b</sup>	179.1±2.65 <sup>c</sup>	76.6±3.53 <sup>b</sup>	659.0±10.1 <sup>d</sup>

Different letters (a,b,c,d) in the same column are statistically different according to Tukey test carried out at 95 % of confidence level

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### Authors' contributions

K. B. Guergoletto designed the study, collected test data and drafted the manuscript. C. S. I. Saori participated in improving the experiment and S. Garcia designed the experiment and revised the manuscript.

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