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Acemannan and Fructans from Aloe vera (*Aloe barbadensis* Miller) Plants as Novel Prebiotics

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ABSTRACT: The nutraceutical properties of Aloe vera have been attributed to a glucomannan known as acemannan. Recently information has been published about the presence of fructans in Aloe vera but there are no publications about acemannan and fructans as prebiotic compounds. This study investigated *in vitro* the prebiotic properties of these polysaccharides. Our results demonstrated that fructans from Aloe vera induced bacterial growth better than inulin (commercial FOS). Acemannan stimulated bacterial growth less than fructans, and as much as commercial FOS. Using qPCR to study the bacterial population of human feces fermented in a bioreactor simulating colon conditions, we found that fructans induce an increase in the population of *Bifidobacterium* spp. Fructans produced greater amounts of short chain fatty acids (SCFA), while the branched-chain fatty acids (BCFA) did not increase with these polysaccharides. Acemannan increased significantly acetate concentrations. Therefore, both Aloe vera polysaccharides have prebiotic potentials.

KEYWORDS: *Aloe vera, fructans, prebiotics, bifidobacterium, qPCR, SCFA, BCFA*

INTRODUCTION

According to Ajose,[1] the World Health Organization states that 80% of the world population uses medicinal plants for the treatment of a large number of diseases. Many components derived from plants are currently used in drug preparations by pharmaceutical companies.[1] One of the most used plants is *Aloe barbadensis* Miller, also known as Aloe vera, a crassulacean acid metabolism (CAM) plant. As a CAM plant, Aloe vera is adapted to arid and semiarid environments and is well-known for its potential health-promoting properties, such as immunostimulation and cell regeneration.[2,3] These properties are mainly attributed to its mucilaginous leaf gel, which has pharmacological, cosmetic, and food industrial importance with great economic projections.[4−7] This gel contains various polysaccharides, including an acetylated glucomannan known as acemannann, which is the most abundant polysaccharide. Mannose constitutes the backbone of the polysaccharide intercepted with glucose units. Mannose and glucose are linked by β-(1→4) glycosidic bonds.

In this study we investigated the prebiotic properties of fructans and acemannan; the latter exhibits antibacterial, anti-inflammatory, and healing properties.[3,8−10] It is very surprising that fructans have been recently described in Aloe vera,[11] considering that this plant belongs to the monocot group whose members generally synthesize these polysaccharides.[12] Fructans are fructose polymers mainly built from sucrose by adding fructose units through β-(2→1) or β-(2→6) glycosidic linkages. For a more compressive description of the acemannan and fructan structures see Figure 1.

Fructans are found in the green part of the leaf tissue (leaf cortex) of Aloe vera plants. We recently reported that the molecular structure of Aloe fructans changes when plants are subjected to water stress. By GC-MS analyses of the glycosidic linkages, we observed that well-watered plants of Aloe vera synthesize inulin, neo-inulin, and neo-levan types of fructans; while plants subjected to water deficit also synthesize branched fructans, the neo-fructans. The fructans of stressed plants have a higher degree of polymerization (DP).[11,13] The amounts of fructans and acemannan also increased in the plant subjected to water stress.[14,15]

The effects of prebiotic compounds have been widely described in the literature.[16−19] Prebiotics promote the growth of beneficial bacterial populations such as *Lactobacillus* and *Bifidobacterium* species in the colon, accompanied by the production of short-chain fatty acids (SCFAs) through fermentation processes. These events have been associated with a lower risk of nontransmissible chronic diseases, including some types of cancer such as colorectal cancer.[20,21] The best-studied prebiotics are the inulin oligosaccharides and galactooligosaccharides[22]; while the use of acemannan and fructans from Aloe vera as prebiotics has been poorly studied, and there are no studies of the prebiotic potential of fructans synthesized by Aloe vera plants subjected to water stress. There is only a recent publication on Aloe vera leaf gel as a prebiotic.[23]

The aim of this study was to determine the prebiotic potential of acemannan and fructans (alone and combined) isolated from water-stressed Aloe vera plants on the growth of beneficial colon bacteria in pure cultures. We also determined, using qPCR the effect of these polysaccharides on the bacterial populations of human feces and in the synthesis of SCFAs produced during fermentation of human feces.

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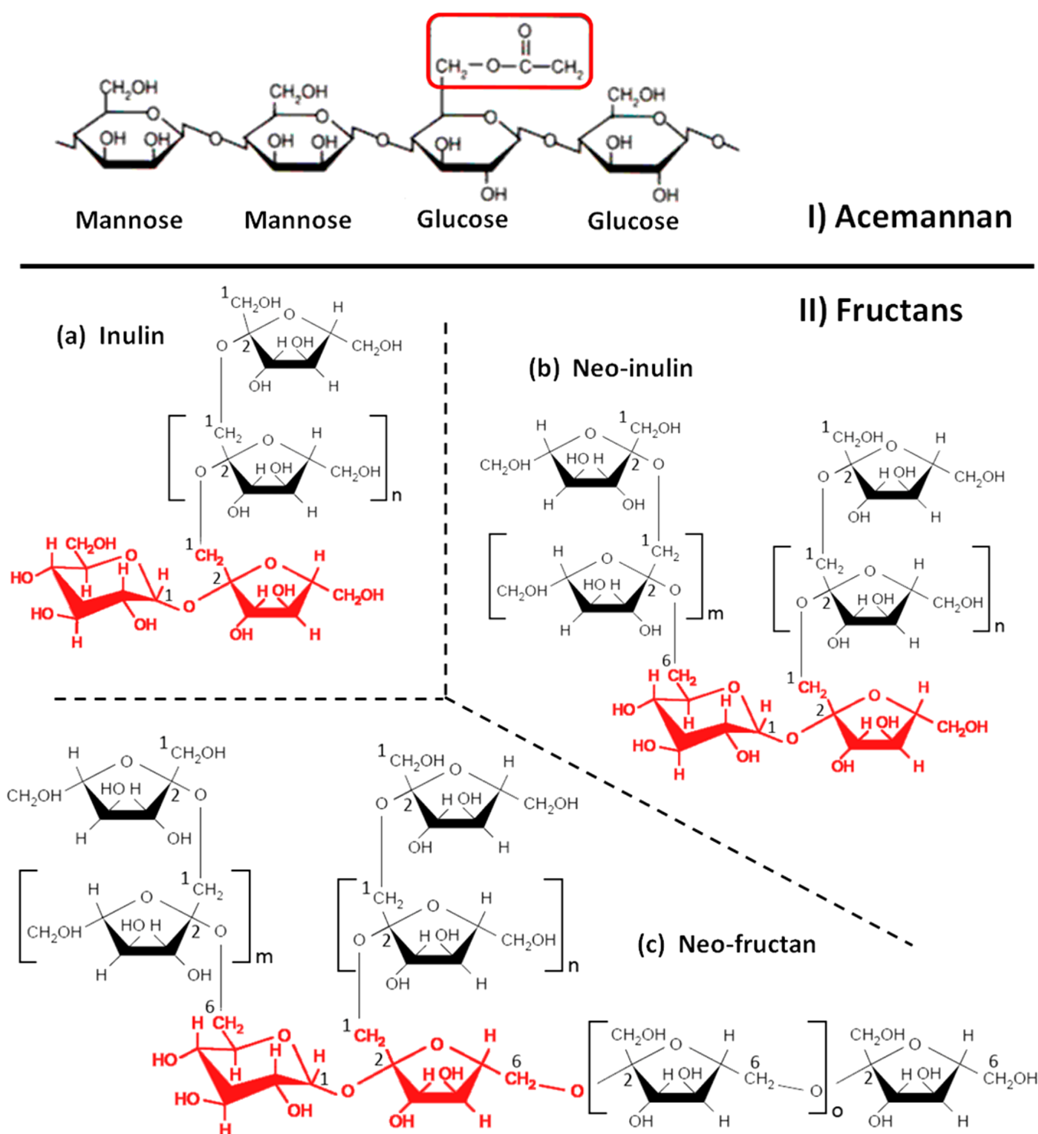

Figure 1. Polysaccharide structures from Aloe vera plants. (I) Acemannan. An acetyl group is highlighted inside a red line. (II) Fructans found in water-stressed plants of Aloe vera. The terminal sucrose (glucose + fructose) unit is shown in red.

■ MATERIALS AND METHODS

Plant Material. All the experiments were performed with plants of *Aloe barbadensis* Miller (Aloe vera) cultivated in Parcela Piedra Redonda, located in La Higuera, Coquimbo Region, IV Region of Chile. Plants were irrigated at 25% field capacity, submitted to a severe water deficit. They were one year old when used for polysaccharide extraction. Mature leaves from four different plants were removed and stored at −80 °C.

Polysaccharide Extraction. For the extraction of polysaccharides, whole leaves were thawed at 4 °C and cleaned with a disinfectant solution of sodium hypochlorite (50 mg/L). It was necessary to process at least 10 leaves (obtained from 4 different plants) to get the amount of polysaccharides required for this study. The photosynthetic cortex of the leaf was separated from the internal pulp or gel corresponding to the parenchyma under a laminar flow hood using sterilized material. Once the parts were separated, fructans were extracted from the cortex and acemannan from the gel, using different extraction protocols.

Fructan Extraction and Depigmentation. Fructan extraction was carried out according to the methodology described by Cairns and Pollock,[24] with modifications. Five grams of cortex were boiled with 10 mL 80% ethanol (v/v) for 5 min in a water bath. The tissue was homogenized in a UV pre-sterilized blender for 20−25 min, the homogenate was centrifuged at 5000 rpm for 5 min at room temperature and the supernatant withdrawn and saved. The pellet was resuspended in 80% ethanol, heated again at 70 °C under agitation for 5 min and centrifuged at 5000 rpm for 5 min. The supernatants were mixed and kept frozen at −20 °C. The pellet was then resuspended in 10 mL deionized water, heated at 60 °C for 15 min and centrifuged at 5000 rpm for 5 min. The supernatant was withdrawn and this step was repeated once. The supernatants were mixed and depigmented by adding 5% (w/v) activated carbon and maintaining the solution under agitation at room temperature for 15 h. Then the mix was centrifuged at 9000 rpm for 1h at 5 °C and the supernatant filtered with Whatman *N*°1 and *N*°3 filters under vacuum. Celite 560 (calcined diatomaceous earth) from Sigma-Aldrich at 5% (w/v) was added to the filtrate and left under agitation at room temperature for 15 h. After centrifugation at 9000 rpm for 60 min at 5 °C, the supernatant was collected and kept frozen at −20 °C until used.

Acemannan Extraction and Depigmentation. The frozen gel was cut into small pieces of approximately 4 × 3 cm and placed in a solution of 0.5 M KCl under gentle stirring at room temperature for 4 h. The pigmented solution was discarded and the pieces of gel were homogenized in a blender (previously sterilized under UV light for 20−30 min) at maximum speed. The gel solution was centrifuged at 8000 rpm for 30 min at 4 °C and the supernatant collected and stored

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at 4 °C. The pellet was resuspended in 50 mL distilled water and stirred for 3h at room temperature. The solution was then centrifuged under the same conditions. The supernatants were mixed and depigmented by incubating with 5% Celite 560 (w/v) under agitation at room temperature for 15 h. The mixture was centrifuged at 9000 rpm for 1 h at 4 °C, the supernatant was withdrawn and mixed with activated carbon 5% (w/v) and left under agitation at room temperature for 15 h, then centrifuged at 9000 rpm for 60 min at 5 °C. The supernatant was recovered and filtered with Whatman *N*°1 and *N*°3 filters under vacuum. The Celite and filtration steps were repeated when the gel solution remained pigmented. Two volumes of 100% cold ethanol were added to the filtered acemannan solution and the mixture was incubated at −20 °C overnight. The solution was centrifuged at 9000 rpm at 5 °C for 1 h and the pellet was resuspended in deionized water (1:10, v/v).

The fructans and acemannan solutions were evaporated with a rotary evaporator to their minimum volume and lyophilized.

Fructans and Acemannan Quantification. Fructans were quantified using a modified anthrone method optimized to increase sensitivity to kestoses.[25,26] Fructans were also analyzed and quantified by GC-MS analysis as has been previously described by Salinas et al.[11] For this, sugars standards of fucose, arabinose, xylose, mannose, galactose, and glucose (from Merck and Sigma-Aldrich) were derivatized to alditol acetates and used to carry out the respective calibration curves, going from 0 to 600 μg. The sugar standards were prepared by serial dilutions ranging from 4.6667 μg/μL to 0.03733 μg/μL. Myo-inositol (20 μg) was added as an internal standard. The extracted polysaccharide was hydrolyzed to monosaccharides in 2N TFA for 60 min at 60 °C, and derivatized to per-O-acetylated alditols previous reduction with sodium borohydride ($NaBH_4$). The alditol acetates were extracted in ethyl acetate and 4 mL water. Fifty microliters were taken for analysis by GC-MS using a Supelco SP-2380 column (Sigma-Aldrich, 30 m x 0.25 mm x 0.20 μm). The GC-MS was a Varian GC-MS System with an automated sample injection, (Varian CP-3800 GC with a CP-8400 Automatic Liquid Sampler) coupled with a Varian Saturn 2200 Ion Trap MS. Under this method, fructose tautomerizes into mannose and glucose.[13] Therefore, free glucose, as a contaminant, could not be quantified as described by Salinas et al.[11] and Salinas.[13]

Acemannan was initially quantified using Congo Red reagent as described by Eberendu et al.[27] A standard solution (2 mg/mL) of Konjac glucomannan (Megazyme) was used to carry out the calibration curve, going from 0 to 25 μg. Acemannan was also quantified by GC-MS using the same derivatization procedure as described for fructans[11] except the acid hydrolysis was performed at 121 °C for 90 min. Sugars standards and the myo-inositol were the same used as for fructan analysis.

Thin Layer Chromatography (TLC) of Fructans. To confirm the effectiveness of the depigmentation process, the fructan solutions were analyzed by TLC. An aliquot equivalent to 12 μg of the purified fructan samples was applied on a 20 × 20 cm silica gel 60 F254 TLC[28] The plates were then sprayed with urea−phosphoric acid[29] and heated at 150 °C for 10 min. The plates were photographed under UV light (366 nm) using a Canon PowerShot A460 digital camera. TLC quantitation of free sucrose and fructose and of oligofructans was performed by MCID Analysis software version 7.0 to estimate the amounts of fructans and free sugars, as described by Salinas et al.[11]

***In Vitro* Fermentation of Aloe vera Polysaccharides with Pure Bacterial Cultures.** Four strains each of *Lactobacillus* and *Bifidobacterium* species from the strain bank of the Institute of Nutrition and Food Technology (INTA), Universidad de Chile were used. The strains were isolated from maternal milk and infant stool samples obtained in previous studies carried out by our laboratory in Chile.[30−32] A commercial strain (*B. lactis* BB-12) was also included. The bacteria species and strains of this study and their origin are shown below: *Lactobacillus fermentum* L55-2-33, maternal milk; *Lactobacillus casei* L54-2-33, maternal milk; *Lactobacillus plantarum* L46-1-12, maternal milk; *Lactobacillus plantarum* N223-3, stools from healthy infants; *Bifidobacterium catenulatum* N173-2, stools from healthy infants; *Bifidobacterium longum* N180-3, stools from healthy infants; *Bifidobacterium bifidum* N364-3, stools from healthy infants; *Bifidobacterium animalis* ssp. *lactis* BB-12, commercial.

Lactobacillus strains were cultured on Man Rogosa Sharpe (MRS) agar plates[33] and *Bifidobacterium* strains on Columbia-Beerens agar supplemented with propionic acid. For the assays, the glucose (10g/L) in the MRS medium was replaced by the tested polysaccharides: fructan (10g/L), acemannan (3g/L), fructan (10g/L) + acemannan (3g/L), from water-stressed Aloe vera plants. Acemannan was limited to 3g/L because higher concentrations were insoluble. Inulin oligofructose, referred to as commercial FOS (10g/L, Beneo P95, Orafti, Pemuco, Chile) was used as a positive control, instead of fructans from well irrigated plants. This was due to the fact that fructan isolation and purification is very time-consuming. Besides the commercial FOS has a 95% linear inulin structure, plus 4% fructose, and 1% sucrose+glucose.[34] Growth curves for *Lactobacillus* species were performed in sterile 96-well microplates, in a Synergy HT microplate reader (BioTek Instruments, VT, USA) by automatically measuring changes in optical density at 600 nm (O.D) every 30 min for 18 h. The plates were incubated at 37 °C. The growth curves for *Bifidobacterium* strains were performed for 72 h under anaerobic conditions (GENbox/GENbag, bioMérieux, Santiago, Chile), measuring the OD every 12 h with the Synergy HT, also incubated at 37 °C. All measurements were made in triplicate.

***In Vitro* Fermentation of Aloe vera Polysaccharides with Human Fecal Bacteria.** Fresh stools from three healthy volunteers (23−26 years old; body mass index between 18 and 24.9 kg/m^2 and without previous intake of antibiotics, prebiotics or probiotics) were collected in sterile, plastic, hermetic recipients which were maintained refrigerated at 4 °C under anaerobic conditions (GasPak, BD, Santiago, Chile) until their delivery to the laboratory. Five grams of stool were homogenized in reduced culture medium (10% w/v) with a stomacher and the suspension was added to the vessel of a bioreactor (B-Braun Scientific MU-200). The culture medium contained 5 g/L yeast extract (MoBio), 10g/L ascorbic acid, 10g/L sodium acetate, 5g/L $(NH4)_2SO_4$, 2g/L urea, 0.2 g/L $MgSO_4$, 0.01g/L $FeSO_4$, 0.007g/L $MnSO_4$, 0.01g/L NaCl, 1 mL/L Tween 80, 0.05g/L hemin, 0.5 g/L cysteine (all from Merck, Santiago, Chile), and the tested Aloe vera polysaccharides (10g/L fructan or 3g/L acemannan), pH 7.0.[35] The suspension was incubated under a nitrogen atmosphere (anaerobic conditions) at 37 °C in the dark under constant stirring for 48 h. The pH of the cultured suspension was controlled regularly. Samples were obtained at the end of the fermentation period and stored at −20 °C. Measurements were made in triplicate.

Microbiota Quantification from Human Feces by qPCR. From the stool samples incubated in the bioreactors of each individual, the bacterial genomic DNA was extracted using the commercial kit QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany, following the manufacturer recommendations. The concentration of total DNA was determined by the absorbance at 260 nm in a spectrophotometer Infinite M200 PRO NanoQuant (Tecan). The purity of the DNA was determined through the absorbance ratio 260 nm/280 nm. The samples obtained were maintained at -80 °C. The bacterial populations of *Bifidobacterium* spp., *Lactobacillus* spp. and total bacteria were determined. For this, 10 ng of DNA was amplified using the corresponding primers of each bacterial population using the kit LightCycler FastStart DNA Master SYBR Green I in a thermocycler LightCycler (Roche Diagnostics, Mannheim, Germany).

The qPCR reactions were performed with 10 ng of DNA, 2 μL LightCycler FastStart DNA Master SYBR Green I 10x (Roche. Diagnostics), 1 μL of each primer (sense and antisense for the 16S rRNA gene) in a concentration of 0.5 μM each. Ultrapure water was added to a final volume of 10 μL. The PCR reaction for *Bifidobacterium* spp. was done with the following thermal program: 1 preincubation cycle at 95 °C (10 min.), 45 amplification cycles consisting of an initial dissociation at 95 °C (10 s), hybridization at 66 °C (10 s), and extension at 72 °C (25 s), followed by one cycle for the melting curve at 95 °C, 65 °C (15 s) and 95 °C, and 1 cooling cycle at 40 °C (30 s).

For *Lactobacillus* spp. the thermal program was: a preincubation cycle at 95 °C (10 min.), 45 cycles of amplification with 95 °C of

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Table 1. Recovery of Aloe vera Fructans and Acemannan after Extraction and Purification[a]

polysaccharide	leaf samples	amount obtained (mg)	mg polysaccharide/g FW	mg polysaccharide/g DW	% recovered from FW	% recovered from DW
fructan	4	774.9 ± 67.9	5.25 ± 0.32	27.36 ± 1.69	0.53 ± 0.03	2.74 ± 0.17
acemannan	6	428.2 ± 5.8	3.25 ± 0.18	93.58 ± 5.14	0.32 ± 0.02	9.36 ± 0.51

[a]The polysaccharides were quantified by GC-MS analysis as described in Materials and Methods Section. The figures represent the average of four samples, in the case of fructans and of six samples in the case of acemannan ± standard deviations. FW = fresh weight, DW= dry weight.

Table 2. Sugar Composition of Aloe vera Fructans and Acemannans by GC-MS[a]

polysaccharide sample	fucose (Fuc)	arabinose (Ara)	xylose (Xyl)	mannose (Man)	galactose (Gal)	glucose (Glc)	total
fructans	n.d.	n.d.	n.d.	41.73 ± 2.17	n.d.	58.27 ± 2.17	100 ± 4.34
acemannans	n.d.	0.39 ± 0.54	n.d.	86.87 ± 2.53	0.05 ± 0.07	12.68 ± 2.98	99.99 ± 6.12

[a]Both polysaccharides extracted from Aloe vera were analyzed by GC-MS to determine their respective sugar composition. Results are given in molar percentage (molar %) with its respective standard deviation (±S.D.). n.d.: the corresponding monosaccharide was not detected in the sample.

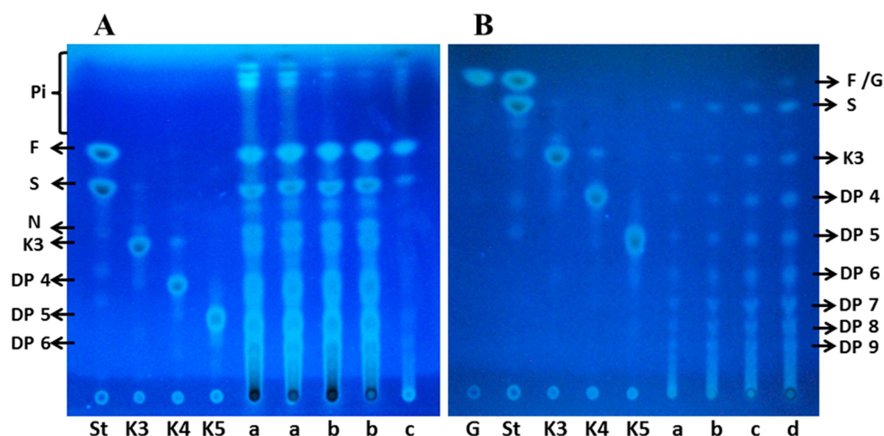

Figure 2. TLC analyses of the fructans obtained from water-stressed Aloe vera plants (A) and commercial FOS inulin (B). Both plates are observed under UV light (366 nm). Standards (St) of fructose (F) and sucrose (S). K3, K4, K5: fructan standards of the inulin series. K3:1-kestose (trisaccharide), K4: tetrasaccharide, K5: pentasaccharide. DP: Degree of polymerization of Aloe vera fructans and commercial FOS inulin. N: Neo-kestose, trisaccharide of neo-fructans; Pi: pigments. In (A) a: fructans from water-stressed plants without depigmentation. b: Fructans from water-stressed plants after depigmentation with AC and Celite 560. c: Fructans from water-stressed plants purified with a Dowex ion-exchange resin column, used as a control of depigmentation. In (B) lanes a, b, c, and d were loaded with 5, 10, 20, and 40 μg of commercial FOS inulin, respectively. Key shows the TLC area were the pigments appear.

dissociation (10 s), hybridization at 59 °C (10 s) and extension at 72 °C (14 s), followed by one cycle for the melting curve at 95 °C, 65 °C (15 s) and 95 °C, and 1 cooling cycle at 40 °C (30 s). To determine the total bacterial population the program was: a preincubation cycle at 95 °C (10 min.), 45 cycles of amplification with 95 °C of dissociation (10 s), 60 °C hybridization (10 s) and 72 °C extension (25 s), followed by one cycle for the melting curve at 95 °C, 65 °C (15 s), and 95 °C, and 1 cooling cycle at 40 °C (30 s).

Measurements for total bacterial, *Bifidobacterium* spp. and *Lactobacillus* spp. populations were done at 0 and 48 h of *in vitro* fermentation.

The primer sequences used were F: 5′-TCCTACGGGAGGCAG-CAGT-3′ and R: 5′- GGACTACCAGGGTATCTAATCCTGTT-3′ for total bacteria.[36] For *Lactobacillus* spp, the primers were F: 5′-AGCAGTAGGGAATCTTCCA-3′ and R: 5′-CACCGCTACA-CATGGAG-3′.[37,38] For *Bifidobacterium* spp the primers were: F: 5′-GGGTGGTAATGCCGGATG-3′ and R: 5′-CCACCGTTA-CACCGGGAA-3′.[39]

Quantification of SCFAs and BCFAs from *in Vitro* Fermentation of Human Feces in the Presence of Aloe vera Polysaccharides. Fecal culture samples (1 mL) obtained from the bioreactor at the beginning and end of the 48 h fermentation process were used to determine SCFA and BCFA concentrations according to Zhao et al.,[40] modified by Morales et al.[41] The pH of the samples was adjusted to 3.0 with 5 M HCl and they were left at room temperature for 10 min. The suspension was then centrifuged at 5000 rpm for 20 min at 4 °C and the supernatant removed. Ethyl butyric acid was added at a final concentration of 1 mM as an internal standard. SCFA and BCFA concentrations were determined in a gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a Stabilwax capillary column (Restek, Bellefonte, PA, USA) and a FID detector. The oven temperature program was as follows: 0.5 min at 100 °C, increased to 180 °C by 8 °C per min, and 5 min at 200 °C. The injector temperature was 200 °C; ultrapure nitrogen was used as a gas carrier. Calibration curves were carried out for acetic, propionic, butyric, isobutyric, valeric and isovaleric acids using commercial standards (Restock).

Statistical Analyses. Differences between groups in the bacterial growth experiments were analyzed by one-way ANOVA and Tukey posthoc tests for repeated samples. To compare maximum and minimum values of the total SCFA concentration the data were analyzed by a paired one-tailed Student *t* test. Data were processed using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Fructan and Acemannan Recovery after Depigmentation. Table 1 shows fructan and acemannan recovery from Aloe vera leaf cortex and gel, respectively, after the different steps of extraction, purification, and lyophilization. The average yield was 2.74% of dry weight for fructans and 9.36% of dry weight for acemannan.

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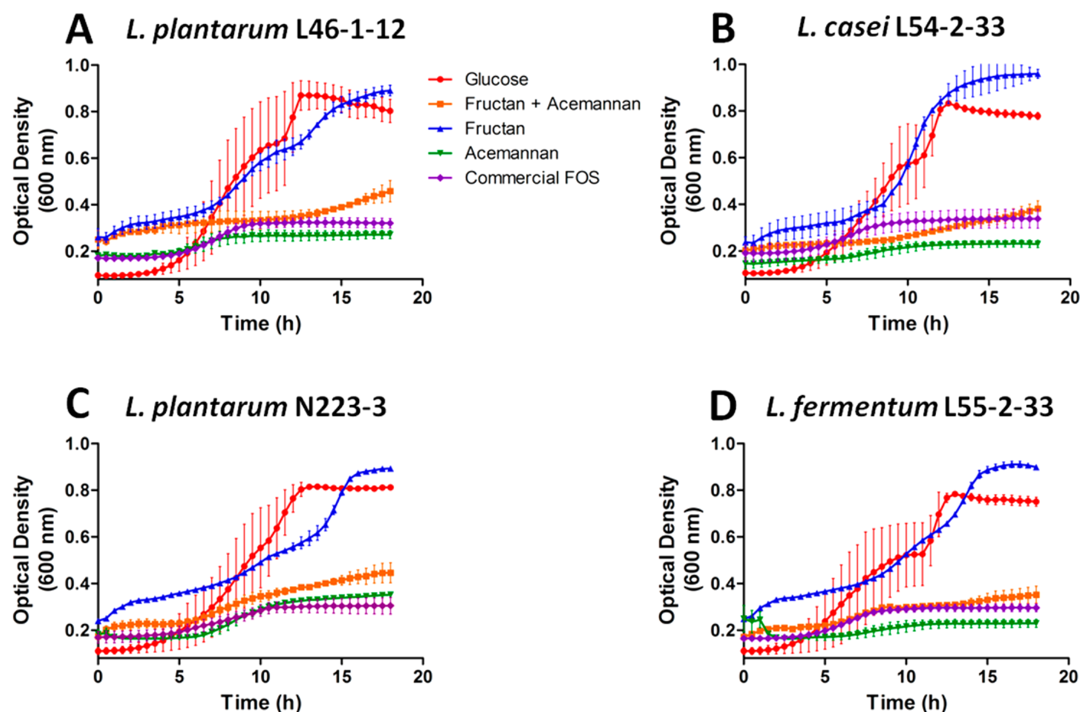

Figure 3. Kinetics of bacterial growth for Lactobacillus species. The growth was measured as the increase in optical density (OD) from the beginning (0 h) and during 18 h of culture at 37 °C. (A) *Lactobacillus plantarum* L46-1-12. (B) *Lactobacillus casei* L54-2-33. (C) *Lactobacillus plantarum* N223-3. (D) *Lactobacillus fermentum* L55-2-33. The OD was at 600 nm versus time (hours) using as carbon source: 10 g/L of glucose (red circle); mixture of fructans + acemannan, (orange square), 10 g/L and 3 g/L, respectively; fructans (blue triangle), 10 g/L; acemannan (green triangle), 3 g/L and oligofructans (pink diamond), 10 g/L (commercial FOS).

Sugar Composition of Fructans and Acemannan. Table 2 shows the sugar components of fructan and acemannan from leaves of water-stressed Aloe vera plants. The results indicate that the fructans are free of cell wall contaminants and the analysis reveals only mannose and glucose as components, due to tautomerization of fructose as glucose and mannose.[13]

The acemannan sugar components reveal that there are very little sugar contaminants, probably from cell walls. Mannose is the major sugar component being 86.87% and glucose 12.68%, followed by arabinose, 0.38% and galactose 0.05% all percentages are expressed in molar percent. Mannose is 6.85 times the amount of glucose (Table 2).

The linkage analyses of both polysaccharides from Aloe vera were performed by GC-MS of the partially methylated alditol acetates. The fructan linkages were reported previously in Salinas et al.[11] Respect to acemannan, the linkage analyses indicated the presence of glucose linked to mannose by (1→4) glycosidic bonds. A polysaccharide analysis by carbohydrate gel electrophoresis (PACE) of acemannan showed that galactose was not present as a sugar component (data not shown).

TLC Analyses of the Extracted and Purified Fructans. Figure 2 shows a thin layer chromatography performed to corroborate that the purified polysaccharides were free of pigments. Figure 2A, lane a shows fructans obtained from leaves of plants subjected to water deficit previous to the depigmentation procedure. Figure 2A, lane b shows depigmented fructans using Celite 560 and activated carbon (AC). In this lane no pigments can be observed in the upper part of the plate, which can be seen in lane a before depigmentation. Figure 2A, lane c shows a control sample of Aloe vera fructans purified with Dowex, an ion-exchange resin. The isolated fructans are less pigmented than those purified by Dowex ion exchange column chromatography.

Figure 2B shows a TLC analysis with increasing concentrations of commercial FOS. Sucrose is present in all lanes and fructose was also detected in three lanes of higher concentrations.

Results published by our group,[11] indicate that the sum of sucrose and fructose present in the depigmented fructans is 42%. Lower percentages of sucrose and fructose were detected in commercial FOS, estimated at about 10%.

***In Vitro* Fermentation of Aloe vera Polysaccharides with Pure Bacterial Cultures.** The effect of Aloe vera fructans and acemannan on the growth of pure cultures of *Lactobacillus* and *Bifidobacterium* species was evaluated *in vitro*. The growth was compared to the widely used prebiotic commercial FOS. The growth kinetics of the four *Lactobacillus* species is shown in Figure 3. When grown only with glucose the strains grew rapidly, reaching a plateau at about 13 h. The bacterial growth of the four *Lactobacillus* strains was more progressive in the presence of Aloe vera fructans and reached a plateau at higher OD values than those observed with glucose (except for *L. plantarum* L46-1-12). Less bacterial growth was observed with acemannan (except for *L. fermentum* L55-2-35); interestingly, bacterial growth in the presence of commercial FOS was much less than with Aloe vera fructans, but commercial FOS has less soluble sugars (fructose and sucrose).

Table 2 demonstrates that glucose is the best carbon source for *in vitro* growth of *Lactobacillus* species. The table also compares commercial FOS (inulin oligofructan) with Aloe vera fructans extracted from water-stressed plants. Aloe vera fructans increased bacterial growth 2.58−3.12 times, while commercial FOS increased it 0.8−0.9 times.

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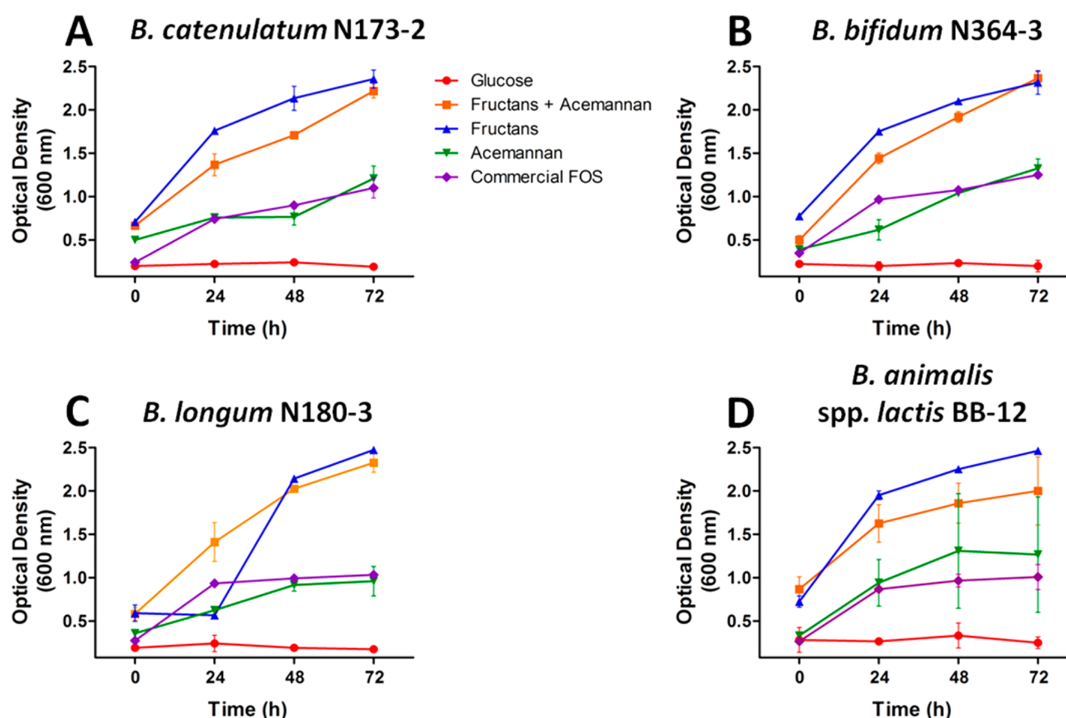

Figure 4. Kinetic of bacterial growth in *Bifidobacterium* species. The rate of growth of the strains was measured by the increase in the optical density (OD) from the beginning (0 h) and during 72 h of culture at 37 °C. In (A), *Bifidobacterium catenulatum*, strain N 173-2. In (B), it was *Bifidobacterium bifidum*, strain N 364-3. In (C), *Bifidobacterium longum*, strain N 180-3. In (D), *Bifidobacterium animalis*, spp. *lactis* BB12. The OD was at 600 nm versus time (hours) using as carbon source: glucose (red circle), 10 g/L; mixture of fructans + acemannan, (orange square), 10 g/L and 3 g/L, respectively; fructans (blue triangle), 10 g/L; acemannan (green triangle), 3 g/L and oligofructans (pink diamond), 10 g/L (commercial FOS).

Table 3. Comparison of the Fold Increment of the *Lactobacillus* Species in Three Carbon Sources, Glucose, Commercial FOS, and Fructans, After 18 h of Culture at 37 °C[a]

	Lactobacillus plantarum L46-1-12			*Lactobacillus casei* L54-2-33			*Lactobacillus fermentum* N-22-33			*Lactobacillus fermentum* L-55-2-33		
carbon source	OD 0 h	OD 18 h	fold increase	OD 0 h	OD 18 h	fold increase	OD 0 h	OD 18 h	fold increase	OD 0 h	OD 18 h	fold increase
glucose	0.10	0.80*	**7.32 a**	0.11	0.78*	**6.43 a**	0.11	0.81***	**6.59 a**	0.11	0.75***	**5.99 a**
commercial FOS	0.17	0.32*	**0.90 c**	0.19	0.34**	**0.82 c**	0.17	0.30*	**0.83 c**	0.17	0.30**	**0.80 c**
Aloe vera fructans	0.26	0.89**	**2.58 b**	0.24	0.96***	**3.12 b**	0.18	0.89***	**2.74 b**	0.25	0.90***	**2.61 b**

[a]The table shows the folds of growth increment. Significant differences between 0 h, and 18 h of culture for the same strain of *Lactobacillus* within the same carbon source, are given by asterisks (*) (Student's *t* test, *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$). Significant differences between different carbon sources among the same strains are indicated by different letters according to Tukeýs Test. Values at 0 and 18 h are the readings at OD 600 nm.

Figure 4 shows the growth kinetics of the *Bifidobacterium* species cultivated in the presence of all the carbon sources used. Since *Bifidobacteria* are anaerobic bacteria their growth is slower than that of the *Lactobacillus* species; for this reason, growth kinetics was monitored for 72 h. As shown in Figure 4, the tested strains did not grow in the medium with glucose, while their growth was moderate with commercial FOS and Aloe vera acemannan, and maximum with Aloe vera fructans.

Table 3 shows that growth increment was species-dependent. No significant differences in fold increment were detected between *B. longum* and *B. animalis* with fructans, the combination of fructan + acemannan or commercial FOS. For *B. bifidum* the greatest increase was with fructans + acemannan, with a 3.76-fold increment. The greatest increase for *B. catenalatum* was 3.56 fold with commercial FOS. However, the final counts of the *Bifidobacterium* species were greater with fructans and the combined mixture of fructans and acemannan (Figure 4).

Quantification of Bacterial Populations from Human Feces by qPCR. The results in Figure 5A show that the total bacterial population does not change after 48 h of *in vitro* fermentation with any carbon source used. Similar results were obtained for *Lactobacillus* spp, Figure 5B. In the case of *Bifidobacterium* spp, there is a significant increase only with fructans of 13.73% with a $P = 0.044$, Figure 5C.

Quantification of Short Chain Fatty Acids by *in Vitro* Fermentation of Human Feces in the Presence of Aloe vera Polysaccharides. Figure 6A shows the concentration of total SCFAs at 0 and 48 h of fermentation. It also shows the concentration of the linear SCFA (Figure 6B) and BCFA (Figure 6C). Due to the small sample size, the figure has a high dispersion of values with large standard errors. Figure 6A shows the total concentration of SCFA with glucose, Aloe vera fructans, and acemannan, commercial FOS, and without a carbon source, which is the negative control. The highest concentration means were obtained with Aloe vera fructans

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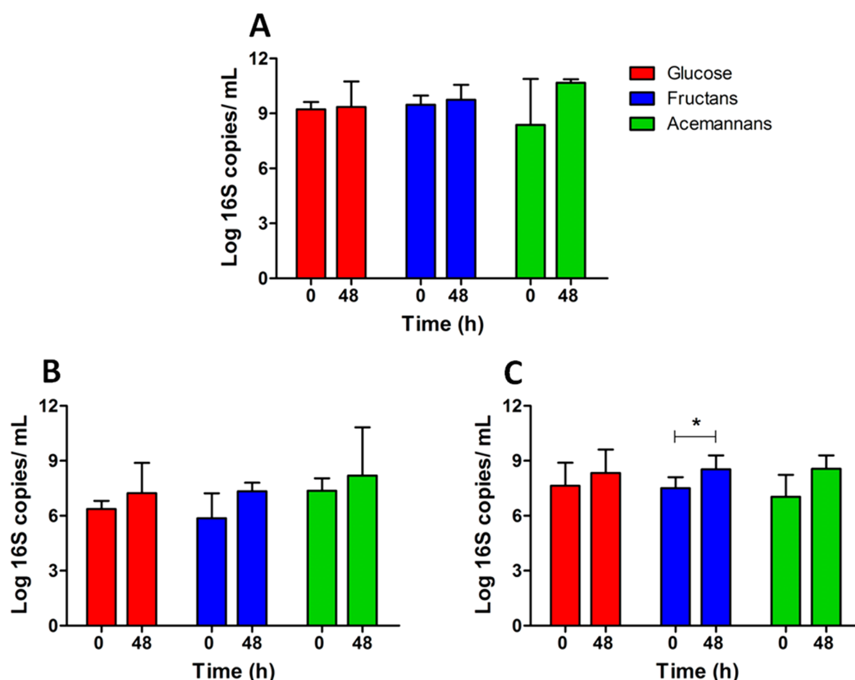

Figure 5. Quantification of the microbiota from human stool after 48h of fermentation. Quantification was performed by qPCR using selected primers at 0 and 48h of fermentation in the presence of glucose, fructans and acemannan, as described in Materials and Methods Section. (A) shows total bacterial population. (B) shows *Lactobacillus* spp. population. (C) shows *Bifidobacterium* spp. population. Each bar is the average of three biological samples and three replicates of each sample ± SD. The asterisk indicates statistical differences between 0 and 48 h by Student's *t* test.*: $P < 0.05$.

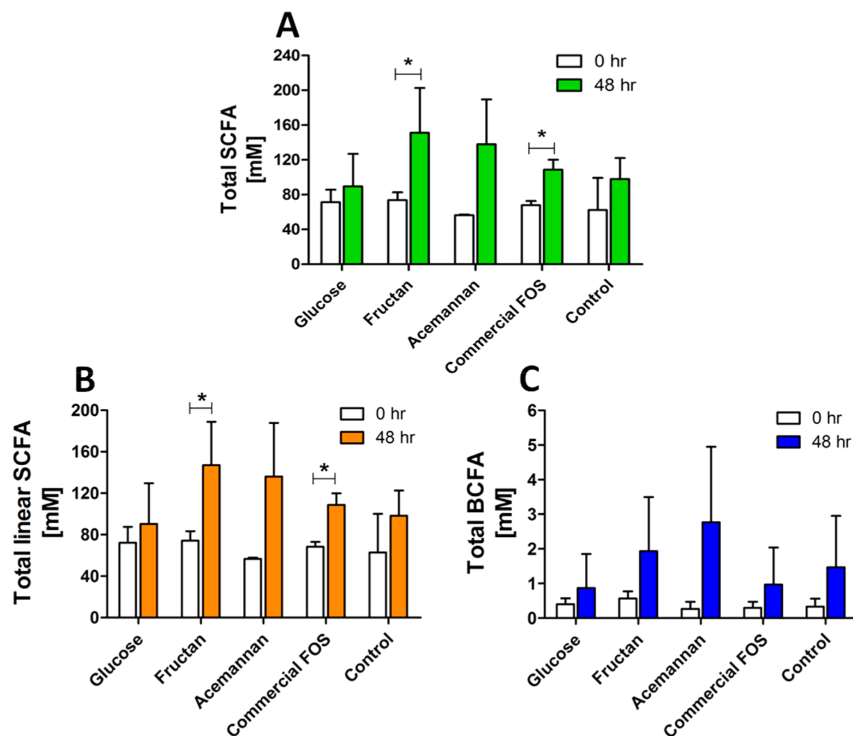

Figure 6. Concentration of SCFA and BCFA produced by fermentation of human feces in the presence of different carbon sources. The human feces were fermented in bioreactors and the SCFA and BCFA produced were quantified by gas chromatography as described in Material and Methods Section, using different carbon sources. The graphs show: (A) the concentration of total fatty acids in mM. (B) Total linear SCFA. (C) The quantification of BCFA. Each bar corresponds is the average of three biological samples and three replicates of each sample ± SD. The asterisk indicates a statistical difference between 0 and 48 h by Student's *t* test.*: $P < 0.05$.

with $P = 0.0395$ and acemannan with $P = 0.0607$, followed by commercial FOS with $P = 0.0176$. Again, in the case of total linear SCFA (Figure 6B), significant differences were found using Aloe vera fructans and commercial FOS, with P values of

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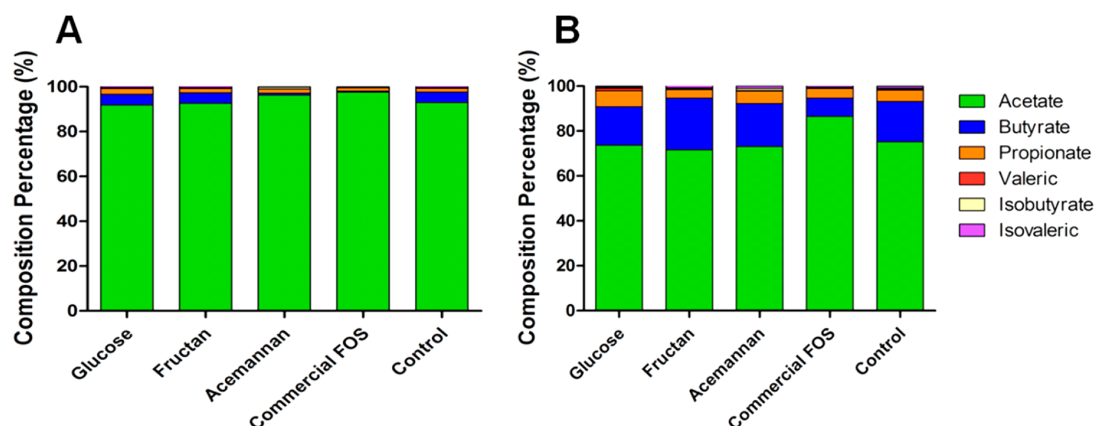

Figure 7. Percentage composition of the SCFA and of BCFA produced by fermentation of human feces in a bioreactor. (A) Percent of each SCFA and BCFA in the feces before fermentation (0 h). (B) Percent of each SCFA and BCFA after 48 h of fermentation.

Table 4. Comparison of the Fold Increment of the *Bifidobacterium* Species in Three Carbon Sources, Commercial FOS, Fructans+Acemannan, and Fructans, After 72 h of Culture at 37 °C[a]

	Bifidobacterium catenalatum N 173-2			*Bifidobacterium bifidum* N 364-3			*Bifidobacterium longum* N 180-3			*Bifidobacterium animalis, spp. lactis* BB-12		
carbon source	OD 0 h	OD 72 h	fold increase	OD 0 h	OD 72 h	fold increase	OD 0 h	OD 72 h	fold increase	OD 0 h	OD 72 h	fold increase
commercial FOS	0.24	1.10**	**3.56 a**	0.35	1.25***	**2.61 b**	0.28	1.03***	**2.78 a**	0.27	1.01*	**2.89 a**
Aloe vera fructans + acemannan	0.67	2.22***	**2.33 b**	0.50	2.37***	**3.76 a**	0.58	2.33***	**3.02 a**	0.87	2.00[ns]	**1.41 a**
Aloe vera fructans	0.71	2.36**	**2.33 b**	0.78	2.32**	**1.99 b**	0.59	2.47***	**3.25 a**	0.73	2.46***	**2.42 a**

[a]The table shows the folds of growth increment. Significant differences between 0 h, and 18 h of culture for the same strain of *Bifidobacterium* within the same carbon source are given by asterisks (*) (Student's *t* test, *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$, ns, not significant). Significant differences between different carbon sources among the same strains are indicated by different letters according to Tukeýs Test. Values at 0 and 18 h are the readings at OD 600 nm.

0.0395 and 0.0187, respectively. Total BCFA (Figure 6C) did not change significantly with any carbon source.

Figure 7 shows the percentage composition of SCFAs and BCFAs obtained from *in vitro* fermentation. Figure 7A and B corresponds to the percentage of all fatty acids (both linear SCFAs and BCFAs) at 0 and 48 h of fermentation. The results show that acetate is the major SCFA present with all carbon sources, as also in the negative control, at 0 h and after 48 h of fermentation. With fructans, acetate constituted 71% and with acemannan 73% of the total SCFAs, both values were lower than with commercial FOS (86%). Butyrate increased with all carbon sources as well as in the negative control after 48 h of fermentation; however, the percentages of butyrate generated with fructans and acemannan were greater, 23% and 19%, respectively. In contrast, propionate was 3.8% and 5.5% of the total SCFAs with fructans and acemannan, respectively.

The BCFAs and valeric acid were produced in very low concentrations. Isobutyrate, isovaleric, and valeric acids each constituted less than 1% of total SCFAs and did not show a significant increment with any carbon source.

DISCUSSION

The structure of fructans from Aloe vera plants subjected to water stress (shown in Figure 1) has been recently characterized by Salinas[13] and Salinas et al.[11] In these publications we reported that with water deficit fructans increased in concentration and in their DP, detecting new glycosidic linkages. These modifications are probably due to the osmotic adjustment that Aloe vera plants undergo when suffering water deficit.[15] Water-stressed plants also contain increased concentrations of acemannan.[14,15] Changes in DP, a higher degree of ramification, and the presence of new glycosidic linkages in fructans from Aloe vera would certainly make the fermentation of these polysaccharides by intestinal bacteria more difficult.

To test the prebiotic properties of the polysaccharides, we used pure cultures of *Lactobacillus* and *Bifidobacterium* species, and human fecal microbiota anaerobically cultured in bioreactors. The Aloe vera fructans and acemannan used in the study were free of pigment contaminants, as shown by TLC analyses (Figure 2A). These results suggest that the prebiotic activity reported here was not due to the pigment contaminants present in the samples. However, the depigmented fructan extracts did have free fructose and sucrose present in them.

Our results indicate that the greater amounts of ramified fructans of higher DP obtained from Aloe vera water-stressed plants are more efficient than acemannan and commercial FOS in stimulating the growth of different *Lactobacillus* and *Bifidobacterium* spp. In *Lactobacillus* spp. there was a significant 2.6−3.1 fold increase with fructans, and these fold increases were greater than those obtained with commercial FOS. In *Bifidobacterium* spp. there was also a significant 2.0−3.3 fold increase with fructans. In this case, the fold increases were similar to those produced by commercial FOS (see Tables 3 and 4).

Lactobacillus spp. can grow with soluble sugars such as glucose, fructose, and sucrose,[42] and unfortunately our fructan extracts contain fructose and sucrose in greater amounts than commercial FOS. Therefore, under these conditions we suggest that the greater growth of *Lactobacillus* spp. is caused mainly by

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fructans without neglecting that the free sugars present may also induce growth.

With *Bifidobacterium* spp. the best carbon sources to induce growth were Aloe vera fructans, followed by the combination of fructans and acemannan. Unlike *Lactobacillus* our *Bifidobacterium* species did not grow with glucose. Therefore, the growth of *Bifidobacteria* cannot be attributed to the free fructose, glucose, and sucrose present in the fructan extracts. While acemannan by itself only slightly stimulated the growth of *Lactobacillus* spp, in *Bifidobacterium* the effect was as much as commercial FOS. It is most likely that the high molecular weight of Aloe vera acemannan makes it difficult to be digested by *Lactobacillus* species. This idea is supported by previous reports that indicate unhydrolyzed konjac glucomannan has a less prebiotic effect than the hydrolyzed konjac glucomannan.[43] In addition, when acemannan was combined with Aloe vera fructans it decreased the growth of *Lactobacillus* spp. induced by fructans. We can speculate that the high viscosity of Aloe vera acemannan decreases the solubility of fructans in the medium, therefore, decreasing the prebiotic properties of fructans. This does not happen in *Bifidobacterium*, which indicates that *Bifidobacterium* species can ferment the mixture of fructans+acemannan more easily. This may be due to the presence of more efficient mannanases. Indeed the *Bifidobacterium* strains ferment the acemannan better than the *Lactobacillus* strains, as shown in the growth kinetic curves.

The *Lactobacillus* species reached their stationary phase more rapidly with glucose than with fructans, even though fructans isolated from Aloe vera have sucrose, glucose, and fructose as contaminants. The media with only glucose is more easily absorbed and metabolized by *Lactobacillus* than fructans. Fructans are complex polysaccharides made of different glycosidic linkages that need to be hydrolyzed by bacterial enzymes. Our results are similar to those of the previous study by Roberfroid et al.[44] They observed that different *Lactobacillus* and *Bifidobacterium* species grew significantly more in the presence of glucose followed by oligofructose, and to a lesser extent with inulin. The necessary enzymes are only present in some bacterial populations. Therefore, polysaccharide digestion slows down bacterial growth. In contrast, glucose is depleted earlier when used as the sole energy source.[45] Bacterial growth stagnation may be due to the depletion of essential nutrients, the accumulation of toxic products, because the culture reaches an excessive number of cells for the available space, or a combination of these factors.[46,47] Cardarelli et al.[48] quantified by qPCR the total bacteria and *L. amylovorus* DSM 16698 in human and pig feces fermented with oligofructans and glucomannan oligosaccharides. They reported that the fermentation process was shorter with oligofructans than with glucomannan oligosaccharides, confirming that *Lactobacillus* spp. grow more easily in the presence of fructans compared to acemannan.

Fructans from water-stressed Aloe vera stimulated *Bifidobacterium* growth more efficiently than commercial FOS and better than Aloe vera acemannan. But it is important to point out that fructans were added to the culture medium at a higher concentration than acemannan in our experiments. This is because acemannan in higher concentrations shows a tendency to form a gel which makes bacterial growth *in vitro* more difficult. However, the mixture of the two polysaccharides induces growth almost as well as with just fructans in *Bifidobacterium* spp. Therefore, both polysaccharides combined make a good prebiotic preparation; the mixture was better than the commercial FOS when evaluated with these specific *Bifidobacterium* species.

These results were partially corroborated by the qPCR analysis of bacterial population. The population of *Bifidobacterium* spp. present in human feces increases significantly in the presence of fructans. Fructans also increase, not significantly, the population size of *Lactobacillus* spp. with a $P = 0.0537$. Acemannan and glucose, on the other hand, did not increase significantly the population sizes of either probiotic genus. But, since our sample size was small ($n = 3$), by increasing the number of individuals in further studies, we could probably find significant population increments with acemannan and/or glucose.

Here we also present preliminary results of short-chain fatty acids (SCFAs) produced by human stool fermentation in the presence of Aloe vera polysaccharides. Aloe vera fructans and acemannan seem to increase the production of total SCFAs during the stool fermentation. The high dispersion of values, due to a low number of samples, did not allow us to demonstrate significant differences among the carbon sources used in the analyses. However, significant increments were found with fructans ($P = 0.0365$) and commercial FOS ($P = 0.0176$). With acemannan, there was only a tendency to increase total SCFA ($P = 0.0612$). The greatest variation in total SCFA production was found with acemannan followed by fructans (145% increase with acemannan and 98% increase with fructans).

The SCFA composition percentages show that butyrate and propionate increased in all conditions, with and without carbon sources. After 48 h of fermentation, butyrate composition was higher with fructans and acemannan. The figure of composition percentages does not show that the acetate concentration increases significantly with acemannan. The acetate produced from commercial FOS was 86% of the total SCFAs, which was the highest value detected under all the conditions used. Acetate is important since it has been shown to help reduce obesity and body fat.[49−51]

The BCFAs are the fermentation products of some amino acids. There were no significant differences in the total amounts of BCFAs with any carbon source. Indeed, the BCFAs produced by the negative control were similar to those generated by the other carbon sources, confirming that purified Aloe vera polysaccharides are free of amino acid contaminants.

Fermentation of complex polysaccharides and eventually some amino acids can be used as an energy source by the beneficial colon bacteria generating SCFAs and BCFAs.[45,52−54] Among the SCFAs, propionate decreases bowel inflammatory diseases and is associated with the production of lipogenic enzymes.[55−57] Acetate, in contrast, enters the peripheral circulation and is metabolized by the peripheral tissues. Butyrate is the main substrate and principal energy source of the colonocytes, promoting the normal phenotypes of these cells avoiding the formation of polyps[58] and, therefore, preventing colorectal cancer. Butyrate with propionate induce apoptosis of colorectal cancer cells.[59,60] These benefits would potentially be induced by Aloe vera polysaccharides. Indeed in our results, fructans and acemannan stimulate the production of acetate, propionate, and butyrate, which in turn possibly prevents the formation of polyps. Further studies are needed to corroborate these health benefits from Aloe vera polysaccharides.

The results of this study confirm that fructans and acemannan from Aloe vera are very promising prebiotics, that

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had not been studied previously in detail. The growth kinetics of single bacterial strains, corroborated by bacterial population qPCR, and SCFA quantifications, indicate that ramified fructans are better prebiotics than commercial FOS which is a linear fructan.

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Notes

The authors declare no competing financial interest.

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