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EVALUATION OF PREBIOTIC POTENTIAL OF AGAVE FRUCTANS FROM DIFFERENT REGIONS OF COLIMA AND ZACATECAS, MEXICO

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ABSTRACT: ***Prebiotic effect of randomly selected fructan samples from Agave tequilana Weber var. azul plants from the states of Colima and Zacatecas, Mexico, were evaluated in vitro and compared with commercial chicory fructans in order to explore new alternatives for the use of this agave plants from Mexico not useful for the elaboration of the alcoholic beverage Tequila. A logistic model was used to obtain an estimated specific growth rate (μ_{max}) from turbidimetric data of Bacillus subtilis and Lactobacillus delbrueckii sbp bulgaricus, all data was statistically analyzed by one-way ANOVA. Raw agave fructans from the state of Zacatecas exhibit growth rates for L. delbrueckii higher than chicory fructans. Hence these fructans were purified by ion exchange permeation and filtered by tangential flow with membranes of 3 and 1 kDa, obtaining three samples: A, B and C with a degree of polymerization (DP) >10, <10 without glucose nor fructose, <10 and >10 with glucose and fructose. These purified samples were also evaluated in vitro, with B. subtilis and L. delbrueckii subp. bulgaricus. Results showed similar prebiotic effect of sample C, 0.138±0.01 h^{-1}, 0.475±0.06 h^{-1} for B. subtilis and L. delbrueckii respectively, as with commercial chicory sample (Synergy1™) 0.107±0.01 h^{-1}, 0.404±0.06 h^{-1} for the same strains.***

KEY WORDS: Agave, *Bacillus,* Fructans, *Lactobacillus*, Prebiotic, Probiotic

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INTRODUCTION

Mexico is the unique producer of the spirituous beverage called tequila. This tequila is produced in selective regions of Mexico and protected by a Mexican Official Norm, NOM-006-SCFI-1994, that include the states of Jalisco, Guanajuato, Michoacán, Nayarit and Tamaulipas. Despite of it, Agave (*Agave tequilana* Weber var. *azul*) crops have extended to other states like Sinaloa, Colima, Zacatecas and Queretaro. Thus, there is an overproduction of unprotected crops of *Agave tequilana* Weber var. *azul* in Mexico, which upturn the interest in research about different uses for this plant, such as soluble fiber, sweetener and food supplement ingredient due of its high fructan concentration (Gomez *et al.*, 2010). Fructans are polysaccharides with b (2-1) and b (2-6) bounds and branch moieties whose main functions are energy storage before flowering and osmoprotection during drought (Wang and Nobel, 1998).

Some reports show that agave fructans have potential prebiotic effect (Gomez *et al.*, 2010, Márquez-Aguirre *et al.,* 2013) and fructans from *A. tequilana* have potential in dietary products and in health prophylactic treatment against steatosis, glycaemia and enteric infections, also technological properties as a fat substitute or texture improver (Urías-Silvas *et al.*, 2008).

Prebiotics are non-digestible food ingredients that beneficially affect the digestive process of the host by selectively stimulating the growth or metabolism, or both, of colonic microbiota (Gibson, 2004). Now a days the more consumed inulin-type- fructans (ITF) as prebiotic worldwide are chicory´s, this could have a degree of polymerization (DP) >10, long chain inulin, fructooligosaccharides (FOS, DP<10)

or the mixture of both, which all present a linear chain of fructose with β (2-1) linkages with a terminal glucose unit. Like agave fructans, are non-digestible in the upper gastrointestinal tract, reaches the caeco-colon intact where it is used by select groups of beneficial bacteria. These bacteria are part of a healthier microbiota composition, obtained when population of Firmicutes, Lactobacilli and Bacilli increase in the gut and their metabolism can positively affect the host, biochemically, physiologically or immunologically, minimizing the risk of many diseases (Urías-Silvas *et al.*, 2008; Angelakis and Raoult, 2010).

Bacillus species have been used as probiotics for at least 55 years in commercial products like Enterogermina®, but only in the last 17 years there is scientific interest on this bacteria species as probiotics. The most extensively examined are: *Bacillus subtilis, Bacillus clause, Bacillus cereus, Bacillus coagulans* and *Bacillus licheniformis*, which have advantages over non-spore forming bacteria such as *Lactobacillus* spp. because the spore is capable of surviving the low pH of the gastric barrier (Cutting, 2011). Besides, *Lactobacillus delbrueckii* sbp *bulgaricus* have shown that improve host digestion and enhance host immune response (Guglielmotti *et al.*, 2007). Owing to it, in this work we used one strain of bacillus and lactobacillus with the aim to evaluate *in vitro* the effect of *A. tequilana Weber* var. *azul* fructans over them.

Also based on the reports (Mancilla-Margalli and López, 2006; Arrizon *et al.*, 2010) that stablished that agave species have differences in the polymerized glucose proportion of the main chain. Furthermore cultivation methods, soil nutrients, plant variety, seasonal changes, water regime, harvest time and agave age have influence over fructan structure; likewise same agave specie grown in different regions may present different structure (Mancilla-Margalli and López, 2006).

As there are only one previous study about the relationship between the origin of the agave fructans from the same plant specie and their capability to estimulate the growth of bacteria consider as probiotic (Márquez-Aguirre *et al.,* 2013), we made this study with the purpose to evalute the influence of the chain length, polymerization degree (DP), of the agave fructans due to where it was cultivated and the relationship with their prebiotic potential *in vitro*, and to compare with commercial chicory fructans, now a days the most use polysaccharide as prebiotic world wide.

MATERIALS AND METHODS

Agave tequila Weber var. *azul* fructans obtainment and purification processes.

Full agave heads or "piñas" were randomly obtained from *A. tequilana* Weber var. *azul* plants harvested in different municipalities from the states of: Colima and Zacatecas. The age of the plants collected were 6 years old, according to the information provided by the producers. Agave juice was obtained as reported before (Arrizon *et al.,* 2010; Márquez-Aguirre *et al.,* 2013). Briefly the agave heads were cut transversally into two halves, smashed and mixed with purified water at a ratio of 10:6 to extract the water-soluble carbohydrates (WSC). The mixture was blended in a mechanical device of stainless steel and then stirred at 70 °C for 7 h. The WSC suspension was filtered through an 80-100 mesh until a final concentration of 10-15 °Brix, then spray dried with an inlet temperature of 90 °C and an outlet temperature of 170-190 °C, to obtain a white-cream powder with a relative humidity of 4.1 %w/w. The powder obtained was labeled as raw samples (RS) and with the first three letters of the corresponding state where it was obtained and a number that correspond to the spray dried batch (*i.e.* Zac L1, correspond to Zacatecas batch 1). All raw samples, were stored in a cool dry place until further determinations, randomly 9 where selected for this study. Besides three commercial chicory fructans, selected by its degree of polymerization DP≥10 (long chain), DP<10 (oligosaccharides) and a mixture of inulin and oligofructose (long chain and oligosaccharides) were also obtained: OraftiGR™ (Orafti® GR™, Tienen Belgium, inulin with DP≥10), OraftiP95™ (Orafti®BeneoP95™, Tienen Belgium, oligosaccharides with DP 2-9) and Synergy1™ (Orafti®BeneoSynergy1™, Tienen Belgium, oligosaccharides with DP 2-9 and inulin with DP≥10). Raw samples were passed thru an ionic exchange column, giving a product with no color or minerals, labeled as Sample C. These C sample were also processed by a tangential flow filtration (TFF) and passed thru a 3 kDa molecular weight cut off membrane (MWCO), which separates fructans with a degree of polymerization >10 (Sample A), from fructans with DP<10, fructooligosaccharides (FOS) rich in glucose and fructose. The fraction rich in FOS were then passed thru a 1 kDa MWCO to obtained a retentate of FOS without any monosaccharide (Sample B), and a permeate full of glucose and fructose not used for any evaluation, samples used are describe in Table 1.

Microorganisms

Bacillus subtilis (ATCC 6633 Manassas, USA) and *Lactobacillus delbrueckii* sbp *bulgaricus* (NRRL-734 from USDA ARS Culture Collection) were separately cultivated in MRS medium (DIBICO, Mexico) for 24 h at 37 °C and kept at 4 °C as stock.

Prebiotic assay

Aliquots of 5 mL of each stock of bacteria was inoculated into 50 mL of MRS medium separately and incubated at 37 °C for 24 h. A modified MRS medium was prepared for each RS and purified sample, substituting dextrose from the original formula of MRS medium with agave samples (raw or purified) or with commercial chicory samples in a concentration of 20 g L^{-1}; thus having 9 batches of modified MRS medium of RS, 3 batches of commercial chicory samples and one control with sucrose, aside 3 batches of the modified MRS medium with the purified agave samples (A, B and C), 3 of commercial chicory samples (GR™, Synergy1™ and OraftiP95™) and a control, sucrose. Then 200 µL of each

MRS-modified medium and the control with sucrose were transferred to a 96-well micro-plate in quadruplicate, then inoculated by triplicate, leaving one blank per assessed sample. Inocula used were 9 X10^7 CFU mL^{-1} of *B. subtilis* and 1.2 X10^6 CFU mL^{-1} of *L. delbrueckii*. Optical densities (OD) were measured every hour up to 15 h using a micro-plate reader (X-Mark Microplate Absorbance Spectrophotometer, Bio-rad California, USA) at 590 nm, kept at 37 °C and shaken in the same micro-plate reader before every measurement for 10 s at high speed. Turbidimetry experimental data were adjusted to a Logistic function (Equation 1), using OriginPro 7.0 (OriginLab Corporation Northampton, MA, USA) in order to obtain an estimated maximum specific growth rate (μ_{max}), based on reports elsewhere (Harris and Kell, 1985; Dalgaard *et al.*, 1994; Begot *et al.*, 1996; Mytilinaios *et al.*, 2012):

$$Y(t) = \frac{a}{1+e^{-kx}}$$

Where y is the absorbance at time t (optical density, O.D. in arbitrary units, A.U.), a is the initial absorbance value ($O.D._0$), k is the growth rate (m in h^{-1}), and x is the time (in h).

Statistic analyses

Normal distribution of data was confirmed by Shapiro-Wilk test. To evaluate the prebiotic effect of agave fructans a comparasion of optical density of each treatment at 15 hours was realized, means were compared using one-way ANOVA followed by Tuckey test post-hoc. The maximum specific growth rate means ($\mu_{max;}$ as described previously) were compared between samples using one-way ANOVA followed by Tuckey test post-hoc. p values <0.05 were considered statistically significant. For this analysis were used OriginPro 7.0 (OriginLab Corporation Northampton, MA, USA).

TABLE 1. Purified agave samples description. DP= Degree of polymerization

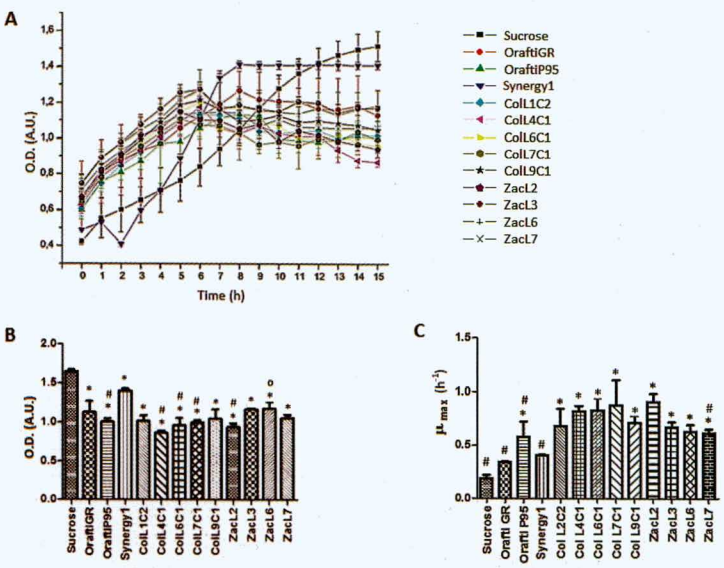
Sample	DP	Agave age (years)	Glucose/ Fructose
Sample A	>10	6	None
Sample B	<10	6	None
Sample C	<10, ≥10	6	Yes
OraftiGR™	>10	-	Yes
OraftiP95™	<10	-	Yes
Synergy™	<10, ≥10	-	Yes

RESULTS

Assay of prebiotic activity of agave fructans and chicory.

Nine randomly selected raw samples from Zacatecas and Colima states stimulated the growth of both probiotic strains, *B. subtilis* and *L. delbrueckii*, Figure 1 and 2, shows demeanor of *L. delbrueckii* and *B. subtilis* respectively after 15 h of incubation; the prebiotic maximum effect for *L. delbrueckii* is given by Sucrose (O.D. = 1.65 ± 0.023 A.U., figure 1B), this result was statistically significant compared with the some of the samples (Table 2). From samples of raw agave fructans (Fig 1A), ZacL6 showed the maximum prebiotic activity (O.D. = 1.18 ± 0.086 A.U.) and when it was compared vs. the references samples (commercial chicory fructans) its activity is significantly higher compared to OraftiGR™ (O.D. = 1.143 ± 0.143 A.U.) and OraftiP95™ (O.D. = 1.0 ± 0.044 A.U.), there were not statistically significant differences compared with Synergy1™ (O.D. = 1.41 ± 0.021 A.U.). However, when the maximum specific growth rate was evaluated (μ_{max}, Figure 1C) ZacL6 presented a μ_{max} (0.635 ± 0.057 h^{-1}) higher than Synergy1™ (0.404 ± 0.008 h^{-1}) statistically significant $p<0.05$, results shows that *L. delbrueckii* in presence of Sucrose presented the smallest μ_{max} (0.191 ± 0.033 h^{-1}) compared with all samples evaluated herein. From all batches of agave raw fructans, ZacL2 presented the maximum μ_{max} (0.905 ± 0.289 h^{-1}) and when it was compared with the rest of the samples, only significant differences were found with that sample, sucrose.

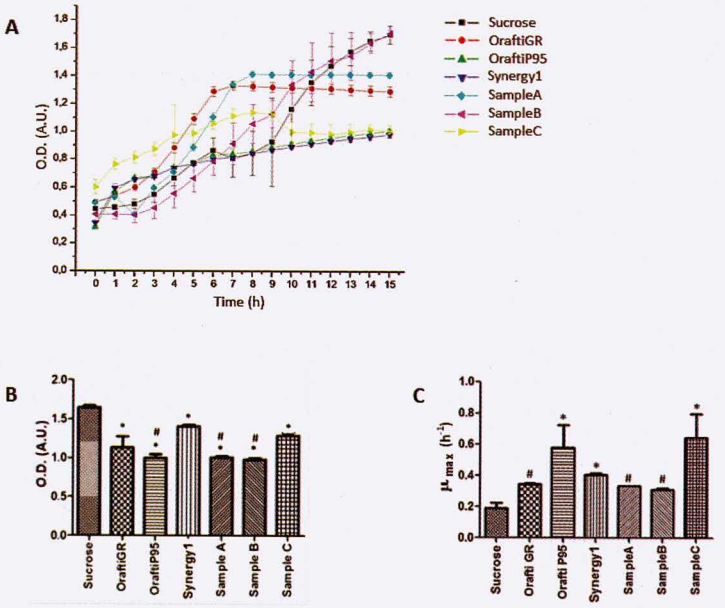
FIGURE 1. 1.2X10^6 CFU mL^{-1} of *L. delbrueckii* inoculated in modified MRS medium (without dextrose) with raw samples of agave fructans at concentration of 20 g L^{-1} from Colima and Zacatecas States. Commercial chicory samples OraftiGR™, Synergy1™ and OraftiP95™ were used as reference samples and sucrose were used as positive control. A) Growth kinetics, results obtained was expressed as optical density (O.D.); each curve corresponds to two independent experiments by triplicate, each bar corresponds to mean ± SD. B) Prebiotic effect evaluated at 15 h, each bar corresponds to two independent experiments by triplicate, mean ± SD. * $p<0.05$ vs. Sucrose, # $p<0.05$ vs. ZacL6, ° $p<0.05$ vs. OraftiGR™ and Synergy1™ C) Maximum specific growth rate (μ_{max}) evaluated at 15 h, each bar corresponds to two independent experiments by triplicate, mean ± SD. * $p<0.05$ vs. Sucrose, # $p < 0.05$ vs. ZacL2.



The prebiotic effect on *L. delbrueckii* evaluated with purified samples of agave fructans, Figure 2, shows that with all samples the biomass related with the turbidity of the sample, was lower than Sucrose (O.D. = 1.648 ±

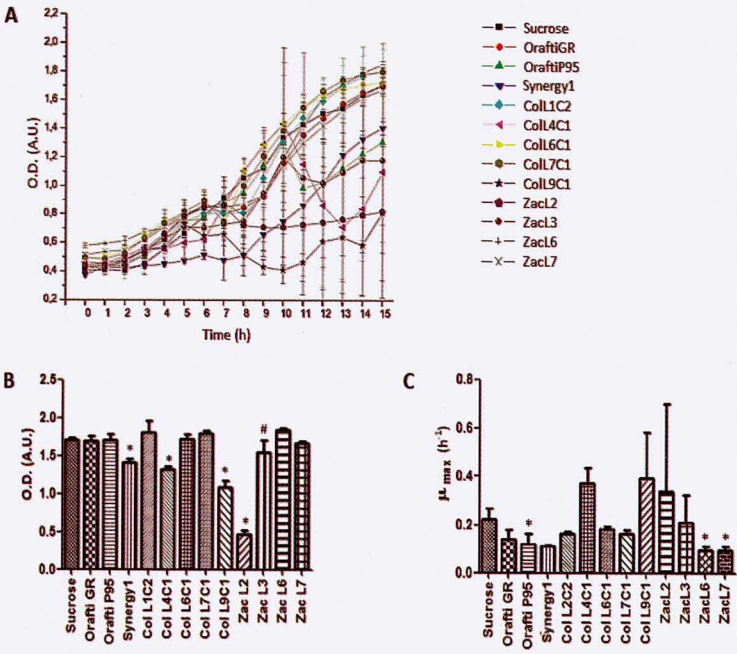
0.023 A.U.), however when sample C (O.D. = 1.29 ± 0.037 A.U.) was compared with the reference sample, OraftiGR™ (O.D.=1.13±0.143 A.U.) and OraftiP95™ (O.D.=1.00±0.044 A.U.) showed no significant differences (figure 2B), but with Synergy1™ (1.41 ± 0.022 A.U.) there were differences being the chicory fructan almost similar to sucrose. When compare this sample C (O.D. = 1.29 ± 0.037 A.U.) with the rest of the purified samples A and B (O.D. = 1.007 ± 0.015 and 0.980 ± 0.02 A.U. respectively) there were significant differences statistically, but between sample A and B there were not significant differences. When the maximum specific growth rate (μ_{max}) was analyzed from *L. delbrueckii* (figure 2C) with purified samples was observed that the highest μ_{max} is given by Sample C (μ_{max} = 0.475 ± 0.066 h^{-1}). Samples A (μ_{max} = 0.332 ± 0.055 h^{-1}) and B (μ_{max} = 0.308 ± 0.054 h^{-1}), OraftiGR™ (μ_{max} = 0.343 ± 0.042 h^{-1}) and Sucrose (μ_{max} = 0.184 ± 0.017 h^{-1}) reached a significantly lower μ_{max} compared to Sample C (μ_{max} =0.644±0.155 h^{-1}), besides there were no differences between Sample C vs OraftiP95™ and Synergy1™.

FIGURE 2. 1.2x10^6 CFU mL^{-1} of *L. delbrueckii* was inoculated in modified MRS medium (without dextrose) with purified agave samples (A, B, C) at concentration of 20 g L^{-1}. Commercial chicory samples OraftiGR™, Synergy1™ and OraftiP95™ were used as reference samples and sucrose were used as positive control. A) Growth kinetics, results obtained expressed as optical density (O.D.); each curve corresponds to two independent experiments by triplicate, each bar corresponds to mean ± SD. B) Prebiotic effect evaluated at 15 hours, each bar corresponds to two independent experiments by triplicate, mean ± SD. * p<0.05 vs. Sucrose, # p<0.05 vs. Sample C. C) Maximum specific growth rate (μ_{max}) evaluated at 15 hours, each bar corresponds to two independent experiments by triplicate, mean ± SD. * p<0.05 vs. Sucrose, # p<0.05 vs. Sample C.



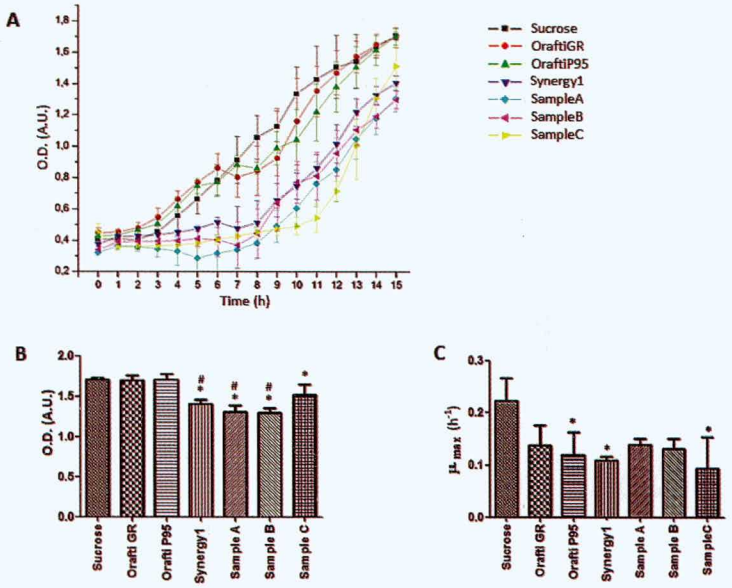
Prebiotic effect with *B. subtilis* using the same raw samples of agave fructans of 6 years old, Figure 3B, showed that five out of nine samples had the same prebiotic activity that Sucrose (no statistically significant differences), from these five raw samples the sample ZacL6 reached the highest prebiotic effect obtained by turbidimetric methods (O.D. = 1.851 ± 0.024 A.U.) no statistically significant differences respect to the positive control, Sucrose (O.D. = 1.708 ± 0.024 A.U.), however when evaluated the maximum specific growth rate (μ_{max}, figure 3C) no agave fructan sample was statistically significant respect to Sucrose; ColL9C1 sample presented the highest growth rate (μ_{max} = 0.390 ± 0.192 h^{-1}).

FIGURE 3. 9x10^7 CFU mL^{-1} of *B. subtilis* inoculated in modifie MRS medium (without dextrose) with raw samples of agave fructan at concentration of 20 g L^{-1} from Colima and Zacatecas State Commercial chicory samples OraftiGR™, Synergy1™ and OraftiP95 were used as reference samples and sucrose were used as positive contro A) Growth kinetics, results obtained was expressed as optical densit (O.D.); each curve corresponds to two independent experiments b triplicate, each bar corresponds to mean ± SD. B) Prebiotic effec evaluated at 15 h, each bar corresponds to two independent experiment by triplicate, mean ± SD. * p<0.05 vs. Sucrose, # p<0.05 vs. ZacL6 C) Maximum specific growth rate (μ_{max}) evaluate at 15 h, each ba corresponds to two independent experiments by triplicate, mean ± SD p>0.05 all treatments vs. Sucrose, * p<0.05 vs. Col L4C, Col L9C1.



Prebiotic activity of purified samples of agave fructans analyzed within *B. subtilis* (Figure 4), samples A, B and C (O.D. = 1.303 ± 0.083 A.U., 1.299 ± 0.056 A.U., 1.513 ± 0.133 A.U. respectively, figure 4B) were lower than the positive control, Sucrose (O.D.=1.708 ± 0.023 A.U.) with statistical significance, when compare these samples with the reference samples, only sample C presented the same activity that Orafti95™ (O.D. = 1.702 ± 0.053 A.U.) and OraftiGR™ (O.D. = 1.698 ± 0.063 A.U.) samples with no significant differences. And when the maximum specific growth rate (μ_{max}) was determined (Figure

FIGURE 4. 9x10^7 CFU mL^{-1} of *B. subtilis* inoculated in modified MRS medium (without dextrose) with different purified samples (A, B, C) of agave fructans (2 years old) at concentration of 20 g L^{-1}. Commercial chicory samples OraftiGR™, Synergy1™ and OraftiP95™ were used as reference samples and sucrose were used as positive control. A) Growth kinetics, results obtained was expressed as optical density (O.D.); each curve corresponds to two independent experiments by triplicate, each bar corresponds to mean ± SD. B) Prebiotic effect was evaluate at 15 h, each bar corresponds to two independent experiments by triplicate, mean ± SD. * p<0.05 vs. Sucrose, # p<0.05 vs. OraftiGR, OraftiP95. C) Maximum specific growth rate (μ_{max}) was evaluate at 15 h, each bar corresponds to two independent experiments by triplicate, mean ± SD. * p<0.05 vs. Sucrose.



4C) it was found that Sucrose (μ_{max} = 0.226 ± 0.019 h^{-1}) has the highest growth rate against all purified agave samples, but there were no significant differences statistically when compare to commercial chicory samples.

DISCUSSION

The prebiotic effect of the raw as well as the purified agave samples evaluated, showed acceptable growth rates for both strains used herein, based on the obtained results here as well with commercial chicory fructans and with the positive control, sucrose (Table 2).

In the case of the strain of *B. subtilis*, it follows the oxidative branch of the pentose phosphate pathway, which is thought to supply pentose precursors for nucleoside biosynthesis (Bai *et al.*, 2004) when carbon source is in abundance. These precursors allow the generation of essential metabolites for the synthesis of biomass components (Rowley and Wolf, 1991). The synthesis of 6-phosphogluconate from glucose-6-phosphate is a precursor to riboflavin biosynthesis (Dauner *et al.*, 2002; Zamboni *et al.*, 2004), hence all samples used herein could be used as efficient carbon source.

On the other hand, when *L. delbrueckii* was cultivated on different oligosaccharides, production of lactic acid and ethanol may vary, due to the fermenting capacity of this *Lactobacillus* strain, especially in presence of fructooligosaccharides (FOS) because ordinarily these strains are non-fermenting FOS (Ignatova *et al.*, 2009), when raw agave samples where used as carbon source an increase in the biomass of *L.delbreuckii* was observed, this might be due to the presence of extra micronutrients like Iron and Zinc from the cultivated soil of the agave plant as reported else where (Márquez-Aguirre *et*

TABLE 2. Estimated μ_{max} from optical densities of evaluated samples by a logistic model.

	L. delbrueckii			*B. subtilis*		
Sample	**O.D.**	**μ_{max} est.**	**R^2**	**O.D.**	**μ_{max} est.**	**R^2**
Sucrose	1.65 ± 0.023	0.184 ± 0.017	0.9904	1.708 ± 0.024	0.226 ± 0.019	0.9906
OraftiGR™	1.13 ± 0.143	0.343 ± 0.042	0.9678	1.698 ± 0.063	0.126 ± 0.026	0.9754
OraftiP95™	1.00 ± 0.044	0.573 ± 0.139	0.8568	1.702 ± 0.053	0.116 ± 0.016	0.9907
Synergy1™	1.41 ± 0.022	0.404 ± 0.066	0.9399	1.408 ± 0.047	0.107 ± 0.007	0.9567
ColL1C2	1.016 ± 0.072	0.684 ± 0.141	0.8915	1.804 ± 0.154	0.154 ± 0.029	0.9724
ColL4C1	0.866 ± 0.024	0.822 ± 0.347	0.6652	1.102 ± 0.377	0.012 ± 0.192	0.5705
ColL6C1	0.959 ± 0.093	0.833 ± 0.283	0.7538	1.726 ± 0.057	0.183 ± 0.041	0.9495
ColL7C1	0.996 ± 0.029	0.831 ± 0.197	0.8627	1.798 ± 0.033	0.164 ± 0.031	0.9678
ColL9C1	1.046 ± 0.125	0.714 ± 0.155	0.8807	0.809 ± 0.471	0.390 ± 0.192	0.6701
ZacL2	0.941 ± 0.046	0.905 ± 0.289	0.7781	0.819 ± 0.599	0.462 ± 0.149	0.7742
ZacL3	1.169 ± 0.014	0.678 ± 0.130	0.9046	1.180 ± 0.648	0.180 ± 0.045	0.9334
ZacL6	1.182 ± 0.086	0.712 ± 0.203	0.8249	1.851 ± 0.024	0.092 ± 0.006	0.9614
ZacL7	1.059 ± 0.042	0.812 ± 0.261	0.7908	1.672 ± 0.024	0.085 ± 0.009	0.9727
A	1.007 ± 0.015	0.333 ± 0.055	0.9414	1.303 ± 0.083	0.133 ± 0.012	0.9284
B	0.980 ± 0.02	0.308 ± 0.054	0.9386	1.299 ± 0.056	0.112 ± 0.008	0.9415
C	1.29 ± 0.037	0.475 ± 0.066	0.9515	1.513 ± 0.133	0.138 ± 0.019	0.8305

al.,2013), hence for this agave fructans their prebiotic effect is acceptable without any purification process. This could be confirmed with the results obtained when purified samples were used, where minerals were removed thru the ionic exchange column. However, both strains *B. subtilis* and *L. delbrueckii* have similar carbohydrate approach in relation with its primary metabolism; accordingly agave fructans could be used as effective carbon source for these probiotic strains. Furthermore, Zacatecas and Colima states are not protected by the Mexican legislation (NOM0006SCFI-1994) for Tequila production, therefore this report might give an option for the use of *A. tequilana Weber* var. *azul* cultivated in this states as an effective prebiotic.

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