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Non-dairy cashew nut milk as a matrix to deliver probiotic bacteria

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Abstract
Cashew nut milk was evaluated as a matrix to deliver commercial probiotic strains (*Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus plantarum*) in order to develop a non-dairy probiotic beverage. The first step was to evaluate the viability of the probiotic strains. All evaluated bacteria were able to survive in this food matrix under 4 °C for 30 days. So, *B. animalis* was the selected strain to perform a stability test of the beverage by monitoring the pH, color, sensory and microbiological quality during refrigerated storage for 30 days. During storage, *B. animalis* counts were above 10^7 CFU.mL^{-1}, the microbiological quality was maintained and there was no significant difference in whiteness. A significant pH decrease was observed, but it did not affect the beverage's sensory acceptance, indicating that cashew nut milk is an adequate vehicle for delivering probiotics.

Keywords: plant-based beverages; non-dairy milk; dairy substitute.

Practical Application: Development of a probiotic cashew nut milk.

1 Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014), mainly through the process of replacing or including beneficial bacteria in the gastrointestinal tract (Ranadheera et al., 2017). Many probiotic cultures are available for application in food matrices with the objective to develop new functional products (Champagne et al., 2018; Santos et al., 2018). Although the most common vehicle for delivery of probiotics are dairy products (Murtaza et al., 2017; Tomar, 2019), there is currently a demand for probiotics in non-lactic matrices based on fruits, vegetables and cereals (Ranadheera et al., 2018).

Cashew nuts present high protein levels (23%) with all the essential amino acids for humans, and lipids (44%) (Freitas et al., 2012), with mono and polyunsaturated fatty acids being the major components (Adjepong et al., 2017). As such, they are a distinct matrix possibility for probiotic delivery.

Cashew nuts are one of the most important edible nuts in international trade. However, its commercial processing yields up to 40% of broken kernels (Lima et al., 2017), which have a low commercial value when compared with the entire nut. Aiming at using the buts, splits and pieces of nuts, *Embrapa Agroindústria Tropical* has been developing a non-dairy milk for people who cannot or do not want to consume lactose (Lima et al., 2018). This product is a sugar added water soluble extract which resembles milk in appearance, with good nutritional content and pH around 6.5 (Lima et al., 2018), reinforcing its use as a promising matrix for the delivery of probiotics.

Most commercial probiotics available on the food market are *Lactobacillus* and *Bifidobacterium* species (Shori, 2016). However, their incorporation in plant based beverages is a great challenge, mainly considering cell viability maintenance during the production, shelf life and consumption (Céspedes et al., 2013; Shori, 2016).

Thus, the aim of this study was to evaluate cashew nut milk as a food matrix of probiotic delivery by incorporating an adequate commercial probiotic strain into a non-dairy beverage and to assess the cell viability and physicochemical and sensorial characteristics during refrigerated storage for 30 days.

2 Materials and methods

2.1 Bacterial strains

Three commercial probiotic strains - *Bifidobacterium animalis* BB-12® (Christian Hansen, Hørsholm, Denmark), *Lactobacillus acidophilus* Howaru® Dophilus (Danisco, Copenhagen, Denmark,) and *Lactobacillus plantarum* Lyofast SP-1 (Sacco S.R.L., Cadorago, Italy,) were used. Freeze dried strains were reconstituted according to the manufacturer's instructions. Next, a working stock was prepared for each strain as following: *Lactobacillus* cells were cultivated in MRS broth (Difco, Le Pont de Claix, France) while *Bifidobacterium* was grown in MRS broth with cysteine 0.1% (24h, 37 °C, anaerobiosis). Next, an aliquot (0.1 mL) of each probiotic was activated in MRS or MRS with cysteine for 16hs for two consecutive times. Then the cultures were washed twice with phosphate saline buffer (1.0 M, pH 7.4), centrifuged (3300 *g*, 15 min, 5 °C) (EBA 12R, Hettich, Tuttlingen, Germany)

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and resuspended in a solution of 10% maltodextrin and 10% lactose to a final concentration of 10^{10} $CFU.mL^{-1}$. Aliquots of 1 mL of each suspension were frozen at -80 °C and used directly in the beverage manufacture.

2.2 Milk manufacture

Broken cashew nuts (buts, splits and pieces) were obtained from a local supplier in Fortaleza, in the Northeastern region of Brazil. Nuts were triturated in a food processor (Robot Coupe R201 Ultra E, Jackson, MS, USA) with the use of a stainless steel cutter. Refined cane sugar was purchased in the local market. The cashew nuts were ground with water (1:10) and 3% sugar in a colloid mill (Meteor Rex Inox I-V-N, São Paulo, Brasil) for 4 minutes. The extract was sterilized by ultra-high temperature (140 °C, 4 s) in a tubular heat exchanger (Armfield FT74, Ringwood, England), cooled at 80 °C, packed into glass bottles (200 mL) and sealed with plastic screw caps. After reaching 30 °C, probiotics were aseptically inoculated to a final concentration of $10^8$$CFU.mL^{-1}$ and the beverage was stored at 4 °C for 30 days.

2.3 Probiotic viability

In order to determine the survival of different commercial probiotics in cashew nut milk, three strains - *Bifidobacterium animalis* BB-12®, *Lactobacillus acidophilus* Howaru® Dophilus and *Lactobacillus plantarum* Lyofast SP-1 - were inoculated separately after preparing the milk. Samples were taken just after inoculation (time 0) and after 30 days of storage at 4 °C. A beverage without probiotic was used as control. The survival of each probiotic in refrigerated cashew nut beverage as well as the control were evaluated by counting lactic acid bacteria (LAB) at days 0 and 30 in MRS agar at 37 °C/48h and anaerobiosis (Harrigan, 1998).

2.4 Beverage stability

The stability test (pH, color, sensory acceptance and microbiological quality) was carried out with the cashew nut milk with the probiotic strain selected in the previous test added. Samples were stored at 4 ± 2 °C, and analyzed after processing (time 0) and at 15 and 30 days of storage.

The pH was measured according to Association of Official Analytical Chemists (1997) guidelines. The color was assessed using a colorimeter (Chroma Meter CR-400, Konica Minolta Sensing Inc., Osaka, Japan) to determine the L*a*b* values using D65 illuminant, and the whiteness index was calculated using the formula: $WI=100-[(100-L^*)^2 + (a^*)^2 + (b^*)^2]^{1/2}$ (Hirschler, 2012). Analyses were performed in triplicate.

Sensory acceptance tests were applied to 50 judges at each evaluation time using a 9-point hedonic structured scale, in which 1 was extremely dislike and 9 was extremely like for the overall acceptability of the beverage (Meilgaard et al., 1999). Judges were also asked about their purchase intent using a 5-point structured scale ranging from 1 (I certainly wouldn't buy it) to 5 (I certainly would buy it). Results are shown as mean values.

Results from pH, color and sensory acceptance were submitted to variance analysis using the SAS statistical program for Windows (Statistical Analysis System, 2009) and were compared with the Tukey test when significant ($p<0.05$).

Microbiological stability was monitored by counting of LAB (Harrigan, 1998). Analyses of coliforms (Feng et al., 1998), *Staphylococcus aureus* (Bennett & Lancette, 1998) yeasts and molds (Tournas et al., 1998) and *Salmonella* detection (Andrews et al., 1998) were performed.

3 Results and discussion

3.1 Probiotic behavior in the cashew nut milk

All strains remained viable during 30 days of refrigerated storage (Table 1), therefore evidencing that the survival of microbial cells was not affect by the food matrix. Considering that the BB-12® strain has been successfully used in commercial products with recognized functional effects in clinical studies (Garrigues et al., 2010), it was chosen to be added to the probiotic cashew nut milk in the stability test.

3.2 Probiotic milk stability during refrigerated storage

There was a significant decrease in pH value ($p < 0.05$), which changed from 6.45 to 5.65 (Table 2) after 30 days of storage. Pimentel et al. (2015) studied the supplementation of clarified apple juice with a commercial *Lactobacillus paracasei* ssp. *paracasei* probiotic and also verified a decrease in pH during 28 days of refrigerated storage. According to these authors, this occurred because probiotic microorganisms could have metabolized the simple sugar present in the juice or because the juice sugars were hydrolyzed by the enzymes (hydrolases) released from dead bacteria.

Although pH decreased during the cashew nut beverage storage, BB12 remained viable for all of the evaluated period (Table 3). In fact, the growth of species from *Bifidobacterium* genus only stops at pH values below 4.5 (Holt et al., 1994).

Table 1. Viability of commercial probiotic strains in cashew nut milk during 30 days.

Probiotic	Day 0 (log CFU/mL)	Day 30 ((log CFU/mL)
	0	0
Control (cashew nut milk without probiotics)	0	0
Bifidobacterium animalis BB-12®	8.72	8.49
Lactobacillus acidophilus Howaru® Dophilus	8.17	8.89
Lactobacillus plantarum Lyofast SP-1	8.04	8.38

Table 2. pH and color monitoring of cashew nut milk during storage.

Time (days)	pH	L*	a*	b*	Whiteness index
0	6.45 a	81.66 b	-0.95 b	9.39 a	79.37 a
15	6.02 b	83.95 a	-0.57 a	9.95 a	81.10 a
30	5.65 c	83.89 ab	-0.36 a	9.49 a	81.30 a

Samples followed by the same letter in columns are not significantly different (Tukey, $p>0.05$).

Table 3. Microbiological characterization of nut beverage during storage.

Sample	LAB* (CFU.mL^{-1})			Coliforms (MPN.mL^{-1})			*Staphylococcus aureus* (CFU.mL^{-1})			*Salmonella* sp./25 mL			Yeast and mold (CFU.mL^{-1})		
	0	15	30	0	15	30	0	15	30	0	15	30	0	15	30
1	5.2 x 10^8	1.8 x 10^8	1.4 x 10^8	<3	<3	<3	<100	<100	<100	-	-	-	<100	<100	1.3 x 10^4
2	5.8 x 10^7	2.2 x 10^8	3.9 x 10^9	<3	<3	<3	<100	<100	<100	-	-	-	<100	<100	<10
3	2.3 x 10^7	4.1 x 10^8	1.7 x 10^8	<3	<3	<3	<100	<100	<100	-	-	-	<100	<100	<10

Counts; Time (days).

*LAB = Lactic acid bacteria.

Nualkaekul et al. (2011) studied the survival of *Bifidobacterium longum* NCIMB 8809 during refrigerated storage for 6 weeks in model solutions and constructed a mathematical model describing cell survival as a function of pH, citric acid, protein and dietary fiber. According to these authors, all four factors had a significant negative effect ($p < 0.05$) on the bacterial viability, with pH and citric acid being the most influential.

The whiteness index is one of the most important quality parameters for milk, and there were no significant differences observed in the index during the storage time (Table 2). It was also observed that the mean value obtained (80.59) was similar to the value reported for bovine milk (81.89) (Jeske et al., 2017). Salmerón et al. (2015) studied the effect of probiotic lactic acid bacteria on the physicochemical composition and acceptance of fermented cereal beverages, and related that the color of probiotic fermented cereal beverages was characteristic to the cereal substrate used during their formulation.

The slight changes observed in pH during storage did not affect the sensory acceptance of the milk which remained at an average value of 6.92 throughout the 30 days of storage, corresponding to an evaluation of "moderately like" on the hedonic scale. The mean value for purchase intent was 3.73, between the answers "I'm not sure if I would or would not buy it" and "I would probably buy it".

Microbial counts during 30 days of storage remained below 3 MPN.mL^{-1} for fecal and total coliforms; 10^2 CFU.mL^{-1} for *Stapylococcus aureus*; 10^2 CFU.mL^{-1} for yeasts and moulds; and there was no detection of *Salmonella* in 25 mL of cashew nut milk. Because there is no specific legislation for this kind of product in Brazil, the parameters for pasteurized and refrigerated juices (10 MPN.mL^{-1} coliforms 45 °C and absence of *Salmonella* sp/25 mL) were considered and the nut beverage is classified as microbiologically safe for human consumption (Brasil, 2001). Moreover, the LAB count remained above 10^7 CFU.mL^{-1}, showing that BB12 survived in the beverage matrix during all 30 days of storage. Céspedes et al. (2013) determined cell viability in non-dairy drinks of two commercial probiotics, *Lactobacillus casei* LC-01 and *L. casei* BGP 93, and found at least one non-dairy drink to offer cell counts around 7 log orders until the end of the storage period for both strains. Pimentel et al. (2015) detected a rapid loss of viability of probiotic culture LC-01 during probiotic and symbiotic apple juice refrigerated storage; however they demonstrated the product shelf life would be 14-28 days under 4 °C, depending on the type of product (probiotic or symbiotic) and package used. Lastly, Lupien-Meilleur et al. (2016) studied the viability of three different commercial probiotics in maple sap, and highlighted the importance of testing the probiotic viability in novel food carriers.

4 Conclusion

Considering the viability maintenance of microorganisms for 30 days at 4 °C, cashew nut milk was shown to be a good matrix for probiotic (*Bifidobacterium animalis*, *Lactobacillus acidophilus* and *L. plantarum*) delivery. It was also demonstrated that it was possible to obtain a probiotic cashew nut beverage by adding a *Bifidobacterium animalis* BB-12® strain as probiotic culture to the nut milk without significant changes in whiteness, and with good sensorial acceptance and microbiological quality.

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