Inovação em Ciência e Tecnologia de Alimentos 2

Vanessa Bordin Viera
Natiéli Piovesan

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APRESENTAÇÃO

O *e-book* Inovação em Ciência e Tecnologia de Alimentos – Vol 1, 2 e 3, traz um olhar integrado da Ciência e Tecnologia de Alimentos. A presente obra é composta por 86 artigos científicos que abordam assuntos de extrema importância relacionados às inovações na área de Ciência e Tecnologia de alimentos.

No volume 1 o leitor irá encontrar 28 artigos com assuntos que abordam a inovação no desenvolvimento de novos produtos como sucos, cerveja, pães, *nibs*, doce de leite, produtos desenvolvidos a partir de resíduos, entre outros. O volume 2 é composto por 34 artigos desenvolvidos a partir de análises físico-químicas, sensoriais, microbiológicas de produtos, os quais tratam de diversos temas importantes para a comunidade científica. Já o volume 3, é composto por 25 artigos científicos que expõem temas como biotecnologia, nutrição e revisões bibliográficas sobre toxinfecções alimentares, probióticos em produtos cárneos, entre outros.

Diante da importância em discutir as inovações na Ciência e Tecnologia de Alimentos, os artigos relacionados neste *e-book* (Vol. 1, 2 e 3) visam disseminar o conhecimento e promover reflexões sobre os temas. Por fim, desejamos a todos uma excelente leitura!

Vanessa Bordin Viera
Natiéli Piovesan

SUMÁRIO

CAPÍTULO 34

VIABILIDADE DE *BACILLUS CLAUSII, BACILLUS SUBTILIS E BACILLUS SUBTILIS VAR NATTO* EM NÉCTAR E POLPA DE CAJU

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RESUMO: Este estudo teve como objetivo verificar a viabilidade do gênero *Bacillus* como microrganismo probiótico em néctar e polpa de caju. Foram avaliadas as cepas de *Bacillus clausii, Bacillus subtilis* e *Bacillus subtilis* var natto em néctares e polpas de caju comerciais ao longo de 60 dias em conservação refrigerada à 4°C para os néctares e -12°C para as polpas. Além disso foram avaliados esses mesmos produtos numa conservação de atmosfera acelerada de 24°C para néctares e 4°C para as polpas. Os resultados foram expressos em média média ± desvio padrão da média e avaliados a significância das diferenças entre as médias através da análise de variância e teste de Tukey ao nível de 99% de confiança. Tanto na conservação normal como acelerada de néctares e polpas de caju, tiveram como resultados a efetiva viabilidade dos microrganismos probióticos. Os resultados foram em média de 10^9, 10^{10} e 10^{10}UFC/mL para *Bacillus clausii, Bacillus subtilis* e *Bacillus subtilis* var natto, respectivamente. Assim, comprovou-se que os néctares e polpas de caju são substratos viáveis para microrganismos probióticos.

PALAVRAS-CHAVE: viabilidade; probióticos; não-lácteos; caju.

VIABILITY OF *BACILLUS CLAUSII, BACILLUS SUBTILIS AND BACILLUS SUBTILIS VAR NATTO* IN CASHEW NECTAR AND PULP

ABSTRACT: This study aimed to verify the viability of genus *Bacillus* as probiotic microorganisms in cashew nectar and pulp. Strains of *Bacillus clausii, Bacillus subtilis* and *Bacillus subtilis* var natto were evaluated in commercial cashew nectar and pulp along 60 days in refrigerate conservation at 4°C for nectars and -12°C for pulps. Besides, the same products were evaluated in conservation of accelerated atmosphere of 24°C for nectars and 4°C for pulps. Results were expressed in mean, mean ± standard deviation of the mean and evaluated the significances of differences between means by variance analysis and Tukey's test at a level of 99% of confidence. Both in normal as well as in accelerated conservation of cashew nectar and pulps had as results the effective viability of the probiotic microorganisms. Results were in average 10^9, 10^{10} and 10^{10}CFU/mL for *Bacillus clausii, Bacillus subtilis* and *Bacillus subtilis* var natto, respectively. Therefore, it was proved that cashew nectar and pulps are viable substrates for probiotic microorganisms.

KEYWORDS: viability; probiotic; non-dairy; cashew.

1 I INTRODUCTION

Functional food has potential to promote health by means of mechanisms not previewed by conventional nutrition (SANDERS, 1998), being important to highlight that this effect is restricted to promoting health and prevention of diseases, not to heal them. Thus, there is interest in developing functional food and beverages in order to improve health and the well being of consumers, mainly the one that creates a beneficial effect to the intestine, which dominates the market of functional food (LUCKOW et al, 2004).

This market niche has been representing a high growth in the last years, reflecting the concern and interest of consumers in taking food that bring benefits to health. Among the functional food, highlight the ones having probiotic cultures, those being internationally defined as living microorganisms that, when administered in proper amounts, bring benefits to the health of the host (SHEEHAN; ROSS; FITZGERALD, 2007; FAO/WHO, 2002). In this context, the intake of sources of probiotic microorganisms is growing as a global trend (RAIZEL et al., 2011).

Among the factors influencing the viability of probiotic bacteria in the elaborated product, must be highlighted genus, species and strains of the microorganism; the formulation and composition of the food (acidity, content of usable carbohydrates; nitrogen sources, mineral content and water activity) to which they were added; the physical condition of storing (time and temperature), and possible interactions of probiotics (bacteriocins, antagonism, synergism) (DEL PIANO et al., 2006).

Probiotic cultures have been added, mainly, to yogurts and to other fermented milk products. The introduction of those microorganism in non-dairy products would allow its consumption by lactose intolerant, allergic to milk proteins, hypercholesterolemic people who refuse to ingest dairy products due to particular reasons, such as vegetarian people or when those products are not reachable (RIVERA-ESPINOZA AND GALLARDO-NAVARRO, 2010).

According to Sheehan, Ross and Fitgerald (2007), fruit juices may represent an ideal vehicle of probiotic cultures to consumers, since they are not regularly consumed, this being an essential factor for the probiotics to exercise their functions. Luckow et al (2006) comment that juice fruits have been suggested as a proper mean for adding probiotic cultures, since they are considered as healthy food products and are consumed by a large parcel of the population, besides not containing starter cultures which compete by substrates with the probiotics; they are usually supplemented with ingredients that promote anaerobic, such as ascorbic acid; and have sugars fermentable by the probiotics.

According to Fernandes et al. (2009) and the Brazilian Fruits Institute (IBRAF, 2015), the production of juice fruits in the scene of the national and international agribusiness is seen as one of the most promising activities of the food sector, being Brazil considered as one of the greatest world producers of tropical fruits.

In the northeast region of Brazil there is a great cashew fruit farming, which normally is consumed as fresh or processed fruits (ARAÚJO, SILVA, MOREIRA, NARAIN and SOUZA, 2011). From its pseudo fruit different products and sub products can be obtained such as integral juice, reconstituted juice, tropical juice, pulp, among others (MATTA et al., 2010).

Considering the relevance of functional food in human health, the benefits caused by ingesting probiotic microorganisms and the fact that sources of probiotic food are still very limited to dairy products, it is essential that new probiotic food products are researched, in order not only to widen the market of those products, but also to have an option to the ones that cannot or do not enjoy the consume of dairy products. Considering this, the present study had the objective of evaluating the viability of Bacillus clausii, Bacillus subtilis and Bacillus subtilis var natto in cashew nectar and pulp.

2 I MATERIAL AND METHODS

2.1 Material

Samples

Nectars (50% of pulp) and pulp (pasteurized) of fruits used in analyzes were obtained from donation of companies acting in this sector, seated in the state of Sergipe. All samples were stored complying with the adequate temperatures of conservation and hygienic-sanitary conditions (FELLOWS, 2006).

Microrganisms

Vegetative cells of Lactobacillus casei were obtained in the form of flakes (Christian Hansen®).

Spores of Bacillus clausii (B.claussi) were commercially bought in drugstore in the form of little bottles (Enterogermina®), containing according to information from the manufacturer 10^9CFU of spores of the microorganism by bottle unity (5mL).

Bacillus subtilis (B. claussi) was obtained from strains cultivated in laboratory. Bacillus subtilis var. natto was isolated from natto (japanese fermented food) marketed in the "Companhia de Entrepostos e Armazéns Gerais de São Paulo (CEAGESP)" in São Paulo-Brazil.

2.2 Activation, inoculation and analysis of viability of Lactobacillus casei and Bacillus subtilis

The Lactobacillus were activated in soup MRS (From Man, Rogosa and Sharpe) during 12 hours at 37°C, statically. While the vegetative cells of Bacillus subtilis were activated in medium BHI (Brain Heart Infusion) during 24hs at 37°C in incubator under stirring of 200 rotations per minute (rpm), described by Pereira et al. (2011). The initial counting of the microorganisms was made using the technique of pour plate for L.casei and plate spread for B. subtilis. After this initial counting, inoculations in concentration of 10^6CFU/mL of probiotic microorganisms were made in cashew nectars and pulp, packed at temperature of 4°C (nectars) and -12°C (pulps).

For analysis of viability serial dilutions until 10^{-3} were executed and aliquots of 100μL taken, adding them in plates with medium MRS for L. Casei and MH (Muller Hilton) for Bacillus subtilis. After this procedure, plates were incubated in greenhouse at temperature of 37°C during 24h, for counting the unities forming colonies, made manually.

2.3 Bacterial sporulation of B. subtilis and Bacillus subtilis var natto

First, was executed the Bacillus activation in medium BHI during 24h at 37°C in incubator under stirring of 200rpm.

The bacterial sporulation of probiotic cultures were made in growth medium as described by Tavares et al. (2013), in F medium, composed by 1% of glucoses, 0.1% of L-glutamate de diethyl, 0.05% of yeast extract, 0.5% of KH_2PO_4, 0.1% of $(NH_4)_3PO_4$, 0.02% of $MgSO_4$, 0.01% of NaCl, 0.005% $CaCl_2$, 0.0007% $MnSO_4$, 0.001% of $ZnSO_4$, and 0.001% $FeSO_4$.

Reagents were dissolved in 1L of distilled water sterile and submitted to sterilization in autoclave model AV AV (Phoenix Luferco®). The culture of B. Subtilis was added to F medium and kept in orbital stirrer brand Superohm for 7 days. Posteriorly, the isolation of spores was executed.

The same procedure was made for Bacillus subtilis var natto.

2.4 Isolation of spores of B. Subtilis and B.subtillis var. natto

For isolation of B. subtilis, and B. subtilis var. natto, the cultures of microorganisms were centrifuged at a speed of 10,000rpm for 10 minutes (centrifuge Megafuge 16R of Datamed) as described by Nicholson and Setlow (1990) and adapted. The supernatant was discharged after centrifugation for obtaining the pellet. The pellet was resuspended in 25mL of sterile distilled water, being submitted to new centrifugation under the same previous conditions and discharged the supernatant. This stage was repeated 6 times in order to get a cultivation of spores, free from vegetative cells, which were stored under freezing temperature of -12°C.

2.5 Inoculation of spores of Bacillus in nectar and cashew pulp

Packages of cashew nectar containing 200mL and cashew pulp containing 100mL, were sanitized with alcohol at 70% and opened with sterilized scissors. The samples of cashew nectars and pulp were distributed (in duplicate) in glass flasks, type Shott, previously sterile. In each flask containing 200mL of cashew nectar were added 200μL of each inoculum (B.claussi with initial spore counting of 10^9CFU/mL and B. subtilis and B. subtilis var natto with initial spore counting of 10^{10}CFU/mL). And in each flask containing 100mL of cashew pulp, were added 100μL of each inoculum.

The samples of cashew nectars were conserved at refrigeration temperature at 4°C, while cashew pulps were conserved at conservation temperature of -12°C in the first stage. In the second stage, the samples of cashew nectars were conserved in accelerated atmosphere at environmental temperature of 24°C, while cashew pulps were conserved at accelerated refrigeration temperature at 4°C.

2.6 Viability analysis of B. clausii, B. subitilis and B. subtilis var natto

Serial dilutions of cashew nectars and pulps containing microorganisms were executed in saline solution at 0.9% until obtaining the dilution of 10^{-4} for B. Clausii (initial counting of 10^9CFU/mL) and 10^{-5} for B. subtillis e B. subtilis var. natto (initial counting of 10^{10} CFU/mL). It was used aliquot of 0.2mL of sample in test tubes with 2mL of saline solution.

Posteriorly were made platings in triplicate for each sample, in different periods of storage along 60 days. The plates with culture medium Muller Hilton (MH), received aliquots of 50μl of the nectar or pulp diluted and it was spread in the plate (technique of spreading in plate) with handle Drigalski until the culture medium absorbed the whole content. After this procedure, plates were incubated in oven at temperature of 31°C for 24 hours, for manually counting the unities forming colonies.

For analyzing the conformity of Colony Forming Unities in the products, the Brazilian legislation was used as parameter, fermented dairy probiotics must have a minimum of 10^8 to 10^9 CFU by portion of product (BRAZIL, 1999; BRAZIL, 2002; BRAZIL, 2008).

2.7 Statistical Analysis

Results were analyzed with the statistics program GRAPHPAD PRISM 5 Software and were expressed in mean ± standard deviation of the mean. In order to evaluate the significance of the differences between the means were used the variance analysis (ANOVA) and Tukey's test at a level of 99% of reliability.

3 I RESULTS AND DISCUSSION

First, tests were made with probiotic bacteria as vegetative cells. Two species were studied: Lactobacillus casei and Bacillus subtilis. The microorganisms analyzed did not have viability in both products of the study (cashew nectar and pulp). The strains of L. Casei did not survive 24 hours in the products, while B. subitilis survived to 72 hours in the samples of cashew nectar, while in cashew pulp the survival was 48 hours. The concentration of B. subtilis found both in the cashew nectar as well as in cashew pulp was not enough to give to those products' functional properties from the added probiotics, as can be observed in Figure 1.

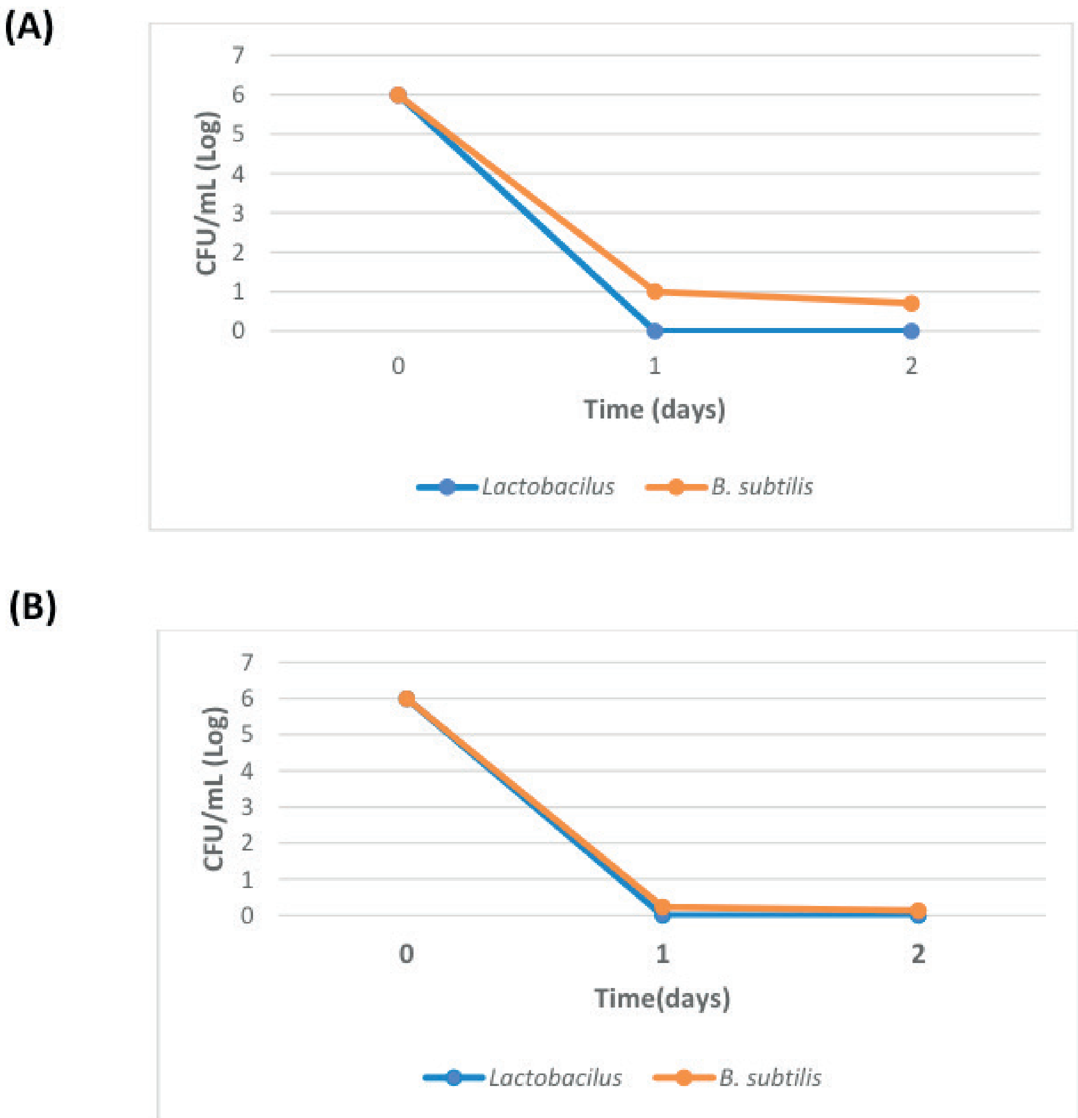

Figure 1. Viability (CFU/mL) of vegetative cells of L. casei and B. subtilis innoculated with cashew nectar (A) and cashew pulp (B).

It is possible to suppose that the inviability of vegetative cells in cashew products was due to the concentration of tannin present in products elaborated from its pseudo fruit. Tannin is known as an antioxidant and antimicrobial which may be the cause of the death of the probiotic bacteria. An alternative to increase the concentration of probiotic vegetative cells in these products would be the clarification of products, a technique that removes a part of the fibers and tannins. However, with this clarification, the products had their nutritional value decreased, and this would not be interesting for the consumers (PEREIRA et al., 2011; PIMENTEL, 2014).

Souza (2014), evaluated the viability of Lactobacillus acidophilus in fermented mango and grape juice, and after 6 hours of fermentation, the microorganisms were no longer feasible in the products. Anekella and Orsat (2014) used Lactobacillus rhamisus and Lactobacillus acidophilus in raspberry juices and after 30 days of storage at 4°C, the microorganisms did not have adequate feasibility to consider the juice as probiotic. Those authors corroborate this study trying to use probiotic microorganisms of genus Lactobacillus.

From those preliminary results and aiming to keep the nutritional characteristics of the products, not reducing their nutritional values but aggregating a functional property, tests were made with spores of genus Bacillus, due to the resistance that the spore shape of a microorganism has.

The counting of unities forming colonies of probiotics studied during the storage time of cashew nectars and pulps, stayed over the concentration determined by the Brazilian law.

In Figure 2 are demonstrated the graphics with the means of the counts of unities forming colonies of cashew nectars during the shelf life conserved at refrigeration temperature of 4°C and at environmental temperature of 24°C (accelerated atmosphere) along 60 days. The conservation under accelerated atmosphere allows to suppose that in normal storage conditions of the product, this may double its lifetime in shelf. With that, it is possible to infer that the period of 60 days under conservation of accelerated atmosphere corresponds to 120 days under conservation at normal conditions of the product.

The probiotic microorganisms inoculated in cashew nectars, kept the feasibility during the whole storage time, having in average concentration of 10^9CFU/mL, without significant difference between times analyzed at 99% of reliability, for B. clausii; 10^{10}CFU/mL, without significant difference between times analyzed, for B.subitilis cultivated in lab and between 10^9 and 10^{10} UFC/mL for strains of B. subtilis var natto, also without significant difference between analyzed times (Figure 2).

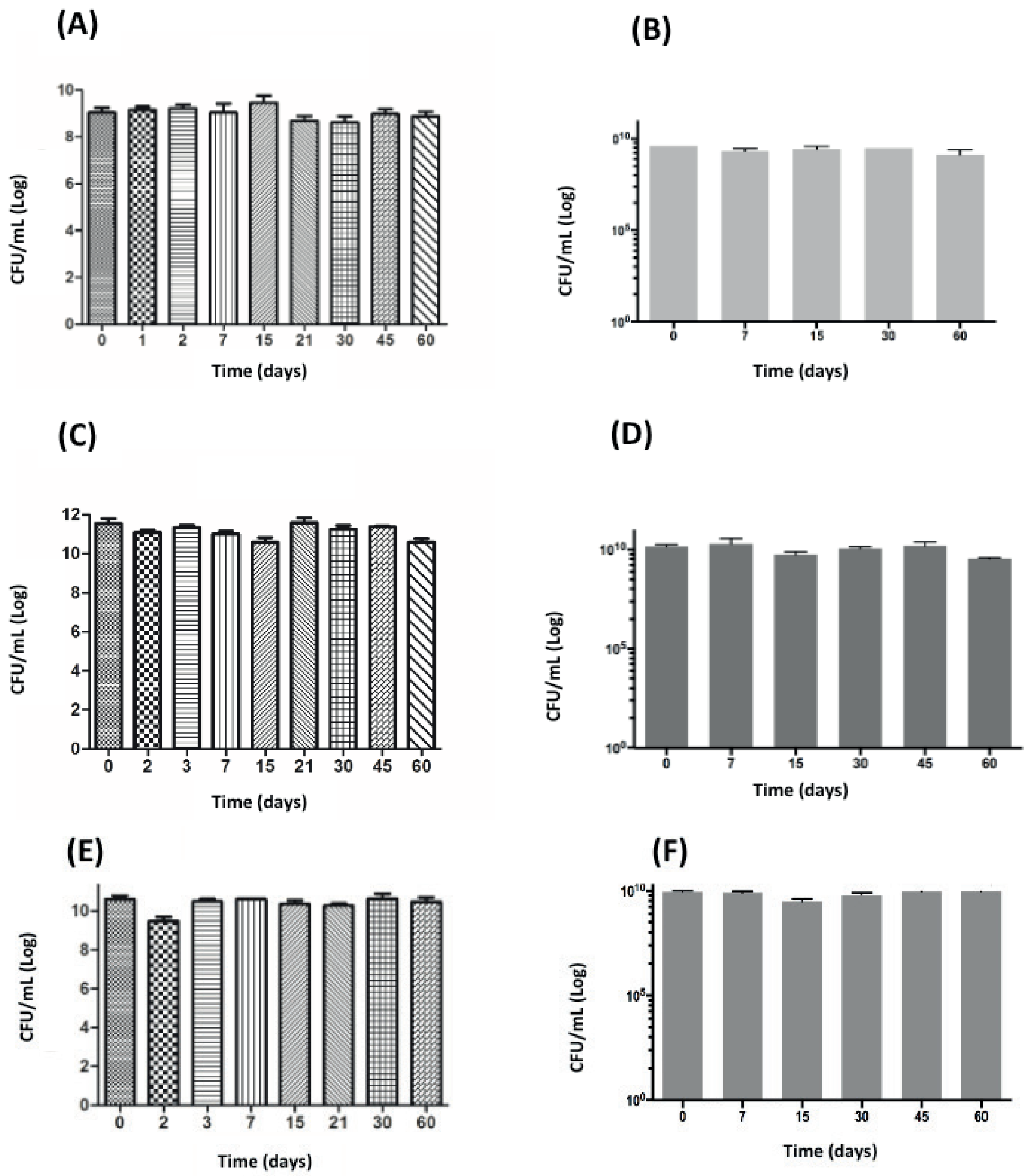

Figure 2. Viability (CFU/mL) of spores of *Bacillus* genus inoculated in cashew nectar along 60 days. (A) *B. clausii* – refrigeration temperature at 4°C; (B) *B. claussi* – environment temperature at 24°C; (C) *B. subtilis* – refrigeration temperature at 4°C; (D) *B. subtilis* – environment temperature at 24°C; (E) *B. subtilis* var natto – refrigeration temperature at 4°C; (F) *B. subtilis* var natto – environment temperature at 24°C.

B. clausii was used as control of tests in order to behave like a probiotic of the same genus already sold in market. While *B. subtillis* cultivated in lab were analyzed for standardizing analyzes with this species and the *B. subtilis* var natto that was isolated from a food, is the main probiotic when someone thinks about food products.

Pimentel et al. (2011) analyzed peach nectar using microorganism of genus *Lactobacillus* for 28 days, conserved at 4°C. It had, as a result, an average of 10^7CFU/mL, being one value below the recommended by Brazilian legislation. While Antunes et al. (2013) evaluated nectar of acerola added with probiotic microorganism of genus *Bifidobacterium* for 30 days and stored at 5°C, finding 10^8CFU/mL, according to the current legislation. This study found values well above the ones of unities forming colonies of probiotic microorganisms than studies found in literature about fruits nectar (Figure 2). The greater viability of probiotic microorganism in fruit nectars than in fruit juices, is possibly related with the concentration of tannins present in those products, being of 27mg/100g and 136mg/100g, respectively (VIDAL, 2016).

In literature some studies are found using fruit as food matrix for producing probiotic beverages, most of the studies are made with fruit juices and using the genus *Lactobacillus* as probiotic microorganism with different processing techniques (BETORET et al., 2012; ALMEIDA et al., 2012; COSTA et al., 2013; ANEKETTA et al., 2014; DIMITROVSKI et al., 2015; ALVES et al., 2016; FARIAS et al., 2016).

Fermentation is one of the processing techniques more used for elaboration of probiotic beverages, having fruits as food matrix. The greater disadvantage of this technique is the production of aromas and flavors unpleasant to the consumer. Santos et al. (2008), Coelho (2009), Pereira et al. (2011), Dimitrovski (2015), Farias et al. (2016), studied probiotic fruit juices by means of fermentation made by the microorganism, finding that the viability of the microorganisms stayed during the whole shelf life. However, from sensorial tests, Coelho (2009) says that products sweetened after fermentation are more accepted by masking products resulting from fermentation.

Another very present technique is the microencapsulation allowing the protection of the microorganism regarding the acidity of the product or presence of antimicrobial substances, despite being a more expensive technique. And another alternative for improving the feasibility of probiotic microorganisms in fruit beverages is the clarification that eliminates, mainly, fibers and tannins from the original beverages.

Another option for improving the viability of the probiotic microorganism in the elaborated food is the addiction of prebiotics. Maltodextrin and fructooligosaccharides (FOS) are more present in studies. Barbosa et al. (2015) and Alves et al. (2016) evaluating probiotic fruit juices, added probiotic substances for increasing the growth of vegetative cells. This work had no need of improving the cost of the elaborated product for increasing the growth of microorganism, by using microorganisms as spores.

This study did not use fermentation or any more elaborated technique for protecting the microorganism from the acidity of the product, neither removed antimicrobial substances that are naturally present in cashew, such as the tannin, being a product of lower cost for the food industry, as well as, probably, more accepted by consumers.

The values of CFU found in tests of the three strains of genus *Bacillus* proves the resistance of the spore shape of the bacteria to antimicrobial substances, as well as the acidity of the product (Figure 3). Products elaborated from the peduncle of cashew, have acidity around 0.26g of citric acid/100g for nectar and 0.94g of citric acid/100g for pulp (FIGUEIRA, PILON, DUCATTI, GASTONI e FILHO, 2015).

In Figure 3 they are expressed in mean ± mean of the standard deviation of the triplicate, not having significant difference in any one of the samples at 99% of confidence, during shelf life. Spores of *B. clausii* had a mean of 10^9CFU/mL, both in the sample conserved in freezing temperature as well as in the sample kept at refrigeration temperature.

Cashew pulps used in the study, inoculated with probiotics from genus *Bacillus*, had viability during the storage of the product, in both types of conservation (frozen at temperature of -12°C and refrigerated at temperature of 4°C).

Concentration of *B.subitilis* stayed between 10^{10}CFU/mL in pulps conserved under freezing and 10^9 and 10^{10}CFU /mL in pulps conserved at refrigerated temperature, even with this reduction, it is still inside the concentration recommended by Brazilian legislation (Figure 3);

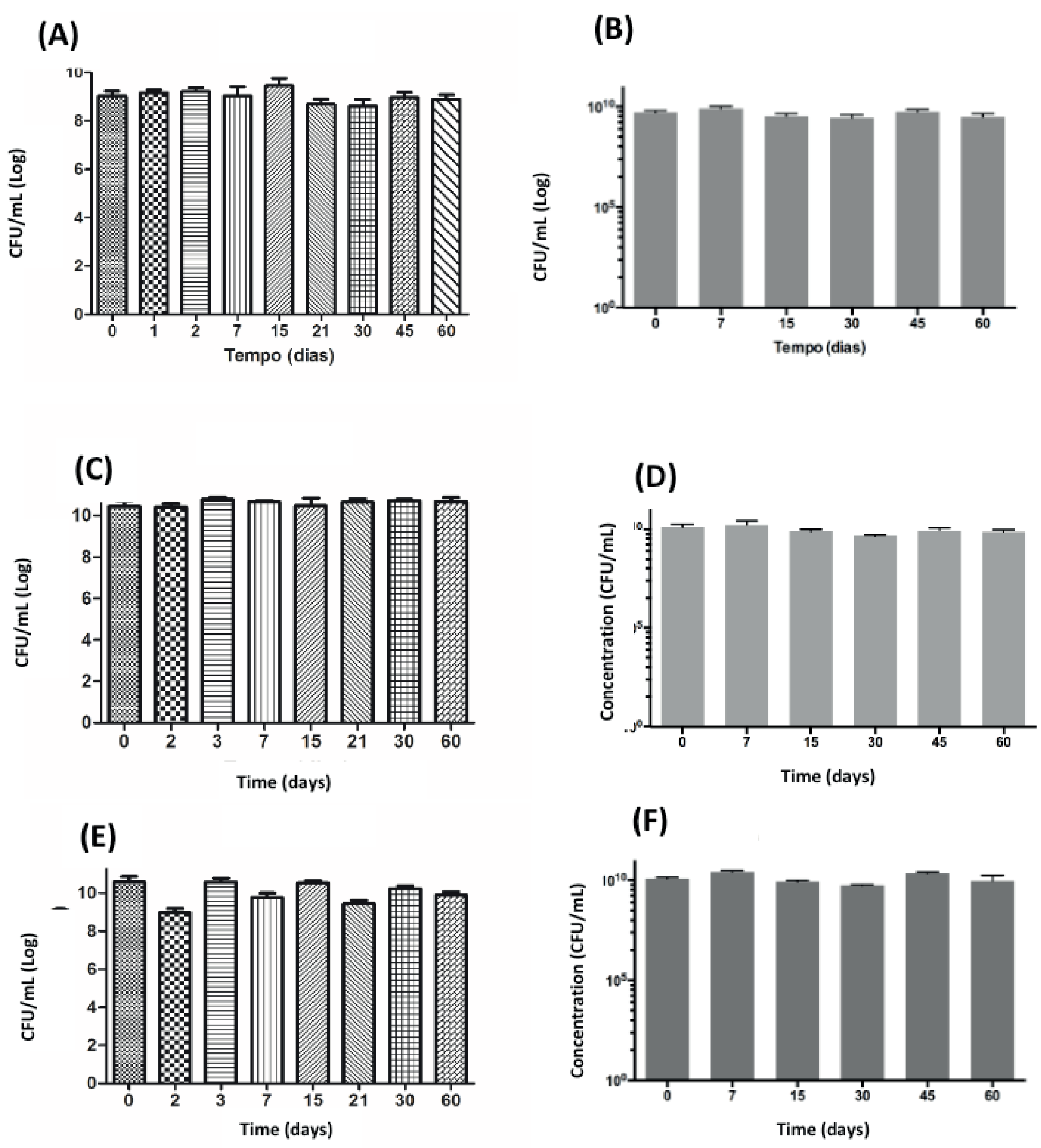

Figure 3. Viability (CFU/mL) of spores of *Bacillus* genus inoculated in cashew pulp along 60 days. (A) *B. clausii* – refrigeration temperature at -12°C; (B) *B. claussi* – environment refrigeration temperature at 4°C; (C) *B. subtilis* – refrigeration temperature at -12°C; (D) *B. subtilis* – refrigeration temperature at 4°C; (E) *B. subtilis* var natto – freezing temperature at

-12°C; (F) *B. subtilis* var natto – refrigeration temperature at 4°C.

Pulps inoculated with *B. subtilis* var natto conserved at freezing temperature, had an average of concentration of 10^9 and 10^{10}CFU/mL and in pulps conserved at 4°C of 10^{10}CFU/mL (Figure 3).

Comparing the feasibility of probiotic microorganisms of spores of genus *Bacillus* in cashew nectars with cashew pulps, the nectars had a greater stability and concentration of probiotics, although, the concentration of cashew pulps being also according with the current law, as mentioned previously. Probably, the greater concentration of probiotics in cashew nectars is due to their chemical composition, cashew pulp having lower pH than the nectars and more concentration of tannins. Both products did not need technological alternatives for protection of microorganisms, such as fermentation and microencapsulation, being advantageous from the economic and sensorial point of view.

The study from Pereira, Maciel and Rodrigues (2011), is the last work published during the last ten years about cashew as a food matrix for feasibility of probiotic microorganisms. After 42 days of refrigerated storage, the concentration of *Lactobacillus casei* was 10^8CFU/mL. In this study, the elaboration of cashew nectar and pulp with spores from genus *Bacillus* obtained superior concentration along the 60 days of storage in normal conditions of those products, as well as in conservation of accelerated atmosphere supposing a viability of 120 days for those products. Thus, it is perceived the efficacy of adding spores from probiotic microorganisms instead of vegetative cells from the microorganisms.

In literature no studies were found analyzing the feasibility of probiotics in pulps of fruits, making this work a starting point for future researches about this theme and in the future the insertion of those products in the market for the consumers.

4 I CONCLUSION

The use of cashew nectar and pulp as substrates for the viability of the probiotic microorganism is a good alternative for the maintenance and feasibility of probiotics. Along the shelf life of cashew nectars and pulps, the probiotics stayed over 10^8CFU/mL. Thus, cashew nectar and pulp with *Bacillus clausii, Bacillus subtilis* and *Bacillus subtilis* var natto are options of food with allegations of probiotic functional properties for consumers with restriction to the ingestion of dairy and/or vegetarian products.

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