Probiotic cashew apple juice
Ana Lúcia Fernandes Pereira³, Tatiane Cavalcante Maciel⁴, Sueli Rodrigues⁵
³Federal university of Ceará, Food Technology Department, Fortaleza, Brazil (e-mail: sueli@ufc.br)

ABSTRACT
Nowadays probiotic beverages are mainly based on dairy products. The development of other probiotic beverages based on non-dairy products is important to diversify the market and to attend those people that for some reason do not want or cannot eat dairy products due to allergy, dietary preferences such as vegetarians, or other healthy issues. The aim of this study was to optimize the conditions of Lactobacillus casei growth in cashew apple juice, as well as, determine the inoculums amount and fermentation time. Moreover, it was investigate the survival ability of L. casei in cashew apple juice during refrigerated storage (4°C) for 42 days. Process optimization was done through an experimental design changing initial pH and fermentation temperature. Response surface methodology (RSM) was applied to the response variables (biomass and cell viability). The optimum conditions for probiotic cashew apple juice production were initial pH 6.4, fermentation temperature of 30°C, inoculums size of 7.48 Log CFU/mL and 16 h of fermentation process. It was observed that the Lactobacillus casei grew during the refrigerated storage. Viable cell counts were higher than 8.00 Log CFU/mL during all storage period (42 days). Cashew apple juice showed to be an efficient as dairy products for Lactobacillus casei growth. The fermented juice with Lactobacillus casei is a good and healthy alternative functional food containing probiotics. Cashew apple juice showed to be as efficient as dairy products for Lactobacillus casei growth.

Keywords: functional food, fermentation, tropical fruits, viability

INTRODUCTION
Anacardium occidentale L. is a tropical tree native to the northern and northeastern regions of Brazil. Its pseudofruit, known as the cashew apple, is the part of the tree that connects it to the cashew nut, the real fruit and a well-known product worldwide. The cashew apple is very popular and highly consumed as ready-to-drink and concentrated juice [1]. Representing 90% of the fruit, the cashew apple has high ascorbic acid and phenols content. Thus, cashew apple is considered a good source of antioxidant compounds [2,3]. The cashew apple is also rich in reducing sugars (fructose and glucose), minerals and some amino acids [4].

Probiotics represent probably the archetypal functional food, and are defined as alive microbial supplement, which beneficially affect the host by improving its intestinal microbial balance [5]. Multiple reports have described their health benefits on gastrointestinal infections, antimicrobial activity, improvement in lactose metabolism, reduction in serum cholesterol, immune system stimulation, antimutagenic properties, anti-carcinogenic properties, anti-diarrheal properties, improvement in inflammatory bowel disease and suppression of Helicobacter pylori infection by addition of selected strains to food products [6-9].

Probiotics have been added to yogurt and other fermented dairy products. However, an increased demand for non-dairy probiotic products comes from vegetarianism; milk cholesterol content, milk allergy and others factors [10]. This fact has led to development of probiotic products from various food matrices including fruits [11] and vegetables [12].

Technological advances have made possible to alter some structural characteristics of fruit and vegetables matrices by modifying food components in a controlled way [13]. This could make them ideal substrates for the culture of probiotics, since they already contain beneficial nutrients such as minerals, vitamins, dietary fibers, and antioxidants [14], while lacking the dairy allergens that might prevent consumption by certain segments of the population [15]. There is a genuine interest in the development of fruit juice based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods [14,16,17]. Sheehan, Ross and Fitzgerald [17] reported viable cell counts of 8.20 ± 0.01 Log CFU/mL of L. casei in pineapple juice. Fruits and vegetables have been suggested as ideal media for probiotic growth because they inherently contain essential nutrients, they are good-looking and have good taste [15,17]. However, the survival of probiotics in fruit-based matrix is more complex than in dairy products, because usually the bacteria need protection from the acidic conditions in these media [18]. Therefore, the aim of this study was...
to optimize the conditions of *Lactobacillus casei* cultivation in cashew apple juice, as well as, to determine the inoculums amount and the fermentation time for processing a probiotic beverage based on cashew apple juice fermented with *L. casei* B-442. Moreover, it was investigated the survivability of *L. casei* in cashew apple juice during refrigerated storage for 42 days [19].

**MATERIALS & METHODS**

**Cashew apple juice**

In this work, cashew apples from Embrapa’s Experimental Station, in Pacajus City (Brazil) were used. The juice was obtained through mechanical process by pressing the peduncles in expeller equipment (INCOMAP, Fortaleza, Ceará, Brazil). Tannins were removed by clarification with gelatin. The clarification process consisted of adding gelatin 1% (w/v) to the juice. After the gelatin dissolution, the juice was allowed to stand at 4 °C for precipitation of the tannins and suspended solids, which were removed by filtration with a cotton cloth. The clarified cashew apple juice was stored frozen (−20 °C) prior to use. No additive was added to the juice.

**Inoculums preparation**

Inoculums were prepared by transferring a glycerol stock culture tube of *L. casei* B-442 to a 250 mL Erlenmeyer flask containing 100 mL of sterile MRS broth. Cell cultivation was carried out statically in an incubator set at 37 °C until the cell density spectrophotometrically determined reach 0.600 that correspond to 9.00 Log CFU/mL, using MacFarland scale [19]. The cell density was spectrophotometrically determined at 590 nm. This culture was used as inoculums to the juice fermentation.

**Optimization of probiotic cashew apple juice production**

The optimum fermentation conditions was studied through a central composite rotated experimental design (CCRD), where the initial pH and temperature were changed from 4.29 to 7.11 and 10.44 to 41.44 °C respectively. The experimental domain was chosen based on the range that *Lactobacillus* can grow: pH from mild acid to neutral values and temperature from 2 to 53 °C [20]. Initial pH values of all experimental points were adjusted to reach the values of experimental design with NaOH (120 g/L). Erlenmeyers flasks containing 100 mL of clarified cashew apple juice were then inoculated with 7.00 log CFU/mL (1 mL of MRS broth containing 9.00 CFU/mL of *L. casei*). This concentration was chosen based on the recommendation for probiotic foods: minimal counts of 7.00 Log CFU/mL for better efficacy in regulating beneficial effects [21].

Fermentations were carried out statically in an incubator set for 24 h at different temperatures of experimental design. Biomass, viable counts and pH analyses were determined at the end of the process. At the optimal initial pH (6.4) and temperature (30 °C), 100 mL of cashew apple juice samples were inoculated with 7.00, 7.30 and 7.48 Log CFU/mL of *L. casei*, which corresponds to adding 1, 2 and 3 mL of activated inoculums prepared as previously described. Measurements of pH, biomass, viable counts and color were done each 2 h, during 24 h of fermentation to determine the best size of the inoculum and the proper fermentation time.

Fermented cashew apple juice containing probiotics was dispensed into sterile Erlenmeyers flasks closed with cotton plugs and stored at 4 °C. Biomass, viable counts, color and pH of cashew apple juice were recorded prior to cold storage and at intervals of 7 days, during 42 days.

**Biomass and cell viability determination**

Biomass was determined by optical density measured at 590 nm (Rodrigues, Lona, & Franco, 2003). The absorbance was measured after a 1:10 dilution of fruit juice with distilled water. The distilled water was used as blank. Biomass was calculated according to a calibration curve. The Results were calculated with Eq. (1) and given as mean value±SD.

Viable cell counts were obtained by serial dilution with sterile peptone water until 10⁻⁶ dilution. Aliquots of 0.1 mL of dilution were plated, in triplicate in plates containing MRS Agar (spread plate method). The plates were incubated for 72 h at 37 °C. Plates containing 20–350 colonies were measured and recorded as colony.
forming units (CFU) per mL of solution. According to Vinderola and Reinheimer [21], \textit{L. casei} has characteristics round white creamy colonies with diameters from 0.9 to 1.3 mm.

\textit{Statistics}

Statistica software version 7.0 (Statsoft, USA) was used to build the experimental design, the surface graphs, and to analyze the results. The results obtained were presented as mean values with standard deviation.

\textbf{RESULTS & DISCUSSION}

\textit{Fermentation Process Optimization}

Data obtained by carrying out the experimental design was fitted to the quadratic models given in Equations 1 and 2 for biomass and cell viability, respectively. F test and ANOVA analysis were used as significance criteria for the fitted models. All models were statistically significant at 95\% of confidence level since the calculated F values (7.39 for Eq. 3 and 20.79 for Eq. 4) were higher than the listed F value (F 5,5=5.05). Good correlation coefficients were also obtained (R^2=0.88 for Equation 1 and R^2= 0.95 for Equation 2).

\begin{align*}
\text{Biomass (g/L)} &= -0.13 - 0.05 \text{pH} - 0.004 \text{pH}^2 + 0.02 \text{T} - 0.0005 \text{T}^2 + 0.0005 \text{pH} \cdot \text{T} \quad (1) \\
\text{Viability (Log CFU/mL)} &= -4.41 + 2.15 \text{pH} - 0.15 \text{pH}^2 + 0.39 \text{T} - 0.006 \text{T}^2 - 0.008 \text{pH} \cdot \text{T} \quad (2)
\end{align*}

Where

- T  temperature (°C)
- pH  pH values

Figures 1 and 2 are the surface graphs built using Equations (1) and (2). The initial pH did not significantly influence the biomass. The increase in temperature caused an increase of \textit{L. casei} biomass followed by a decrease at temperature values above 35°C. Therefore, the optimum temperature for \textit{L. casei} growth in cashew apple juice was around 35°C. High viable cell counts were observed at medium fermentation temperatures, being maximum values at 30°C (Figure 2). Temperatures higher than 30°C caused viability losses. The highest (optimum) microbial viability was obtained at pH 6.4 and fermentation temperature of 30°C (critical point of Equation 2, according to the statistic program).

The optimum process condition for microbial viability (initial pH 6.4 and temperature of 30°C) was chosen for the evaluation of fermentation time and inoculums amount. According to Figure 3, after 2 hours of fermentation, cashew apple juice inoculated with 7.30 and 7.48 Log CFU/mL of \textit{L. casei}, presented a fast growth. At 6 hours of fermentation viable cell counts of 8.04±0.00 and 8.08±0.00 Log CFU/mL, respectively, was observed for these assays. The microbial viability increased until 14 h of fermentation in cashew apple juice.

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{Figure1.png}
\caption{Figure 1. Response surface graph for biomass formation.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{Figure2.png}
\caption{Figure 2. Response surface graph for biomass formation.}
\end{figure}
Although the maximal cell viability was obtained at 14 h of fermentation in the cashew apple juice inoculated with 7.48 Log CFU/mL, the fermentation time of 16 h was chosen because the pH was below 4.6 (data not shown), which inhibit the pathogenic microorganisms’ growth. It has been reported that the decrease in the pH of the medium and accumulation of lactic acid, diacetyl, and acetaldehyde from growth and fermentation are the main factors for viability loss of probiotics added to milk [22]. Therefore, the fermentation time of 16 h was chosen as favorable to maintain the probiotic cashew apple juice conservation and microorganism viability.

Our results are in agreement to the previously reported for vegetable juice. *L. plantarum* and 30ºC *Pediococcus pentosaceus* grew well in carrot juice with initial pH of 6.4 [23]. Yoon, Woodams and Hang [12] found that *L. plantarum*, *L. casei* and *L. delbrueckii* grew rapidly on sterilized cabbage juice without nutrient supplementation reaching nearly 8.00 Log CFU/mL at 48 h of fermentation at 30 ºC. It is important to emphasize that in the present study, no heat treatment was necessary and there was not contamination evidence. Thus, the *L. casei* overlapped and controlled other microbial growth and juice spoilage avoiding costs with heating treatment, as well as, its adverse effects, as nutritional losses and sensory changes.

![Figure 3. Biomass (g/L) and viability (Log CFU/mL) of *Lactobacillus casei*, inoculated at 7.00Log CFU/mL, 7.30 Log CFU/mL and 7.48 Log CFU/mL, in cashew apple juice during 24 h of fermentation.](image)

Storage stability

The probiotic cashew apple juice was produced in the follow conditions: initial pH of 6.4, fermentation temperature of 30 ºC, inoculums size of 7.48 Log CFU/mL of *L.casei* in 100 mL of juice and 16 hours of fermentation. The probiotic juice was stored for 42 days. The viable cell counts increased from 8.41±0.04 Log CFU/mL, at the storage beginning to 8.72±0.07 Log CFU/mL, at the day 21º of storage at 4ºC. From day 35º, there was a slight reduction of viable cell counts to 8.62±0.04 Log CFU/mL (Figure 4). Despite, the reduction of the viable cells at the end of the storage period, the viable cell counts of *L. casei* was higher than 8.00 Log CFU/mL, which is considered a great value for fermented products containing probiotics (Shah, 2007).

The results presented herein are in agreement with those presented by Yoon, Woodams and Hang [24] who evaluated the viability of *L. plantarum* and *L. delbrueckii* in fermented red beets juice (30ºC for 72 h), during refrigerated storage for 28 days. These authors reported that although the lactic cultures in fermented beet juice gradually lost their viability during cold storage, the viable cell counts in the fermented beet juice still remained at 6.00-8.00 Log CFU/mL after 28 days of cold storage at 4 ºC. It is important to have a significant number of viable lactic acid bacteria in the product for maximum health benefits. According to the storage assay carried out in the present study, the viable cell counts were higher than 8.00 Log CFU/mL along the whole storage period (42 days).
CONCLUSION

*L. casei* presented good growth and viability in cashew apple juice and no appreciable viability losses were observed during 42 days of storage. This results makes cashew apple juice a good vehicle for probiotic bacteria. *L. casei* was able to overcome the natural microbiota of cashew apple juice dismissing thermal treatment and, thus, reducing the production costs.

ACKNOWLEDGMENTS

Authors thank CNPq for the financial support through the National Institute of Science and Technology of Tropical Fruit and FUNCAP for the scholarship.

REFERENCES


