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Evaluation of the Potential of *Araucaria angustifolia* Seeds as Source of Oligosaccharides, Resistant Starch and Growth of Probiotic Bacteria

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Abstract

"Pinhão", the seed of *Araucaria angustifolia*, is an important food, being part of the eating habits of Indigenous communities. In this study, we evaluated the oligosaccharide content, resistant starch and the growth of probiotic bacteria. GF4 (1-fructofuranosylnystose) was the main fructo-oligosaccharides found, in higher contents compared to other food sources. Maltooligosaccharides (MOS) represented the main part of the oligosaccharides profile of Brazilian pine seeds. In descending order of importance was maltoheptaose (G7), maltohexose (G6) and maltotriose (G3). The starches from the variety Sanct josephi presented the highest amount of resistant starch that could stimulate probiotic strains, mainly *B. breve* and *L. plantarum*, and may have a prebiotic effect, potentially promoting health benefits. This study advances the understanding of the chemical composition of the main portion of the "pinhão" enhancing awareness of its potential as a healthy food source, contributing to different uses and indirectly with the species preservation.

Keywords

Maltooligosaccharides, Fructooligosaccharides, Resistant Starch, *B. breve*, *L. plantarum*

1. Introduction

"Pinhão", the seed of *Araucaria angustifolia*, is a historical and cultural food in Brazil. It was part of the eating habits of indigenous communities and still nowadays goes on as a preferred food by South Brazilians. In general, these seeds are roasted or boiled in a pressure cooker [1]. However, it has been widely used as an ingredient for preparing dishes such as cakes, risotto, breads, flan, soups and pasta. The seeds contain a hard coat with three layers (exotesta, mesotesta and endotesta), an endosperm and an embryo [2]. Its outer layer coat has colors ranging from yellow, light red, reddish red to dark red and the length varies from 3.0 to 8.0 cm, 1.0 wide and average weight of 8.7 g [3] [4].

Brazilian pine seeds have gained the attention by researchers because of its high nutritional value, and contain resistant starch, dietary fiber and minerals such as magnesium and copper [5]. Although they are rich in starch, they are low in fat and sugar and its intake produces low glycemic index [4]. Also, some compounds present in Brazilian pine seeds have functional properties, for instance, antioxidants and the aforementioned resistant starch [6] and can promote health benefits for consumers. However, there are no studies on the oligosaccharides content of "pinhão". Both resistant starch and other prebiotic compounds may suffer modification by processing. For instance, extrusion reduced resistant starch content and increased slow digestible starch in pine seeds [7].

Oligosaccharides, together with resistant starch, are two of the most important prebiotics [8], acting as bioactives in the body. Prebiotics are short-chain carbohydrates that, during digestion, are not broken down by enzymes present in the digestive system [9] [10]. The functional oligosaccharides are fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), isomalto-oligosaccharides (IMO), soy-oligosaccharides (SO), mannan oligosaccharides (MOS), gentiooligosaccharides, isomaltulose, lacto-sucrose, malto-oligosaccharides (MO), xylooligosaccharides (XOS), and pectin-derived acidic oligosaccharides (pAOS). Fructooligosaccharides, inulin and galactooligosaccharides are the most known prebiotics [11] [12].

Oligosaccharides are naturally abundant in vegetables, roots, tubers and fruit crops. Some studies found oligosaccharides in leek, asparagus, chicory, Jerusalem artichoke, garlic, onion, wheat, oat, soybean, banana, dragon fruit, nectarine, okra, jack fruit, palm fruit and others [8] [13] [14]. Yacon, a millennial tuber consumed by Incas, is one of the most important prebiotic foods in South America [15]. Oligosaccharides not only occur in nature but can also be synthesized by physical, chemical or enzymatic methods [16].

Starch is the most abundant storage polysaccharide in plants and is the major component of diet. Digestibility of starch improves during cooking, but not all of the starch present in a food is digestible [17]. Resistant starch contains a portion that escapes digestion and absorption in the small intestine and is fermented in the large intestine of humans, with the production of short-chain fatty acids (SCFA) [18]. This type of starch is considered a dietary fiber and prebiotic, since

its fermentation in the intestine provides health benefits such as colonic cancer prevention, control of hyperglycemia, better absorption of minerals and reduction in cholesterol [19]. There are five types of resistant starch. The first one called inaccessible (RS1), found in legume seeds with thick cell walls. The type B or C (RS2) is found in native or uncooked starches, such as green banana and high-amylose maize starch [20]. The third, retrograded starch, can be found in cooked, cooled potatoes and fried rice [21], also called resistant starch type 3 (RS3). The fourth is a chemically modified starch (RS4). The last one, amylase-lipid complex (RS5) is found in some tubers [22].

There is great interest by the industries regarding novel sources of prebiotic foods due to the increase in demand for products targeted towards health-conscious consumers. To meet this growing and changing demand, the food industry needs to create new and innovative concepts and food products, using new technologies and raw materials. New raw materials are emerging as superfoods, which gained consumers' attraction over the last years [23]. Most of the plants investigated regarding prebiotic potential come from western origin. It is very important to explore other parts of the world that have a wide variety of plant species [24], for example, Brazil.

The goal of this study was to investigate the oligosaccharides and resistant starch contents, and the biometric parameters of three varieties of *Araucaria angustifolia* that differ in the coloration and maturation season of the seeds. The *Sancti josephi* variety has an early maturation season (February-March), popularly known (in Brazil) as "São José". The *Angustifolia* variety, known as "comum", ripens from April to May. Finally, the Caiova or "kayuvá" variety, known as larger seeds, ripens from June to August [25]. Additionally, we evaluated probiotic bacterial growth using starch as the carbohydrate source.

We hypothesize that these seeds, known as "pinhão", contain significant amounts of these prebiotic compounds with differences among the varieties since the stage of maturity causes modifications in the composition of the seeds. We also expect the presence of resistant starch to stimulate selectively probiotic bacteria.

This study might bring awareness to the importance of these seeds as a nutritious and potential food, thus assisting in the preservation of the species.

2. Materials and Methods

2.1. Materials

The Brazilian pine seeds "pinhão" were harvested in the germplasm bank of *Araucaria angustifolia* (Bertoloni) Otto Kuntze, aged 31 years old, located in Embrapa Forestry (25˚16'36"S and 49˚04'58"W), Colombo, State of Paraná, Brazil. Samples of each variety were harvested (**Figure 1**): *Sanct josephi* (March), *Angustifolia* (May) and *Caiova* (July), at the stage IV-megastrobili with predominantly brown surface and undergoing dehiscence, red/orange seeds and embryos from 150 to 200 mg fresh [26]. The lactic acid bacteria used in this study came

from UFVCC (Federal University of Viçosa Culture Collection, Brazil) and commercially available. The probiotic strains were *Bifidobacterium longum* (*B. longum*), *B. breve*, *B. adolescentis*, *Lactobacillus acidophilus* NCFM® (*L. acidophilus* NCFM), *L. acidophilus* ATCC 4962, *Lactiplantibacillus plantarum* (*L. plantarum*), *Lacticaseibacillus rhamnosus* (*L. rhamnosus*).

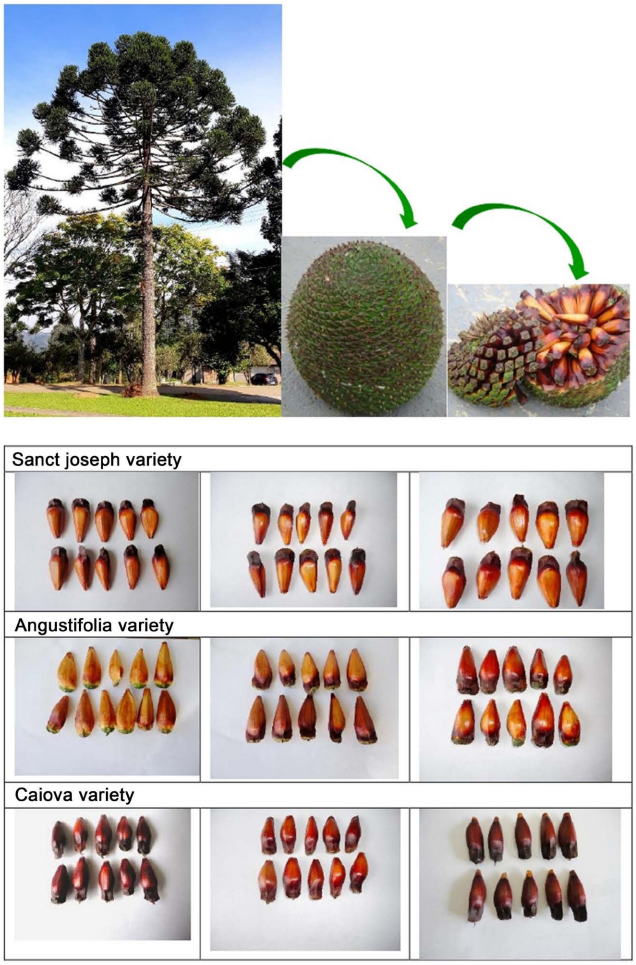

Figure 1. Brazilian pine seeds of different varieties.

2.2. Evaluation of Biometrical Parameters

Sixty randomized seeds of each variety were evaluated for length, width, diameter using a pachymeter. The weight was obtained in a semi analytical scale (Shimadzu BL3200H).

2.3. Analysis of Oligosaccharides

2.3.1. Extraction of Saccharides

Only the endosperm of the seeds was grinded in a blender (IKA A11 basic). A sample of 2 g was mixed with 20 mL of pure water type 2 and thoroughly stirred (Vortex-Genie2). The resulting solution was placed in an ultrasonic bath for 10 minutes (Unique T28220) and incubated in a shaking water bath at 80˚C, during

60 min, with intermittent mixing 150 RPM (Nova Técnica NT 232). The extract was then centrifuged (Fanen 204NR) at 5000 RPM during 20 min for separation of insoluble solids. The supernatant, was collected in a separate tube and stored at −18˚C.

2.3.2. Chromatographic Analysis of Oligosaccharides

The samples were thawed and immediately diluted. The solution was filtered in 0.22 μm and analyzed by a high-performance anion exchange chromatography, coupled to a pulsed amperometric detection (PAD) (Thermo ICS-5000). Sugars and oligosaccharides separation were achieved by a CarboPac PA 100 guard (50 mm × 4 mm i.d., particle size 8.5 μm, Thermo Fisher Scientific®, Waltham, MA, USA) and analytical column (250 mm × 4 mm i.d., particle size 8.5 μm, Thermo Fisher Scientific®, Waltham, MA, USA) using a 25 μL looping for sample injection [27]. Eluent flow rate was 1.0 mL·min^{-1} and the temperature at 30˚C. The mobile phase consisted of a gradient elution of 100 mM NaOH (eluent A), 500 mM sodium acetate containing 100 mM NaOH (eluent B). The elution gradient was performed as follows: 0 - 5 min, 3% B; 5 - 25 min, 3% - 40% B; 25 - 30 min, 100% B; and 30 - 35 min, 3% B. Gold electrode was used and the reference electrode in Ag/AgCl mode, and data collection rate was 1 Hz. The sugars and oligosaccharides were identified comparing the retention times with the standards. The oligosaccharides were converted to compound mass using an external calibration curve in the range of 1 to 40 mg·L^{-1}. All solutions were prepared with deionized water, and the content of individual compounds was expressed in mg·g^{-1} [28]. Data acquisition and processing were carried out with the Chromeleon v 7.0 software. The experiment was set up in a completely randomized design. Differences were tested using one-way analysis of variance (ANOVA) and the average test (Duncan $p < 0.05$) was used to verify the difference between the samples.

2.4. Analysis of Resistant Starch

A 100 mg sample was homogenized in a mixer and placed in a 50 mL Erlenmeyer flask. Then, 10 mL of KCl/HCl buffer, pH 1.5 (0.2 M) and 0.1 mL of pepsin solution (0.1 g of Sigma P-7012 pepsin in 10 mL of KCl-HCl buffer, pH 1.5) were also added. The sample was kept in a water bath at 40˚C during 60 minutes, under constant agitation. After this period, the sample was cooled to room temperature.

Once cooled, 9 mL of Tris-maleate buffer (Acros 26497-0260), pH 6.9 (0.1 M) and 1 mL of α-amylase solution (4 g of Sigma A-3176 *α*-amylase in 100 mL of tris-maleate buffer) were added to the sample. The sample was incubated in a water bath at 37˚C for 16 hours, with constant agitation and filtered through 11.0/12.5 filter paper, discarding the liquid. The residue was washed with 10 mL of distilled water and the liquid was discarded again.

In a beaker, with the help of a spatula, the filter residue was transferred together with 3 mL of distilled water. The beaker sample was transferred again to a 50 mL Erlenmeyer flask and 3 mL of KOH (2 M) was added. The sample was stirred at room temperature for 30 minutes and 5.5 mL of HCl (1 M), 3 mL of sodium

acetate buffer, pH 4.75, and 80 μL of amyloglucosidase (0.144 g of Sigma A-7255 amyloglucosidase in 10 mL of water) were added. The sample was taken to a water bath at 60˚C for 45 minutes, under constant agitation. The sample was filtered through 11.0/12.5 filter paper and the residue washed with 10 mL of distilled water. The residue was discarded.

The glucose concentration of the remaining liquid was determined by the glucose oxidase method (LABORLAB) in a spectrophotometer. For this, 20 μL of sample and 2 mL of the working glucose-oxidase reagent were placed in each test tube. The tube was then capped and placed in a water bath at 37˚C for 10 minutes. After this period, the tube was cooled in running water. The reading was performed in a spectrophotometer with a wavelength of 505 nm, previously zeroed with the blank (20 μL of distilled water and 2 mL of the working reagent). To evaluate the absorbance readings, a glucose standard curve with the working reagent used in the analyses [29].

2.5. Starch Extraction

The starch was extracted from the Brazilian pine seeds according to Costa *et al.* [30]. Briefly, the main coat (hard) of seeds was removed as well as the second coat (a thin layer) adhered to the surface. Isolated seeds were milled, and an equal mass of distilled water was added. The suspension was mechanically stirred for 10 min, sieved (200 mesh or 0.075 mm), and centrifuged (5000 rpm for 10 min). The obtained starch was carefully dried in an oven with forced air circulation at 40˚C for 24 h. Finally, the purified starch was kept in a desiccator over anhydrous calcium chloride up to constant mass.

2.6. Evaluation of the Growth of Probiotic Bacteria

2.6.1. Bacteria Inocula

Probiotic bacteria were precultivated in their respective broth media three consecutive times for activation and biochemical stability under anaerobic conditions at 37˚C for 48 hours. *Lactobacillus* cultures were activated in MRS (De Man, Rogosa e Sharpe, DIFCO, Detroit, Michigan) and *Bifidobacterium* cultures in MRS modified with agar 0.075%, sodium carbonate 0.02%, calcium chloride dihydrate 0.01% and 1% of L-cysteine hydrochloride solution (0.05% concentration). The inocula were prepared by centrifuging and resuspending the cells in peptone water and the concentration varied between 105 to 107 UFC·mL^{-1} in the final medium at the beginning of the experiments.

2.6.2. Media Preparation

The MRS standard broth medium (1 L) was prepared with 10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g dextrose (Merck, Darmstadt, Germany), 1 g Tween 80.2 g ammonia citrate, 5 g sodium acetate, 0.05 g manganese sulfate, 0.1 g magnesium sulfate and 2 g disodium phosphate.

Three Brazilian pine seed starches were used to prepare the experimental broth media by substituting dextrose for the starches, 2-1-2 (early) (starch 1), 2-5-1

(early) (starch 2), and 3-10-1 (early) (starch 3).

Four formulated broths were prepared: MRS (control broth medium), 2-1-2 (MRS with starch 1), 2-5-1 (MRS with starch 2), and 3-10-1 (MRS with starch 3).

2.7. Statistical Analyses

The Two-Way ANOVA, Student's t-test were performed at 95% confidence using the GraphPad Prism 8.0.2 software for the results of bacterial growth and resistant starch content of the Brazilian pine genotypes (2-1-2, 2-5-1, and 3-10-1). Tukey and Duncan test were performed using software Statistica 7.0.

3. Results and Discussion

3.1. Biometrical Parameters

The Brazilian pine seeds differed in length, width and diameter. *Sanct josephi* seeds presented the shortest length in contrast to the biggest diameter (Table 1). *Caiova* seeds showed the shortest width. A study carried out with Brazilian pine seeds consumers showed that larger and fuller appearance seeds are preferred at purchase [1].

Table 1. Biometrical parameters of Brazilian pine seeds varieties.

Variety	Biometrical parameters			
	Length (cm)	Width (cm)	Diameter (cm)	Weight (g)
Sanct josephi	50.21^{b}	20.38^{a}	16.75^{a}	7.81^{a}
Angustifolia	53.18^{a}	20.59^{a}	15.76^{b}	7.88^{a}
Caiova	52.76^{a}	19.12^{b}	16.17^{ab}	7.54^{a}

Means followed by the same letter on the same column do not differ according to the Tukey test ($p < 0.05$).

3.2. Oligosaccharides Content

Figure 2 shows the chromatographic profile of the oligosaccharides identified in the samples of Brazilian pine seeds. The content of these compounds is summarized and displayed on Table 2.

The first class of oligosaccharides, found in Brazilian pine seeds ("pinhão"), was the fructooligosaccharides. The synthesis of this fructooligosaccharides occurs via the function of 1-SST (sucrose fructosyltransferase) [31]. In nature, fructan metabolism takes place in the vacuole where is carried out by a group of enzymes defined as fructan activeenzymes (FAZY) with fructosyltransferases (FTs) and fructan exohydrolases (FEHs) activities. The fructan content increase with plant age, whereas glucose, fructose, and sucrose decrease [32].

Brazilian pine seeds presented the following fructooligosaccharides (FOS): GF2 (1-kestose), GF3 (nystose) in small contents, less than 10 mg·100g^{-1} and GF4 (1-fructofuranosylnystose) in higher amounts mainly in samples of *Caiova* variety with the average of 93.6 mg·100g^{-1} (Figure 3).

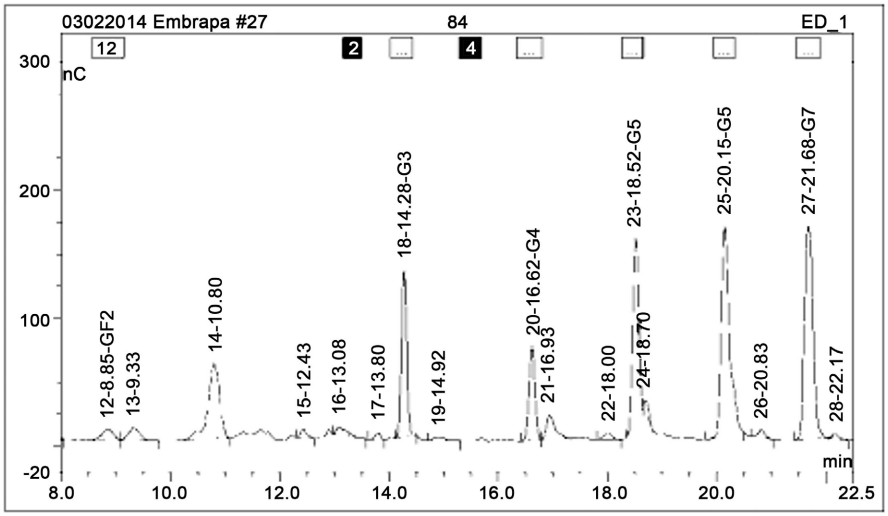

Figure 2. HPAEC–PAD chromatogram showing the oligosaccharides profile in the Brazilian pine seeds, GF2 (1-kestose (1-kestotriose)), G3 (maltotriose), G4 (maltotetraose), G5 (maltopentaose), G6 (maltoexose), G7 (maltoheptaose).

Table 2. Average contents of oligosaccharides in Brazilian pine seeds (mg·100g^{-1}).

Variety	GF2	GF3	GF4	G3	G4	G5	G6	G7
Sanct josephi	8.1^{a}	5.6^{a}	83.7^{b}	108.9^{b}	0.0^{c}	97.8^{a}	197.0^{a}	234.1^{a}
Angustifolia	6.8^{b}	3.3^{b}	60.7^{c}	111.2^{b}	32.5^{a}	58.8^{c}	153.0^{b}	210.7^{b}
Caiova	8.8^{a}	2.7^{b}	93.6^{a}	120.0^{a}	13.9^{b}	68.1^{b}	129.0^{c}	177.1^{c}

GF2 (1-kestose), GF3 (nystose), GF4 (1-fructofuranosylnystose), G3 (maltotriose), G4 (maltotetraose), G5 (maltopentaose), G6 (maltoexose), G7 (maltoheptaose). *Means followed by the same letter in one column indicate that values do not differ statistically (Duncan test) $p < 0.05$.

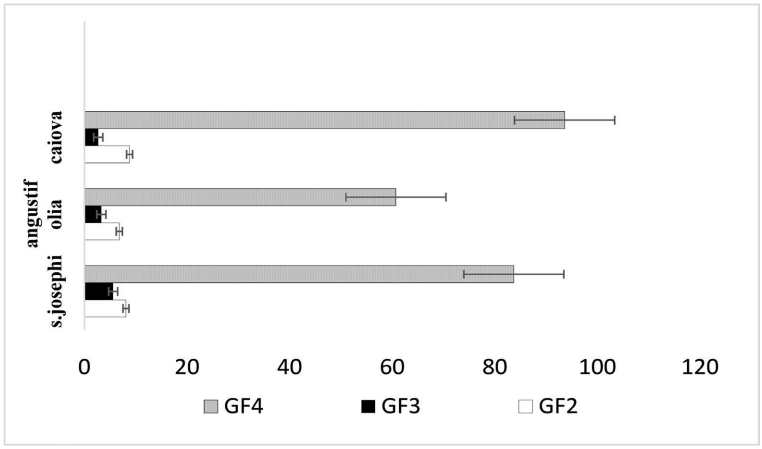

Figure 3. Fructooligosaccharides (FOS) in samples of Brazilian pine seeds varieties.

The amount of GF2 in Brazilian pine seeds varied from 0.0 to 12.5 mg·100g^{-1}. When compared to other sources as leeks 300 - 400 mg·100g^{-1}, raspberry 320, watermelon 290, cherry 220, parsnip 270 and white onion 1150 mg·100g^{-1} (FW),

"pinhão" had the lowest concentration of GF2 [30]. However, if compared to other common Brazilian foods, "pinhão" presented more GF2 than parsnip, white yam and, taro (4.51; 0.30; 0.18 mg·100g^{-1}, respectively), and less than cassava (7.00 mg·100g^{-1}) [33]. In our study, all the varieties presented GF2 in their composition except one sample of Angustifolia. The absence of GF2 in samples from the same species is common according to former studies [32].

The second and the least significant fructooligosaccharides in "pinhão" was the GF3 (nystose), from 6.9 to 10.0 mg·100g^{-1}, predominantly in seeds of *Sanct josephi* variety. Compared to other sources, Brazilian pine seeds present more GF3 than scallion and nectarine (being 3.25 and 1.19 mg·100g^{-1}, respectively) and less than peach and blackberry [34].

The GF4 (1-fructofuranosylnystose) was the largest fructooligosaccharides of Brazilian pine seeds, varied highly from 9.2 to 142.8 mg·100 g^{-1}. Comparing "pinhão" to other Brazilian food sources, it presented more GF4 than sweet potato (2.79 mg·100g^{-1}). Other roots and tubers such as parsnip, white yam, taro and cassava did not present GF4 in their structure [33]. In fruits as peach and watermelon GF4 were 90 and 80 mg·100g^{-1} (FW), respectively. In vegetables, 1-fructofuranosylnystose appeared in white onion and scallion [34].

It is possible that the great variation of fructooligosaccharides among the samples of Brazilian pine seeds is due to the phenological state. Some authors observed the synthesis and accumulation of these molecules only during dormancy [35]. Comparing two varieties of Jerusalem artichoke and different parts of the plant, authors noticed differences not only related to varieties but also to organs, tubers contained the highest content of fructooligosaccharides (GF2, GF3 and GF4) [36]. The health importance is that daily doses of 4 to 5 g of FOS are enough to stimulate the growth of bifidobacteria, a benefit gut bacterium [37].

In particular, the content of maltooligosaccharides (MOS) was more expressive (**Figure 4**). Maltoheptaose (G7) had a higher concentration with an average of 234.210 and 177 mg·100g^{-1} (*Sanct josephi*, *Angustifolia* and *Caiova*, respectively). The second most important MOS was the maltohexaose (G6) with 197.0, 153.0 and 129 mg·100g^{-1} in the same sequence. Maltotriose was more expressive in *Caiova* variety while maltopentaose (G5) in *Sanc josephi*. Maltotetraose also was detected, in small contents.

Maltooligosaccharides (MOS) are composed by glucose units joined by α (1→4) glycosidic bonds and a serie of linear oligosaccharides composed of two (G2), three (G3), four (G4), five (G5) and six (G6) of glucose (G) units. They are produced commercially from starch by the action of debranching enzymes such as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), combined with hydrolysis by various CX-amylases. The majority of maltooligosaccharides produced by these multiple enzymatic processes are a mixture of different degrees of glucose (G) polymerization of maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and maltohexaose (G6) [9] [38].

The quantification of maltooligosaccharides (MOS) in Brazilian pine seeds was significant. Maltoheptaose (G7) had a higher concentration with an average of

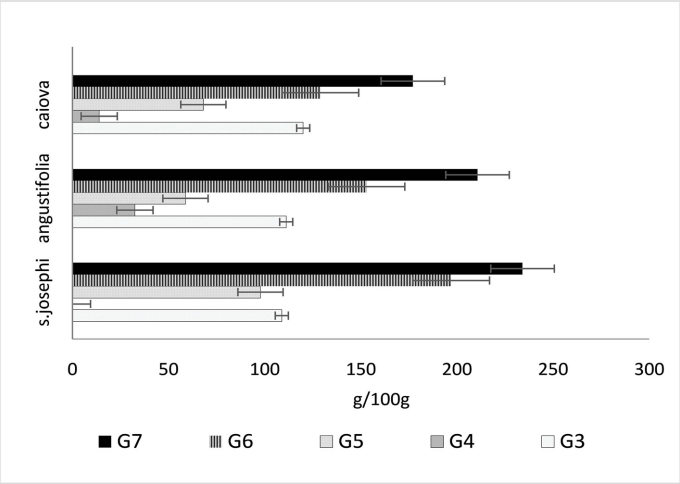

Figure 4. Maltooligosaccharides (MOS) in samples of Brazilian pine seeds varietiess.

234.210 and 177 mg·100g^{-1}; in second place was the maltohexose (G6) with 197.0, 153.0 and 129 mg·100g^{-1} (*Sanct josephi*, *Angustifolia* and *Caiova*, respectively). Other MOS, such as maltopentaose (G5) and maltotriose (G3) were also detected.

The content of maltoheptaose (G7) found in this study (average of 207.3 mg·100g^{-1}) was higher than pieces of sweet potato (0.49 mg·100g^{-1}), parsnip (4.31 mg·100g^{-1}), white yam (6.25 mg·100g^{-1}) and cassava (2.32 mg·100g^{-1}) [33]. In our study, the average of maltohexose (G6) was 159.7 mg·100g^{-1}, higher than other sources as sweet potato (1.11 mg·100g^{-1}), parsnip (2.97 mg·100g^{-1}), white yam (2.85 mg·100g^{-1}) and cassava (2.42 mg·100g^{-1}) [33].

The maltotriose (G3), the third most abundant MOS, was detected in "pinhão" with contents (113.4 mg·100g^{-1}) higher than sweet potato (5.45), parsnip (5.50), white yam (2.43), and cassava (4.76) [33], and equal to cabbage stalk flour and lower than pineapple crown flour [39]. The G4 (maltotetraose) appeared only in the *Angustifolia* and *Caiova* varieties, being considered no relevant for the oligosaccharides profile.

Maltooligosaccharides, in general, do not increase the numbers of bifidobacteria in the human colon. They are hydrolyzed and absorbed in the small intestine and do not reach the colon intact. However, some reports consider that their consumption can reduce the levels of intestinal putrefactive bacteria such as *Clostridium perfringens* and members of the family Enterobacteriaceae [40]. Therefore, maltooligosaccharides may be effective in improving colonic conditions.

Although there is an extensive literature about sugars/oligosaccharides and their potential nutritional and functional properties, there is no referenced data reporting the presence and variation of these compounds in Brazilian pine seeds. This is the first study about the characterization and quantification of different oligosaccharides in the seeds of *Araucaria angustifolia*.

The difference of oligosaccharides among the seeds from different varieties of *Araucaria angustifolia* is probably associated to the genetic diversity [41]. Pre-

germination metabolism can also cause differences between varieties [24].

The methodology used to extract the soluble sugars is important to identify differences in the contents of oligosaccharides. Pereira *et al.* [26] extracted the maximum of oligosaccharides from banana pulp using ethanol 52% (vol/vol). In this study, the soluble sugars were extracted using pure water type 2. The time used in ultrasound bath is another variable to consider in the oligosaccharides extraction [34]. The chromatography methodology for determination is also important to compare values for the same samples. Gangola *et al.* [42], compared outcomes with high performance anion exchange chromatography (HPAEC) with high-performance liquid chromatography (HPLC) and refractive index (RI). They concluded that HPAEC presented higher sensitivity and shorter run time.

Plant foods contain a complex mixture of carbohydrates, which makes the identification difficult [43]. Further studies should be undertaken to characterize the biochemical pathways of oligosaccharides as well as the use of some advanced analytical techniques.

3.3. Resistant Starch

The resistant starch contents of the different Brazilian pine seeds varieties are in Table 3. The data shows that early stages of harvesting (*Sancti josephi*) resulted in higher amounts of resistant starch. This also happened in a study performed by Yang *et al.* [44] where authors analyzed the starch content of potato in different harvesting stages and found that the resistant starch content was the highest at the earliest time.

A study performed by some authors about resistant starch in cooked "pinhão" found values around 3.27 g·100g^{-1}, similar to the *Caiova* variety [4]. It is important to note that resistant starch, in cooked matrix, has already retrograded, being different compared to raw seeds.

Table 3. Average content of resistant starch in raw Brazilian pine seeds from different season of harvesting (g·100g^{-1}).

Varieties	*Sancti josephi*	*Angustifolia*	*Caiova*
Average content of resistant starch (g·100g^{-1})	6.2 ± 1.2^{a}	5.5 ± 1.4^{ab}	3.9 ± 1.4^{b}

Sanct josephi: early harvest between March and April; Angustifolia: middle harvest between May and June and *Caiova*: late harvest between July and August. *Means followed by the same letter in one column indicate that values do not differ statistically (Tukey test) $p < 0.05$.

We also evaluated the percentages of resistant starch in starches obtained from three genotypes belonging to *Sancti josephi* variety, collected in March from cities of Minas Gerais State, Brazil: 2-1-2 (city of Ipuína), 2-5-1 (city of Ipuína) and 3-10-1 (city of Congonhal), showing higher percentages of resistant starch than the pine seed starches, which is explained by the concentration of such fraction in the pure starch samples (Table 3). These data show the highest contents of resistant

starch in genotypes 2-1-2 and 2-5-1, both from the same locality (**Table 4**). This constituent may vary based on the genotype, as well as be influenced by differences in management, locality, and conditions of the environment [45] [46].

The starch from the pine genotypes evaluated showed a higher percentage of resistant starch than other plant sources, also rich in starch, such as corn (2.82%), black beans (16.59%) and yellow sweet potatoes (9.44%). The content of this prebiotic was similar in purple sweet potatoes (22.93%) and lower in green bananas (48.29%) and potatoes (56.43%) [47].

Table 4. Resistant starch in starches obtained of genotypes 2-1-2, 2-5-1 and 3-10-1 (belonging to Sancti josephi variety (g·100g^{-1}).

Sancti josephi (Genotype)	(2-1-2)	(2-5-1)	(3-10-1)
Resistant starch (g·100g^{-1})	21.35 ± 0.6^{a}	20.53 ± 0.6^{a}	17.94 ± 0.7^{b}

*Means followed by the same letter in one column indicate that values do not differ statistically (Duncan test) $p < 0.05$.

3.4. Evaluation of the Growth of Probiotic Bacteria

Probiotic bacteria were cultivated in MRS broth (dextrose - control) and MRS broth with substituted carbohydrate source (starch samples extracted from the three genotypes of the variety *Sancti josephi*, namely 2-1-2, 2-5-1 and 3-10-1) for 72 hours. If all actual growth of the probiotic strains (**Figure S1**, supplementary material) is summed up, there were no differences between the counts of the experimental MRS broth growth and the control MRS ($p > 0.05$). Therefore, for these probiotic strains, the media containing pine seeds starch stimulated bacterial growth in a manner equivalent to the medium with dextrose (MRS). This may be related to the higher percentage of resistant starch present in the starch obtained from the pine seeds of such pine genotypes (**Table 3**), suggesting the potential of these starch samples as an energy source for probiotic metabolism. **Figure 5** shows the actual growth of the probiotic strains individually taken. These data are also available in **Table S1** (supplementary material).

Most of the probiotics evaluated grew similarly in media with pine seed starch and dextrose, except *B. breve*, which showed higher growth using starches (mainly 2-1-2 genotype) as substrate, and *L. plantarum* with outstanding growth in 2-5-1 media (9.76 ± 0.0 log CFU·mL^{-1}) and 2-1-2 (9.70 ± 0.0 log CFU·mL^{-1}) (**Figure 5**). In this sense, it is suggested that microbial growth in the presence of resistant starch is strain dependent.

This happens due to the fact that the fermentation of different carbohydrate sources is related to the metabolic capacity of each microorganism. The metabolization of resistant starch, for example, depends on the type of hydrolysis enzyme produced by the bacteria [48].

In **Figure 6**, this correlation between medium and probiotic is more evident, highlighting the greater effectiveness of *L. plantarum*, a probiotic associated with the fermentation of starch-rich vegetables [49], in metabolizing pine seed starch (2-1-2 and 2-5-1).

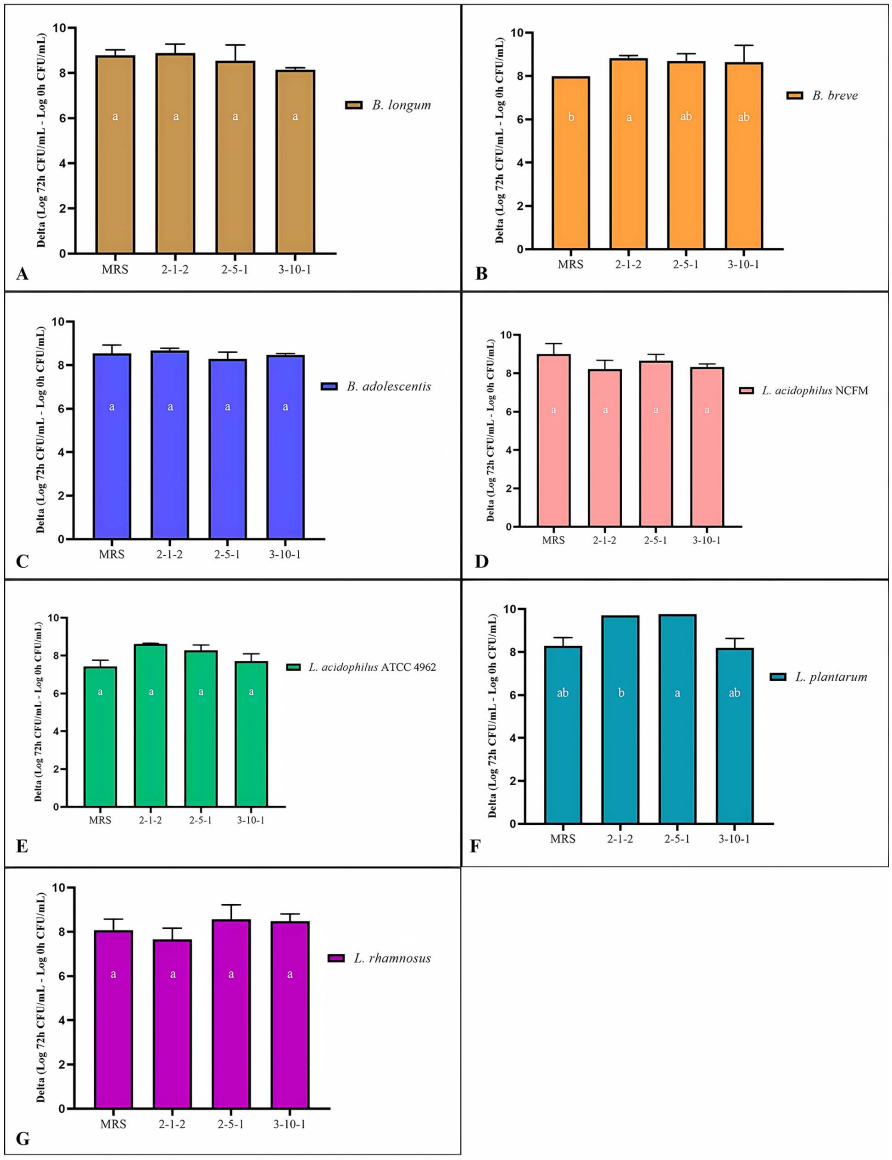

Figure 5. Actual growth of the probiotic strains (CFU·mL^{-1}) in MRS (control), 2-1-2 (MRS plus starch 1), 2-5-1 (MRS plus starch 2), 3-10-1 (MRS plus starch 3).

The impact of resistant starch on the metabolism of probiotic strains has not yet been completely elucidated. Still, this prebiotic is fermentable from beneficial microorganisms present in the intestinal microbiota and capable of promoting the production of short-chain fatty acids such as acetate, butyrate, and propionate, reducing the pH of the environment and modulating the multiplication of beneficial microorganisms [50].

As observed in this study and based on the literature, a prebiotic will promote higher growth of those microorganisms with higher metabolization capabilities. These, in turn, can favor the development of other beneficial strains. *L. plantarum*, with good development in pine seed starches, can promote the multiplication of some *Lactobacillus* strains and reduce the expression of *Escherichia coli* present

in the intestine. Furthermore, it is a strain related to anti-obesity and anti-glycemic effects [51].

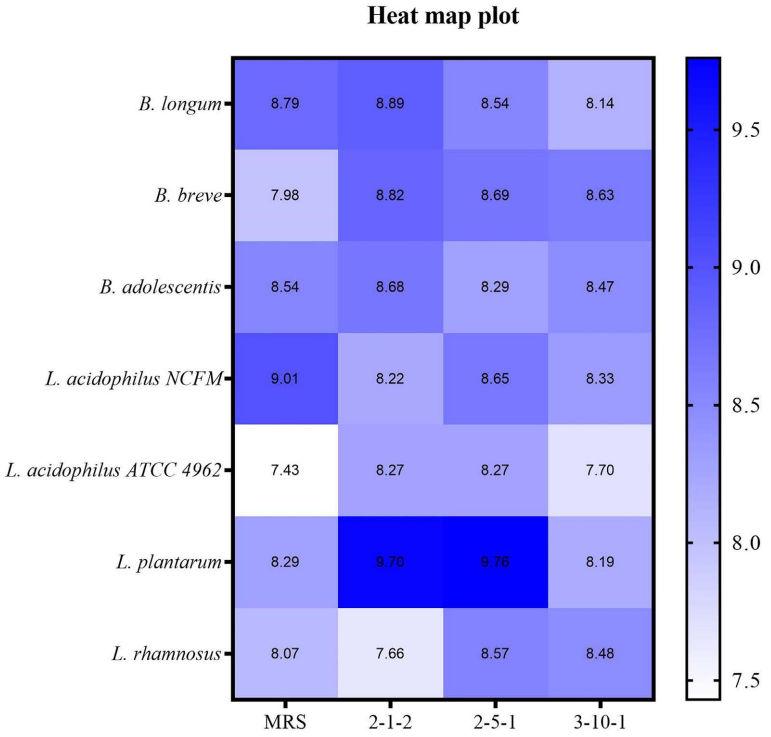

Figure 6. Heat map plot of the probiotic strains counts versus the broths with different carbohydrate source tested, MRS, and experimental broths with pine seed starches (2-1-2, 2-5-1, 3-10-1).

These results corroborate the hypothesis that Brazilian pine seeds starch has the potential to stimulate the growth of probiotic bacteria in a similar way to dextrose. Thus, some genotypes of *Sanct joseph* variety (such as 2-1-2 and 2-5-1) can promote the growth of specific bacteria (*B. breve* and *L. plantarum*) more markedly than others. Finally, complementary studies are necessary to better ascertain the prebiotic effect of this starch, comparing it with other pine seed starches.

4. Conclusion

"Pinhão" seeds present high content of carbohydrate content, most of them, play as prebiotics, promoting health benefits. This manuscript identified and quantified the oligosaccharides and resistant starch present in "pinhão" seed in different varieties (*Sancti josephi*, *Angustifolia* and *Caiova*). In relation to fructooligosaccharides GF4 (1-fructofuranosylnystose) is the main one, presenting higher contents compared to other food sources. Maltooligosaccharides (MOS) represent the main part of the oligosaccharides profile of Brazilian pine seeds. In descending order of importance was maltoheptaose (G7), maltohexose (G6) and maltotriose (G3). Seeds of *Sanct josephi* variety have the shortest size and the highest amount of resistant starch. Starch from *Sanct josephi* genotypes could stimulate probiotic

strains, mainly *B. breve* and *L. plantarum*, and possibly may have prebiotic effect derived from its resistant starch content.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supplementary Material

Table S1. Actual growth (delta = log 72 h CFU·mL^{-1} − log 0 h CFU·mL^{-1}) of probiotic strains in MRS (control) and MRS with substituted carbohydrate source by pine starches (2-1-2, 2-5-1 and 3-10-1).

Probiotic strain	MRS	2-1-2	2-5-1	3-10-1
B. longum	8.79 ± 0.25^{Aa}	8.89 ± 0.39^{Aab}	8.54 ± 0.70^{Ab}	8.14 ± 0.10^{Aa}
B. breve	7.98 ± 0.00^{Bab}	8.82 ± 0.12^{Aab}	8.69 ± 0.35^{ABab}	8.63 ± 0.78^{ABa}
B. adolescentis	8.54 ± 0.39^{Aa}	8.68 ± 0.10^{Aabc}	8.29 ± 0.31^{Ab}	8.47 ± 0.06^{Aa}
L. acidophilus NCFM	9.01 ± 0.54^{Aa}	8.22 ± 0.46^{Abc}	8.65 ± 0.34^{Aab}	8.33 ± 0.16^{Aa}
L. acidophilus ATCC 4962	7.43 ± 0.32^{Ab}	8.61 ± 0.06^{Abc}	8.27 ± 0.30^{Ab}	7.70 ± 0.39^{Aa}
L. plantarum	8.29 ± 0.38^{ABab}	9.70 ± 0.00^{Ba}	9.76 ± 0.00^{Aa}	$8.19 \pm 0.45A^{Ba}$
L. rhamnosus	8.07 ± 0.50^{Aab}	7.66 ± 0.51^{Ac}	8.57 ± 0.65^{Ab}	8.48 ± 0.33^{Aa}

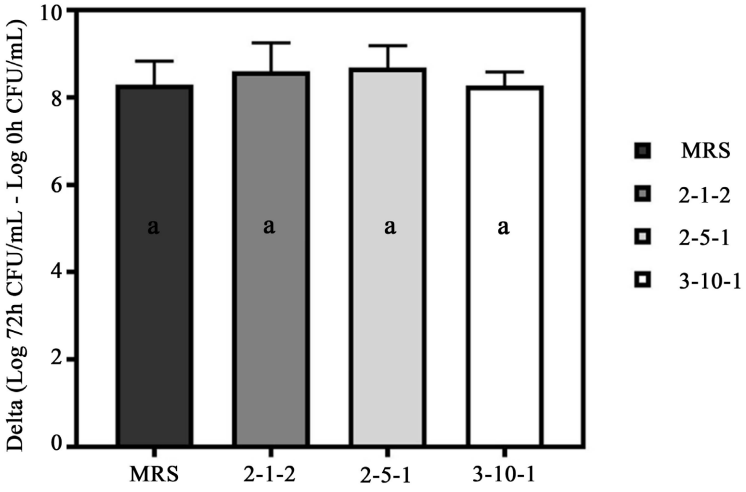

Figure S1. General comparison of the delta (log 72 h CFU·mL^{-1} − log 0 h CFU·mL^{-1}) between the sources of carbohydrates considering all probiotic strains actual growth.