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Isolation and characterization of epiphytic, fructanolytic, homofermentative lactic acid bacteria from *Agave salmiana*

Aislamiento y caracterización de bacterias ácido lácticas epífitas, fructanolíticas y homofermentativas de *Agave salmiana*

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Abstract
Lactic acid is a chemical compound that plays important functions in various biochemical processes and has different applications in industries such as food, cosmetics, among others. Therefore, there is a constant search for this compound which can be obtained by lactic fermentation. Here a collection of *Agave* epiphytic lactic acid bacteria is presented with the purpose to use them for direct homo lactic fermentation of fructans, as an alternative process for silage and industrial fermentations, based on *Agave* plants prevalent in semi desertic lands, instead of the conventional starch crops of temperate weather. This way, abundant *Agave* residues can be used to support cattle and lactic acid production. A set of 260 isolates were screened in Petri dishes and test tubes for fructan homolactic fermentation. Best strains were able to transform more than 70% of a 20 g/L fructan solution in lactic acid solution with little or negligible fermentation by-products. Analysis of 16S DNA segments allowed the identification of most productive strains belonging to genus *Enterococcus*, *LacticaseiBacillus* and *Bacillus*. Direct lactic acid fermentation of fructans, extracted from *Agave* biomass, seems to be an interesting alternative to decrease upstream expenses for industrial lactic acid production as compared to conventional glucose fermentation.
Keywords: Epiphytic bacteria, *Agave salmiana*, *Agave* fructans, lactic acid.

Resumen
El ácido láctico es un compuesto químico que cumple funciones importantes en diversos procesos bioquímicos y tiene diferentes aplicaciones en industrias como la alimentaria, cosmética, entre otras. Por ello, existe una búsqueda constante de este compuesto que pueda obtenerse mediante fermentación láctica. Aquí se presenta una colección de bacterias ácido lácticas epífitas de *Agave* con el propósito de utilizarlas para la fermentación homoláctica directa de fructanos, como proceso alternativo para ensilaje y fermentaciones industriales, a base de plantas de *Agave* prevalentes en tierras semidesérticas, en lugar del almidón convencional cultivado en climas templados. De esta manera, se pueden utilizar abundantes residuos de *Agave* para apoyar la producción de ácido láctico y el ganado. Se analizó un conjunto de 260 cepas en placas de Petri y tubos de ensayo para determinar la fermentación homoláctica de fructanos. Las mejores cepas fueron capaces de transformar más del 70% de una solución de fructano de 20 g/L en una solución de ácido láctico con pocos o insignificantes subproductos de fermentación. El análisis de segmentos de ADN 16S permitió la identificación de las cepas más productivas pertenecientes a los géneros *Enterococcus*, *LacticaseiBacillus* y *Bacillus*. La fermentación directa con ácido láctico de fructanos, extraídos de la biomasa de *Agave*, parece ser una alternativa interesante para disminuir los gastos iniciales de la producción industrial de ácido láctico en comparación con la fermentación convencional de glucosa.
Palabras clave: Bacterias epífitas, *Agave salmiana*, fructanos de *Agave*, ácido láctico.

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1 Introduction

Agavaceae is a family of plants abundant in semi-desertic areas of Central and Northern Mexico and the Southwestern United States These plants have evolved for more than ten million years, adapting to low levels of humidity and a broad temperature range and to grow in a wide variety of environments, distributed throughout the Mexican territory (García-Mendoza *et al*., 2004; Good-Avila *et al*., 2006). They store metabolic energy in branched fructose polymers (fructans) that comprise up to 80% of *Agave* dry mass. Such polymers are known as *Agave* fructans and are fructosyl moieties grafted to sucrose initiators, with β (2 $\rightarrow$ 1) and/or β (2 $\rightarrow$ 6) linkages that may contain terminal or intermediate glucose. *Agave* fructans are produced and blended as prebiotics in a variety of health foods, and can be used as food stabilizers and sweeteners, among other functionalities (Carranza *et al*., 2015; Aldrete-Herrera *et al*., 2019; Velázquez-De Lucio *et al*., 2022). According to Davani-Davari *et al* (2019), prebiotics are a group of nutrients that are degraded by the human intestinal microbiota and are used to improve human health. In the case of *Agave* fructans, the prebiotic effect is related to its high polymerization and the inhibition of pathogenic intestinal bacteria (García-Gamboa *et al*., 2020).

According to "Consejo Regulador del Tequila" (Gschaedler *et al*., 2017) in 2015, nearly two and a half million tons of *Agave* stems were used for tequila production, representing 45% of whole biomass of *Agave tequilana* (Iñiguez-Covarrubias *et al*., 2001) leaving out two million tons of residual leaves in the field with an estimate of 320,000 tons of fructans to recover. It has been shown that minced *Agave* leaves can be ensiled and used as a roughage for lambs (Romero-López *et al*., 2015; Pinos-Rodríguez *et al* 2006; Pinos-Rodríguez *et al*., 2008; Álvarez-Fuentes *et al*., 2015), supporting the widespread existence of epiphytic lactic acid-bacteria (LAB) on *Agave*s, able to transform fructans in lactic acid. Metagenomic analysis of fermented *Agave* sap from *Agave salmiana*, rich in glucose, fructose, and sucrose, was found to be a mixture of *Saccharomyces cerevisiae* and LAB such as *Lactobacillus spp*., and *Leuconostoc mesenteroides* subsp. *mesenteroides* together with *Zymomonas mobilis* (Escalante *et al*., 2004). Such earlier work has been confirmed using a more comprehensive metagenomic analysis (Coleman-Derr *et al*., 2015) that found a wider variety of LAB, including *Enterococcus* spp.

Direct fermentation of fructans requires that LAB produce the hydrolases necessary to catalyze the breakdown of β (2 $\rightarrow$ 1) and/or β (2 $\rightarrow$ 6) bonds of *Agave* fructans (Mancilla-Margalli *et al*., 2006) This phenotype may be different from those found in milk and grain fermentation systems because fructans are structurally different to lactose or starch. Hence, the LAB isolated as epiphytic of *Agave* plants may be different from the epiphytic LAB of milk, grains or a variety of roughages, and such isolates (or the enzymes involved in fructans fermentation) could be used for future development of lactic industrial fermentations based on *Agave* extracts.

Therefore, the aim of this study was to isolate and characterize epiphytic, homofermentative LAB capable to grow on fructans from *Agave* sp. This work would improve our understanding of the fermentation process involved in *Agave* silage and could contribute to the search of new strains with valuable metabolic capabilities for application in the food and bioplastic industries; for example, the development of a lactic acid fermentation platform for the production of food additives and bioplastics (Vink *et al*., 2015) based on fructans from *Agave* crops instead of the conventional approach that uses corn or sugarcane carbohydrates.

2 Materials and methods

2.1 Materials

Glucose, lactic acid, acetic acid and polyethylene glycol were purchased from Sigma-Aldrich (St. Louis, MO., USA). *Agave* fructans, free from monosaccharides, came from NutriAgaves Group, S.A. de C.V. MRS agar, yeast extract, peptone, bacteriologic agar and meat extract were purchased from BD Bioxon (Mexico State, Mexico). Sodium acetate, polysorbate 80, ammonium citrate, magnesium sulfate, manganese sulfate, potassium phosphate, sodium chloride, bromocresol green and sulfuric acid were purchased from J.T. Baker TM (Phillipsburg, N.J., USA).

2.2 Microbial sampling

All samples were obtained from a small *Agave* plantation in the outskirts of Tocatlán, a town in Tlaxcala, Mexico (19°19'8.6" N, 98°11'59.4" O). A mature plant of *Agave salmiana*, grown to produce the sap in a traditional way, was used to collect samples from an artificial cavity ("cajete") in the plant stem. The sap samples were collected in triplicate and stored in sterile plastic tubes. Two pieces of *Agave* leaves were obtained, and each was cut into small pieces (with 2 cm x 2 cm of surface). *Agave* samples were suspended in 10 mL sterile saline solution (NaCl, 0.9 g/L) in sterile plastic tubes. Aliquots of residual stem scraps called *metzal* were also suspended in a sterile saline solution. All samples were stored on ice and transported to the laboratory in less than 8 h.

2.3 Preliminary bacterial screening

MRS agar plates were inoculated by crossed streaking (Madigan *et al*., 2010) from homogenized suspensions of the following samples: *Agave* sap (*aguamiel*), *Agave* scraps (*metzal*), and *Agave* leaves (after 15 min homogenization in Stomacher ® 400 circulator Seward). The agar plates were incubated at 37 °C for 24 h.

Fully grown separate colonies were randomly selected to be reinoculated in fresh agar plates with squared arrays. The culture medium used was MRS (Man-Rogosa-Sharpe) whose composition was: 1.0% peptone, 1.0% meat extract, 0.4% yeast extract, 2.0% glucose, 0.5% sodium acetate, 0.1% polysorbate 80, 0.2% potassium phosphate, 0.2% ammonium citrate, 0.02% magnesium sulfate, 0.005% manganese sulfate and 1.0% bacteriologic agar (de Man *et al*., 1960). Some plates were prepared with standard MRS agar (MG, glucose as carbon source), and others were prepared with industrial *Agave* fructans (MF instead of glucose) with an initial concentration of 20 g/L, free from sucrose, glucose or fructose as confirmed by the HPLC analysis (data not shown). Bromocresol green dye (0.04 % w/v) was added as pH indicator. The plates were incubated at 37 °C for 24 h. Growth patterns (positive or negative) were registered for each colony since it was observed that some colonies were positive on MG but negative in MF broth, as indicated in the results.

2.4 Fermentation conditions

Based on the plate screening experiments, 60 colonies were selected according to the size of the yellow halo associated to its acidity. A sample from each colony was inoculated in a 10 mL test tube that contained MF broth with 0.04% (w/v) bromocresol green, and a Durham bell, as an indicator of gas production. The latter is related to the heterofermentative process. All tubes were incubated for 12 h at 37 ºC. Following that time, color change from green to yellow as well as the presence of gas produced in the Durham bell were recorded for each tube.

The strains that showed color change from blue to yellow (indicating production of organic acids) and no gas production in the Durham bell were selected for Gram smears. One colony of each of the selected strains was inoculated in MF broth and incubated for 24 h at 37 °C. Aliquots were taken every 3 h and centrifuged at 3,500 rpm for 20 min. Following the pH measurement, the samples were stored at -20 ºC until quantitative analysis for carbohydrate, lactic acid and acetic acid were carried out as described below.

2.5 Lactic acid quantification

Lactic acid quantification was performed by HPLC (LabAlliance, State College, PA, USA) using an Aminex HPX87H column (1,300 x 7.8 mm; particle size 25μm; 4% crosslinking) (BIO-RAD; California, USA) at 50 °C, equipped with a diffraction index detector. Sulfuric acid solution (5 mM) was used as a mobile phase with a flow rate of 0.6 mL/min. Concentration of lactic acid was calculated using a standard curve plotted for different concentrations. A standard lactic acid curve of 0.1 to 1 mg/mL was used as a reference.

2.6 Acetic acid quantification

Acetic acid quantification was performed by gas chromatography (Agilent Technologies 7820A), equipped with a capillary column AT1000 (1.2 mm x 0.53 mm x 15 m), with a stationary phase of polyethylene glycol and a flame ionization detector. Samples of 20 μL were injected to the column with a nitrogen flow rate of 5.3 mL/min and temperature ramps as follows: A) 40 ºC during 20 min; B) Starting at 50 °C and a ramp of 2 °C/min; C) Starting at 200 °C and a ramp of 70 °C/min. Concentration of acetic acid was calculated using standard curves plotted for different concentrations solutions of 0.1 to 1 mg/mL.

2.7 Bacterial identification

According to the fermentation results, the strains with higher lactic acid production were selected for sequencing molecular marker 16S rRNA. For that purpose, strains were cultivated in MG broth at 37 °C for 12 h and following that time a 1 ml aliquot of each inoculate was withdrawn and centrifuged at 9000 rpm for 5 min. DNA was extracted from the cell pellet using the Pure Link Genomic DNA Mini Kit^{TM} (ThermoFisher, Mexico) adapted to Gram-positive bacteria (Wang *et al*., 2020). The 16S rRNA gene of the isolated bacteria was amplified using polymerase chain reaction (PCR) with the primer's sequences: 27 F (5'-GAGTTTGATCCTGGCTCAG-3´) and 1492 R (5'-GGTTACCTTGTTACGACT- 3´) (Chen *et al*., 2015; Frank *et al*., 2008). PCR analysis was performed using 30 ng of template DNA under the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. The resulting 16S rDNA PCR amplicons were purified with the Pure Link Quick PCR Purification KitTM (ThermoFisher, México) (Tenover *et al*., 2007). The nucleotide sequence was determined using Sanger chemical sequencing at Labsergen (Sanger *et al*., 1977) (LANGEBIO, CINVESTAV Irapuato, Mexico).

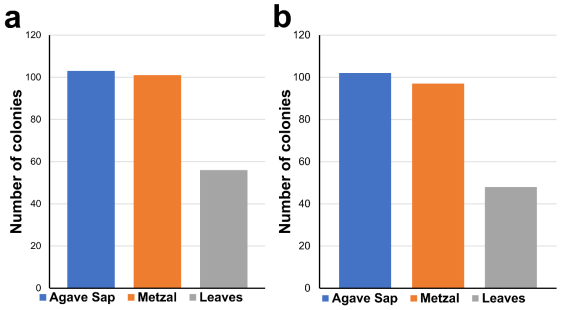

Fig. 1 Number of colonies that grew in MG agar (a) and MF agar (b) corresponding to bacteria isolated from agave sap, *metzal* and agave leaves.

The obtained nucleotide sequences were analyzed using Geneious software and identified sequences were deposited in the GenBank database with a given accession number. Comparison of the obtained sequences with the available data deposited at the NCBI nucleotide database and the Ribosome Database Project (Maidak, 1996) was done using BLASTn (Altschul *et al*., 1990).

3 Results

3.1 Bacterial isolation and phenotypic screening

To verify fermentation patterns and lactic acid production in the bacterial isolates, they were grown on different carbohydrate sources. Fig. 1 shows the growth of randomly selected colonies cultivated in MG and MF media. Overall, 95% of total 260 colonies did grow in MF agar showing the prevalence of fructanolytic bacteria.

Lactic acid production homofermentative (Durham negative) or heterofermentative (Durham positive) (Zúñiga *et al*., 1993) were screened using bacterial isolates from *Agave* sap, scraps (*metzal*) and leaf surfaces. Fig. 2a shows fermentation profiles obtained from 60 colonies grown on MF agar plates selected based on their most pronounced shifts in color of the pH indicator. Twenty-four out of 60 colonies (40%) followed the heterofermentative pathway (Durham +) with positive dye shifts. Of the remaining 36 homofermentative colonies (Durham -), only 13 produced a noticeable dye shift from green to yellow (pH < 4.5). The variation among these homofermentative colonies could be related to differences in the metabolic activities of bacteria (discussed below). A thorough analysis of the Gram smears of these 13 isolates revealed the presence of two yeast colonies that were discarded. Fig. 2b shows the final pH in MF fermentation media measured for each of the 11 strains (identified as DG1-DG11) whose metabolic activity was characteristic for a

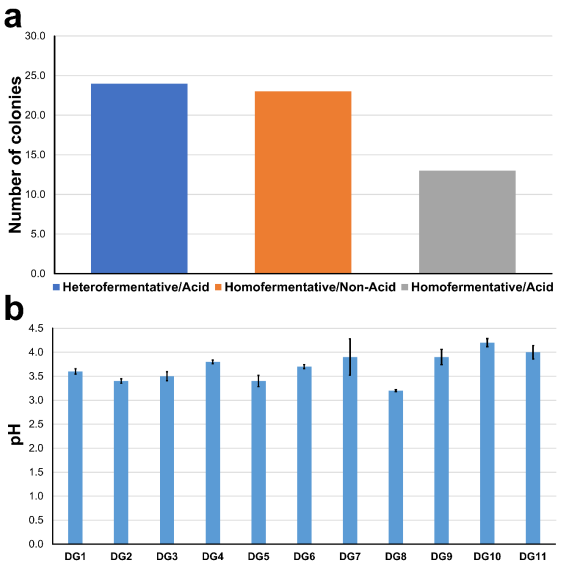

Fig. 2. (a) Number of colonies grouped according to their fermentation profile: homofermentative or heterofermentative; production of organic acids. (b) pH values measured after 24 h fermentation in MF medium for homofermentative/acid strains.

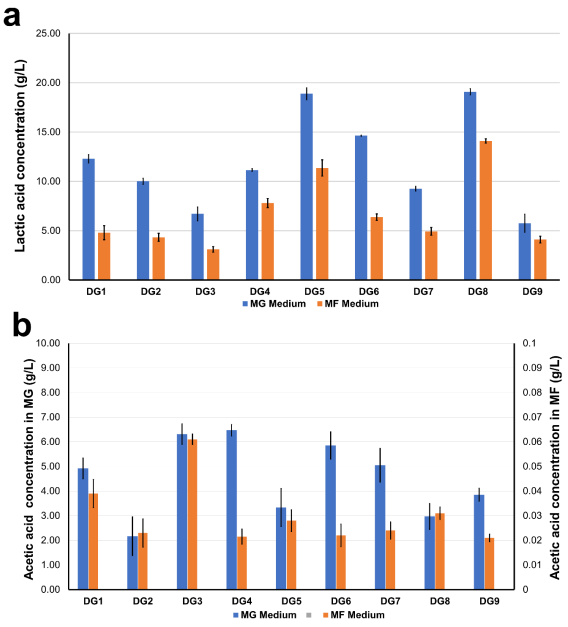

Fig. 3 Maximum lactic and acetic acid production from homofermentative fructanolytic bacteria isolated from *A. salmiana*. Initial carbon sources at 20 g/L. (a) Lactic acid in MF medium (orange) and in MG medium (blue). (b) Acetic acid in MF medium (orange) and in MG medium (blue).

homofermentative pathway. As can be seen, the pH values were in a range between 3.2 ± 0.12 and 4.3 ± 0.12.

Fig. 3 shows lactic and acetic acid production in nine homofermentative fructanolytic bacterial strains capable of lowering pH below 4.0 in MF medium. Fig. 3a shows the maximum lactic acid concentrations

Table 1. Identification of LAB strains isolated from *Agave salmiana*.

Strain code	Microorganism identified	Source	Sequence length (bp)	Similarity (%)	Accession number
DG1	*Enterococcus faecium* ASA.DG1	Agave sap	1494	99.73	OM967267
DG2	*Enterococcus faecium* ASA.DG2	Agave sap	1358	99.93	OM802845
DG3	*Enterococcus faecium* ASA.DG3	Agave sap	1464	94.94	OM967268
DG4	*Enterococcus faecium* ASA.DG4	Agave sap	1456	99.72	OM967269
DG5	*Lacticaseibacillus paracasei* ASA.DG5	Agave sap	1430	99.93	OM802846
DG6	*Enterococcus faecium* ASA.DG6	Agave sap	1440	99.31	OM967270
DG7	*Enterococcus faecium* ASA.DG7	Agave sap	1466	99.59	OM967271
DG8	*Enterococcus faecium* ASP.DG8	Agave leaf	1462	99.79	OM967272
DG9	*Bacillus subtilis* AGM.DG9	Agave metzal	1251	99.85	OM80284

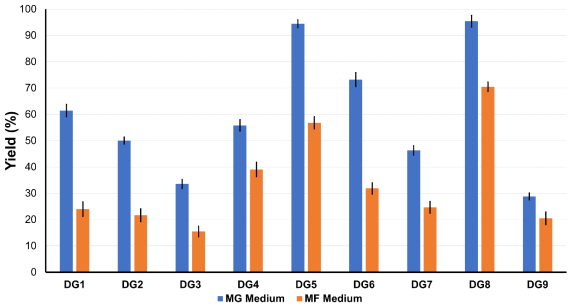

Fig. 4 Yield of lactic acid from homofermentative fructanolytic bacteria isolated from *A. salmiana* in MF medium (orange) and in MG medium (blue).

obtained in both media, i.e., MG and MF. The lactic acid yields were consistently higher for glucose than for fructans. Figure 4 shows the apparent product yield (lactic acid/substrate), as can be seen, varied between the different strains, from 36% (DG9) to 96% (DG8) in the MG medium and 15% (DG3) to 70% (DG8). It should be noted that both media contained significant amounts of peptones, yeast, and meat extracts (25 g/L) and such compounds can be used as partial carbon sources. Moreover, when comparing maximal lactic acid titers in the two media, they were lower in MF medium (Fig. 3a), with a maximum yield of 73% for DG8, compared to MG medium. Fig. 3b shows the production of acetic acid in MG and MF broths. It is worth noticing that acetic acid content in MG was lower than 1 g/L, while it reached nearly 6 g/L in MF. These results support major differences in the metabolic pathways involved in glucose and fructan fermentations.

3.2 Molecular identification of selected strains

All nine bacterial strains (DG1 to DG9) were selected since have the homolactic phenotypic and were Gram-positive rods. Bacterial isolates DG1-DG9 were identified based on the high level of similarity (98-100% identity) of their 16S rDNA sequences with known bacterial sequences from the GenBank database. 16S rDNA sequences from DG1-DG9 strains were deposited in GenBank (Table 1).

4 Discussion

Present results show that homolactic acid, Gram positive bacteria, are natural epiphytic inhabitants of *Agave salmiana* plants and most of them use fructans as a major carbon source which may be due to natural selection, related to the abundance of such carbohydrates in Agavacea. Most of the identified isolates belonged to genus *Enterococcus* and few to *Lacticaseibacillus* and *Bacillus*. Those observations are in contrast with the predominance of *Lactobacillus* spp. and *Pediococcus spp*. in timothy silage (*Phleum pratense* L.) (Pang *et al*., 2011) or the coexistence of *Weissella spp* and *Leuconostoc ssp* with *Lactobacillus spp*. in a variety of common roughages (Cai *et al*., 1998). Such differences may be associated with variations in plant composition from which they were isolated, as well as differences in their respective habitats (including potential environmental contamination), since both factors play a role in shaping the microbial community. There are also major differences between the epiphytic microbiota studied here and the microbiota found at the final step of traditional pulque fermentation (Escalante *et al*., 2004), where *Leuconostoc spp*. are quite prevalent, probably because the differences in the main carbon source in both processes, since pulque is made from "*aguamiel*", an *Agave* sap rich in fermentable sugars, and *Agave* silage is made from *Agave* leaves, where fructans are the predominant fermentable substrate.

In this work hetero-fermenters (Durham +) strains were excluded from further analysis due to the primary objective of identifying homo-fermenters for future lactic acid production based on *Agave* raw materials, and because previous studies have indicated the prevalence of lactic acid fermentation in minced and silage *Agave* leaves, suggesting the dominant

role of homo-fermenters in *Agave* silages (Romero-López *et al*., 2015; Pinos-Rodríguez *et al* 2006; Pinos-Rodríguez *et al*., 2008; Álvarez-Fuentes *et al*., 2015).

Fructan fermentation requires previous hydrolysis of such polymers into smaller sugars, such as fructose, to be metabolized by the bacterial cells. This process involves the presence of fructosyl glycosidases enzymes (Chi *et al*., 2009; Chi *et al*., 2011). In this context, the isolates presented here serve as valuable starting material for genomic analysis aimed at identifying and characterizing the specific genes encoding such enzymes and exploring their functional properties; also, it would allow the exploration of the relationship between the presence of specific fructosyl glycosidases, and the substrates present in *Agave* biomass. This knowledge will enable the optimization of strain screening for fructosyl glycosidases capable of acting on the variety of substrates found in *Agave*. Furthermore, the potential utilization of these enzymes can be explored by either employing the whole strain or by expressing their respective genes into organisms that have GRAS status to obtain recombinant enzymes, thereby enabling acid lactic production from *Agave* waste through various biotechnological approaches. Dien *et al*. (2001) reported the use of recombinant LAB enzymes in *Escherichia coli* to produce lactic acid from carbon sources other than glucose.

It is worth noting that fructans are water soluble polymers abundant in above ground *Agave* biomass (stems or leaves) and are obtained at industrial scale using continuous water extraction systems called diffusers, both in tequila and fructan factories (Ávila-Fernández *et al*., 2009). Therefore, such lixiviates can be pasteurized and fed directly to continuous lactic acid fermenters because, as shown in this work, it is possible to select LABs that transform fructans to lactic acid without previous acid hydrolysis. This is a simplification of the upstream processes of future lactic acid factories based on *Agave*, as compared to lactic acid factories based on glucose derived from maize grains (Abdel-Rahman *et al*., 2013). Hence this small set of fructanolytic and homolactic lactic acid bacteria is a first step in the larger goal to adapt lactic acid production to countries with *Agave* plantations such as Mexico.

Conclusions

Here it is confirmed that the *Agave* sap of *A. salmiana*, is a good source for the isolation of homolactic and fructanolytic bacteria belonging to genus *Enterococcus*, *Lacticaseibacillus* and *Bacillus*. These bacteria were able to produce lactic acid from a culture medium with *Agave* fructans as the only carbon source, without the need to add glucose or any exogenous nitrogen source, so the application of these bacteria in practical fermentations such as the *Agave* stalk silages are quite interesting; since it is a low-cost fermentation that would use agro-industrial waste from the *Agave*. Such microbiota could have certain degree of specificity to Agavacea because it is different to epiphytic microbiota reported by other researchers when studying maize or herbal silages and it thrives using only *Agave* fructans as carbon source. Such specificity could be used for future "omic" studies of present strain collection with the purpose to develop new industrial fermentations for lactic acid production based on *Agave* fructans instead of conventional lactic acid fermentation based on glucose, sucrose, or food crops such as maize or sugarcane. In this context, present collection was a necessary step in the search for proprietary industrial strains registered with specific accession numbers in Gene Data Bank. This work seems to be a positive step in the way to develop the use of *Agave* derivatives as alternative to the local deficit of food crops as raw materials for lactic acid and polylactic acid production in Mexico.

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