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Evaluation of *fruta-do-lobo* (*Solanum lycocarpum* St. Hill) starch on the growth of probiotic strains

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ABSTRACT

Fruta-do-lobo (*Solanum lycocarpum* St. Hill) is a native fruit commonly used in Brazilian folk medicine as a hypoglycemic agent. These properties are attributed to their starch, mainly its resistant fraction. Resistant starch has shown to increases the growth of *Bifidobacterium* and *Lactobacillus* in the gut, even though not being selective for these strains. In this scenario, this study aimed to investigate the potential prebiotic activity of *fruta-do-lobo* starch (FLS). FLS showed around 30% of resistant starch and their prebiotic potential was evaluated with five probiotic strains *L. acidophilus* (LA3 and LA5), *L. casei* (LC01) and *B. animalis* (BB12) and *B. lactis* (BLC1) in a concentration range of 1.0–2.0% of starch. In a preliminary screening, we evaluated, during 48 h, the viability of the starch with promoting growth agent. An increase in the growth of the probiotic strains tested was observed. We also evaluated the microorganism's metabolic activity by assessing the short-chain fatty acid (SCFA) production, using the best starch growth promotion conditions (2% of FLS and strains BLC1, LA5, and LC01). As expected, MRS and lactose were preferentially metabolized by BLC1, with the highest growth rates: 0.231 and 0.224 h^{-1}, respectively. However, for this strain, the FLS growth rate (0.222 h^{-1}) was 65% higher than FOS (0.144 h^{-1}). Also, for LA5 FLS promoted higher growth (0.150 h^{-1}) than FOS (0.135 h^{-1}). Additionally, FLS promoted acetate production. These data are promising and indicate that FLS may have prebiotic potential and more studies need to be done with pathogenic microorganisms.

1. Introduction

Fruta-do-lobo (*Solanum lycocarpum* St. Hill) (wolf fruit) is a native fruit commonly used in Brazilian folk medicine as a hypoglycemic agent. Experimental evidence indicates that *fruta-do-lobo* starch (FLS) is involved in the reduction of glycemic index followed by the attenuation of physiological changes associated with diabetes, such as polyuria and polyphagia (Farina, Piassi, Moysés, Bazzolli, & Bissoli, 2010; Perez, Franca, Daldegan, & Duarte, 2006). These effects are attributed to their resistant starch content, which corresponds to about 30% of the total starch (Clerici et al., 2011; Dall'agnol & von Poser, 2000).

Resistant starch is the total amount of starch as well as the products of starch degradation which resists digestion in upper gastrointestinal tract. Since it is resistant to enzymatic hydrolysis within 120 min of consumption, it does not contribute to postprandial hyperglycemia (Tacer-Caba & Nilufer-Erdil, 2019). Instead, it reaches the colon, and then it is fermented by the gut microbiota (Zaman & Sarbini, 2015). Therefore, resistant starch has the characteristics of a prebiotic compound (Colantonio, Werner, & Brown, 2020; Gibson et al., 2017). The most prominent metabolites of this fermentation are short-chain fatty acids (SCFA) such as acetic, propionic and butyric acids (Cerqueira, Photenhauer, Pollet, Brown, & Koropatkin, 2020).

It is essential to highlight that SCFA are absorved by the host and are subsequently used in several metabolic processes as well as exerting beneficial effects on the human body (Morrison & Preston, 2016). Among these benefits are the improvement of intestinal function and absorption of minerals, regulation of lipid and glucose metabolism, and a reduction in the risk of developing colon cancer (De Paulo Farias,

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Fernandes de Araújo, Neri-Numa, & Pastore, 2019).

The supplementation with resistant starch can also improve the glycemic status, the inflammation markers and lipid profile (Gargari et al., 2015). Still, alterations in gut microbial composition or function, known as dysbiosis, have been associated with health impairment and changes in host immune status (Danneskiold-Samsøe et al., 2019). Therefore, the use of resistant starch could be beneficial to health if eaten raw.

In this sense, FLS could aggregate value to food industry, due to a prebiotic potential effect. So, this study aimed to investigate the effect of *fruta-do-lobo* starch on the growth of probiotic strains, which could exert potential improved of gut microbiota.

2. Material and methods

2.1. Collection and identification of fruit species

About 25.0 kg of *fruta-do-lobo* (*Solanum lycocarpum* St. Hill) were manually collected in August 2016, in Carmo do Rio Claro, Minas Gerais, Brazil, and the exsiccate was deposited in the herbarium of the Institute of Biology of UNICAMP (UEC 197248). Fruits were selected taking into consideration the stage of maturation (totally unripe), uniform size, the color of the peel (intense dark green), stalks trapped, and absence of injuries caused by insects or mechanical shocks.

2.2. Extraction of starch

The extraction of starch was performed according to Clerici et al. (2011), with modifications. Fruits were washed, peeled, and the seeds were removed. Pulp was cut in small pieces and put into a sodium bisulfite solution (200 mg/L). Subsequently, they were washed to remove excess bisulfite and ground in a blender with distilled water (1:5 v/v) until a homogeneous suspension was formed. The suspension was sieved (22 mesh) and the portion retained was characterized as pomace. The liquid was kept under refrigeration overnight for decanted of starch. Starch was dried at 50 °C for approximately 15 h in convective hot-air dried (Ethik, 420-7D, Brazil). For the calculation of the yield of extraction, the relation between the weight of the original raw material and the weight of the final product was established, and the results were expressed in %.

2.3. Characterization of starch

Moisture, protein, ash, and dietary fiber contents of starch were determined in triplicate according to official methods 925.09, 979.09, 923.03, and 991.43, respectively (AOAC, 2006). Lipid content was determined according to Bligh and Dyer (1959), and carbohydrate content was calculated by difference.

2.4. Irradiation starch

The sterility of FLS was achieved through gamma-irradiation. The packed FLS sample were subjected to a dose of gamma irradiation (1.5 kGy) using cobalt-60 as the source of irradiation at room temperature of 25 ± 2 °C. The treatment was performed at Nuclear Energy Center in Agriculture of the University of São Paulo (CENA/USP).

2.5. Resistant starch

The resistant starch content was determined according to the methodology proposed by Goñi, Garcia-Diaz, Mañas, and Saura-Calixto (1996). Briefly, a digestion process was performed using pepsin, α-amylase, and amyloglucosidase enzymes. After the digestion process, the residue was discarded and the supernatant was used to determine the glucose concentration by the glucose oxidase method, using the spectrophotometer with a wavelength of 505 nm. A standard glucose curve was used.

2.6. Effect of fruta-do-lobo starch on the growth of probiotic strains

We evaluated the effect of FLS on the growth of five probiotic strains: *Lactobacillus acidophilus* (LA5), L*actobacillus casei* (LC01) and *Bifidobacterium animalis* (BB12) from Christian Hansen (Denmark); *Bifidobacterium lactis* (BLC1) and *Lactobacillus acidophilus* (LA3) from (Lyofast, Italy). The experiments were done according to Sousa et al. (2015), with modifications.

Before each test, the strains were activated in MRS broth (DeMan-Rogosa-Sharpe) and incubated at 37 °C overnight. In order to prepare stock cultures, a 1 mL aliquot of the new inoculum was transferred to pre-sterilized microtubes and centrifuged (5734*g*, 10 min, 4 °C) (Hettich Rotanta 460R). The supernatant was discarded, and the microorganism maintained in the microtube. Subsequently, 1 mL of sterile freezing medium was added to the microorganism, and each microtube was identified and stored at −80 °C for future use as a stock culture.

2.6.1. Starch concentration evaluation

The cryopreserved strain was reactivated in MRS medium and incubated under anaerobic conditions at 37 °C for 48 h.

The growth promoting activity screening of each strain was performed in microplate assays using three different starch concentration, 1.0, 1.5 and 2.0% (w/v). Each strain was inoculated with the media mentioned above, considering the initial optical density of 0.6. The optical density was measured using a microplate reader (BMG LABTECH, Germany). The microplate was incubated during 48 h at 37 °C and monitored by measuring absorbance at 655 nm every 30 min. Specific growth rates were calculated (by determination of the slope of the trend line, of the viable cell numbers in the log phase of the growth curves) in order to compare the obtained results.

2.6.2. Evaluation of the effect of fruta-do-lobo starch on the growth via viable cell number and metabolic activity

Growth curves and metabolic activity were used to confirm the effect of *fruta-do-lobo* starch on the growth of probiotic strains. MRS with a four different carbon source were used. Each medium was composed of: MRS broth (with glucose), mineral MRS with 2% lactose (w/v) and mineral MRS with 2% FOS, which were used as a control, and mineral MRS with the best starch concentration (2%). Those carbon sources were added in replacement of glucose. Three probiotic strains were selected (BLC1, LC01, and LA5), since the use of the carbon source may differ according to the microorganism. Growth curves were monitored by viable cell counts, as well as by the evaluation of optical density (655 nm). Bacterial metabolism was assessed by changes in pH and SCFA production. All analyses were performed in duplicate and sampled at 0, 4, 8, 12, 24, and 48 h.

2.7. Short-chain fatty acids production

Short-chain fatty acids were identified and quantified according to Zhao, Nyman, and Jönsson (2006) and da Silva, Cazarin, Bogusz Junior, Augusto, and Maróstica Junior (2014) with modifications, as described below:

2.7.1. Preparation of standard solution

Standard solutions were individually prepared and were used for quantification of produced acetic (0.04–10.49 mg/mL), propionic (0.04–9.93 mg/mL), and butyric acids (0.08–9.64 mg/mL). A 2-ethyl-butyric acid solution containing HCl 5 M was used as an internal standard stock solution. All the standard solutions were prepared and immediately used. Results are expressed in mmol/L.

2.7.2. Sample preparation

Samples were collected from each Erlenmeyer at different times (0,

4, 8, 12, 24, and 48 h) and were immediately stored at −18 °C.

At the time of analysis, samples were centrifuged (10 min, 10,000 RPM and 5 °C). The supernatant was filtered using 0.22 μm filters. After that, the pH was adjusted to 2–3 by adding acidified water and then kept at room temperature for 10 min with occasional shaking. The internal standard, 2-ethyl butyric acid solution, was spiked into the supernatant at a final concentration of 1 mM and the solution was injected in the GC for analysis.

2.7.3. Short-chain fatty acids (SCFAs) quantification

Chromatographic analysis was carried out using an Agilent 6890A gas chromatograph system equipped with a flame ionization detector (FID) and a capillary column (30 m × 0.25 mm i.d. × 0.25 μm Nukol™, Supelco, Bellefonte, PA, US). The chromatographic conditions were as follows: injector and detector temperatures set at 250 °C, injected volume 1 μL with the split ratio set to 1:10, and the carrier gas was helium at 1.0 mL/min. The initial oven temperature was 100 °C, maintained for 0.5 min, raised to 180 °C at 8 °C/min and held for 1.0 min, then increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5 min totalizing 17.5 min. The SCFAs were identified on chromatograms by their retention times compared with the standard solution.

2.8. Statistical analyses

Statistical analyses were performed using Statistica® and R programs. Analyses of variance (ANOVA) and Tukey's test were used to determine differences among treatments ($p < 0.05$).

3. Results and discussion

3.1. Process yield and characterization of starch

We obtained a yield of 3.7 ± 0.53% starch, lower than the obtained by Clerici et al. (2011) (4.8%). Given that the same extraction methodology was used, these differences can be explained by the fruit ripeness degree, manipulation, among others.

The proximate composition and resistant starch content of FLS are shown in Table 1. Our results for moisture, protein, ash, and fiber are lower than the values found by Clerici et al. (2011), 12.15, 6.27, 0.38, and 0.93 (g/100 g), respectively. These variations are common and depend on climatic factors and soil types, for example.

Regarding resistant starch content, our results (Table 1) were similar to those found by Clerici et al. (2011) (32.32 g/100 g). However, when compared to green bananas, a recognized source of resistant starch, their starch has a higher content, ranging from 47.3 to 54.2% (Almeida-Junior, Curimbaba, Chagas, Quaglio, & Di Stasi, 2017). It is important to highlight that of the five types of resistant starch, type 1 and 2 resistant starch, present in green fruits, are hydrolyzed with thermal processing, and so its use is not indicated in all types of product (Tacer-Caba & Nilufer-Erdil, 2019). Therefore, to ensure the benefits of FLS, it must be consumed raw.

3.2. Evaluation of the effect of fruta-do-lobo starch on the growth of probiotic strains

3.2.1. Choosing of microorganism and starch concentration

All strains were able to consume the FLS in all concentrations, and the specific growth rates are shown in Table 2. Our results are smaller when compared with the values found by Sousa et al. (2015), who evaluated the potential prebiotic activity of yacon at different concentrations. This finding can be explained because, once the initial media starch solution was cloudy, we measured the microorganism growth indirectly via starch consumption monitoring, due to turbidity decrease. Looking at our results, the higher growth rates were observed for BLC1 when 2.0% FLS concentration was used (0.007 h^{-1}), showing a better ability of this strain in using FLS as a carbon source. For the other strains, there was no difference in the multiplication rate independent of the concentration tested. Therefore, we opted for the highest concentration (2%). In addition to BLC1, we chose to evaluate two more strains of different species (LA5 and LC01), since the use of the carbon source may differ according to the microorganism.

Table 2
Specific growth rate for the *Lactobacillus* and *Bifidobacterium* strains tested in the different concentrations of *fruta-do-lobo* starch (FLS) (1.0–2.0%).

Strain	FLS concentration (%)	Specific growth rate (h^{-1})
LA3	1.0	0.002 ± 0.001
	1.5	0.003 ± 0.000
	2.0	0.003 ± 0.001
LA5	1.0	0.002 ± 0.000
	1.5	0.003 ± 0.001
	2.0	0.004 ± 0.002
LC01	1.0	0.002 ± 0.001
	1.5	0.002 ± 0.000
	2.0	0.003 ± 0.001
BB12	1.0	0.002 ± 0.000
	1.5	0.002 ± 0.000
	2.0	0.002 ± 0.000
BLC1	1.0	0.004 ± 0.002
	1.5	0.003 ± 0.001
	2.0	0.007 ± 0.002

3.2.2. Evaluation of the effect of fruta-do-lobo starch on the growth via viable cell number and metabolic activity

The effect of *fruta-do-lobo* starch on the growth of probiotic strains was compared with a conventional carbon source – MRS; and lactose, a sugar preferentially and quickly metabolized by lactic acid bacteria; and FOS, a non-digestible carbohydrate known for acting as a prebiotic. Prebiotics have been related to an increase in the abundance of *Bifidobacterium* and *Lactobacillus* species, playing a crucial role in maintaining a healthy microbiota (Danneskiold-Samsøe et al., 2019).

Firstly, we evaluate the microorganism growth, and as expected, all strains were able to grow using FLS, MRS, lactose or FOS as the primary carbon source. Fig. 1 shows the growth curves using viable cell number, once the presence of starch prevents the direct access to growth rate using OD measurements (Supplementary material 1). Additionally, in order to prove the difference between carbon sources, growth rates were calculated for each of the strains tested. Glucose (MRS) and lactose were preferentially metabolized by BLC1, with the highest growth rates: 0.231 and 0.224 h^{-1}, respectively. However, for this strain, the FLS growth rate (0.222 h^{-1}) was 65% higher than FOS (0.144 h^{-1}). Also, for LA5, FLS promoted higher growth (0.150 h^{-1}) than FOS (0.135 h^{-1}). These results are promising and point to the possible prebiotic effect of FLS.

The pH reduction was also evaluated during microorganism growth (Fig. 2). The acid production confirmed that there was a reduction for all conditions and no significant difference was observed between them

Table 1
Proximate composition and resistant starch content of *fruta-do-lobo* (*Solanum lycocarpum* St. Hill).

Moisture (g/100 g)	Total carbohydrate (g/100 g)	Lipid (g/100 g)	Protein (g/100 g)	Ash (g/100 g)	Fiber (g/100 g)	Resistant starch (g/100 g)
8.66 ± 0.07	90.3	0.24 ± 0.00	0.68 ± 0.02	0.12 ± 0.02	0.00	29.79 ± 0.61

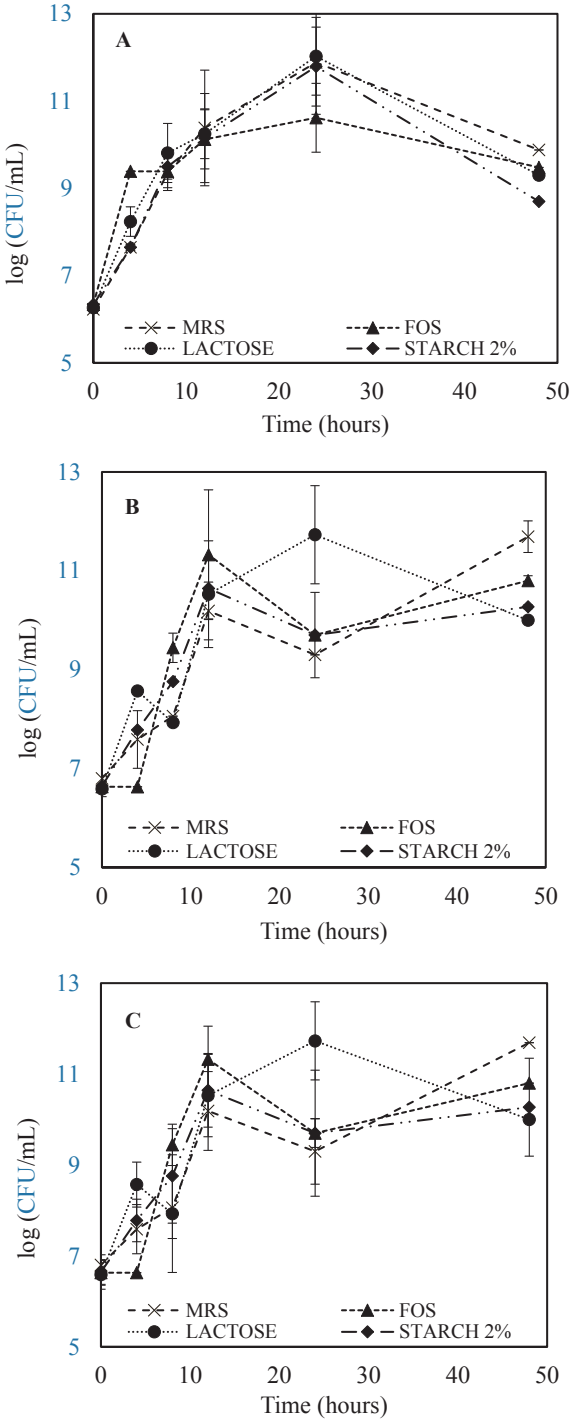

Fig. 1. Growth curve for *Bifidobacterium lactis* BCL1 (A), *Lactobacillus casei* LC01 (B) and *Lactobacillus acidophilus* LA5 (C) using different carbon source. Results are expressed in log (CFU/mL).

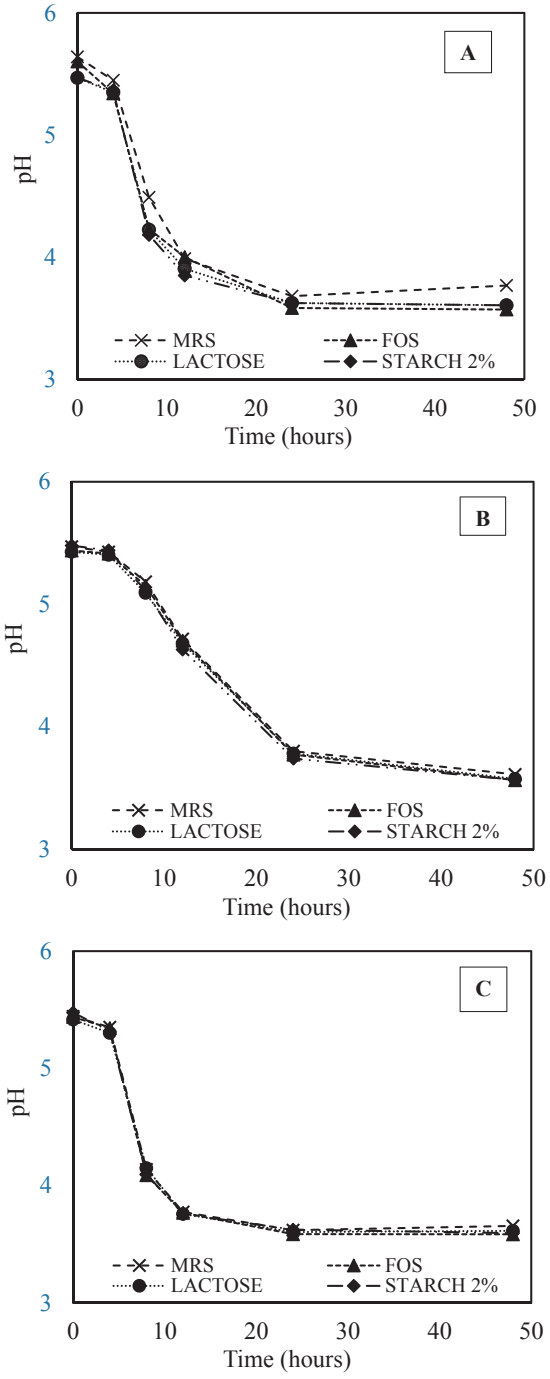

Fig. 2. pH curve for *Bifidobacterium lactis* BCL1 (A), *Lactobacillus casei* LC01 (B) and *Lactobacillus acidophilus* LA5 (C) using different carbon sources. Results are expressed as acidity degrees (pH).

($p < 0.05$). However, the strains BCL01 and LA5 were able to do it faster than LC01 when using FLS as a carbon source.

Regarding acid production at 24 h, our results are similar to those observed by Sousa et al. (2015), who evaluated the prebiotic effect of yacon flour. When 1% of this flour was added to the MRS medium with glucose, a final pH (24 h) of 3.66 was observed for *L. casei* and 4.00 for *L. acidophilus*. Although the pH results found do not reflect the physiological conditions of the colon, we emphasize that this is a preliminary study, which assessed only the ability of probiotic strains to metabolize FLS.

Studies have been shown that when pathogens bacteria are grown together with beneficial bacteria and prebiotics are used, there are a change their population balance, favoring the beneficial one (Jung, Jeon, & Han, 2015). We believe that it may occur because friendly bacteria such as *Bifidobacterium*, while feeding on prebiotics, lower the pH levels by acids production, making the gut less favorable for pathogens growth (Kolida, Tuohy, & Gibson, 2002). In addition, the acid production of *Lactobacillus* and *Bifidobacterium* using prebiotics inhibit the proliferation of harmful bacteria due to increasing in the production of SCFA, IL-10, among others (Colantonio, Werner, & Brown, 2020; Sharma & Tripathi, 2019).

Our results are promising and demonstrate the need for additional

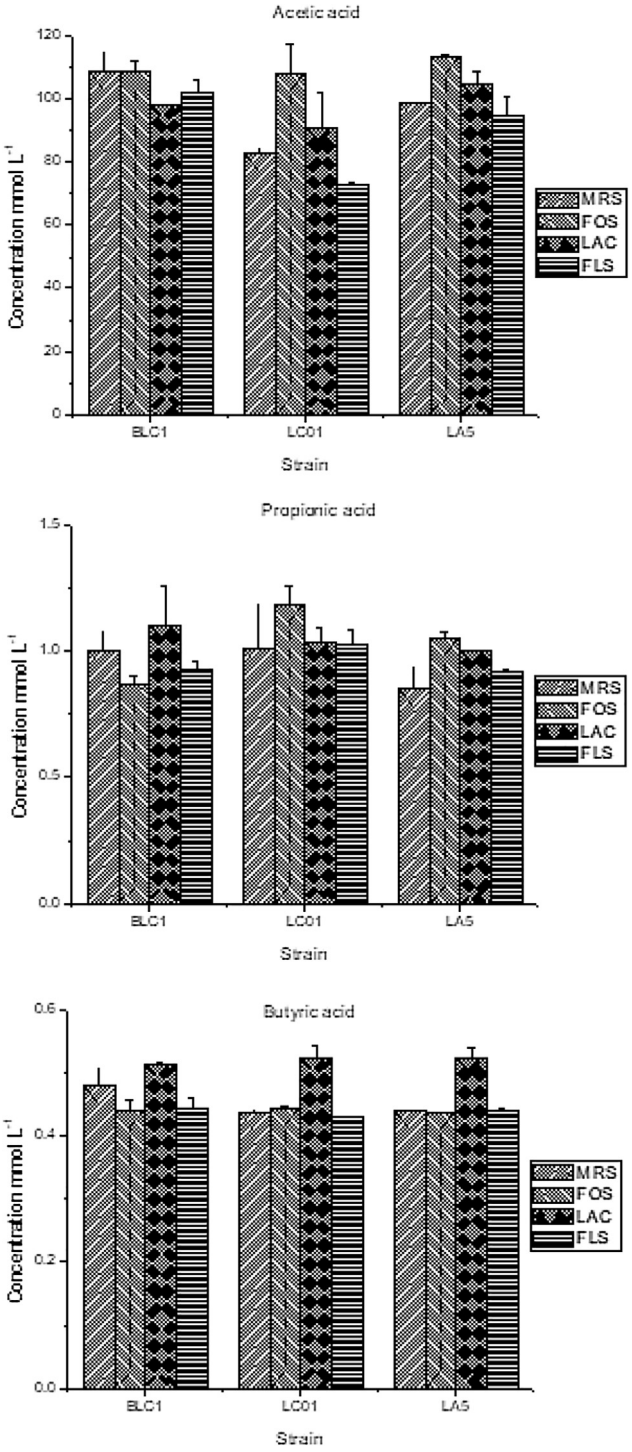

Fig. 3. Final production of SCFA (acetic, propionic and butyric) produced from *fruta-do-lobo* starch (FLS) and others carbon sources for probiotic strains for *Bifidobacterium lactis* BCL1, *Lactobacillus casei* LC01 and *Lactobacillus acidophilus LA5*. Results are expressed as mmol/L of acid.

studies in vivo, which could demonstrate the FLS prebiotic potential.

3.3. Short-chain fatty acids (SCFA) production

SCFA are the primary metabolic products of natural polysaccharides from gut microbiota fermentation in the distal intestine and play an essential role in maintaining the normal function of the colon and in the morphology of their epithelium (Xia, Wang, Yu, Liang, & Kuang, 2019). About 90–95% of SCFA are acetate, propionate, and butyrate, with smaller proportions of valerate and hexanoate (Wang et al., 2019). Relatively minor amounts of branched-chain fatty acids (BCFA), such as isobutyrate and isovalerate, also can be present but originate from the breakdown of proteins rather than carbohydrates (Morrison & Preston, 2016).

The SCFA generated by probiotic strains depends on the type of fiber or substrate consumed as well as the type of bacteria metabolizing it (Lewis & Abreu, 2017). Fig. 3 shows the final production and Supplementary Material 2 shows the production over the monitored period of three SCFA (acetate, propionate and butyrate) produced from FLS and other carbon sources for three strains previously selected (BLC1, LC01, and LA5). In our study, there was a predominance of acetate (about 98%) and low production of propionate and butyrate (approximately 1.5 and 0.7%, respectively), regardless of the source of carbon used. Our results are in accordance with Fukuda et al. (2011) and Bindels, Delzenne, Cani, and Walter (2015), who showed that *Bifidobacterium* and *Lactobacillus* are mainly responsible for acetate production.

Although fermentation of resistant starch is highly related to an increased production of butyrate in the colon, it is mostly done by some specific microorganisms, such as *Ruminococcus bromii, Faecalibacterium prausnitzii, Eubacterium retale* and *Eubacterium hallii* (Morrison & Preston, 2016).

At 48 h time, the production of acetate from fermentation of FLS by strain BLC1 was statistically equal for MRS, FOS and FLS. And, even though acetate production was lower when the LC01 and LA5 strains were tested, values close to those found for other carbon sources were still observed (Supplementary material 2). Production of acetate, as well as a decrease of pH values, demonstrate the *Lactobacillus* and *Bifidobacterium* were able to metabolize FLS.

SCFA play an essential role in the immune system. Previous investigations suggest that acetate amplify the production of the anti-inflammatory cytokine IL-10 and exerts its action on the colonic epithelium (Fukuda et al., 2011; Khangwal & Shukla, 2019). Regarding the role of SCFA on diabetes, it is known that two short-chain fatty acid receptors FFA2 (acetate and propionate) and FFA3 (butyrate) have a direct effect on insulin secretion and β cell proliferation. These receptors were found in pancreatic islets (Tang et al., 2015). FFA3 modulate glucose-stimulated insulin secretion, and FFA2 contributes to the regulation of β-cell mass (Priyadarshini, Wicksteed, Schiltz, Gilchrist, & Layden, 2016). According to Tang et al. (2015), under diabetic conditions, elevated acetate acts on FFA2 and FFA3 to inhibit proper glucose-stimulated insulin secretion and acetate is more likely to regulate insulin secretion through these receptors because it reaches sufficiently high plasma concentrations. Therefore, this may be the mechanism by which FLS acts to reduce blood glucose in an animal model, which has been demonstrated by Farina et al. (2010).

Until now, there is no data on the prebiotic activity of FLS. However, a review study show that a supplementing or increased resistant starch intake (doses of 10–60 g/day) improves meal glucose response, blood glucose and lipid levels, and inflammatory biomarkers, endotoxemia and glycemic, oxidative stress, and antioxidant biomarkers among adults with type 2 diabetes (Colantonio, Werner, & Brown, 2020).

Although there is scientific evidence that SCFA modifies the microbiota and FLS are promoting the SCFA production, further studies using in vivo experimental models are needed to the complete evaluation of these benefits.

4. Conclusion

It was observed that *fruta-do-lobo* starch promoted the growth of probiotic strains of genera *Lactobacillus* and *Bifidobacterium* tested, as well as the recognized prebiotic FOS, to which starch was compared. There was also production of AGCC, mostly acetate. The results of this

study offer a new way to understand how FLS can bring health benefits, as previously demonstrated in animal model. However, further studies are needed using other experimental models to demonstrate its prebiotic potential as well as the safe dose for human consumption. If its prebiotic activity is proven, FLS could be used by the food industry, giving products a healthy appeal.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Publication

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109187.

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