
Ingeniería de alimentos

*Revista Mexicana de Ingeniería Química*
Vol. 14, No. 2 (2015) 281-292

# CHEMICAL COMPOSITION, ANTIOXIDANT CAPACITY AND PREBIOTIC EFFECT OF *aguamiel* (*Agave atrovirens*) DURING *in vitro* FERMENTATION

# COMPOSICIÓN QUÍMICA, CAPACIDAD ANTIOXIDANTE Y EL EFECTO PREBIÓTICO DEL *aguamiel* (*Agave atrovirens*) DURANTE SU FERMENTACIÓN *in vitro*

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Received April 17, 2015; Accepted June 11, 2015

**Abstract**

Characterization of the compounds present in *aguamiel* (*A. atrovirens*) by standard methods can evaluate the potential health effect of *aguamiel* as a functional food or food ingredient. *Aguamiel* contained 89.61% moisture, 3.50% protein, 3.10% ash, and 61.31% total reduced sugars, of which 32.63% was total fructose and 28.68% was total glucose, while the fructooligosaccharides content was 15.51%. The *aguamiel* contained minerals such as potassium, calcium, and sodium in higher proportions, followed by iron, copper, magnesium, selenium and zinc. Were determined water-soluble vitamins B complex vitamins (B1, B2, B3 and B6) and ascorbic acid. It was found a total saponins content of 1.17 g/100 g of sample db, with anti-inflammatory activity. A total of nine essential and eight non-essential amino acids, of the 20 necessary for health, were determined in *aguamiel*. An additional characteristic was the phenolic compounds content, which exhibited antioxidant activity against DPPH and ABTS. The degree of polymerization of fructooligosaccharides was 5-10 determined by MALDI-TOF. The effect fructooligosaccharides prebiotics during *in vitro* fermentation and the quantification of short-chain fatty acids were evaluated.

*Keywords*: *aguamiel*, fructooligosaccharides, prebiotic, antioxidant capacity, *in vitro* fermentation, short-chain fatty acids.

**Resumen**

La caracterización de los compuestos presentes en *aguamiel* (*A. atrovirens*) mediante métodos estandarizados, ayudaron a conocer el efecto potencial de éste en la salud como alimento funcional o ingrediente alimentario. El *aguamiel* tuvo 89.61% de humedad, 3.50% de proteína, 3.10% de cenizas, 61.31% azúcares reductores totales, de los cuales 32.63% es fructosa y 28.68% glucosa, mientras que el contenido de fructooligosacáridos fue 15.51%. El *aguamiel* contiene una mayor proporción de minerales como potasio, calcio y sodio, seguidos de hierro, cobre, magnesio, selenio y zinc. Se determinó el contenido de vitaminas hidrosolubles que incluyen el complejo B (B1, B2, B3 y B6) y ácido ascórbico. Se encontró un contenido de saponinas de 1.17 g/100 g muestra, las cuales tienen actividad anti-inflamatoria. En el *aguamiel* se encontraron además un total de nueve aminoácidos esenciales y ocho no esenciales, de los 20 necesarios para la salud. Una característica adicional fue el contenido de compuestos fenólicos, los cuales exhiben actividad antioxidante contra DPPH y ABTS. El grado de polimerización de los fructooligosacáridos fue de 5-10 el cual se realizó por MALDI-TOF. Durante la fermentación *in vitro* se evaluó el efecto prebiótico de los fructooligosacáridos y se cuantificaron los ácidos grasos de cadena corta.

*Palabras clave*: *aguamiel*, fructooligosacáridos, prebiótico, capacidad antioxidante, fermentación *in vitro*, ácidos grasos de cadena corta.

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# 1 Introduction

Multiple agave species grown in the semi-desert areas of Mexico, such as maguey-*pulquero* (*Agave atrovirens*, *Agave salmiana*, *Agave mapisaga* and *Agave americana*). Agave species are important from an ecological point of view because they reduce soil erosion (Escalante *et al*., 2004). When the maguey is mature, the leaves are cut before the development of the central scape. Then, a cavity of 20 to 30 cm is formed, which stores the agave sap called *aguamiel*. Over a period of 3 to 6 months, the *aguamiel* is collected, and each plant provides approximately 1,500 L (Alanís and González, 2011).

The *aguamiel* is used for the production of *pulque* (a drink with cultural importance in Mexico) contains fructooligosaccharides that are susceptible to fermentation in the colon by colonic microorganisms that produce short-chain fatty acids (SCFAs), which reduce lipid and glucose levels in the blood and decrease the incidence of gastric lesions (gastritis). Storddart *et al*., (2008) reported that SCFAs concentrations are also sensed by specific G protein-coupled receptors (GPRs), which are involved in the regulation of lipid and glucose metabolism. GPR41 and GPR43 have been identified as SCFAs receptors. GPR40 and GPR42 have been found to be receptors for medium- and long-chain fatty acids, respectively. The scarce data available on the effect of SCFAs on glucose metabolism reveal a decrease in plasma glucose levels possibly via multiple mechanisms. The plasma glucose level is determined by uptake via food, gluconeogenesis, and uptake by multiple organs (Jäger *et al*., 2007).

Valadez *et al*., (2012) observed that *aguamiel* is rich in carbohydrates and can be used to obtain polysaccharides, fructans or high fructose syrup. Fructans, such as inulin and fructooligosaccharides (FOS), have gained attention as food additives due to their beneficial health effects. FOS is a common name for these fructose oligomers, and these are typically regarded as inulin-type oligosaccharides. In the food industry, these are known as probiotics when incorporated into food. These compounds used in the food bioprocessing technologies constitute a series of homologous oligosaccharides derived from sucrose; these are usually represented by the formula GFn and are mainly composed of 1-kestose (GF2), nystose (GF3), and 1F-$\beta$-fructofuranosylnystose (GF4), in which two, three, and four fructosyl units are bound at the $\beta$-2,1 position of glucose, respectively (Yun, 1996).

FOS can modify the gut microbiota and their metabolic activities in a beneficial way, and many other helpful and useful effects of prebiotics are being investigated. This include their ability to modulate gut function and transit time, to activate the immune system, to increase the production of butyric acid and other short-chain fatty acids, to increase the absorption of minerals such as calcium and magnesium, and to inhibit lesions that are precursors to adenomas and carcinomas. Thus, prebiotics can potentially reduce some of the risk factors involved in the causes of colorectal diseases and reduce the risk of diseases such as cardiovascular disease, colon cancer, and obesity (Slavin, 2013). Strategies for developing prebiotic products aim to provide specific fermentable substrates for beneficial bacteria (Bifidobacteria and Lactobacilli). These may provide adequate amounts and proportions of fermentation products, especially in the lower part of the colon where the effects are most favorable. Adequate prebiotic administration may exert important prophylactic effects against gut pathogens (Shimizu and Hachimura, 2011).

It is necessary to know about the production of beneficial metabolites in colonic fermentation of indigestible agave ingredients and their potential health effects when incorporated into complex food formulations. Short-chain FOSs are fermented in the proximal colon. The remaining long-chain prebiotics are then available for more distal colonic activity (Singh and Singh, 2010). A number of *in vitro* and *in vivo* studies have confirmed that long-chain FOSs are fermented into lactic acid and SCFAs in the colon. Huebner *et al*., (2007) reported that this fermentation leads to the selective growth of Bifidobacteria. Additionally, FOS stimulates calcium absorption in postmenopausal women, increases iron absorption in children, aids in the prevention of colon cancer and reduces the glycemic index when consumed moderately. SCFAs are end products of luminal microbial fermentation of predominantly non-digestible dietary carbohydrates. SCFAs have different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4)), and the highest concentrations of SCFAs are found in the proximal part of the colon. Butyrate exhibits anti-inflammatory properties mainly through the inhibition of nuclear factor kappa B (NF-$\kappa$B), which controls inflammation by acting as a transcription factor. Propionate possesses the ability to induce histone hyperacetylation, while acetate shows no effects on histone acetylation (Yu Wang, 2010; Willems and Low, 2012).

The aims of this study were to determine the main compounds in *aguamiel* from *A. atrovirens* Karw, their antioxidant capacity and to evaluate the short-chain fatty acid production from *in vitro* fermentation of fructooligosaccharides to determinate their possible hypocholesterolemic and anti-ulcer effects.

# 2 Materials and methods

## *2.1 Materials*

*Aguamiel* was obtained from *A. atrovirens* Karw in the mature stage (8 years) from a village in Michoacan state, Mexico. The samples were filtered to eliminate traces of pineapple and were packaged in 250 mL glass-topped metal bottles that had been sterilized at 121 °C for 20 min. Then, the bottles were cooled and stored (4 °C).

## *2.2 Chemical composition of aguamiel*

The moisture content was determined according to Bidwell-Sterling (Nielsen, 2003) using 10 g of sample and 150 mL of toluene. The samples were heated for 2 h. Ash was analyzed according to the AACC (2000) method 923.03. Proteins were analyzed according to Bradford (1976). The pH was determined with a pH meter (Conductronic, pH120, Mexico). The soluble solids content was obtained using a manual refractometer (ATAGO, PR-101, Japan). The titratable acidity was determined according to method 942.15 (AOAC, 2000) with the titration by 0.1 N NaOH reported as milliequivalents of lactic acid (0.9). Total reducing sugars and total fructose and glucose levels were determined by the method of Ting (1956) with previous hydrolysis of the samples (2 N HCl) for 18 h. For the determination of free sugars (fructose and glucose), sample hydrolysis was not performed. The content of sucrose was calculated from the difference between the reducing sugar content before and after inversion (hydrolysis with 2 N HCl, 18 h) multiplied by a factor of 0.95. The levels of fructooligosaccharides were determined according to the method of McCleary *et al*., (1997).

The mineral content was determined by flame atomic absorption spectroscopy (Varian, SpectrAA 220I, Australia), with previous acid digestion (HCl-$HNO_3$, 3:2) of the samples (45 mL), read at 248.3 nm and 5 mA (lamp) (Alcánzar *et al*., 2014). The determination of lead was according to the method established by NMX-117-SSA1 (1994) by atomic absorption spectroscopy (Varian, SpectrAA 220I, Australia) with previous acid digestion (HCl-$HNO_3$, 3:2) of the samples (45 mL), read at 217 nm and 8 mA (lamp).

The water-soluble vitamins niacin, riboflavin, thiamine and pyridoxine were quantified on a Waters 515 high performance liquid chromatography apparatus coupled to a UV-Vis detector (Waters, 2487, USA) at a wavelength of 254 nm and to a fluorescence detector (Waters, 2475, USA) at a wavelength of 454 nm. Separation was performed on a column (Thermo Scientific, Hypersil ODS, USA) with dimensions of 150 x 4.6 mm. The mobile phase was 0.1 N phosphate buffer-methanol (ratio 85:15) containing 5 mM of sodium hexanesulfonate. The flow rate was 0.8 mL/min (Albalá *et al*., 1997). The vitamin C level was determined by the iodine titration method proposed by Suntornsuk *et al*., (2002). Equation 1 to calculate the amount of vitamin C (ascorbic acid milliequivalents, 0.064 g) present in the *aguamiel*:

$$VitC = \frac{\text{Iodine used} * \text{Normality} * \text{meq of ascorbic acid}}{\text{Sample volume}} \quad (1)$$

The identification and quantification of amino acids were performed according to AOAC, (1995) using HPLC (Dionex system, P680, USA) with a DAD UVD 340U detector (UP5ODB-25QS, 250 x 4.6 column mm). Amino acids were injected into a mixture (78:22) with two solvents: Solvent A: 9 mM sodium dihydrogen phosphate, 4% dimethylformamide and 0.1% triethylamine, pH 6.55; and Solvent B: 80% (v/v) acetonitrile. The mixture was eluted at a flow rate of 1 mL/min.

The total saponins content was estimated using the method described by Hiai *et al*., (1976). The amount of saponins was calculated using a calibration curve with diosgenin (Sigma, D 1634,USA) at concentrations of 0, 25, 50, 75, 100 and 125 g/mL in 80% methanol. The techniques for the characterization of *aguamiel* were performed in triplicate.

The total phenols determination in *aguamiel lyophilized* was assessed according to Abdel and Hucl., (1999). Total phenols were extracted by 80% methanol with shaking for 16 h. Then, the samples were centrifuged at 10,000 x g for 15 min. Finally, the supernatant was used for the quantification of total phenols (Makkar *et al*., 1993). The analysis was performed in triplicate.

### *2.3 Antioxidant activity*

DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma, D9132, USA) radical absorption extinction at 515 nm in a spectrophotometer (Buck Scientific, UV-Vis, 105, USA) was used to determine the antioxidant activity in methanol (80%) extract of *aguamiel* (Brand, 1995). The analysis was performed in triplicate and reported as (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma, 238813, USA) Trolox equivalent antioxidant capacity/g *aguamiel*.

The ABTS method is based on the ability of different substances to scavenge 2,2'-azino-bis[ethylbenzothiazoline-6-sulfonic acid] (Sigma, A1888, USA) radical cations. Radical cations were prepared mixing 7 mM of ABTS stock solution with 2.45 mM of potassium persulfate in ethanol and read by a spectrophotometer (Buck Scientific, UV-Vis 105, USA) at 734 nm (Arts *et al*., 2004). The analysis of antioxidant capacity of methanol (80%) extract of *aguamiel* was performed in triplicate and reported as (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma, 238813, USA) Trolox equivalent antioxidant capacity/g *aguamiel*.

### *2.4 Fructooligosaccharides distribution*

Fructooligosaccharides were characterized to obtain their distribution based on chain length according to Alvarado *et al*., (2014). The *aguamiel* sample was analyzed by MALDI-TOF (Bruker, Daltonics autoflex III TOF/TOF 200, USA) at 500 Hz, an ion source voltage of 19 kV, and a reflector voltage 21 kV.

### *2.5 In vitro fermentation of fructooligosaccharides*

The *in vitro* fermentation assay was performed according to the method described by Serrano *et al*., (2007). The inoculum was prepared with fresh human feces and placed in bags that had been previously filled with $CO_2$, which were then weighed. The inoculums were mixed for 15 min with sterilized anaerobic fermentation medium (10 mL per g of inoculums) and vacuum filtered. The anaerobic fermentation medium was composed of tryptone (2.5 g) diluted in distilled water, a micromineral solution (125 $\mu$L), a buffer solution (250 mL), a macromineral solution (250 mL) and resazurin (1.25 mL) as an anaerobic redox indicator; it was brought to 1 L with water. In glass flasks that had been previously dried, lyophilized *aguamiel* samples (100 mg) were weighed in triplicate; the samples had contents of soluble sugars (free sugars) lower than 1% (data not shown), these were not eliminated, as Wenzel *et al*., (2010) recommended. In contrast, the indigestible fraction (IF) of the sample was not obtained because the fructooligosaccharides, which were in greater amounts in the *aguamiel* sample and were of low molecular weight (less than 12000 Da), were likely lost during the hydrolysis step in the determination of the IF, decreasing the fermentation substrate available. Lactulose was used (100 mg, Sigma, L-7877, USA) as the control, and the blank consisted no sample. The determination was assessed in triplicate. Each flask contained oil cloth plugs and two needles (one lumbar puncture and another of 0.9 x 25 mm). The samples were hydrated with anaerobic fermentation medium (8 mL) at 4 °C for 16 h after 2 mL of inoculum was added, and these were injected with $CO_2$ for 1 min. *Aguamiel* samples, controls (lactulose) and blanks (no sample) were placed in a shaking water bath (37 °C for 0, 6, 12, 24 and 48 h). Samples at t = 0 h (without incubation) were used to correct the values obtained in the controls and *aguamiel* samples after each incubation time (0, 6, 12, 24 and 48 h fermentation). For subsequent pH determination, the fermentation was stopped with 3 mL of 1 M NaOH; the samples were then centrifuged (4 °C at 1,500xg for 15 min), and the supernatants (5 mL) were collected for SCFAs analysis.

#### *2.5.1 Quantification of short-chain fatty acids (SCFAs)*

The SCFAs quantification (during the fermentation) was assessed on a gas chromatograph (Varian, 3900, Australia) equipped with a flame ionization detector and an HP-INNOWax column (Hewlett Packard 19091N-133, 30 mm x 0.25 mm x 0.25 mm, cross-linked with polyethylene glycol) according to Wenzel *et al*., (2010). The starting temperature (95 °C) was maintained for 2 min and then increased to 180 °C at 20 °C/min. The external standards were acetic, propionic, and butyric acids (HPLC grade). SCFAs were synthesized during the *in vitro* fermentation and quantified by interpolation of the standard curve. The molar ratio of SCFAs according to the proportions produced during fermentation was estimated, and SCFAs were expressed as mmol/L. These analyses were performed in triplicate.

### *2.6 Statistical analysis*

The results were expressed as the means ± standard errors. Comparisons were performed with one-way analysis of variance (ANOVA) followed by Tukey's tests with the statistical package Sigma plot version 12.

## 3 Results and discussion

### *3.1 Chemical composition*

Table 1 shows the results for moisture, ash, protein, pH, titratable acidity and soluble solids. The moisture content was 89.61%. The ash (3.1%) and protein (3.5%) contents were similar to that reported by Ortiz *et al*., (2008) (3.3 and 3.0 g/100 g of *aguamiel* from *Agave mapisaga*, respectively), but were higher than that reported by Bautista and Arias, (2008) (1.82 g and 2.38 g/100 g of *aguamiel* from *Agave americana*, respectively). The pH was within values reported by NMX-V-022 (1972) from 4.5 to 7.5, similar to that reported by Bautista and Arias, (2008); the pH is very important during *aguamiel* handling because it indicates the quality of this agave product.

The titratable acidity (0.06 mg/100 g lactic acid) was slightly higher than that reported by Ortiz *et al*., (2008) (0.05 mg/100 g lactic acid), indicating that heat treatment prevented spontaneous *aguamiel* fermentation. Bautista and Arias, (2008) reported a value of 0.24 mg/100 g lactic acid in *aguamiel* from *A. americana*, which is greater than that found in *aguamiel* from *A. mapisaga* reported by Ortiz *et al*., (2008).

Table 1. Chemical composition of *aguamiel* from *Agave atrovirens*

| | Wt % (in dry base)* |
|---|---|
| Moisture | 89.61± 0.02 |
| Protein | 3.50 ± 0.11 |
| Ash | 3.10 ± 0.23 |
| Total fructose | 32.63 ± 0.06 |
| Total glucose | 28.68 ± 0.02 |
| Fructooligosaccharides | 15.51 ± 0.04 |
| Sucrose | 12.90 ± 0.06 |
| Saponins | 1.17 ± 0.10 |
| pH | 6.29 ± 0.02 |
| Titratable acidity | 0.06 ± 0.02 |
| Soluble solids (°Brix) | 11.10 ± 0.10 |

* Per 100g in db. Average of three replicates ± standard error.

This result may be due to a reduction in the accumulation of lactic acid from fermentation caused by lactic bacteria (Escalante *et al*., 2008). The soluble solids (11.10 °Brix) were intermediate to that reported by NMX-V-022, (1972) (7 to 13 °Brix).

The total amounts of reducing sugars (glucose and fructose), sucrose, and fructooligosaccharides are listed in Table 1. *Aguamiel* has a considerable amount of total reducing sugars. The amount of total glucose (28.68 g) was similar to the report by Ortiz y *et al*., 2008. The total fructose (32.63 g) was similar that reported in *aguamiel* from A. *mapisaga*. The amount of total reducing sugars (61.31 g/100 g) was similar to that reported in *A. mapisaga* by Ortiz *et al*., (2008) (58.9 g/100 g), and it was less than that reported by Bautista and Arias, (2008) (71.95 g/100 g) in *aguamiel* from *A. americana*. The amount of sucrose (12.90 g/100 g of dry *aguamiel*) was greater than that reported in *aguamiel* from *A. mapisaga* (8.8 g/100 g of dry *aguamiel*). This suggests that the region in which the agave is located and the particular agave species influence the sugar content. The fructooligosaccharides content was greater than in *A. mapisaga aguamiel* in which Ortiz *et al*., 2008 reported 10.2 g/100 g. Considering these results, the *aguamiel* from *Agave atrovirens* is a good source of fructooligosaccharides that may have potential health benefits during colonic fermentation.

The total saponins content (Table 1) in agave sap was previously unknown. Hernández *et al*., (2005) found 1-2 g/100 g in the leaves of *A. lechuguilla*. A total of 1.17 g/100 g was found in *aguamiel* from *A. atrovirens*. This result is important because Monterrosas *et al*., (2013) reported that saponins have pharmacological properties, such as anti-inflammatory activity. Taking into account these results, we can say that *aguamiel* could have alternative uses. These findings justify the popular use of *aguamiel* for the treatment of gastritis.

Some mineral levels are listed in Table 2. Potassium (120.44 mg), calcium (11.70 mg) and sodium (0.83 mg) per 100 g of *aguamiel* were present in high proportions. The recommended dietary dose of potassium is 3510 mg/day (WHO, 2012), while that of calcium and sodium are 1000 mg/day and 2400 mg/day (FAO, 2004), respectively. *Aguamiel* provides 3.43%, 1.17% and 0.03% of the daily doses of potassium, calcium and sodium, respectively, recommended by the WHO and FAO. The amounts of iron (0.81 mg/100 g) and zinc (0.18 mg/100 g) in *A. atrovirens* were higher than the measurement obtained by Ortiz *et al*., (2008) in *A. mapisaga*

(0.09 mg/100 g). *Aguamiel* may be an important source of these mineral as a natural product or nutraceutical complement, which could be adopted by health programs in developing countries to lower iron and zinc deficiencies (Hambidge and Krebs, 2007). The copper (0.07 mg/100 g) and magnesium (0.055 mg/ 100 g) contents were greater than that reported by Ortiz *et al*., (2008) (0.03 mg and 0.034 mg/100 g, respectively). Selenium, an important mineral due to its excellent cellular antioxidant properties (Cloutier and Baffigo, 1999), was present at 0.047 mg/100 g. The lead content is important because it is considered toxic in foods. According WHO (2012), the maximum tolerable amount of lead is 0.243 mg. In this study, the lead content was 0.015 mg/100 g, which is not considered toxic for human consumption. There is evidence that micronutrient-fortified beverages (containing iron, vitamin C, niacin, riboflavin, folate, vitamin B-12, and vitamin B-6) promote increases in both in hemoglobin concentration and ferritin. Furthermore, these beverages reduced the risk of anemia and iron deficiency in pregnant women and infants (Makola *et al*., 2003), and *aguamiel* could be used in beverages with health benefits from these minerals, especially iron.

The water-soluble vitamin content (Table 3) consisted of B1 0.10 mg/100 g, B2 0.38 mg/100 g, and B6 0.57 mg/100 g. However, vitamins B3 and B1 have not been reported in the literature but were found in the *aguamiel* of *A. atrovirens* in this study. The B complex vitamins participate in the regulation of energy metabolism by modulating the synthesis and degradation of carbohydrates, fats, proteins, and bioactive compounds (Lukaski, 2004). Niacin was present in high amounts (4.77 mg/100 g *aguamiel*). This compound is very important as a cofactor in the mitochondrial respiratory chain, facilitating the release of energy from foods and the transformation of NAD to NADP, which play key roles in oxidation-reduction reactions in all cells (Huskisson *et al*., 2007). The vitamin C content (17.99 mg/100 g) was higher than reported by Gentry (1998) (7-11 mg/100 g *aguamiel*) and by Bautista and Arias, 2008 (14.82 mg/100 g *aguamiel*), indicating that *aguamiel* is a good source of the antioxidant vitamin C (Walingo, 2005). According to the human vitamin requirements by the FAO (2004), it is necessary to consume 1.2 mg/day of B1, 1.3 mg/day of B2, 14 mg/day of B3, 1.3 mg/day of B6 and 20 mg/day of ascorbic acid. *Aguamiel* provides 8.33%, 29.23%, 34.07% and 89.95% of the daily requirements of B1, B2, B6, and vitamin C, respectively.

Table 2. Minerals in *aguamiel* from *Agave atrovirens*

| Mineral | mg/100 g in db* |
|---|---|
| Potasium (K) | 120.44 ± 0.10 |
| Calcium (Ca) | 11.70 ± 0.30 |
| Lead (Pb) | 0.015 ± 0.00 |
| Zinc (Zn) | 0.18 ± 0.01 |
| Iron (Fe) | 0.81 ± 0.20 |
| Sodium (Na) | 0.83 ± 0.06 |
| Copper (Cu) | 0.07 ± 0.03 |
| Magnesium (Mg) | 0.55 ± 0.07 |
| Selenium (Se) | 0.047 ± 0.00 |

*Average of three measurements ± standard error.

Table 3. Water-soluble vitamins in *aguamiel* (*Agave atrovirens*)

| Mineral | mg/100 g in db* |
|---|---|
| Tiamine (B1) | 0.10± 0.01 |
| Riboflavin (B2) | 0.38± 0.03 |
| Niacin (B3) | 4.77± 0.30 |
| Pyridoxine (B6) | 0.57± 0.15 |
| Ascorbic acid (C) | 17.99± 0.20 |

*Average of three replicates ± standard error.

Table 4. Amino acid profile in *aguamiel* from (*Agave atrovirens*)

| Amino acids | mg/100 g in db* |
|---|---|
| Aspartic acid | 7.91 ± 0.50 |
| Glutamic acid | 20.08 ± 0.22 |
| Serina | 4.48 ± 0.01 |
| Glycine | 2.51 ± 0.08 |
| Histidine | 1.84 ± 0.04 |
| Arginine | 3.97 ± 0.09 |
| Threonine | 2.30 ± 0.06 |
| Alanine | 2.38 ± 0.04 |
| Proline | 7.38 ± 0.36 |
| Tyrosine | 3.58 ± 0.16 |
| Valine | 16.35 ± 0.42 |
| Methionine | 2.18 ± 0.06 |
| Cysteine | 0.48 ± 0.05 |
| Isoleucine | 4.68 ± 0.12 |
| Leucine | 4.67 ± 0.11 |
| Phenylalanine | 10.03 ± 0.15 |
| Lysine | 5.17 ± 0.15 |

*Average of three replicates ± standard error.

The amino acid profile of *aguamiel* is presented in Table 4. The amino acids present in greater proportions were glutamic acid (20.08 mg), valine (16.35 mg) and phenylalanine (10.03 mg) per 100 g of *aguamiel*; valine and phenylalanine are essential

amino acids. Valine is an amino acid necessary for the growth and maintenance of body tissues, and phenylalanine is used in the production of tyrosine and three hormones (epinephrine, norepinephrine and thyroxine) (WHO, 2007). In addition, *aguamiel* contained many essential amino acids (lysine, phenylalanine, isoleucine, leucine, valine, and methionine) and some non-essential amino acids (serine, proline, threonine, alanine, tyrosine and histidine). The total amino acid content was 0.20 g/100 g of *aguamiel*. These results agree with Ortiz *et al*., 2008 and demonstrate that agave sap could also be a source of amino acids and support the daily requirements of an adult: 20 mg of isoleucine, 39 mg of leucine, 18 mg of lysine, 13 mg of methionine, 33 mg of phenylalanine, 15 mg of threonine, 5 mg of tryptophan, 26 mg of valine and 14 mg of histidine per kg per day (WHO, 2007).

### *3.2 Total phenols and antioxidant activity*

Table 5 presents the total phenol content in lyophilized *aguamiel* (3.02 gallic acid equivalent/g *aguamiel*). Tovar *et al*., (2011) reported 2.26 gallic acid equivalent/g *aguamiel* in *A. salmiana*. The antioxidant activities in agave sap from *A. atrovirens* assayed by DPPH and ABTS were 8.72 and 8.88 Trolox equivalent antioxidant capacity/g, respectively. Compared with commercial beverages, such as coffee (472.5 Trolox equivalent antioxidant capacity /g) and grape juice (38.9 Trolox equivalent antioxidant capacity/g), *aguamiel* had a low antioxidant capacity. In addition, *aguamiel* had higher antioxidant activity than other beverages such as orange juice/nopal (6.7 Trolox equivalent/g) and half the activity of *pulque* (13.7 Trolox equivalent/g) (Tovar *et al*., 2011). Studies in rabbits were the first reports of the effect of the antioxidant capacity of *aguamiel* on an animal system without adverse effects (Tovar *et al*., 2011).

Table 5. Total phenolics and antioxidant capacity of *aguamiel* from *Agave atrovirens*

| Determination | g in db |
|---|---|
| Total phenols* | 3.03 ± 0.47 |
| Antioxidant capacity | |
| DPPH** | 8.72 ± 0.10 |
| ABTS** | 8.88 ± 0.20 |

Average of three replicates ± standard error.

* Gallic acid equivalent.

**Trolox equivalent antioxidant capacity (TEAC).

Despite this, more studies are necessary to understand this beneficial effect and to identify other possible biochemical agents in *aguamiel* proving this antioxidant capacity.

### *3.3 Fructooligosaccharides distribution in aguamiel*

The MALDI-TOF distribution of *aguamiel* fructooligosaccharides (FOS) contained peaks from 867 Da to 1840 Da (Figure 1) that corresponded to molecules with a degree of polymerization between 5 to 10 DP, according to reports Arrizón *et al*., (2010) (500 Da to 1824 Da) in *Agave spp*. and Alvarado *et al*., (2014) (851 Da to 1985 Da) in *Agave tequilana*. However, predominantly short-chain FOSs, which have a prebiotic effect during fermentation in the colon, were in higher proportions. Gómez *et al*., (2010) have demonstrated the prebiotic activity of agave fructans and fructooligosaccharides and provided information on the dynamics of their fermentation in different regions of the colon. Stewart *et al*., (2008) mentioned that the chain length affects the *in vitro* fermentability, in which fructooligosaccharides with a low DP are rapidly fermented and fructooligosaccharides with longer chains are steadily fermented to SCFAs with different end-product profiles and time dependences.

### *3.4 In vitro fermentation of aguamiel*

Figure 2 presents the decrease in pH during the fermentation of FOSs by the production of fatty acid chains (SCFAs). During *in vitro* fermentation, the pH decreased between 6 and 48 h of incubation. The pH of lactulose was lower (6.45 at 48 h) than the pH of *aguamiel* (6.68 at 48 h) ($p < 0.05$). Lactulose is considered to be 100% fermentable. The SCFAs values produced during *in vitro* fermentation (Table 6) were due to the formation of three SCFAs (acetic, propionic and butyric acid) that increased over time. Acetic acid was the most abundant SCFA (86.51 and 49.31 mmol/L in lactulose and *aguamiel*, respectively), followed by propionic acid (41.21 and 15.29 mmol/L, respectively). Butyric acid (18.57 and 12.62 mmol/L, respectively) was significantly different ($p < 0.05$) from lactulose, which was utilized as a control. A greater amount of butyric acid, which could play a protective role against colon cancer, was produced from *aguamiel* compared to other substrates, such as $\beta$-gluco-oligosaccharides (Sarbini *et al*., 2012) or legumes (Hernández *et al*., 2010).

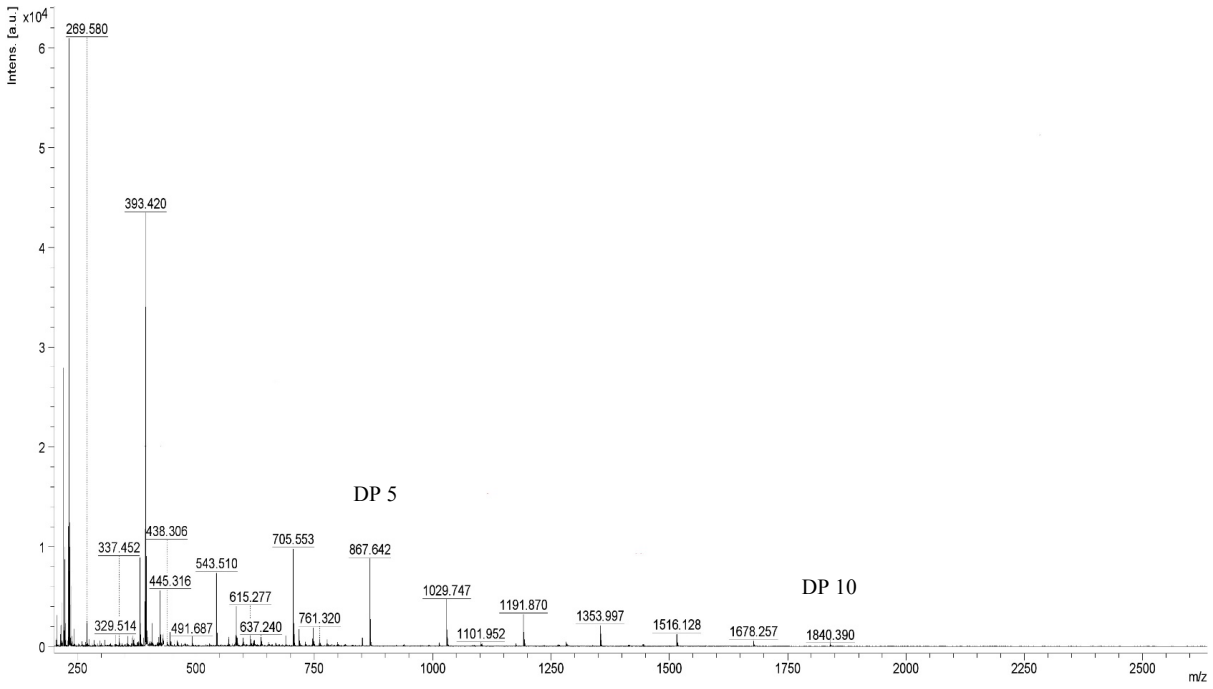


Fig 1. Mass spectrum of *aguamiel* FOS by MALDI-TOF Bruker autoflex III TOF/TOF 200. DP: Degree of polymerization.

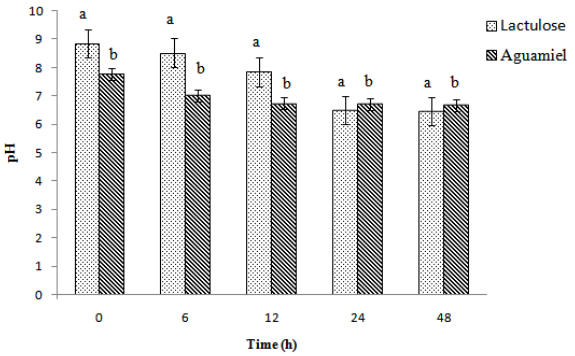


Fig 2. pH changes during in vitro fermentability of *aguamiel*. Different letters between samples indicate significant differences ($p$ <0.05), sample compared with control (Lactulose) at different time.

The molar ratio of SCFAs (acetic-propionic-butyric acid) produced after 48 hours of fermentation was 64:20:16, indicating good production of these compounds with possible beneficial effects of *in vivo* models. The amount of SCFAs produced and absorbed from the colon (acetic, propionic and butyric acids) can account for up to 15% of the total energy uptake of humans. Butyrate is the preferred energy source for colonocytes. The formation of SCFAs, especially propionate and butyrate, at high physiological levels in the colon contributes to the defense mechanisms of the intestinal wall. The anti-inflammatory and anti-carcinogenicity capacities of SCFAs are some of the indirect benefits of absorption of these compounds (Havenaar, 2011). SCFAs are end products of luminal microbial fermentation of predominantly non-digestible dietary carbohydrates. SCFAs have different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4)), and the highest concentrations of SCFAs are found in the proximal part of the colon. Butyrate has anti-inflammatory properties mainly from inhibition of nuclear factor kappa B (NF-$\kappa$B) that controls inflammation by acting as a transcription factor. Propionate possesses the ability to induce histone hyperacetylation, while acetate shows no effects on histone acetylation (Hamer *et al*., 2008).

## Conclusions

The moisture, ash, protein, and soluble solids (°Brix) contents, the pH and titratable acidity were similar to the values reported in *aguamiel* from other agave species (*A. mapisaga* and *americana*), suggesting that the sample from *A. atrovirens* is a significant source of compounds with potential health benefits such as antiulcer and hypocholesterolemic activities due to the

Table 6. Short chain fatty acids (SCFAs) produced in the *in vitro* fermentation of *aguamiel* (mmol/L) from *Agave atrovirens*

| Sample | Time (h) | Acetic | Propionic | Butyric |
|---|---|---|---|---|
| Lactulosa | 0 | $7.06 \pm 0.10^a$ | $1.15 \pm 0.44^a$ | $0.93 \pm 0.20^a$ |
| | 6 | $74.63 \pm 0.20^a$ | $19.47 \pm 0.10^a$ | $12.47 \pm 0.60^a$ |
| | 12 | $81.61 \pm 0.40^a$ | $35.72 \pm 0.20^a$ | $14.79 \pm 0.20^a$ |
| | 24 | $83.59 \pm 0.10^a$ | $40.65 \pm 0.10^a$ | $17.37 \pm 0.10^a$ |
| | 48 | $86.51 \pm 0.10^a$ | $41.21 \pm 0.15^a$ | $18.57 \pm 0.30^a$ |
| *Aguamiel* | 0 | $6.60 \pm 0.54^b$ | $1.77 \pm 0.66^b$ | $0.46 \pm 0.10^b$ |
| | 6 | $46.95 \pm 0.85^b$ | $12.08 \pm 0.90^b$ | $9.15 \pm 0.17^b$ |
| | 12 | $47.13 \pm 0.34^b$ | $13.21 \pm 0.42^b$ | $9.87 \pm 0.98^b$ |
| | 24 | $49.02 \pm 0.68^b$ | $13.68 \pm 0.21^b$ | $10.68 \pm 0.10^b$ |
| | 48 | $49.31 \pm 0.94^b$ | $15.29 \pm 0.33^b$ | $12.62 \pm 0.17^b$ |

Results expressed as average of three replicates ± standard error. Different letters between samples indicate significant differences ($p$ <0.05), samples compared with control (Lactulose) at different time.

presence of fructooligosaccharides, reducing sugars and phenolic compounds. The mineral content was greater than of *A. mapisaga*, and the potassium levels were ideal for the recommended consumption for good health. Furthermore, the lead levels are not toxic for the consumption of *aguamiel*. Vitamins B1, B2, B3, B6 and C in *aguamiel* were similar to the literature values. However, according to the daily requirement of these vitamins, the amount present in *aguamiel* is low, but may be considered a complementary source of vitamins. The amino acid content of *aguamiel* represents an alternative source of these compounds. Saponins were found in low concentrations; these saponins can provide health benefits, such as anti-inflammatory effects. The total phenol content and antioxidant capacity in *aguamiel* (*A. atrovirens*) were comparable to other beverages. The fructooligosaccharides present in *aguamiel* were susceptible substrates to fermentation by colonic microbiota in an *in vitro* model. SCFAs produced acetic, propionic and butyric acids that decreased the pH, suggesting an *in vivo* hypocholesterolemic effect attributed to *aguamiel* by the fructooligosaccharides content.

## Acknowledgements

The author Romero-Lopez acknowledges the support of the Consejo Nacional de Ciencia y Tecnología (CONACYT) through a scholarship to pursue a Ph.D. The authors RME and POD acknowledge the funding provided by National Polytechnic Institute (SIP, COFAA and EDI).

## Nomenclature

| | |
|---|---|
| ABTS | 2,2'-azino-bis (ethylbenzothiazoline-6-sulfonic acid) |
| db | dry base |
| DP | degree of polymerization |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| FOS | fructooligosaccharides |
| g | grams |
| GF | glucose-fructose |
| h | hours |
| L | liters |
| MALDI-TOF | Matrix-Assisted Laser Desorption/Ionization-Time of Flight |
| mg | milligrams |
| mL | milliliters |
| NF-$\kappa$B | nuclear factor-$\kappa$B |
| mmol | millimoles |
| $\mu$mol | micromoles |
| pH | hydrogen potential |
| SCFAs | short-chain fatty acids |
| t | time |

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