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Viability of probiotics and bioactive compounds in avocado paste

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Abstract
We evaluated the viability of *Lactobacillus rhamnosus* in avocado paste during storage period by verifying the content of bioactive compounds in it. Therefore, the following analyses were performed: pH; centesimal composition; color; *L. rhamnosus*, yeast and mould count; standard plate count of mesophilic aerobic bacteria and psychrotrophic microorganisms. The quantification of bioactive compounds, such as flavonoids, was expressed as mg rutin 100 g^{-1}; total antioxidant activity was expressed as % reducing DPPH; and total phenolic compounds were expressed as mg gallic acid per 100 g sample. *L. rhamnosus* remained constant during refrigerated storage period with a mean of $9.03x10^7$ $CFUg^{-1}$ for 40 days. Thus, it was possible to develop avocado paste with probiotic potential and commercial viability using *L. rhamnosus* with desirable amounts of bioactive compounds and no microbial contamination.

Keywords: *Lactobacillus rhamnosus*, Hass avocado, antioxidant activity, irradiation

1. Introduction
Avocado fruit is prized for its high nutritional value, such as vitamins (B and E), minerals and fibers. Also, it is rich in oleic acid and beta-sitosterol, which helps to prevent cardiovascular diseases. In addition, it contains high levels of bioactive phytochemical compounds, such as carotenoids, sterols, phenolic compounds, flavonoids and others [1, 2]. Some studies have evidenced that phytochemicals found in avocado have growth inhibitory functions, besides it can induce apoptosis in tumour and premalignant cell lines [1].

Probiotic cultures are often found in dairy foods, but there is a consumer demand for non-dairy probiotic products, such as vegans, people who are lactose intolerant or those with milk allergies. Thereby, avocado fruit has been incorporated into beverages, as well as dietary supplements like capsules, tablets and lyophilized preparations. Thus developing fruit-based products become a good option for consumers, since they are rich in vitamins, minerals and phytochemicals, despite being dairy-free [3].

There are evidences supporting the ingestion of *Lactobacillus rhamnosus* and its beneficial effects in the prevention and treatment of acute diarrhea, acute atopic dermatitis, as well as immune system booster [4, 5]. Yet *L. rhamnosus* GR-1 is a probiotic strain that increases resistance when incorporated into fruit juices with low pH [6, 7].

Therefore, the development of avocado paste with probiotic potential is innovative and tends to reach a large consumer market, since the paste contains avocado and cacao, which are extremely rich in nutrients and bioactive compounds, besides being inoculated with probiotic culture. Thus, this study aimed to evaluate the viability of *L. rhamnosus* in avocado paste during storage period by verifying the levels of bioactive compounds in it.

2. Material and Methods
2.1 Raw material, ingredients and bacteria strain
Fruits of Hass avocado were harvested at maturation stage. Afterwards, fruits were selected to compose a homogenous lot, in terms of size, colour and absence of injuries and defects. Then fruits were rinsed in running tap water to remove dirt and residues. Subsequently, fruits were immersed in a bleach bath, containing sodium hypochlorite aqueous solution with an active chlorine concentration of 2.5% (8 $mL.L^{-1}$) for 15 minutes; therein all fruits were placed on a bench surface covered with paper towel to dry. Besides that demerara sugar, soybean lecithin powder and cocoa powder were purchased at the confectionery store. Lyofast® LRB (SACCO Brasil) was applied in products as culture lactic acid bacteria, consisting of a strain of *Lactobacillus rhamnosus*, which is capable of producing bacteriocins, a bioprotective culture that inhibits unwanted bacteria, moulds and yeasts of the medium.

2.2 Processing of avocado paste
Before starting the preparation, acidification curve (Figure 1) was calculated to establish the correct amount of citric acid for the paste [8]. Then, 50 g of avocado pulp were weighed into an erlenmeyer flask, adding 50 mL of distilled water. The solution was titrated with 2% citric acid, and pH measured at each 0.5 mL titration. Considering that the optimum pH for *Lactobacillus* is between 5.5-5.8 [9], 0.30 g of citric acid was used in 100 g of avocado pulp. Initially, food processor and citric acid ground pulp for five minutes, then soybean lecithin powder was added and shaken in a domestic mixer for five minutes; therein cocoa powder and demerara sugar were added in the mixer for the same period of time. All utensils and equipment were properly cleaned and sanitized before use. Since there was no cooking

involved, avocado paste was stored in white opaque storage containers to reduce microbial contamination at a dose rate of 5 kGy, using a Co-60gamma at Multipurpose Gamma Irradiation Facility of the Radiation Technology Centre (CTR), in the Nuclear and Energy Research Institute (IPEN), University of São Paulo.

The lyophilized *L. rhamnosus* was rehydrated with 10 mL of pre-autoclaved mineral water per kg of pulp, and 10^{11} CFU was inoculated into the irradiated paste. The lactic culture amount was based on the number of viable cells present in lyophilized commercial culture required to achieve the minimum value of 1 x 10^8 CFU per portion in the paste. The pulps remained refrigerated throughout storage period in a domestic refrigerator at 2 ± 2°C and 45.5 ± 3% relative humidity.

2.3 Variables analyzed

Analyzes were performed every five days in nine storage times: 0, 5, 10, 15, 20, 25, 30, 35, 40 days. The analysis of pH [10]; moisture content; ash content [10]; crude protein content; crude fiber content; grease content [11]; sugars [12, 13], counts of *L. rhamnosus* on Man Rogosa Sharp (MRS) - vancomycine agar (Difco - Becton Dickinson and Company, Franklin Lakes, NJ) and incubation at 35ºC for 48h [14, 15]. The yeast and mould counts were performed using potato dextrose agar (PDA – Difco) with tartaric acid to lower the pH to 3.5, in order to inhibit bacterial growth [9] and incubation at 25°C for 7 days. The count of mesophilic aerobic bacteria was performed in Plate Count Agar (PCA-Difco), incubated promptly for 5 days at 35°C [9]; and counts of psychrotrophic microorganisms, in PCA and incubated for 7 days at 4°C.

The quantification of flavonoids followed the methods proposed by Santos, & Blatt (1998) [16] and Awad, de Jager, & van Westing (2000) [17]. The results were expressed as mg rutin 100 g^{-1}. The extract for the quantification of total antioxidant activity was expressed as % reducing DPPH [18]. The total phenolic compounds results are expressed as mg gallic acid per 100 g of sample [19], and it was performed according to Wang, Bostic, & Gu (2010) [20], with some adaptations using different extractors (i.e. 50% methanol, 80% methanol and acidified acetone). Therefore, 0.1 g of sample were weighed in falcon tubes and added 10 mL of acidified acetone (acetone, water and acetic acid - 70: 29.7: 0.3), homogenized using a Turrax, placed in an ultrasonic bath for 15 min and centrifuged at 6000 rpm for 20 minutes. Then the supernatant was completely removed and stored for a maximum of three days in amber glass.

The color was evaluated in triplicate using a colorimeter Chroma Meter CR-400 (Konica Minolta®), with illuminant D-65 and the following parameters were determined: L, indicates brightness values (0% = black and 100% = white), the angle Hue is the value in degrees corresponding to the three-dimensional color diagram: 0 ° (red), 90 ° (yellow) and 270 ° (blue). The ° Hue varies from: 0 to 12 ° for red, 13 to 41 ° for orange, 42 to 69 ° for yellow, 70 to 166 ° for green, 167 to 251 ° for blue, 252 a 305 ° to violet and 306 to 359 ° to red, making 360 °; C * is represented by the Choma that defines the intensity of color, which varies from 0 (less intense color) to 60 (more intense color) [21].

A 2x9 (treatment x storage) factorial scheme arranged in a completely randomized design was used. Data were submitted to analysis of variance. Polynomial regression was performed for storage period and probiotic counts and the results were evaluated using SISVAR statistical system [22].

3. Results & Discussion

The potential of hydrogen (pH) is a simple measure that provides useful information about the potential chemical changes that are occurring in the product during processing and storage, as well as influencing the viability of probiotics [23, 24]. The pH is crucial for feasibility of probiotics. Studies have demonstrated that the higher the pH of fruit juices are, the better the viability of *Lactobacillus* is. The viability of *L. casei* was higher in orange juice corrected at pH 6.0 than 3.85 [24]. Yet the growth and number of viable cells of different *Lactobacillus* was higher in fruit juices at pH 4.2 than at a pH range from 3.6 to 4.0 [7]. *L. casei* presented excellent growth in cashew juice at pH 6.5 [25]. In this study, the pH was close to 6.0; therefore, optimal viability for *Lactobacillus*. However, no difference for pH was observed between avocados pulps in the presence or absence of probiotic, showing that *L. rhamnosus* did not influence pH during forty days under refrigerated storage.

In avocado paste, pH decreased as the storage time elapsed, i.e. from 5.8 to 5.4 (Figure 2). Some authors reported different pH decrease, as they considered the presence of acid-lactic bacteria during fermentation and refrigerated storage. In 28 days, there was a pH drop in milk that was fermented with *L. casei* from pH 4.50 to 4.15 [26]. Another study that evaluated the same strain and refrigeration period, showed a more significant pH decrease, from 5.59 to 4.60 [27]. After 30 days of refrigeration, there was a pH reduction from 4.72 to 4.45 in buffalo milk with *L. casei* [28]. According to de Oliveira (2002), small pH variations during storage period show good microbiological stability of the product [29].

The decline in pH during storage is due to gradual migration of organic acids from intracellular locations to the avocado pulp matrix. The processing induces morphological alterations of plant cells, resulting in rupture of membranes and leaching of intracellular constituents, as well as occurred in avocado paste submitted to high hydrostatic pressure stored for 45 days at 4 ° C [30].

Several factors interfere in the structure, chemical and nutritional composition of the processed food, such as oxygen, humidity, environment pH, light, temperature, metal ions and reducing and oxidizing agents. These changes may be negative, leading to loss of nutrients and organoleptic changes, either as positive, forming new desirable compounds or destroying inhibitory substances [31].

Humidity remained unchanged between probiotic and non-probiotic pastes during the storage period (Table 1), probably due to refrigeration, which is a positive result because one of the factors of shelf-life is the susceptibility to loss of moisture [23].

Inoculation of probiotics did not interfere with the ash content, which is indicative of mineral content in the avocado paste (Table 1). During the storage period of the avocado paste (Figure 3), the ash values decreased during the 40 days of storage, from 1.74 to 1.67%, with discrete oscillations during this period. Similar behavior was observed by Villa-Rodrígues *et al*. [32].

No influence was observed in the fiber content by the addition of probiotics in the avocado paste and in the interaction of the factors evaluated (Table 1). The presence of fibers in the avocado paste is of extreme importance,

since it is not digested by enzymes but rather fermented by the intestinal flora, serving as food for *Lactobacillus*, thus acting as a prebiotic [33].

For the protein content (Table 1) there was no statistical difference for pulps with and without *L. rhamosus*, storage and in the interaction (treatment x storage). The fruits contain low protein content, not being nutritionally significant, but it has functions in the metabolic mechanisms [23].

There was a significant difference in the lipid content between the interaction of the factors studied (Figure 4), however, only the pulp without probiotics was significant. There was a decrease in lipid content in this formulation (14.28 to 12.26%), while the avocado paste +probiotic kept its contents statistically equal during storage. Several factors can initiate oxidative decomposition, such as heat treatments, ionizing radiation, or chemical initiation through metal ions. The main effect of oxidation on storage that may have occurred in avocado paste is lipid peroxidation, since avocado is a rich lipid fruit [34].

According to Villa-Rodrígues *et al*. [32], after harvest the production of reactive oxygen species continues normally. Therefore, the reduction in lipid content could be related to the protection conferred against oxidative reactions, which did not occur in the product with probiotic [32].

Total sugars are the sum of the soluble sugars present in the plant tissue (reducing and non-reducing) and contribute to flavor sweet taste of the product [23]. The averages of 20% total sugars from avocado pastes represent a sweet product, which tends to be pleasing to the palate of consumers.

There was interaction of the avocado pulps with the storage period (Figure 5), therefore, the presence of *L. rhamnosus*, as well as the time, interfered in the sugar concentration, presenting an increase in the avocado paste without probiotic (18,86 to 22.09%) and enriched with probiotic (19.21 to 20.83%).

The increase of total sugar during time may be due to biosynthetic processes or degradation of polysaccharides [23]. In a study by Mattietto; Lopes and Mezezes (2007), there was a decrease of total sugars of cajá and umbu nectar, unlike in the present work [35].

Probiotics influenced the lipid, sugar, flavonoid, luminosity and chroma contents due to the fact that they consume energy reserves for the production of lactic acid.

From a safety point of view, the risk produced by the growth of pathogenic microorganisms, as well as the toxins produced, is more important than the physical and chemical deterioration of products [36]. Yet there were no growth of moulds, yeasts, mesophilic and psychrotrophic microorganisms throughout storage period, suggesting that irradiation was effective to control microbial contaminations in avocado paste.

Furthermore, pulps were irradiated with intermediate dose of 5 kGy, which is safe for food. According to Food and Agriculture Organization of the United Nations (FAO), International Atomic Energy Agency (IAEA) and World Health Organization (WHO), a radiation dosage up to 10 kGy is considered as being unconditionally safe for human consumption [5, 37]. Moreover, the dose of 5 kGy is applied to reduce or eliminate spore-forming pathogens [38]. But irradiation is not enough on good manufacturing practices whether utensils or equipment were not appropriately sanitized and cleaned, especially the pots in which the avocado paste were stored in, as well as the refrigerator used. After irradiation, the aseptic enclosure must be kept in foods to avoid further contamination [39], as performed in this study. In addition, refrigeration can stop microbial growth and retard chemical and enzymatic reactions, therefore, an aseptic condition despite its mild cold temperature [40].

L. rhamnosus used in this study produces bacteriocins, which are small and thermostable peptides that can inhibit the growth of gram-positive pathogenic bacteria, some species of gram-negative bacteria and yeasts. Such strain may also have kept the avocado paste away from contamination until the end of storage [41]. The *L. rhamnosus* count had no statistical difference during the whole period of refrigeration, with a mean of $9.03x10^7$ $CFUg^{-1}$ (Figure 6). Thereby, a partion of avocado paste (20 g) presented on average $11.8x10^9$ CFU of *L. rhamnosus*, such quantity is within the range recommended by Brazilian legislation (from 10^8 to 10^9 CFU/portion) [42]. Moreover, this amount is also above the recommendations of food industry, which is at least 10^6 $CFUg^{-1}$ [43]. Thus, *L. rhamnosus* is viable in avocado paste for 40 days under refrigerated storage.

One of the factors that may have influenced this optimal viability of *L. rhamnosus* in paste was the pH range (5.4 to 5.8), as the lactic bacteria develops well at pH near neutrality (from 5.5 to 7.5) [25]. Shah (2000) stated that the growth of lactic acid bacteria starts stopping at pH 4.0 [44]. In the storage of products enriched with lactic acid bacteria, there is production of lactic acid and is often referred to as "post-acidification", but this behaviour was not observed in this paste. During post-acidification, probiotic bacteria lose viability, and it is important that the cells remain viable throughout product life, since cell viability is crucial to guarantee enough number of viable cells at the moment of consumption [44]. Even there was a slight increase in titratable acidity (from 0.38 to 0.44 mg acid $100g^{-1}$), this was insufficient to prevent adequate amount of lactic acid bacteria in the paste.

Although *Lactobacillus* is commonly found in yogurts, studies have shown that many branded yogurts contain low concentrations of bacteria (< 3 $\log_{10}$ CFU g^{-1}) [44]. This study showed a different outcome, as it presented a high amount of lactic acid bacteria (>6 $\log_{10}$ CFU g^{-1}) in the avocado paste.

Alongside this study, some studies on fruit juices inoculated with *Lactobacillus* presented good evidence in this culture viability in fruit-based products. *L. casei* was also viable, with more than 10^8 CFU/ mL of orange juice for 45 days under refrigerated storage at 4°C [24]. Different species of *Lactobacillus* remained viable in tomato juice for 4 weeks under refrigerated storage at 4°C, with values above 10^6 CFU/mL of juice [45]. In fruit juices stored for 80 days at 4°C, distinct *Lactobacillus* remained viable, that is, above 10^6 CFU/mL [7]. Moreover, different species of *Lactobacillus* remained viable (i.e. above 10^8 CFU/mL) in beet juice for 4 weeks at 4°C [46]. Furthermore, *L. acidophillus* was viable in ice creams enriched with different types of dietary fibre. Unlike this study, there was a decline in *L. acidophillus* count at the end of the storage period [47].

It is important to consider the analysis of fruit chemical traits, as they may be showing an inhibition of probiotic cultures. In guava pulp mousses with *L. acidophillus*, viability was only ideal above 10^6 CFU g^{-1}; as in this study (Table 2); while passion fruit mousses presented a count of 4.7 log CFU g^{-1} at the end of 21 days of storage [48].

Regarding to the contents of phenolic compounds, no influence was observed in the presence of probiotics and studied factors interaction. In the storage period, the phenolic content reduced from 212.37 to 171.06 mg of gallic acid $100g^{-1}$ (Figure 7). The content of phenolic compounds and the antioxidant activity showed the same behaviour, suggesting that phenolic compounds present in plants are mainly responsible for antioxidant activity [49, 50], as verified in this study.

Concerning the levels of antioxidant activity, there was no statistical difference between avocado pastes in the presence or absence of probiotic and interaction factors. Thus, antioxidant activity was observed in paste formulations from 31.01 to 57.8%, with a general mean of 49%, being higher as compared to some fruits such as melon (43.5%) and mango (35.8%) [51]; and similar to orange (51.88%) [52] and passion fruit (48%) [51]. According to Richard, Kefi, Barbe, Bausero, & Visioli (2008), avocados contain mostly monounsaturated fat, which can act with antioxidants depending on the degree of instauration [53].

During storage period, antioxidant activity was reduced (Figure 8) and may have been due to lipid peroxidation that can be described as the oxidative degradation of lipids, a process that free radicals "steal" electrons from the lipids in cell membranes [31]. Furthermore, antioxidant activity may be affected during processing and storage, since natural antioxidant compounds of the plant are lost [54], as verified in this study. Bioactive compounds come from the plant's secondary metabolism and tend to increase under stress conditions [55], such as processing. According to Campos, Martino, Sabarense, & Pinheiro-Sant'ana (2008), stress is provoked by cutting the vegetables, as there is disarrangement in tissue structure, leading to antioxidants degradation by enzymes that are naturally present in fruits, besides intense light and oxygen exposure [56].

For flavonoid contents, there was a significant difference between pulps that inoculated or not *L. rhmanosus*, as well as storage period interaction (Figure 9). There was an increase in the contents during storage in avocado paste, that is, from 60.7 to 88.12 mg rutin $100g^{-1}$ (without probiotic) and 63.69 to 79.73 mg rutin $100g^{-1}$ (with probiotic). Flavonoids are an important part of the Brazilian antioxidant consumption, since their daily intake is about 23 mg, higher than the consumption of other antioxidant sources such as beta-carotene (2 to 3 mg day^{-1}) and vitamin E (7 to 10 mg day^{-1}) [57]. During experiment period, the concentration of flavonoids in avocado paste was higher than the total daily intake of flavonoids by a Brazilian consumer.

With the storage period, the luminosity decreased regardless addition of probiotics (32.95 to 31.01) (Figure 7), which is possibly due to processing of the avocado. This behavior was also observed by other authors, who justify this fact to the presence of light and oxygen that can oxidize thermosensitive pigments and/or non-enzymatic dimming [58, 59, 60].

There was interaction for avocado pastes with and without probiotics and storage days in the chroma evaluation (Figure 7), showing tendency to decrease at the end of days for both at 18.7 and 13.8 %, respectively. Since the chroma represents color intensity, the color of the avocado paste became less intense.

The hue angle presented a decrease in the avocado paste with the storage, corroborating with the other attributes of the color evaluation (Figure 7), presenting a reading of 57.92 at the experiment installation and in the day 40, the value was 54, 64. In a study regarding conservation of guacamole produced with avocado, onion, salt and acidulants, it was also observed a decrease in the hue angle during storage, decreasing the green tone due to degradation of chlorophyll [58].

The same behavior was reported by Villa-Rodrígues *et al.*, 2011 in 'Hass' avocados stored at 15 ° C for 14 days that had decreased brightness, chroma and hue hue over time [32].

Table 1: Moisture, ash, fiber and protein (average) in avocado pastes with and without probiotics during 40 days of refrigerated storage (2 ± 2 ° C and 45.5 ± 3%).

Analyses	Avocado paste with probiotcs	Avocado paste without probiotcs	Coefficient of variation (%)
Moisture (%)	53,88	55,36	14,54
Ash(%)	1,59	1,61	6,88
Fiber (%)	4,96	5,07	17,29
Protein (%)	2,44	2,45	3,51

Treatment and interaction averages were not significant at 5% significance by the Tukey test.

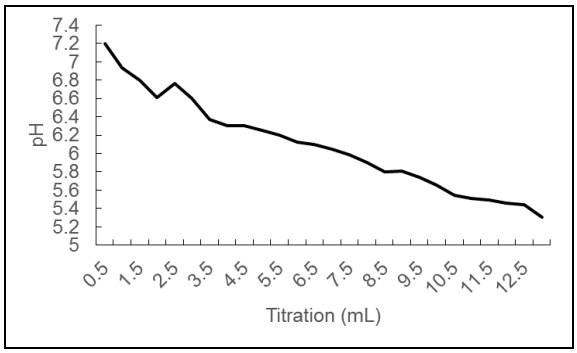

Fig 1: Hass avocado pulp acidification curve

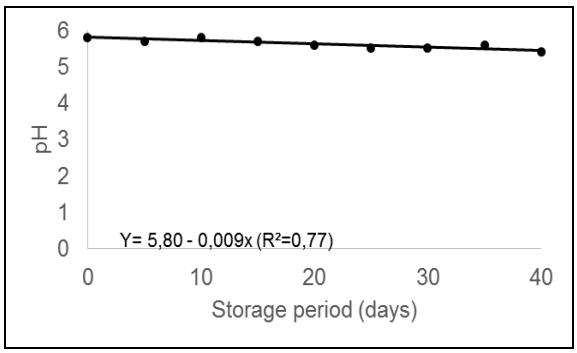

Fig 2: Potencial of hydrogen (pH) in avocado paste with and without probiotics during 40 days refrigerated storage (2 ± 2 °C e 45,5 ± 3%).

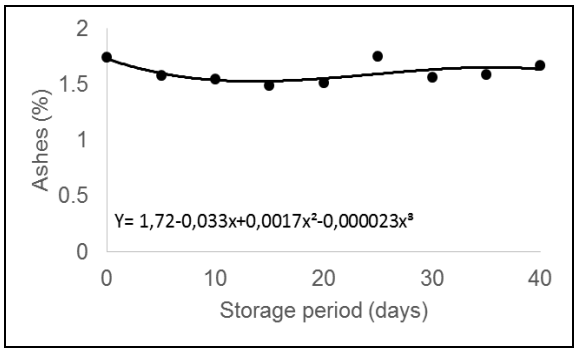

Fig 3: Ashes (%) in avocado paste with and without probiotics during 40 days of refrigerated storage (2 ± 2 °C e 45, 5 ± 3%).

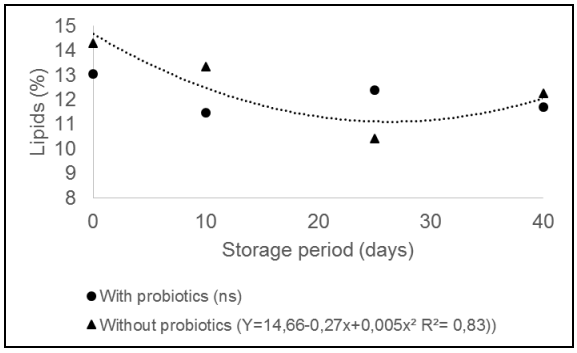

Fig 4: Lipids (%) in avocado paste with and without probiotics during 40 days of refrigerated storage (2 ± 2 °C e 45,5 ± 3%).

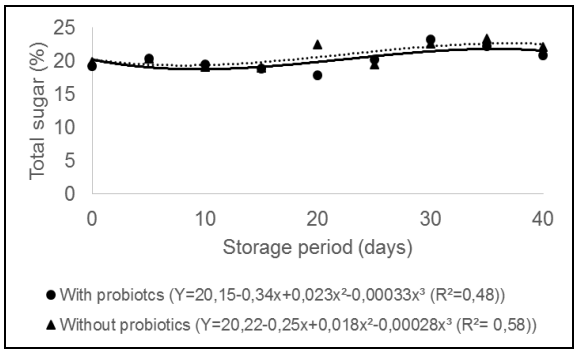

Fig 5: Total sugar (%) in avocado paste with and without probiotics during 40 days of refrigerated storage (2 ± 2 °C e 45, 5 ± 3%).

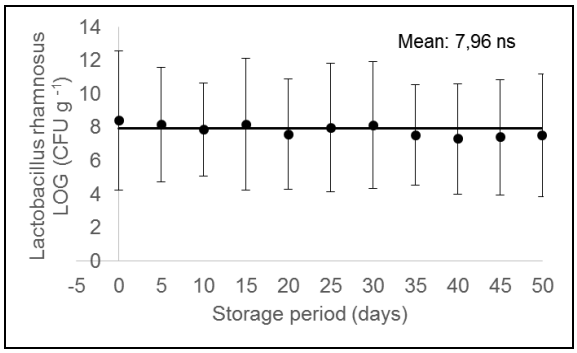

Fig 6: *Lactobacillus rhamnosus* count in avocado paste during 50 days refrigerated storage (2 ± 2 °C e 45, 5 ± 3%).

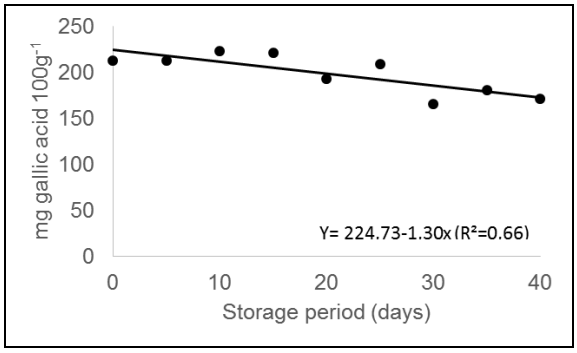

Fig 7: Phenolic compounds (mg gallic acid 100g $^{-1}$) in avocado paste with and without probiotics during 40 days of refrigerated storage (2 ± 2 °C e 45,5 ± 3%).

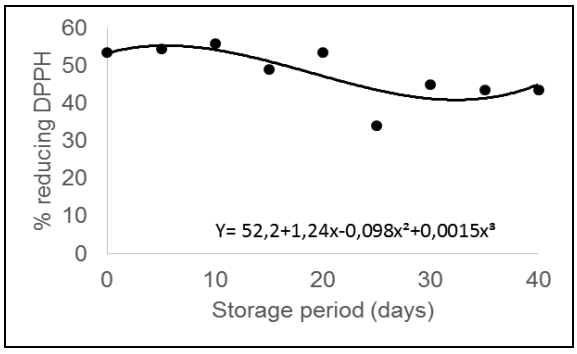

Fig 8: Antioxidant activity (% reducing DPHH) in avocado paste with and without probiotics during 40 days of refrigerated storage (2 ± 2 °C e 45, 5 ± 3%).

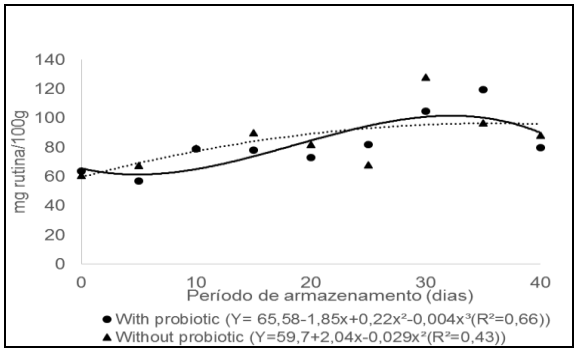

Fig 9: Flavonoids (mg rutin 100g $^{-1}$) in avocado paste with and without probiotics during 40 days of refrigerated storage (2 ± 2 °C e 45,5 ± 3%).

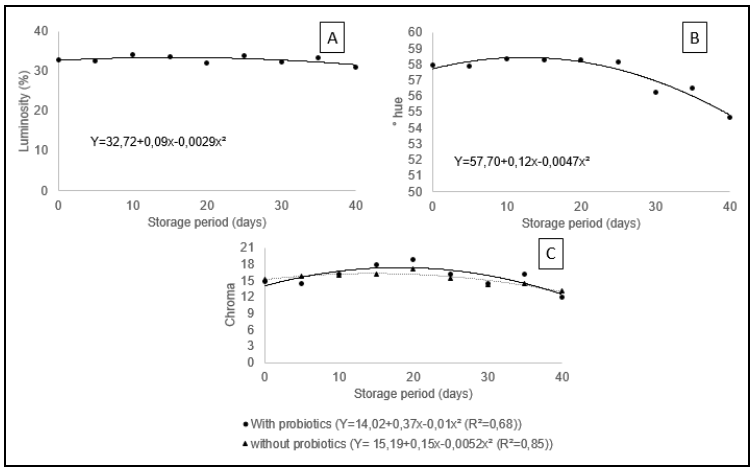

Fig 10: A) Luminosity in avocado paste with and without probiotics during 40 days refrigerated storage (2 ± 2 °C e 45, 5 ± 3%). **B)** Hue in avocado paste with and without probiotics during 40 days refrigerated storage (2 ± 2 °C e 45, 5 ± 3%). **C)** Chroma in avocado paste with and without probiotics during 40 days refrigerated storage (2 ± 2 °C e 45, 5 ± 3%).

5. Conclusion

It is feasible to produce avocado paste with probiotic potential and commercial viability using *L. rhamnosus*, demerara sugar, cocoa powder, soybean lecithin powder and citric acid without pathological microorganisms' contamination. The viability of *L. rhamnosus* remained constant for up to 40 days under refrigerated storage. In this period, there were enough bacteria to comply with Brazilian legislation and Food industry requirements. Probiotic avocado paste proved to be efficient for *L. rhamnosus* viability, as dairy products are, besides containing desirable amounts of bioactive compounds.

6. Acknowledgements

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