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Fresh cut fruit salads as a promising vehicle for *Lactobacillus acidophilus* and *Lactobacillus plantarum*

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Abstract

This work aimed to study the potential use of fresh cut fruit salads as carriers for *Lactobacillus acidophilus* LA-5 and *Lactobacillus plantarum*. The viability of *L. acidophilus* LA-5 and *L. plantarum* in fruit salads was above 7.83 Log CFU/g during 120 h of storage. Scanning electron microscopy showed that the fruit tissues served as shelter for probiotic bacteria. These tissues allowed bacterial adhesion, especially on pineapple, banana, papaya, mango and apple. Fruit salads from different treatments had no differences regarding pH, acidity and °Brix ($p>0.05$). *L. acidophilus* caused changes to apple texture. Fruit salads with *L. acidophilus* presented psychrotrophic counts of at least 3.0 Log CFU/g less than control fruit salads for up to 120 h of storage at 8°C. *L. acidophilus* and *L. plantarum* reduced coliform counts at 30°C in treated fruit salads, improving biopreservation. Therefore, fruit salads have potential for use as promising carriers of probiotic bacteria and could constitute an alternative option for consuming healthy and functional food products.

Introduction

The growing demand for new probiotic products has encouraged development of non-dairy food products, such as those based on fruits (Espírito-Santo *et al*., 2011; Martins *et al*., 2013), with potential benefits for human health (Champagne *et al*., 2011). Probiotic bacteria have shown promising results in juices and fermented beverages made with fruits and vegetables. These studies aimed to develop new food products to satisfy consumer demand for probiotic food products without cholesterol, lactose or other allergenic substances present in dairy products (Yoon *et al.*, 2005; Sheehan *et al*., 2007; Champagne and Garder, 2008; Nualkaekul *et al.*, 2011; Ellendersen *et al.*, 2012; Fonteles *et al.*, 2012; Antunes, 2013).

However studies regarding the impregnation of probiotic bacteria in minimally processed food products are limited in the literature (Rößle, Auty, Brunton *et al.*, 2010; Rößle, Brunton, Gormley *et al.*, 2010; Silva *et al.*, 2013). According to Rojas-Graü *et al*. (2011), the market for minimally processed or ready-to-eat fruits has increased worldwide as a result of growing demand for healthy and convenient food products. Thus, it has become viable to use probiotic cultures to offer foods with dual functionality, with the intrinsic characteristics of fruits allied to potentially beneficial probiotic microorganisms to generate healthy food products with high added value.

Moreover, products of plant origin, such as fruits, can be used as a promising substrate for growing probiotic cultures since they have nutrients (Yoon *et al.*, 2004; Soccol *et al*., 2010) and specific morphological structures favoring microbial growth (Sapers, 2001; Martins *et al*., 2013). Fruits also do not have the same allergenic substances typically found in dairy products that restrict consumption by some individuals, but at the same time they have other allergens once some people are allergic to apples.

Espírito-Santo *et al.* (2011) reported that probiotic foods containing fruits are increasingly preferred by consumers. Therefore, considering that minimally processed fruits are popular and healthy food products, this work aimed to assess the use of fruit salads as carriers for the probiotic microorganisms *L. acidophilus* LA-5 and *L. plantarum.*

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Materials and Methods

Minimal processing of fruit and salad preparation

Pineapple, banana, guava, apple, papaya and mango at commercial maturity were bought at a local market. Fruits were washed in water to eliminate impurities and dirt and immersed for 20 min at 5°C in water with sodium dichloroisocyanurate (Sumaveg® Diversey Lever) at a concentration of 200 mg/L of residual active chlorine to inactivate the microorganisms. The fruits were peeled and manually cut with stainless steel knives into cubes of approximately 1x1 cm^2. Fruit salads were prepared with all fruits in equal proportion.

Probiotic microorganism inoculation and antibrowning agent addition

Fruit salads were immersed in solution containing approximately 10^{10} CFU/mL of the probiotic microorganisms *L. acidophilus* LA-5 and *L. plantarum* (Christian Hansen®), separately. The cultures were prepared and added individually to the salad according to Rößle, Auty, Brunton *et al.* (2010). The probiotic cultures were grown twice in Man Rogosa Sharpe (MRS) broth, incubated at 37°C for 18 h, and again activated in MRS broth for 16 h. The cultures were centrifuged at 5°C for 15 min at 7000 x g. The supernatant of the culture medium was discarded and the probiotic cell pellets were aseptically resuspended in a buffer solution of citric acid:sodium citrate at a 1:1 ratio and pH 3.8 and centrifuged again at the same conditions. Then, the obtained pellets were resuspended in the buffer solution (citric acid:sodium citrate), pH 3.8, at a ratio of 1:10; i.e., for every gram of cells, 10 ml of buffer solution was added to obtain at least 10^{10} cells/mL of each probiotic.

Thus, to obtain fruit salads containing probiotic cultures, 1 ml of the previously prepared probiotic cell solution was added for each gram of fruit salad. This suspension was kept in contact with the fruit salads for 15 min.

Fruit salads were drained for 3 min and immersed in an antibrowning solution containing 1% ascorbic acid (w/v) for 3 min. The ratio of this solution to fruit salad was 3:1. The control treatment was a minimally processed fruit salad without *L. acidophilus* LA-5 and *L. plantarum* and 1% ascorbic acid. The control was immersed in water in order to observe if the buffer would promote change in the flavor of the fruit salads. Salads were packaged in polypropylene containers with lids.

Counts of L. acidophilus *LA-5 and* L. plantarum *in minimally processed fruit salad*

Samples of 25 g from each fruit salad treatment were homogenized in 225 mL of peptone saline solution (0.85% NaCl and 0.1% peptone) followed by serial dilutions. The pour plate method was used to count the probiotic microorganisms, with 1 mL of each dilution placed on a Petri dish with a small amount of Rogosa SL agar (HIMEDIA, India). The Petri dishes were incubated in anaerobic jars at 37°C for 72 h. Analysis were carried out in duplicate after 0 h, 24 h, 72 h and 120 h of storage at 8°C.

Evaluation of L. acidophilus *LA-5 and* L. plantarum *present in fruit salads by Scanning Electron Microscopy*

Control fruit salads and those treated with *L. acidophilus* LA-5 and L. plantarum were examined by Scanning Electron Microscopy (SEM). In this procedure, each fruit component of salad was used to verify microbial adhesion, distribution and morphology of probiotic cultures on plant tissue. These were analyzed at 0 h and after 120 h storage at 8°C.

Fruits were sliced into 0.5x0.5 cm^2 sections, 1-2 mm thick. To fix plant tissue cells, fragments from each fruit were transferred to a 5% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer at 1:1 ratio. The final concentration of both reagents was 2.5% glutaraldehyde and 0.05 M phosphate buffer. Fruit fragments were kept in this solution for 18 h at 7°C, washed for 1 min in sodium phosphate buffer (0.05 mol/L, pH 7.2), then dehydrated with acetone at 30°GL (Gay-Lussac), 50°GL, 70°GL and 90ºGL for 10 min. Afterward, the fragments were treated three times with acetone at 100°GL for 10 min. Fruit fragments were transferred to the critical point dryer (CPD020 model, Balzers, Liechstenstein) for total dehydration and samples were metalized using the Sputter Coater equipment (model FDU 010, Bal-Tec, Balzers, Liechtenstein) for observation with the SEM (LEO 1430 VP model Zeiss, Cambridge, England) and image capture.

Physicochemical evaluation

Acidity, pH and soluble solids (°Brix)

Acidity and pH were determined after 0 h, 24 h, 72 h and 120 h storage at 8°C, according to AOAC (2010). Samples of 1.67 g from each fruit were combined to achieve a total sampling mass of circa 10.00 g, for the control and fruit salads impregnated with *L. acidophilus* LA-5 and *L. plantarum*. Samples from the control and treated fruit salads (10 g

each) were diluted in 100 mL of distilled water and analyzed.

The soluble solids of the control and treated fruit salads were determined by refractometry, using an Abbe refractometer (Model 100 RTA) at 0 h, 24 h, 72 h and 120 h storage at 8°C. The refractometer was calibrated prior to use. The scale was set to zero using the refractive index of water according to Rößle, Auty, Brunton *et al.* (2010).

In Brazil there is no specific legislation for minimally processed products. The shelf life of fruit salad was evaluated at 120 h, which is generally the maximum storage time that these products tolerate to the conditions of the Brazilian market.

Determining fruit firmness

The firmness of fruits from the control and fruit salad impregnated with *L. acidophilus* LA-5 and *L. plantarum* was determined by compression test using a texture analyzer (CT3, Brookfield, USA) set with a load cell of 25 kg. Analyses were done after 0 h, 24 h, 72 h and 120 h post-processing. Results were expressed in Newtons (N). Three samples from each fruit were selected from each fruit salad treatment and analyzed by placing them individually on a flat surface compressed by a 3.5 cm diameter probe (SMSP/35). The distance between the sample and the probe was 60 mm and the test speed was 5 mm/s.

Microbiological analysis

Total and thermotolerant coliforms were determined using samples from control and fruit salad treated with probiotic cultures. Analyses were done using the most probable number (MPN) method according to Kornacki and Johnson (2001). Lauryl Sulfate Tryptose broth was used for the presumptive test, brilliant green lactose bile broth was used to confirm total coliforms and EC broth was used to confirm thermotolerant coliforms. Obtained results were expressed in MPN per gram of fruit salad.

To quantify *Salmonella* sp., samples of each treatment of fruit salad (25 g) were homogenized in 225 mL of buffered peptone water, according to Andrews *et al*. (2001). Psychrotrophic bacteria were counted according to Cousin *et al*. (2001) with Plate Count Agar (PCA). Petri dishes were incubated for 10 days at 7°C and this microbiota was counted by selecting plates containing 25-250 colonies. The results were expressed in CFU (colony forming units) per gram of fruit salad.

All microbiological analyses were performed in duplicate for the control treatment and the fruit salads containing *L. acidophilus* LA-5 and *L. plantarum*, at 0 h and after 120 h storage at 8°C, to assess whether the products met Brazilian microbiological quality standards (National Health Surveillance Agency, 2001).

Sensory analysis

The acceptance test was performed on the control treatment and the fruit salads treated with *L. acidophilus* and *L. plantarum* by 20 tasters at 0 h and after 120 h storage at 8°C. Each taster received a form containing a nine-point hedonic scale, ranging from "like extremely" (score 9) to "dislike extremely" (score 1). Each untrained tasters evaluated the samples regarding flavor attribute.

The results of the acceptance test of fruit salads were analyzed with a 3x2 completely randomized factorial design. The independent variables analyzed were three treatments (control and fruit salads treated with *L. acidophilus* and *L. plantarum*) and two storage times (0 h and 120 h).

Statistical analysis

To study the viability of *L. acidophilus* LA-5 and *L. plantarum*, as well as the texture, pH, acidity and soluble solids of fruit salads, we used a completely randomized statistical design with three replications. Results were subjected to analysis of variance (ANOVA) followed by the Tukey test.

All statistical procedures were performed considering the level of 5% probability and using the statistical software STATISTICA 7.0 software-(StatiSoft, Inc., Tulsa, Okla., USA).

Results and Discussion

Viability of L. acidophilus *LA-5 and* L. plantarum *in minimally processed fruit salads during the storage*

The average viability of *L. acidophilus* LA-5 in fruit salads was 7.96 Log CFU/g, while for *L. plantarum i*t was 7.83 Log CFU/g after 120 h storage at 8°C, being the food product considered probiotic once it contained probiotic microorganisms above 7.00 Log CFU/g (Vinderola and Reinheimer, 2000; Lourens-Hattingh and Viljeon, 2001). Storage time did not influence the viability of tested probiotic microorganisms ($p>0.05$), with no significant interaction between probiotic bacteria and storage time ($p>0.05$).

Therefore, a package containing 100 g of fruit salad developed in this study offered the consumer a concentration of 9.0 Log CFU of *L. acidophilus* or *L. plantarum*. This probiotic concentration should be enough to promote beneficial effects on the host organism (Prado *et al*., 2008).

Rößle, Brunton, Gormley *et al.* (2010) prepared

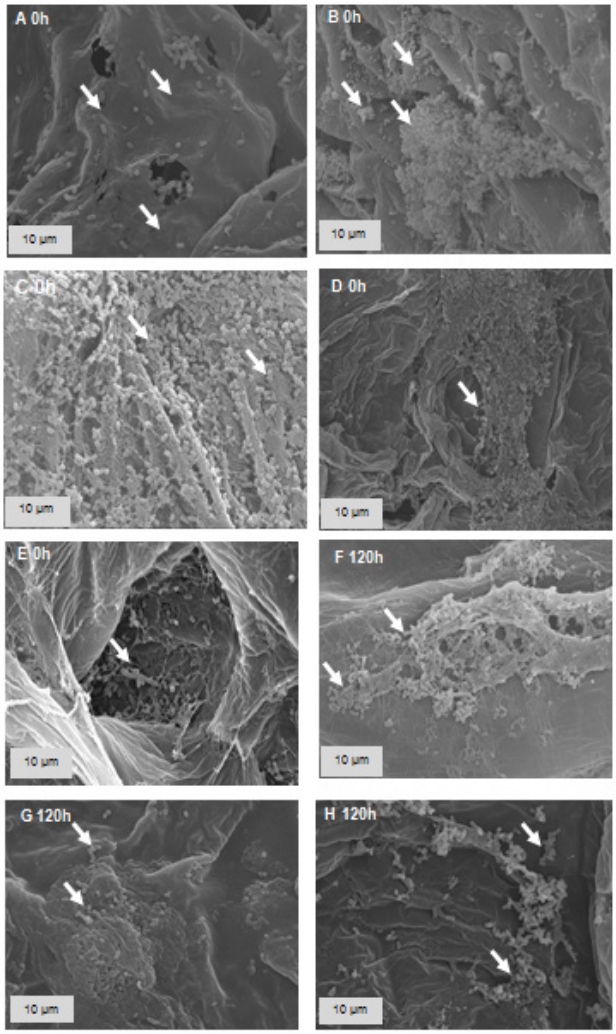

Figure 1. *Lactobacillus acidophilus* adhered to the surface of pineapple (A), banana (B), papaya (C) and mango (D) after processing fruit salads (time 0), and *Lactobacillus plantarum* adhered to the surface of guava (E) (time 0) and adhered to apple (F), papaya (G) and mango (H) after 120 h storage. White arrows indicate bacterial cells adhered to the vegetal tissue

minimally processed symbiotic apples, with probiotic (*L. rhamnosus* GG) and prebiotic (oligofrutose and inulin) ingredients, observing that symbiotic apples contained 10^7 to 10^8 CFU/g of probiotic bacteria after 14 days storage at 2 and 4°C. Thus, they considered the developed apples a good alternative to available dairy food products.

Scanning Electron Microscopy (SEM)

It was not found cells attached to fruits of the control treatment, which shows the efficiency of sanitization and good manufacturing practices used in the minimally processing. SEM imaging allowed verification of the colonization of *L. acidophilus* and *L. plantarum* in fruit tissue after impregnation. Moreover, SEM imaging showed a high number of *L. acidophilus* and *L. plantarum* (Figure 1) adhered to the surfaces of treated fruits.

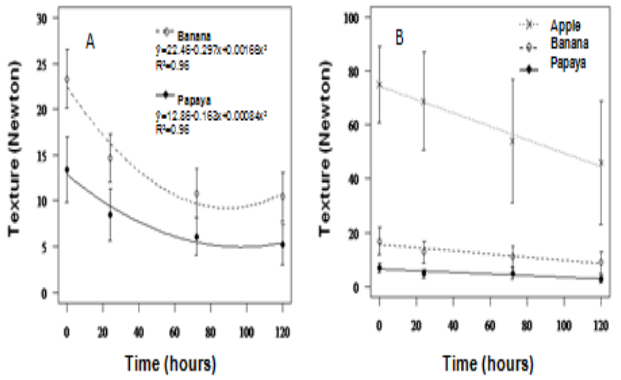

Figure 2. Regression equation and coefficient of determination of firmness of banana and papaya from the control treatment and fruit salad with *L. plantarum* as a function of storage time (A), and firmness of apple, banana and papaya from the control treatment and fruit salad with *L. acidophilus* as a function of storage time (B). Regression equation and coefficient of determination (B) of firmness of apple ($y = 74.39 - 0.2491x$; $R^2 = 0.98$), banana ($y = 15.59 - 0.0592x$; $R^2 = 0.89$) and papaya ($y = 6.44 - 0.0303x$; $R^2 = 0.83$). Data averaged from 3 replicates

SEM images showed that probiotic cells were well distributed in the tissues of treated fruits. However, some areas had agglomerated cells forming on the surface of the pineapple, banana (Figure 1A, 1B), papaya, mango (Figure 1C, 1D, 1G, 1H) and apple (Figure 1F).

Probiotic cells presented typical morphology, with the rod shape being predominant. Moreover, probiotic bacteria presented good adhesion in all tested fruits. The adhesion of probiotic microorganisms to fruit tissues might be attributable to the internal structure of plant tissues that harbor bacteria in places that allow their survival. Figures 1A and 1E show bacterial cells in protected areas, such as pores, which were previously damaged by the minimal processing.

Some fruits, such as apple, papaya and mango inoculated with *L. plantarum,* presented an increased population of probiotic cells after 120 h, showing that a certain time is necessary for bacteria to colonize fruit tissues. Some probiotic cells were also found to adhere to banana surface and were likely formed biofilms (Figure 1B).

According to Fahn (1989) and Passam *et al.* (2011), sugar, soluble carbohydrates and nitrogenous substances can be found in vacuoles. Since minimal processing causes damage to plant tissue, resulting in cellular content leakage (Oliveira *et al.*, 2011), these nutrients are available to microorganisms, thus contributing to probiotic viability as we observed in our study. Impregnation at atmospheric pressure occurs when solutions with probiotic culture are maintained in contact with fruit salad, where the

Table 1. Mean values (n=3) of pH, acidity and soluble solids (°Brix) of minimally processed fruit salads of control treatment and containing probiotic bacteria

Treatment	pH	Acidity	Soluble solids
Control	4.15[a]	0.319[a]	11.84[a]
L. acidophilus	4.11[a]	0.387[a]	12.08[a]
L. plantarum	4.11[a]	0.337[a]	12.00[a]

[a]Means values followed by the same letter in the column do not differ statistically according to the Tukey test (P > 0.05).

Table 2. Mean values (n=12) of firmness (N) of fruits in control fruit salads and containing *L. plantarum* or *L. acidophilus*

Treatment	Mango	Papaya	Banana	Guava	Apple	Pineapple
Control	10.30[a]	8.55[a]	15.24[a]	59.8[a]	115.20[a]	26.45[a]
L. plantarum	16.67[a]	8.04[a]	14.38[a]	68.3[a]	112.21[a]	29.92[a]
Control	18.00[a]	5.05[a]	13.15[a]	35.55[a]	71.49[a]	20.15[a]
L. acidophilus	22.35[a]	4.55[a]	11.63[a]	24.91[a]	50.41[b]	20.97[a]

[a,b]Means values followed by the same letter in the column when it was compared control with the respective probiotic do not differ statistically according to the F test (P> 0.05).

substrates present in the cells are lost or changed by the probiotic-containing solution, because of the hydrodynamic process (Derossi *et al*., 2011).

Studies conducted by our research team showed that incorporating probiotic bacteria into processed fruits is highly advantageous, due to the high amount of nutrients available to microorganisms that favor their permanence and adhesion, in addition to the health benefits preferred by the majority of consumers compared to dairy products. According to Espírito-Santo *et al*. (2011), although there are few studies showing the effect of fruit as a food matrix on the viability and activity of probiotic microorganisms, these studies indicate the positive effect of fruit on probiotic microorganisms and their interaction with the host.

Physicochemical characteristics of fruit salads

Physicochemical parameters, such as pH, acidity and soluble solids content had no significant difference among treatments (Table 1). Moreover, storage time had no influence (p>0.05) on the assessed physicochemical parameters of any treatment. Similar to our results, Rößle, Brunton, Gormley *et al.* (2010) verified there were no changes in minimally processed apple inoculated with *L. rhamnosus* GG and stored at 2 and 4°C for 14 days.

Moreover, our results showed that adding *L. plantarum* did not cause significant changes to the texture of treated fruits (p>0.05) compared to the control (Table 2). However, the texture of apple was altered (p<0.05) when *L. acidophilus* was added (Table 2).

The texture of papaya and banana in fruit salads impregnated with *L. plantarum* was influenced (p<0.05) by storage time (Figure 2A), but there was no difference between treatments, and the interaction of storage time and treatment was not affected (p>0.05). Therefore, the softening of texture of these fruits was determined by the mean values of both treatments (Figure 2A). Papaya, banana and apple in fruit salad impregnated with *L. acidophilus* (Figure 2B) lost firmness during storage (p<0.05). However, only the texture of apple (Table 2) was affected by adding *L. acidophilus* (p<0.05). There was no significant interaction (p>0.05) between storage time and the probiotic treatment for the fruits.

Climacteric fruits, such as papaya, banana and mango presented short shelf life, with papaya being the most perishable fruit. Tapia *et al.* (2008) in their study verified that the firmness of minimally processed papaya was approximately 2 N, indicating little firmness, during 8 days storage at 4°C. For the authors, the softening of papaya occurred mainly due to the hydrolysis of pectic acids in the cell wall, promoting the loss of firmness.

Table 3. Mean values (n = 3) of psychrotrophic microorganisms count (Log CFU/g), most probable number of total and thermotolerant coliforms (MPN/g) and *Salmonella* sp. results in controls fruit salad, fruit salad with *L. acidophilus* LA-5 and *L. plantarum*

Time (h)	Microbial group	Control salad without *L. acidophilus* LA-5	Salad with *L. acidophilus* LA-5	Control salad without *L. plantarum*	Salad with *L. plantarum*
0	Psychrotrophic	< 1,0	< 1,0	2,4	2,2
	Total coliforms	< 3,0	< 3,0	< 3,0	< 3,0
	Thermotolerant coliforms	< 3,0	< 3,0	< 3,0	< 3,0
	Salmonella sp.	Absence	Absence	Absence	Absence
120	Psychrotrophic	4,0	< 1,0	2,7	2,2
	Total coliforms	12,1	< 3,0	6,7	< 3,0
	Thermotolerant coliforms	< 3,0	< 3,0	< 3,0	< 3,0
	Salmonella sp.	Absence	Absence	Absence	Absence

Microbiological characteristics of fruit salads

Psychrotrophic growth was higher in the control treatment than in fruit salad treated with *L. acidophilus* LA-5 which presented counts of at least 3.0 Log CFU/g lower than the control treatment after 120 h storage at 8°C (Table 3). Alegre *et al.* (2011) evaluated the addition of L. rhamnosus GG in minimally processed apple and its effect on pathogen growth, such as *L. monocytogenes* and *Salmonella* from different strains and verified that the count of *L. monocytogenes* was reduced by 1 Log cycle in apples inoculated with probiotics. The fruit salads developed in this work followed the microbiological standards established by the Brazilian law (National Health Surveillance Agency, 2001). Thus, the developed probiotic fruit salads were appropriate for consumption for a period of 120 h after minimal processing.

Sensory analysis

Sensory analysis was done after processing and at the end of shelf life of fruit salads to determine whether there was a difference regarding sample acceptance by tasters for flavor attribute. Acceptance notes for all treatments (control and fruit salads with probiotics bacteria) were above 7.0, "like moderately" on a nine-point hedonic scale, indicating that control fruit salad and fruit salads with *L. acidophilus* and *L. plantarum* had a good acceptability and no significant differences ($p>0.05$). However, there was significant impact of time on the taste of fruit salad ($p<0.05$). After processing, this food had an average score of 7.70 and after 120 h of storage at 8°C the average score for flavor attribute was 7.21.

Conclusion

Minimally processed fruit salad can be considered a promising carrier for probiotic bacteria, since counts of *L. acidophilus* LA-5 and *L. plantarum* in the product were similar to those found in fermented dairy products, and the developed fruit salads followed the microbiological requirements of the Brazilian legislation.

Fruit salad has promise in the minimally processed food product market, with health benefits promoted by impregnated probiotic microorganisms. One of the main advantages of probiotic fruit salad is that it can be consumed by members of the population, with no allergy-related restrictions. Although the results show that the fruit salad is a promising vehicle for probiotics, does not exist in Brazilian legislation standard for non dairy-based probiotic foods.

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