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Prebiotic Effect of Agave Fructans and Mixtures of Different Degrees of Polymerization from *Agave angustifolia* Haw

Patricia Araceli Santiago-García[1] • Mercedes G. López[2*]

[1] Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional CIIDIR-IPN-U, Oaxaca, 68030, Oaxaca, Mexico
[2] Departamento de Biotecnología y Bioquímica, Centro de Investigación y de Estudios Avanzados del IPN, CINVESTAV-Irapuato, Km. 9.6 Libramiento Norte Carretera León-Irapuato, 36821. Irapuato, Guanajuato, Mexico

Corresponding author: * **mlopez@ira.cinvestav.mx**

ABSTRACT

Agave fructans are complex and highly branched molecules, which cannot be digested in the upper gastrointestinal tract. As a result, when they reach the large intestine, they serve as fermentative substrates for bacterial growth. Previous reports have shown that fructans of the inulin-type, through both *in vitro* and *in vivo* assessments, are effective prebiotics, increasing the content of bifidobacteria and lactobacilli in the gut and, consequently, inhibiting the growth of pathogenic bacteria. Oaxaca has the largest diversity of *Agave* species in Mexico. *Agave angustifolia* contains high amount of fructans with potential health benefit for humans. The aim of this work was to investigate the growth rate of six bifidobacteria and four lactobacilli strains when fructans and mixtures with different degrees of polymerization (DP) from *A. angustifolia* were used as an energy source. We observed that agave fructans stimulated the growth of bifidobacteria and lactobacilli more efficiently (2-fold) that commercial inulins. Bacterial growth, pH drop and SCFA's production, mainly acetate, were different among strains; while *in vitro* fermentation revealed that mixtures of different degrees of polymerization and short-DP (< 10) fructans were highly fermented. Biomass and pH drop were larger when the substrate contained mostly short-DP fructans. In conclusion, the presence in the mixtures of short-DP fructans, influenced significantly the rate of fermentation by the probiotic bacteria.

Keywords: *in vitro* fermentation, bifidobacteria, lactobacilli, short chain fatty acids
Abbreviations: **ATCC**, american test culture cells; **DP**, degree of polymerization; **FOS**, fructooligosaccharides; **GC**, gas chromatographer; **MRS**, Mann Rogosa Sharpe; **OD**, optical density, **SCFA**, short chain fatty acids

INTRODUCTION

Fructans are fructose polymers present in plants as a heterogeneous mixture presenting in their structure a moiety of terminal glucose, possessing mainly β(2-1) linkages, and their structure can be linear or branched. According to the type of linkage, fructans are classified into several categories including inulin, β(2-1) linkage, levan, β(2-6) linkage, and graminans which have both links (Vijn and Smeekens 1999). Other types also exist such as inulin neo-series, levano neo-series and mixed types (Ritsema *et al*. 2003). These carbohydrates are found in plants such as chicory, onion, garlic, and agaves among others. The molecular structures of *A. tequilana* fructans are very complex and highly branched, containing principally β(2-1) and β(2-6) linkages and with internal glucose, predominantly the neofructan series (López *et al*. 2003). Mancilla-Margalli and López (2006) showed the existence of at least two types of fructans in agaves: neoseries (agavins) and those with terminal glucose (graminans) moiety.

Previous reports showed that fructans of the inulin-type, through both *in vitro* and *in vivo* assessments are effective prebiotics, increasing the content of bifidobacteria and lactobacilli in the gut. This generates short chain fatty acids (SCFA), lactic and formic acids, and gases including H_2, CO_2, and CH_4 as products of an anaerobic metabolism (Roberfroid 2005) favoring the maintenance and development of the colonic microbiota (Roberfroid 1998; Gibson *et al*. 2004).

Fructans of the inulin-type possess only β(2-1) linkage that escape the action of digestive enzymes, therefore they reach the large bowel unchanged, and serving consequently as fermentative substrates to the colonic microbiota (Cummings and Macfarlane 2002; Kolida *et al*. 2002; Roberfroid 2005).

It is known that several pathogenic species can cause acute gastroenteritis and certain species may be involved in chronic gut disorders like ulcerative colitis, bowel cancer, and others diseases (Gibson *et al*. 2004). Previous reports indicated that fructans selectively stimulate the growth and activity of bifidobacteria and lactobacilli in the guts and consequently inhibit the growth of pathogenic bacteria by providing some protection against infections (Steer *et al.* 2000). The criteria used for the classification of food ingredient as prebiotic are as follows: resistance to digestion, hydrolysis and fermentation by colonic microbiota, and most importantly, selective stimulation of growth of one or a limited number of bacteria in the colon. One of the most important aspects of prebiotics ingestion is the fortification of the gut microbiota to resist acute infections (Gibson *et al*. 2004). Inulin-type fructans are an example of such carbohydrates that typically has a degree of polymerization (DP) between 3 and 60 and is extracted from chicory roots. Fructooligosaccharides (FOS) compounds have a DP between 2 and 20 and reach the colon unabsorbed and are utilized selectively as a substrate for the endogenous bacteria and by fermentation producing short chain fatty acids (SCFAs) mainly acetate, propionate, butyrate as well as lactate (Roberfroid 1993; Roberfroid *et al*. 1998; Roberfroid 2005).

Human trials have established that the addition of FOS or inulin to the diet leads to an increase in bifidobacteria (Gibson *et al*. 1995; Kolida *et al*. 2002) and several studies have described *in* vitro fermentation of FOS in pure cultures of *Bifidobacterium* (Gibson and Wang 1994; Kaplan and Hutkins 2000; Perrin *et al*. 2001, 2002). Nevertheless, little

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is known regarding the influence of differing DP of fructans on fermentation capability. A single *in vitro* study on three *Bifidobacterium* strains suggested that short chains FOS were fermented at a higher rate than longer chains and resulted in a higher biomass yield (Perrin *et al*. 2002). The results of *in vitro* studies indicated the specificity of *Bifidobacterium* except for *B. bifidum* to utilize FOS, but not inulins (Kaplan and Hutkins 2000; Biedrzycka and Bielecka 2003; Rossi *et al*. 2005).

Higher concentration levels of GLP-1 and its precursor, proglucagon mRNA, in different colonic segments of male mice (C57B1/6J) were observed when their diet was supplemented with agave fructans from *A. tequilana* and *Dasylirion* sp. These might have promising effects on glucose metabolism, body weight and fat mass development in humans (Urías-Silvas *et al.* 2008).

Agave angustifolia are succulent plants with spirally arranged leaves forming a rosette. Belonging to the family Agavaceae, they are nearly stemless. The leaves are bluish green, lanceolate-shaped over 1.87 m long in mature plants, and end in a sharp brown thorn. *A. angustifolia* is of great importance due to the report of the presence of fructans (Mancilla-Margalli and López 2006).

Based on the well-known health benefits of fructans and the differences among agave fructans and inulins, this study was carried out to determine the prebiotic effect of agave fructans and mixtures of different degrees of polymerization from *Agave angustifolia*.

MATERIALS AND METHODS

Plant material

Eight-year-old *A. angustifolia* were harvested from Santiago Matatlán, Mexico. The leaves were cut off, keeping the stems or "heart" of the plant (*piñas*). The *piñas* were milled and the pulp fraction was stored at -76°C until they were analyzed.

Extraction of agave fructans

One hundred grams of agave pulp were extracted twice with 100 mL of 80% w/v ethanol and shaken for 1 h at 55°C. The sample was filtered and re-extracted with 100 mL of water at 55°C for 1 h. The filtered liquids were combined and were washed with chloroform. The organic phase was concentrated at 80 mL by rotatory evaporation under reduced pressure and the separation of fructans in long and short-DP was achieved by precipitation of long-DP fructans by the addition of 130 mL of absolute ethanol and short-DP fructans were obtained from the supernatant. Samples were spray-dried and stored in a humidity-free container.

Experimental design

In this study, seven different types of fructans were compared: long-DP (LAA) and short-DP (SAA) agave fructans and mixtures of different degrees of polymerization from *A. angustifolia* (A=75% LAA + 25% SAA; B=50% LAA + 50% SAA; C=25% LAA + 75% SAA) and as controls, Raftiline GR (RAF1) and Raftilose Synergyl (RAF2) manufactured by Orafti. *Bifidobacterium* strains were obtained from ATCC (American Type Culture Collection, Rockville, Md.). (A) *Bifidobacterium adolescentis*; (B) *B. animalis*; (C) *B. bifidum*; (D) *B. breve*; (E) *B. infantis*, and (F) *B. longum* and four lactobacilli strains (A) *Lactobacillus acidophillus*; (B) *L. casei*; (C) *L. paracasei* and (D) *L. rhamnosus* were tested as probiotics by following the method reported by Gibson and Wang (1994). Optical densities (OD630), pH and short chain fatty acids production were measured after 20 h incubation in Mann Rogosa and Sharpe (MRS) containing 10 gL^{-1} of fructans. Three independent determinations were done and mean values from the measurement were compared using Tukey's test. Orthogonal contrasts were also used to determine significant differences. Differences were considered statistically significant when $P \leq 0.05$.

Bacterial growth

Culture broth of Mann, Rogosa, and Sharpe (MRS) for bacterial growth was used; in the case of bifidobacteria the culture broth was supplemented with L-cysteine. To inoculate, two anaerobic subcultures of 48 h at 37°C were carried out in 10 mL of MRS medium containing glucose as the sole energy source. Bacteria were inoculated in 10 mL of the culture broth. The transferred inocula were always 1%. The incubation conditions were 20 h at 37°C under an anaerobic atmosphere. To evaluate the effect of the different fructans on bacterial growth the broth was prepared without glucose and with a fructans concentration of 10 g/L as a carbon source. Bacterial growth was measured by optical density (OD) at 630 nm and the uptake of fructans by bacteria (probiotics) was evaluated measuring the pH change.

Determination of short chain fatty acids (SCFA's)

To determine acidic metabolites, samples were centrifuged (16,000 g for 10 min) and the amounts of acetic, butyric, lactic and propionic acids in the cell-free culture supernatants were determined by gas chromatography (GC). One milliliter of bacterial culture broth was extracted with diethyl ether according to Prieto-Femia *et al*. (2002). In brief, an internal standard solution (2-methyl-valeric acid 0.01%) was added to the broth, the solution was acidified with 0.5 mL H_2SO_4 and SCFA's were extracted by shaking with 2 mL of diethyl ether and subsequent centrifugation at 3500 rpm for 5 min. Two µL of the organic phase were injected directly into the capillary column (HP-FFAP) of the GC. The initial temperature was 50°C and the final temperature was 200°C. Helium was used as carrier gas at a flow rate of 18 mL min^{-1}. Quantification of the samples was carried out using calibration curves for acetic, butyric, lactic and propionic acids.

RESULTS AND DISCUSSION

Effect of different fructans on the growth of *Bifidobacterium*

1. Long-chain agave fructans

The growth of bifidobacteria after 20 h of fermentation is given in **Fig. 1**. LAA showed the best growth for *B. animalis* (**Fig. 1B**) that had an OD_{630} 0.301 compared to the *B. breve* and *B. infantis* with values close to 0.168. Slightly lower growth values were obtained with *B. longum*, whereas *B. adolescentis* and *B. bifidum* almost did not utilize LAA as substrate. In agreement with this observation, *B. animalis* grew on both SAA and LAA (**Fig. 1B**), being able to hydrolyze and ferment LAA. We observed that bifidobacteria exhibited better growth in LAA than Raftiline®GR (inulin type fructan) in almost all cases. This suggests that bifidobacteria hydrolyzed better agave fructans branched with β(2-1) linkages, but it has a greater capacity to hydrolyze β(2-1). Such differences were remarkable with *B. bifidum.*

In general, bifidobacteria possess intracellular β(2-1)-fructan-fructan hydrolyase activity making the fructan molecules an efficient substrate (Roberfroid *et al*. 1998); however, long-DP fructans were slowly metabolized by *B. adolescentis* and *B. bifidum*. This difference in fermentability may be also reflected by the type of the enzymes that each evaluated strain contains or it lacks an adequate transport system (Van der Meulen *et al*. 2004).

2. Short-chain agave fructans

The fermentation with 1% (w/v) SAA showed the best growth for *B. animalis* and *B. breve* (**Figs**. **1B, 1D**). In SAA culture medium, *B. breve* had an OD_{630} of 0.514 which was significantly different and 3.2 times greater than *B. bifidum.* This was one of the strains with the lowest biomass yields on SAA. *B. animalis* grew in short-DP fructans best and had the second highest growth level observed. *B. infantis* occupied third place. The results showed that the majority of

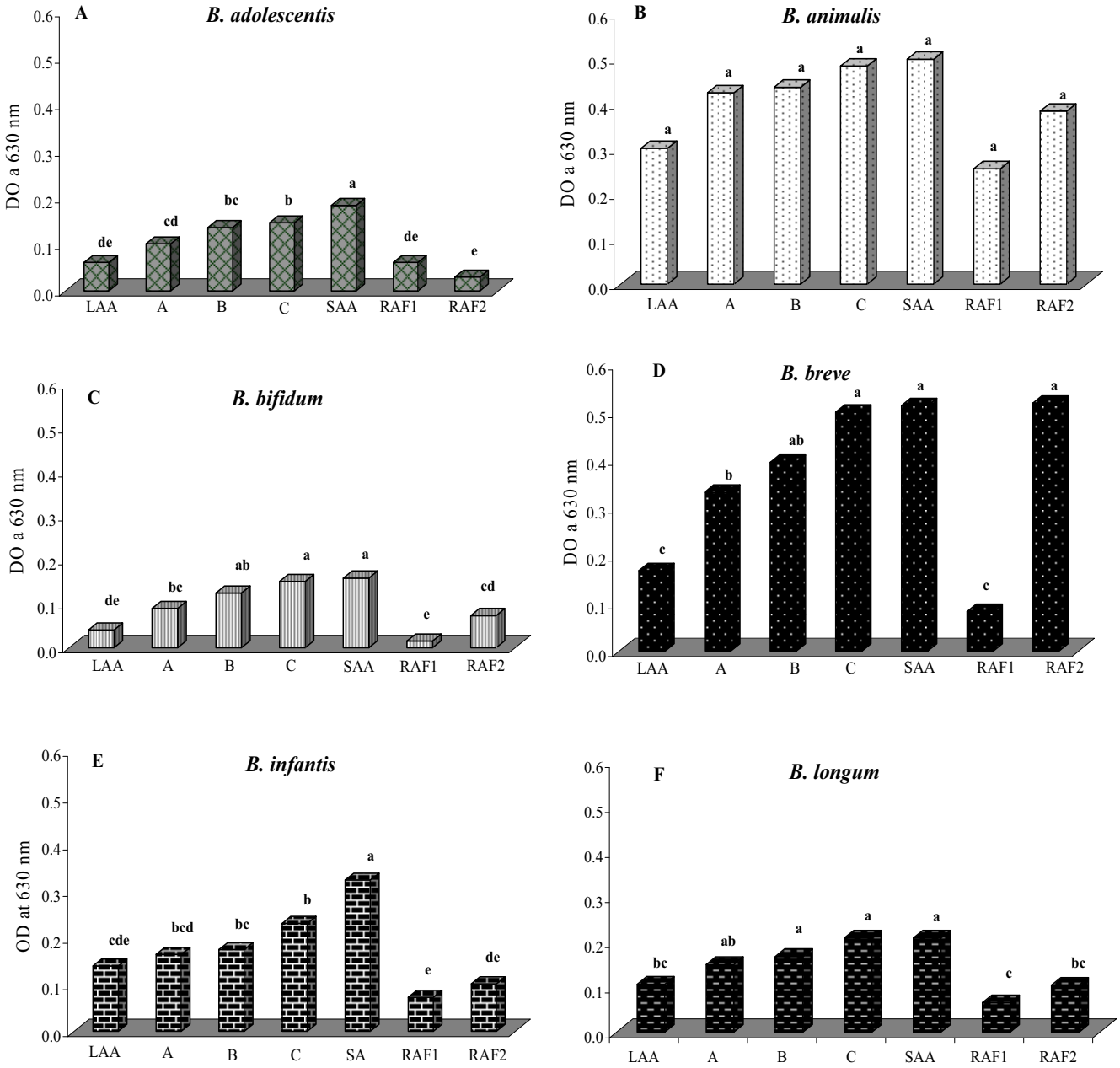

Fig. 1 Effect of different fructans as the sole carbon source on the growth of bifidobacteria. (**A**) *Bifidobacterium adolescentis*; (**B**) *B. animalis*; (**C**) *B. bifidum*; (**D**) *B. breve*; (**E**) *B. infantis*; and (**F**) *B. longum.* Optical densities (OD_{630}) were measured after 20 h incubation in Mann Rogosa and Sharpe (MRS) containing 10 g L-1 of fructans. LAA, Large-DP fructans of *A. angustifolia*; A=75% LAA plus 25% SAA; B=50% LAA plus 50% SAA; C=25% LAA plus 75% SAA; SAA, short-DP fructans of *A. angustifolia*; RAF1, Raftiline®GR; RAF2, Raftilose®Synergyl. Differences were considered significant at $P \leq 0.05$.

Bifidobacterium strains studied utilized SAA. Moreover, in comparison to RAF2 as control, the growth of strains *B. adolescentis*, *B. bifidum*, *B. infantis*, and *B. longum* (**Figs. 1A, 1C, 1E, 1F**) were stimulated between 2 to 3 times more.

High specificity of short-DP as a substrate for bifidobacteria results from the activity of the specific cell associated β-fructosidase which hydrolyse fructose monomers from the non-reducing end of the chain in which the fructose residue occurs at the β(2-1) position. The preferential growth in SAA observed in all strains probably was produced by β-fructosidase enzymes activity since these enzymes are most effective on short-DP fructans and hydrolyze β(2-1) and α(1-2) glycosidic bonds (O'Sullivan 1996). Van der Meulen *et al*. (2004) reported similar results in *B. animalis* and argued that the preferential metabolism of FOS by bifidobacteria strains may be due also to the presence of phosphotransferases which occur also in *B. breve*. These results are in agreement with other studies that conclude that maximum growth is higher on oligosaccharides moiety (Gibson and Wang 1994; Hopkins *et al*. 1998; Kaplan and Hutkins 2000; Biedrzycka and Bielecka 2003; Kim *et al*. 2003; Van der Meulen *et al*. 2004).

Mixtures

The growth of bifidobacteria in mixtures of different degrees of polymerization from *Agave angustifolia* after 20 h of fermentation is given in **Fig. 1**. It was observed that growth was proportional to the concentration of SAA in each of the mixtures. The highest growth occurred in mixture C (25% long-chain and 75% short-chain agave fructans) in the 6 tested species. *B. animalis* (**Fig. 1B**) showed no significant difference between the mixtures from *A. angustifolia* (A, B, and C) and their growth rates were similar to those obtained in SAA. No statistically significant differences were observed between mixtures C and SAA with *B. breve* (**Fig. 1D**). *B. bifidum* and *B. longum* (**Figs. 1C, 1F**) showed the same trend but with lower growth rates, whereas *B. adolescentis* and *B. infantis* (**Figs. 1A, 1E**) showed values in mixture C significantly lower ($P \leq 0.05$) than SAA. All the mix-

tures show more significant growth than the controls except *B. breve*. The six *Bifidobacterium* strains differed significantly ($P \leq 0.05$) in their ability to grow on mixtures of different DP from *A. angustifolia*.

The increase utilization of the agave fructans by *B. adolescentis*, *B. bifidum* and *B. longum* as compared to commercial fructans type inulins (RAF1 and RAF2) suggests that these can also hydrolyze β(2-6) bonds, extra- and intracellular hydrolyzed FOS and simultaneously metabolize fructose. Perrin *et al*. (2002) reported the higher specificity of *B. infantis* for short-DP fructans and Imamura *et al*. (1994) attributed it to the activity of the β-fructosidasa as well as to *B. adolescentis* GI (Amaretti *et al*. 2006). The results suggest that these species have a higher specificity for short-DP. On the other hand the progressive release of glucose and fructose induces β-fructosidase activity which causes hydrolysis of the short-DP fructan fraction with β(2-1) and β(2-6) bonds. The results of *B. adolescentis* are similar to those reported by Marx *et al*. (2000) who reported that *B. adolescentis* produced the best ferment of levans with β(2-6) fructosyl bonds among four different strains. This is similar to the results of Amaretti *et al.* (2006), who reported maximum growth yield in disaccharides and oligosaccharides. As noted, the prebiotic effectiveness of agave fructans not only depends on the on the DP, as mentioned by Van Loo (2004), but also on the type of link. The agave fructan of longer-DP are more resistant to fermentation. Hence, the presence of short-DP fructans in the mixtures highly influenced the rate of fermentation by the probiotic bacteria (Kaplan and Hutkins 2000; Biedrzycka and Bielecka 2003).

On the other hand the superior growth observed in LAA fructans, as compared with the control Raftiline®GR (RAF1), could be the result of induction of a β-fructosidase that hydrolyzes long-chain branched fructans with β(2-1) and β(2-6) bonds, therefore releasing FOS and fructose. The progressive release of glucose and fructose from agave fructan induced the β-fructofuranosidase activity responsible for the hydrolysis of these oligosaccharides (Perrin *et al*. 2001; Rossi *et al*. 2005).

The best bacterial growth in agave fructans could be an indication of the predominance of the branched types in the fructan molecule from *A. angustifolia*, allowing a faster hydrolysis of inulin, β(2-1) linkage and levan, β(2-6) linkage, being three times faster for *B. longum*, *B. infantis*, *B. bifidum* and *B. adolescentis* than commercial fructans. Amaretti *et al.* 2006 reported that *B. adolescentis* simultaneously displayed α-galactosidase, β-galactosidase and β-fructofuronosidase on FOS and other sugars.

Orthogonal contrasts showed that the growth of bifidobacteria was significantly different ($P \leq 0.01$) than that noted between agave fructans and the control. A significant difference was also found in the effects of long and short fructans DP on the growth of bifidobacteria.

pH drop

Another important parameter during fermentation was the pH drop due to the production of SCFAs. In the culture broth containing LAA fructans as substrate, the pH drop was slight for all bifidobacteria, being higher for the *B. animalis* but no significant differences were found with the control (**Table 1**). In SAA fructans the pH drop was ≥ 1 for all bifidobacteria. The level of pH drop with *B. adolescentis*, *B. bifidum*, *B. infantis* was significantly higher on SAA fructan than that observed ($P \leq 0.5$) in control cultures. pH drop in the mixtures was proportional to the concentration of SAA fructans. In all cases bacterial growth was directly related to pH drop: *B. breve* and *B. bifidum* had an $r^2 = 0.88$ between growth and pH drop (**Table 1**), which has been reported to have an increased beneficial effect by inhibiting the growth of pathogenic bacteria (Gibson and Wang 1994).

Production of short chain fatty acids

Acetic, butyric, lactic and propionic acids were monitored

Table 1 Culture broth pH drop due to fermentation of fructans by *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*.

Fructan	*B. adolescentis*	*B. animalis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
LAA	0.58 bc	0.92 a	0.48 bc	0.68 b	0.61 ab	0.49 bc
A	0.47 cd	1.15 a	0.49 bc	0.89 ab	0.69 ab	0.49 bc
B	0.48 cd	1.10 a	0.57 bc	0.96 ab	0.85 c	0.55 ab
C	0.65 bc	1.25 a	0.68 b	1.14 ab	0.89 ab	0.75 bc
SAA	0.98 a	1.50 a	0.94 a	1.43 ab	1.22 a	0.86 a
RAF1	0.33 d	0.71 a	0.37 c	0.63 b	0.55 b	0.40 c
RAF2	0.76 ab	1.28 a	0.69 ab	1.70 a	0.90 ab	0.66 ab

Differences were considered significant at $P \leq 0.05$. The drop in pH of the cultures was measured directly in culture tube.

Table 2 Short chain fatty (mM) liberated by bifidobacteria during fermentation on *Agave angustifolia* and commercial fructans. BA, *Bifidobacterium adolescentis*; BAN, *B. animalis*; BBF, *B. bifidum*; BBR, *B. breve*; BI, *B. infantis*; BL, *B. longum*.

Fructan	ACID	BA	BAN	BBF	BBR	BI	BL
LAA	Acetic	60.94 a	58.33 a	45.41 bc	48.61 b	84.67 a	92.89 a
	Lactic						
	Propionic						
A	Acetic	55.22 a	46.85 ab	47.09 abc	51.03 b	51.34 b	63.38 bc
	Lactic						
	Propionic						
B	Acetic	53.02 a	45.49 ab	48.44 abc	54.17 b	58.00 b	57.47 c
	Lactic						
	Propionic						
C	Acetic	54.73 a	56.75 a	46.37 abc	44.23 b	56.05 b	53.17 c
	Lactic						
	Propionic				18.50		
SAA	Acetic	59.91 a	47.93 ab	54.27 ab	71.30 ab	54.77 b	45.49 c
	Lactic				3.95		
	Propionic						
RAF1	Acetic	34.25 ab	51.47 a	40.03 c	63.69 ab	49.25 b	78.95 ba
	Lactic						
	Propionic				2.00		
RAF2	Acetic	15.06 b	30.77 b	58.06 a	83.13 a	66.32 ab	55.02 c

Different letters indicate significant differences ($P \leq 0.05$) compared with the commercial fructans of each strain, and SD n=3

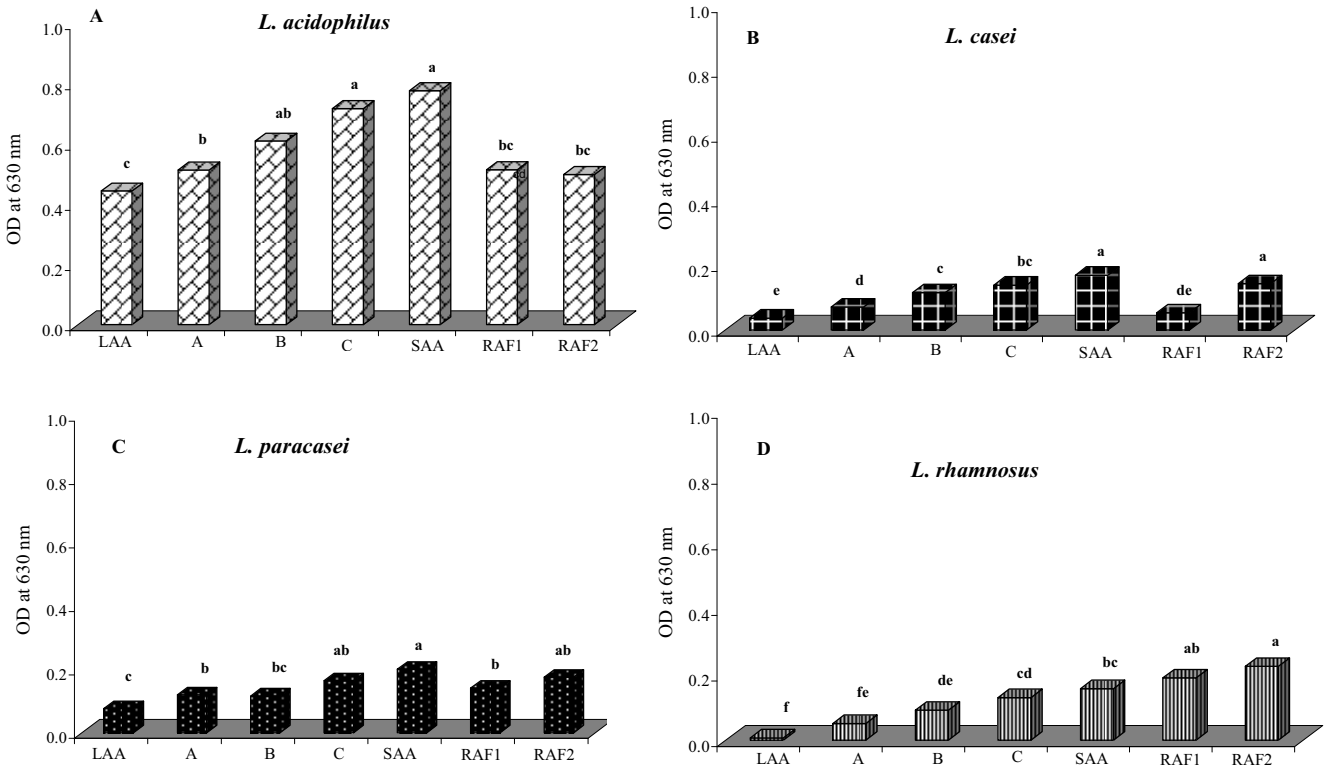

Fig. 2 Effect of different fructans on the growth of lactobacilli. (**A**) *Lactobacillus acidophilus*; (**B**) *L. casei*; (**C***) L. paracasei*; (**D**) *L. rhamnosus.* Optical densities (OD_{630}) were measured after 20 h incubation in Mann Rogosa and Sharpe (MRS) containing 10 g L-1 of fructans. LAA, large-DP fructans of *A. angustifolia*; A=75% LAA plus 25% SAA; B=50% LAA plus 50% SAA; C=25% LAA plus 75% SAA; SAA, Short-DP fructans of *A. angustifolia*; RAF1, Raftiline®GR; RAF2, Raftilose®Synergyl. Differences were considered significant at $P \leq 0.05$.

during fermentation (**Table 2**). Acid production was much higher in cultures containing LAA fructan that in control culture. *B. infantis* and *B. longum* exhibited the same behaviour observed in LAA fructans. *B. breve* had increased production of acetic acid when the substrate was SAA fructan. *B. adolescentis* and *B. bifidum* produced an average 60 mM of acetic acid in SAA fructans. These two species grew slowly but attained notable metabolite production. Acetic acid was the main metabolite in all cases, which was also the case for bifidobacteria in general. No production of lactic and propionic acids were observed during fermentation in cultures with *B. animalis*, *B adolescentis*, *B. bifidum*, *B. infantis* and *B. longum*. Lactic and propionic acids were only produced with *B. breve* (3.5 and 18.5 mM, respectively) in short-DP fructans and mixture C. Acetic acid production was slightly higher in cultures containing agave fructans than in control cultures concomitant with the pH reduction. Similar results were reported by López and Urías-Silvas (2007). Butyric acid was not observed in any treatment.

Effect of different fructans on the growth of lactobacilli

The growth of lactobacilli after 20 h of fermentation is given in **Fig. 2**. The results show that long-DP fructan (LAA), are only used by *L. acidophillus* (**Fig. 2A**) that had an OD_{630} 0.504. Lower values were obtained with *L. paracasei* (**Fig. 2C**), whereas *L. casei* (**Fig. 2B**) and *L. rhamnosus* (**Fig. 2D**) almost did not utilize LAA. This difference in fermentability may also be reflected by the type of enzymes contained in each strain evaluated. *L. acidophillus* grew effectively on all the evaluated fructans, although the highest level of growth was observed with SAA fructans (**Fig. 2A**). The growth of *L. acidophillus* (OD_{630} = 0.77) was significantly greater ($P \leq 0.01$) than that recorded for *L. casei*, *L. paracasei*, and *L. rhamnosus* (OD_{630} = 0.173). In other words, the ability of *L. acidophillus* to ferment graminans and neo-series type highly branched fructans was 4.6 times greater than that of *L. casei*, *L. paracasei*, and *L. rhamnosus* on the SAA fructans.

On the other hand, the mixtures B and C showed a higher growth with *L. acidophillus*, *L. casei* and *L. paracasei* (**Figs. 2A**, **2B, 2C**) than commercial fructans, while *L. rhamnosus* (**Fig. 2D**) growth was significantly higher in the cultures supplemented with commercial fructans. The degree of polymerization in the mixtures had a highly significant effect ($P \leq 0.01$) on growth since the larger the amount of short-DP fructan content, the greater the growth of the microorganisms evaluated. When the degree of polymerization (DP) decreased in the mixture, optical density (OD_{630}) increased and pH decreased; also as DP increased, growth decreased. Biomass concentration for *L. casei*, *L. paracasei* and *L. rhamnosus* varied depending on the fructans DP. The observed behaviour of these micro-organisms in the tested culture media is a result of each species metabolism, which is not yet well established for some of them. The growth of lactobacilli on the agave fructans indicates that they probably ferment the fructans by producing β-fructosidases; more specifically, *L. acidophillus* probably extracellularly hydrolyzes, long-chain and highly branch fructans releasing (FOS) and fructose which enter the cell to promote fermentation. According to Kaplan and Hutkins (2000) this species does not require an induction period to use fructans. In contrast, the other species did not use fructans as effectively to increase biomass, suggesting that fructans metabolism and transport in *L. casei* and *L. paracasei* must be induced and requires an ATP-dependent transport system to ingest short-chain fructan; that is, their ability to transport fructans depends on the availability of intracellular ATP (Kaplan and Hutkins 2003). This indicates that an extracellular enzyme is needed for the degradation of long-DP and highly branched agave fructans, which then allows the accumulation of short-DP fructan which can be hydrolyzed within the cell.

pH drop

The pH drop during fermentation with lactobacilli due to SCFA production is shown in (**Table 3**). Bacterial growth and pH drop presented a 0.95 correlation. The largest pH

Table 3 pH drop due to fermentation of fructans by *Lactobacillus acidophilus*, *L. casei*, *L. paracasei*, and *L. rhamnosus.*

Fructan	*L. acidophilus*	*L. casei*	*L. paracasei*	*L. rhamnosus*
LAA	2.16 c	0.68 d	0.64 c	0.73 c
A	2.31 c	0.74 bc	0.74 c	0.73 c
B	2.53 b	0.76 bc	0.86 b	0.84 bc
C	2.69 ab	1.01 b	0.98 ba	0.94 ba
SAA	2.79 a	1.17 a	1.06 a	1.09 a
RAF1	1.68 d	0.47 e	0.43 d	0.81 bc
RAF2	1.80 d	0.84 b	0.73 c	0.81 bc

The drop in pH induced by fructans fermentation is expressed as pH (means of 3 independent determinations) at the end of the substrate fermentation minus the pH at the beginning of the fermentation.

Table 4 Short chain fatty (mM) liberated by lactobacilli during fermentation on *Agave angustifolia* and commercial fructans. LA, *Lactobacillus acidophilus*; LC, *L. casei*; LPC, *L. paracasei*; LR, *L. rhamnosus*.

Fructan	ACID	LA	LC	LPC	LR
LAA	Acetic	32.12 c	110.94 b	45.72 b	64.60 a
	Lactic				
	Propionic				
A	Acetic	33.86 c	95.55 cb	54.23 b	54.84 b
	Lactic				
	Propionic				
B	Acetic	36.49 c	93.67 cb	70.41 a	53.78 b
	Lactic				
	Propionic				
C	Acetic	83.81 a	163.76 a	62.57 a	57.58 a
	Lactic	7.50			35.80
	Propionic				
SAA	Acetic	58.78 b	76.22 cd	37.39 c	54.84 b
	Lactic				
	Propionic				
RAF1	Acetic	38.90 cb	148.19 a	67.37 a	52.92 b
	Lactic				
	Propionic				
RAF2	Acetic	42.98 cb	69.86 d	36.39 c	52.92 b

Different letters indicate significant differences ($P \leq 0.05$) compared with the commercial fructans of each strains, and SD; n=3

drop with LAA was *L. acidophillus* with *L. rhamnosus*, *L. casei* and *L. paracasei* it was less. pH drop was significantly higher on SAA fructans in all species compared to the controls. At the beginning of the fermentation period of *L. acidophillus* with SAA fructans the pH was 7.0 but decreased to 4.21 after 20 h. In a culture that contained mixture C the pH drop was significantly larger ($P \leq 0.05$) than in control cultures. Among the mixtures evaluated, the mixture C showed the largest drop after SAA, while mixtures A and B had a lower drop and that was related to the amount of SAA present in the mixture. Greater bacterial growth, therefore, was directly related to pH decrease which can have a beneficial effect by inhibiting the growth of pathogenic bacteria (Gibson and Wang 1994). López and Urías-Silvas (2007) reported similar results on pH drop with *Dasylirion* sp. fructans as the energy source. The SCFA values for the lactobacilli cultures showed acetic acid to be the main metabolic product of the fermentations. Differences between acetic acid concentration on LAA and SAA fructans were statistically significant (**Table 4**). Little acetic acid was accumulated with *L. acidophillus* while significantly higher concentrations ($P \leq 0.05$) were produced by *L. casei.* Acid production was much higher in cultures containing mixtures of different degrees of polymerization concomitant with the pH reduction. Short-chain agave fructans were a better carbon source for the production of metabolites, similar to results reported by Perrin *et al.* (2002). In particular, acetic acids were the major products of fructans fermentation. No production of lactic acid was observed during fermentation with controls, whereas the mixture C led to a slight accumulation of this acid with *L. acidophillus* and *L. rhamnosus.* Propionic and butyric acids were absent in all treatments.

CONCLUSIONS

From the results obtained in our work, it can be concluded that utilization of agave fructan by bifidobacteria and lactobacilli depends on the degree of polymerization of the fructooligomeric chains, chemical structure and linear or branched structure. Our *in vitro* experiment showed that the majority of *Bifidobacterium* strains studied utilized SAA. *B. animalis*, *B. breve* and *L. acidophillus* showed the highest growth with agave fructans and fructans type inulin. *B. animalis* showed the greatest hydrolysis capability and also fermented the longest chains of agave fructan but did not exhibit stringent selectivity based on DP. The response of the other strains varied depending on the fructan characteristics. The difference in behaviour of agave fructans as compared with RAF1 and RAF2 could be attributed to the linkage type, DP and the highly branched structural features of these fructans. These consist of a complex mixture of fructans containing principally β(2-1) linkages, but also some β(2-6) with terminal o internal glucose denominated graminans and agavins respectively. This assumes similarity with those structures previously reported for *A. tequilana*. The results found in this research, confirmed the bifidogenic effect of *A. angustifolia* fructans. Biomass and metabolite production by the bifidobacteria and lactobacilli tested in this work corroborate the preference to ferment short-DP fructans over long-DP fructans. Long-chain fructans are fermented more slowly. In conclusion, the presence of short-DP fructans in the mixtures highly influenced the rate of fermentation by the probiotic bacteria.

Acetic acid was the main fermentation product in all the tested energy sources and there were significant differences in its production. Agave fructans and mixtures offer possible prebiotic potential, thus opening new alternatives as food ingredients.

The prebiotic concept with its overall of its implications for health is generating a great amount of interest in the search for new sources of fructans to be used in a wide range of food products. Fructans from *Agave angustifolia* could be one of them.

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