In vitro fermentation of the extract of *Agave fourcroydes* (henequen) by lactic acid bacteria

Yanelys García[1], Mercedes G. López[2], R. Bocourt[1], Zoraya Rodríguez[1], Judith Urias-Silvas[2] and Magali Herrera[1]

[1]*Departamento de Ciencias Biofisiológicas. Instituto de Ciencia Animal, Apartado Postal 24,San José de las Lajas, Mayabeque, Cuba*

[2]*Departamento de Biotecnología y Bioquímica. Centro de Investigaciones y Estudios de Avanzadas del IPN, Campus Guanajuato. Apartado Postal 629, Irapuato, Guanajuato, 36 500 México*

Email: ygarcia@ ica.co.cu

In order to determine the growth and fermentation capacity of *Lactobacillus spp.* and *Bifidobacterium spp.* in presence of dried extract of *Agave fourcroydes* (henequen) under *in vitro* conditions, three strains of Bifidobacterium (*B. adolescentes*, *B. bifidum*, *B. breve*) and three of Lactobacillus (*L. acidophilus*, *L. casei*, *L. paracasei*) were selected. The commercial prebiotics Raftilosa P95, Raftiline HP, and the dried extract of *A. fourcroydes*, rich in oligosaccharides of fructans, were used as energy source. The MRS medium, without glucose, was used as control. Cysteine was added when necessary. Higher microbial growth ($P < 0.001$) and pH decrease in the medium ($P < 0.001$) were obtained in all the groups of bacteria in presence of dried extract of *A. fourcroydes*. It was superior to the sources of commercial fructans under use, with high production of organic acids. The lactic acid bacteria fermented efficiently the dried extract of *A. fourcroydes*, thereby being a proper probiotic candidate to be used in animal feeding. These results could generate further studies with the aim of obtaining new functional feeds, such as the symbiotics, which would enhance the effects.

Key words: *fructans, prebiotic, lactobacilli, bifidobacteria*, *Agave fourcroydes*.

The application of prebiotics, as growth promoter additives in animal feeding, constitutes an efficient and safe alternative to intensive production systems, due to the beneficial effects on the health and the productive behavior of animals subject to several stressing factors (Chuanlai *et al*. 2005 and Janssens and van Loo 2006).

The search for new sources of prebiotics is gaining ground at present, because they are little available at the international market and their productions do not fulfill the current demands, making the prices keep high (Carro and Ranilla 2002). The Institute of Animal Science, in Cuba, obtained a dried extract of *A. fourcroydes*, rich in oligosaccharides of fructans (García 2010) that can be used as energy source by lactic acid bacteria.

In the gastrointestinal tract, *Lactobacillus spp.* and *Bifidobacterium spp.* are beneficial microorganisms related to nutritional, metabolic and protection functions in the complex microbial ecosystem and in the host (Guo *et al*. 2004). Therefore, it is necessary to favor their growth to maintain the intestinal eubiosis. Different scientific studies reveal, in detail, the capacity of these microorganisms of using the prebiotics, among them the fructans (García *et al*. 2007 and Roberfroid 2007). This is an important tool to assess the effect of these carbohydrates and their potentials of use *in vivo* as prebiotic in animal feeding.

The aim of this work was to determine the growth and fermentation capacity of *Lactobacillus spp.* and *Bifidobacterium spp.* on the dried extract of *Agave fourcroydes* under *in vitro* conditions.

Materials and Methods

Microorganisms and culture media. Three strains of Bifidobacterium were used: *Bifidobacterium adolescentes* ATCC 460 (BA), *Bifidobacterium bifidum* ATCC 12400 (BBF) and *Bifidobacterium breve* ATCC 15700 (BBR). Besides, three of Lactobacillus: *Lactobacillus acidophilus* ATCC 250 (LA), *Lactobacillus casei* ATCC 393 (LC), and *Lactobacillus paracasei* ATCC 25302 (LPC). All came from the international strain bank of the Research Center and Advanced Studies, Irapuato Unit, Mexico.

As culture medium, MRS (de Mann *et al*. 1960) was used without glucose and substituting glucose by three sources of fructans: dried extract of *A. fourcroydes* (rich in oligosaccharide of fructan) (García 2010) and the commercial prebiotics Raftilose P95 and Raftiline HP, of the ORAFTI brand. The substitutions were performed independently, at concentration of 20 g L^{-1}. The initial pH of the media was fitted to 6.20. For the culture of the bifidobacteria, hydrochloric cysteine (0. 05 %) was added as reducing agent. The culture media, without the carbon source, were sterilized at 121 ºC for 15 min. The methodology of Urías-Silvas (2008) was followed for the preparation of the MRS-fructan medium.

Experimental procedure. The activation of the strains of lactobacilli and bifidobacteria was performed in liquid MRS medium. They were incubated at 37 ºC for 48 h. Aliquots of 1 mL of each strain to be assessed were collected from the culture tubes and cultures in all the culture media. The ratio of inoculation was of

1/10 (v/v). The incubation was conducted in atmosphere of CO_2, for 24 h, at 37 ºC.

At the end of the experiment, the microbial growth capacity was determined estimating the optical density through UV-Visible spectrophotometer, at 630 nm wave length. In the culture media, the pH was calculated with the utilization of digital pH-meter (Sartorius Meter PP-25) and the concentration of organic acids, through high performance liquid chromatography, according to Al-Tamimi *et al*. (2006).

Statistical analysis. A completely randomized design was used, with three treatments and three repetitions per strain. An analysis of variance was performed for data processing. In order to find out significant differences, the test of Duncan (1955) was applied, when necessary. The statistical software INFOSTAT version 1 (Balzarini *et al*. 2001) was used.

Results and Discussion

The strains of *Lactobacillus spp.* and *Bifidobacterium spp*. did not grow on the medium without glucose. However, the bacteria under study, in the culture media with fructans, grew (figures 1 and 2), showing that they were able of using them as energy source.

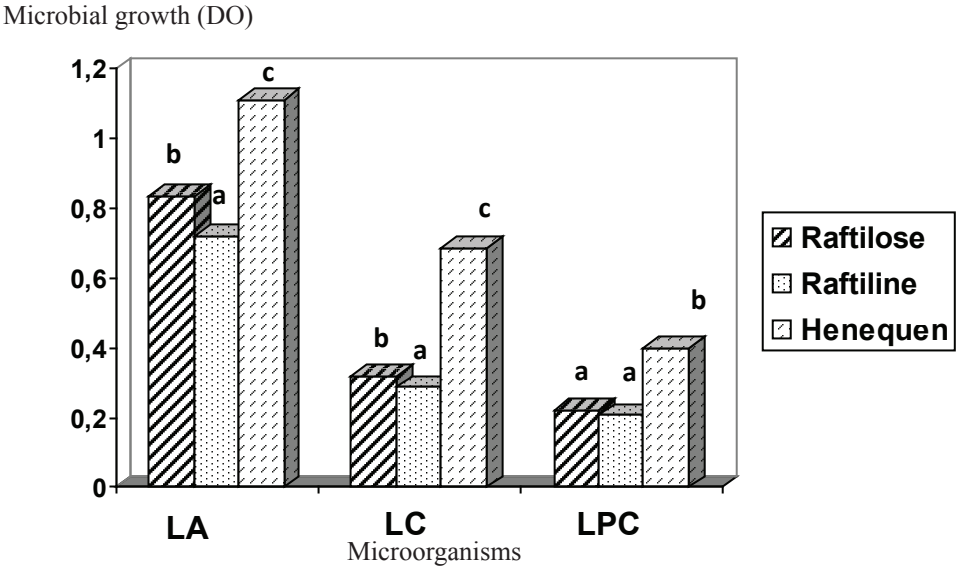
The genera *Lactobacillus spp.* and *Bifidobacterium spp*. have fructosilfuranosidases enzymes, able of degrading the β bonds present in the chemical structures of the fructans (Probert and Gibson 2002 and Ehrmann *et al*. 2003). This characteristic permits the growth in the media that uses these carbohydrates as energy source.

When comparing three sources of fructans, the greatest growth of the strains was in the medium with dried extract of *A. fourcroydes* ($P < 0.001$), whereas in the media with Raftiline HP, fructans of high degree of polymerization, the growths were the lowest. This could be associated with the chemical composition of the dried extract and with the structural diversity present in the fructans, as well as with the length of the chains that, by being made up by oligosaccharides, are easily degraded by part of the lactic acid bacteria, compared with the polysaccharides (van den Broek *et al*. 2008).

Several studies demonstrated that the presence of oligofructans with heterogeneous structures and different types of bonds, similar to those in the fructans of *A. fourcroydes*, induce the synthesis of larger number of enzymes involved in the degradation of these carbohydrates (van der Meulen *et al*. 2004 and van den Broek *et al*. 2008). Nevertheless, the presence of monosaccharides and disaccharides in the dried extract could also contribute to the stimulation of the bacterial growth (García 2010).

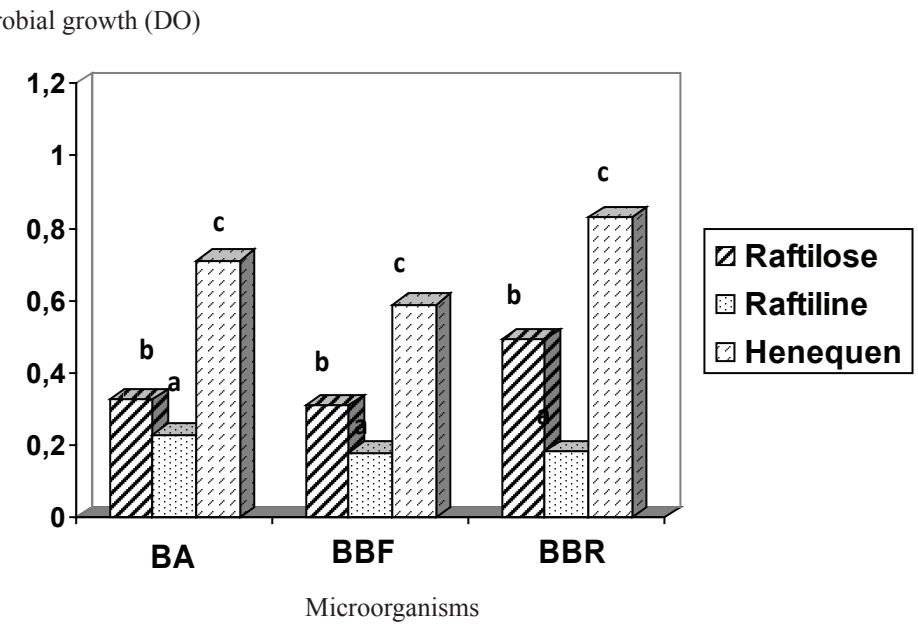
The previous results coincid with studies of Wang and Gibson (1993) and Van der Meulen *et al*. (2004), when obtaining increments in the growth of bifidobacteria in presence of mixtures of oligofructans, in respect to inulins and monosacchacarides of glucose and fructose. Also, they are similar to the studies of Urías-Silvas and López (2004), who used different extracts of plants of the Agavaceae family. These authors obtained higher growths of *Bifidobacterium breve*, *Lactobacillus casei* and *Lactobacillus paracasei* with extracts contining oligofructans from Dasylirium. However, with those including fructans of high degree of polymerization, extracted from *Agave tequilana*, the growth was lower.

In the metabolism of the fructans there are intra and extracellular inducible enzymes (Lambertus *et*



[abc]Columns with different letters differ at $P < 0.05$ (Duncan 1955)
Lactobacillus acidophilus (LA) $P < 0.001$, SE ± 0,02; *Lactobacillus casei* (LC) $P < 0.001$, SE ± 0. 01 and *Lactobacillus paracasei* (LPC) $P < 0.001$, SE ± 0.01)

Figure 1. Growth capacity of *Lactobacillus spp.* at 24 h in MRS medium with Raftilose P95, Raftiline HP and dried extract of *A. fourcroydes* as energy source



[abc]Columns with different letters differ at P < 0.05 (Duncan 1955)
Bifidobacterium adolescentes (BA) P < 0.001, SE ± 0.01; *Bifidobacterium bifidum* (BBF) P < 0.001, SE± 0.01 and *Bifidobacterium breve* (BBR) P < 0.001, SE ± 0.02

Figure 2. Growth capacity of *Bifidobacterium spp.* at 24 h in MRS medium with Raftilose P95, Raftiline HP and dried extract of *A. fourcroydes* as energy source.

al. 2008). Ventura and Turroni (2008) noted that the genera of lactobacilli and bifidobacteria produce larger number of intracellular enzymes and also, have various transport systems that facilitate the input of the oligos to the inner part of the cell. Therefore, the degradation of the oligoschacarides is faster, because the utilization of polysaccharides includes the external enzymatic hydrolysis to oligosaccharides. They penetrate to the interior of the cell and are later metabolized, which may delay their fermentation.

Table 1 presents the results corresponding to the performance of the pH in the medium at 24 h, with the strais of *Lactobacillus spp.* and *Bifidobacterium spp.*, respectively. In all the instances, the cultures with dried extract of *A. fourcroydes* showed lower pH, compared with Raftilose P95 and Raftiline HP (P < 0.01). Rycroft *et al*. (2001) stated that low values of pH indicate higher acidogenic metabolic activity of the strains in the culture medium.

The variations in the pH values of the medium at 24 h in all the treatments were related to the concentrations of organic acids. Several authors found correspondence between these indicators when using prebiotics, because these compounds constitute the energy source of lactic acid bacteria, that in their fermentative processes produce, primarily, acids that diminish the pH (Kneifel *et al*. 2000 and Van der Meulen *et al*. 2006).

The lactic acid was the principal product of the fermentation for *Lactobacillus acidophilus* (figure 3) and the responsible for the differences between the media for this strain, being superior in the dried extract of *Agave fourcroydes* (P < 0.01), whereas for the rest of the microorganisms the differences were determined by the productions of lactic and acetic acid (P < 0. 05) (figures 3 and 4).

The differences in the concentrations of organic

Table 1. Effect of the energy source on the pH values of the medium at 24 h in the cultures of *Lactobacillus spp.* and *Bifidobacterium spp.*.

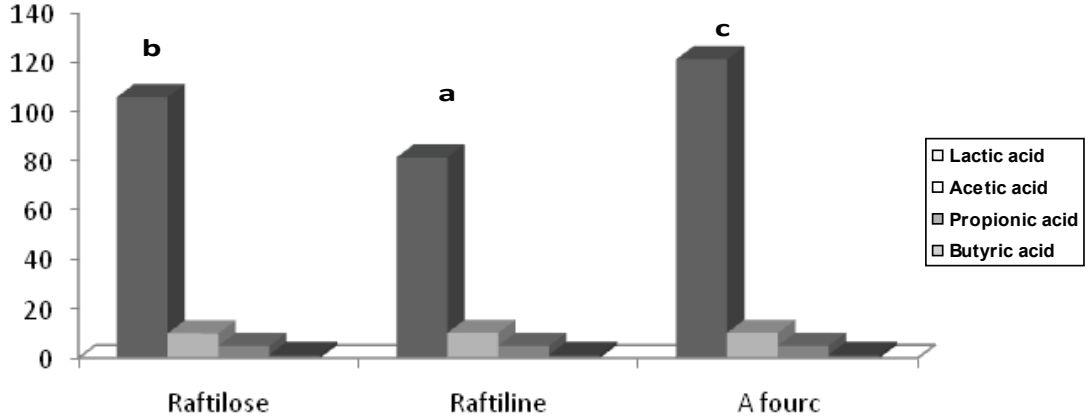
Microorganisms	Culture media			
	MRS-Raftilose P95	MRS-Raftiline HP	MRS-*A. fourcroydes*	SE ±
L. acidophilus	4.09[b]	4.61[a]	3.74[c]	0.03***
L. casei	5.81[b]	6.14[a]	5.07[c]	0.01***
L. paracasei	5.74[b]	6.03[a]	5.28[c]	0.02***
B. adolescentes	5.79[b]	6.13[a]	5.05[c]	0.01***
B. bifidum	5.72[b]	6.09[a]	5.06[c]	0.01***
B. breve	4.54[b]	5.68[a]	4.19[c]	0.02***

[abc] Means with different letters in the same column differ at P < 0.05 (Duncan 1955) ***P < 0. 001

acids between the strains could be due to the type of fermentation, because *L. acidophilus* is homofermentative, while the rest of the bacteria under study were heterofermentative (Kandler and Weiss 1986). The fermentation patterns of the carbohydrates are specific for each microorganism (Poolman 1993 and Sghir *et al*. 1998).

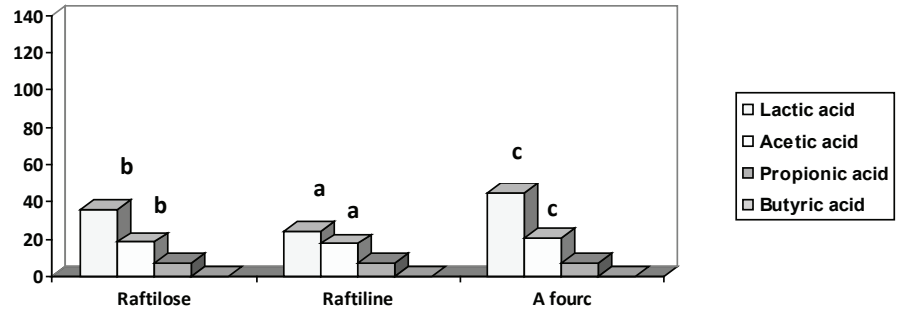
The largest productions of acids in the medium wit

(a) Concentration (mmol L^{-1})



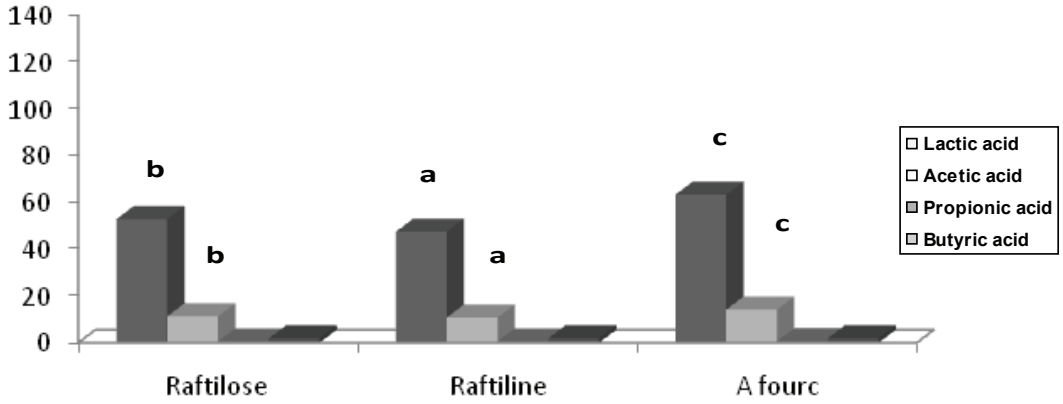
[abc] Columns with different letters differ at $P < 0.05$ (Duncan 1955)
Lactic acid $P < 0.01$ SE ± 1.45

(b) Concentration (mmol L^{-1})



[abc] Columns with different letters differ at $P < 0.05$ (Duncan 1955)
Lactic acid $P < 0.05$ SE ± 0.52; acetic acid $P < 0.05$ SE ± 0.40

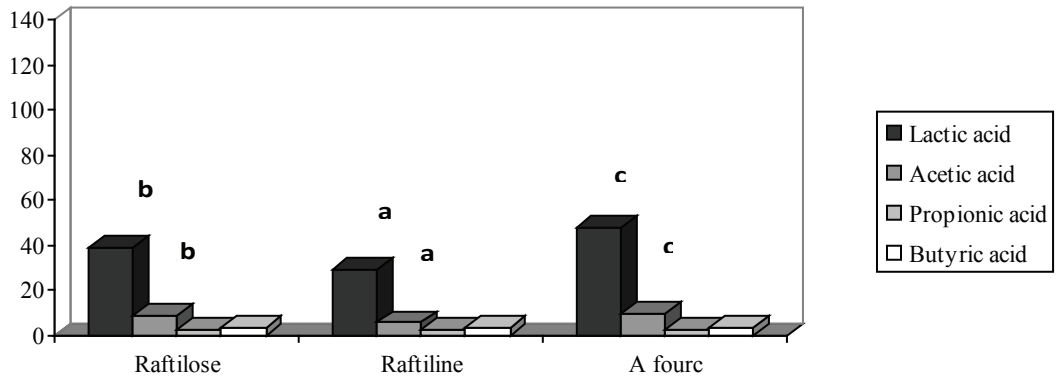
(c) Concentration (mmol L^{-1})



[abc] Columns with different letters differ at $P < 0.05$ (Duncan 1955)
Lactic acid $P < 0.05$ SE ± 0.75; acetic acid $P < 0.05$ SE ± 0.35

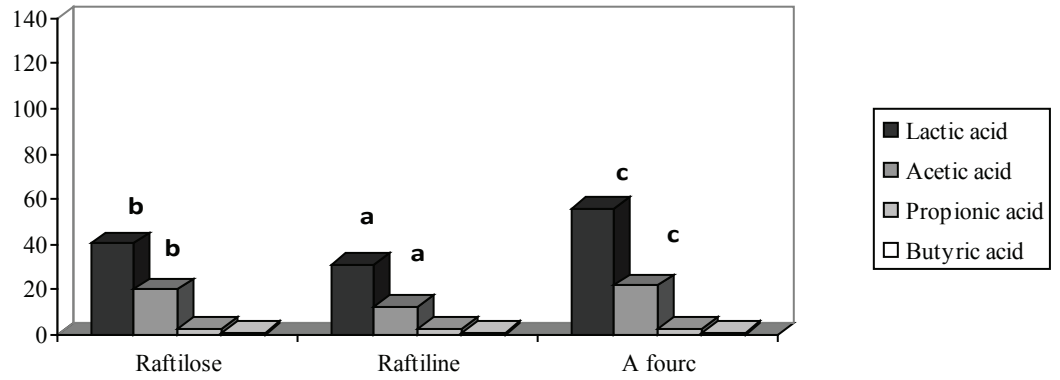
Figure 3. Production of organic acids by *Lactobacillus acidophilus* (a), *Lactobacillus casei* (b) and *Lactobacillus paracasei* (c) in MRS medium with Raftilose P95, Raftiline HP and the dried extract of *Agave fourcroydes*

(a) Concentration (mmol.L^{-1})



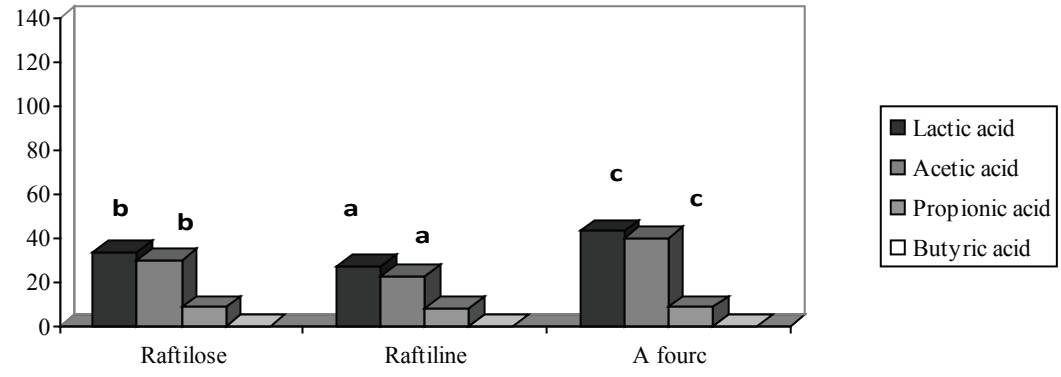
[abc] Columns with different letters differ at P < 0. 05 (Duncan 1955)
Lactic acid P < 0.05 SE ± 0.50; acetic acid P < 0. 05 SE ± 0.24

b) Concentration (mmol.L^{-1})



[abc]Columns with different letters differ at P < 0.05 (Duncan 1955)
Lactic acid P < 0.05, SE ± 0.67; acetic acid P < 0.05, SE ± 0.35

(c) Concentration (mmol.L^{-1})



[abc] Columns with different letters differ at P < 0.05 (Duncan 1955)
Lactic acid P < 0.05 SE ± 0.22; acetic acid P < 0.05 SE ± 0.18

Figure 4. Production of organic acids by *Bifidobacterium adolescentes* (a), *Bifidobacterium bifidum* (b) and *Bifidobacterium breve* (c) in MRS medium with Raftilose P95, Raftiline HP and dried extract of *Agave fourcroydes*.

the dried extract of *A. fourcroydes* could be associated with the chemical and structural differences between the fructan sources under use. This propitiated that the extract, by having a mixture of structures, was better used as energy source, because it attained higher microbial growth and fermentative activity.

Marx *et al*. (2000) found increments of lactic and acetic acid, by fermenting oligofructans by different species of bifidobacteria. However, van der Mulen *et al*. (2004), when evaluating th strain of *Bifidobacterium animalus* DN 173010, they only noted rises of the acetic acid in the medium with oligofructans, and they did not find growth in the presence of inulin, with high degree of polimerization. Kneifel *et al*. (2000) and Crittenden *et al*. (2002) proved that there are differences in the patterns of fermentation of prebiotics that depend, mainly, on the structure and composition of the carbohydrates, as well as on the microbial species under study.

According to Lyons (1997), the concentrations of organic acids could favor the processes of digestion and absorption of nutrients, as well as the leubiosis of the intestinal microbial ecosystem (Lyons1997). It is determinant that the sources selected as prebiotics provoke in the benefical bacteria high capacity of production of organic acids, because it would reduce the intestinal pH. This, together with other factors, causes the inhibition of the growth of enteropathogens such as *Salmonella ssp.* (Adams 2000 and Chaveerach *et al*. 2002).

The utilization of dried extract of *A. fourcroydes* stimulates the growth and the microbial fermentation of lactobacilli and bifidobacteria, thereby making it a good prebiotic candidate to be used in animal feeding. Besides, further works could be generated in the obtainment of new functional feeds, such as the symbiotics, potentiating the effects.

Acknowledgments

Thanks are due to the Research Center and Advanced Studies of the IPN, Guanajuato Campus, by providing the facilities to develop the experiments.

References

Adams, C. 2000. Asistencia con Resistencia. Avicultura y ácidos en la dieta. Rev. de Alimentos Balanceados para Animales. marzo-abril. p. 14

Al-Tamimi, M., Palframan, R., Cooper, J.M., Gibson, G. & Rastall, R. 2006. *In vitro* fermentation of sugar beet arabinan and arabinooligosaccharides by the human gut microflora. J. Appl. Microbiol. 100: 407

Belzarini, M.G., Casanoves, F., Di Rienzo, J., González, L. & Robledo, C. 2001. INFOSTAT, Versión 1.0. Universidad Nacional de Córdoba. Argentina

Carro, M.D. & Ranilla, M.J. 2002. Aditivos antibióticos promotores de crecimiento de los animales: Situación actual y posibles alternativas. Exopol. Circular 90, 7 pp.

Chaveerach, P., Keuzenkamp, D., Lipman, L. & van Knapen, F. 2004. Effect of organic acids in drinking water for young broilers on Campylobacter infection, volatile fatty acid production, gut microflora and histological cell changes. Poult. Sci. 86: 2466

Chuanlai, X., Xudong, Ch., Cheng, J., Qiugang, M. & Kai, H. 2005. Study of the application of fructooligosaccharides in piglets. Asian-Australasian J. Anim. Sci. 18:1011

Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerström, R., Mättö, J., Saarela, M., Mattila-Sandholm, T. & Poutanen, K. 2002. *In vitro* fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. J. Sci. Food Agric. 82:781

De Mann, J., Rogosa, M. & Sharpe, M. 1960. A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23:130

Duncan, P.B.1955. Multiple range and multiple F test. Biometrics 11:1

Ehrmann, M., Korakli, M. & Vogel, R. 2003. Identification of the gene for beta-fructofuranosidase of *Bifidobacterium lactis* DSM 10140T and characterization of the enzyme expressed in *Escherichia coli*. Curr. Microbiol. 46:391

García, Y. 2010. Obtención de fructanos a partir del *Agave fourcroydes* (Lem.),caracterización estructural y evaluación biológica como prebiótico. PhD Thesis. Instituto de Ciencia Animal, Mayabeque, Cuba. 100 pp.

García, Y., Bocourt, R., Alvelo, N. & Nuñez, O. 2007. Inulin fermentation by lactic acid bacteria with probiotic characteristics. Cuban J. Agric. Sci. 48:251

Janssens, G. & Van Loo, J. 2006. Prebiotics improve mineral metabolism. World Poult. 22:14

Kandler, O. & Weiss, N. 1986. Bergey´s manual of systematic bacteriology. Eds. Williams y Wilkin. Baltimore. Vol. 2. p. 1208

Kneifel, W., Rajal, A. & Kulbe, K.D. 2000. *In vitro* growth behaviour of probiotic bacteria in culture media with carbohydrates of prebiotic importance. Microb. Ecol. Health Dis. 12:27

Lambertus, A., Van de Broek, K. & Vorange, A. 2008. Bifidobacterium glycoside hydrolases and (potencial) prebiotics. Innov. Food Sci. Emerging Technol. 9:401

Lyons, P. 1997. Opinan los hombres de negocios. Avic. Prof. 15:22

Marx, S.P., Winkler, S. & Hartmeier, W. 2000. Metabolization of β-(2,6) linked fructose-oligosaccharides by different bifidobacteria. Federation of European Microbiological. Soc. Microbiol. Lett. 182:163

Poolman, B. 1993. Energy transduction in lactic acid bacteria. Federation of European Microbiological. Soc. Microbiol. Lett. 12:125

Probert, H.M. & Gibson, G.R. 2002. Investigating the prebiotic and gas-generating effects of selected carbohydrateson the human colonic microflora. Lett. Appl. Microbiol. 35: 473

Roberfroid, M. 2007. Prebiotics: The concept revisited. J. Nutr. 137:830

Rycroft, C.E., Jones, M.R., Gibson, G.R. & Rastall, R. 2001. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J. Appl. Microbiol. 91:878

Sghir, A., Chow, M. J. & Mackie, R. 1998. Continuous culture selection of bifidobacteria and lactobacilli from human faecal samples using fructooligosaccharide as selective substrate. J. Appl. Mirobiol. 85: 769

Urías-Silva, J. & López, M. 2004. Prebiotic effects of Agave fructans. Fifth International Fructan Symposium. Havana. Cuba. p. 73

Urias-Silvas, J. 2008. Efecto prebiótico de los fructanos de Agaves y Dasylirium y su implicación en el metabolismo de glucosa y lípidos en ratones. PhD Thesis. CINVESTAV-IPN. Unidad Irapuato, México

Van der Broek, L.A., Hinz, S., Beldman, G., Vincken, J. & Voragen, A. 2008. Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. Mol. Nutr. Food 52:146

Van der Meulen, R., Avonts, L. & De Vuyst, L. 2004. Short fractions of oligofructose are preferentially metabolized by *Bifidobacterium animalis* DN-173 010. Appl. Environ. Microbiol. 70:1923

Van der Meulen, R., Makras, L., Verbrugghe, K., Adriany, T. & De Vuyst, L. 2006. *In vitro* kinetic analisis of oligofructose consumption by Bacteroides and *Bifidobacterium ssp*. indicates different degradation mechanisms. Appl. Environ. Microbiol. 72:1006

Ventura, M. & Turroni, F. 2008. How bifidobacterial genomics could help inunderstanding probiotic-prebiotic functionality. Microbial Ecol. Health Dis. 20: 177

Wang, X. & Gibson, G. 1993. Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. J. Appl. Bacteriol. 75:373

Received: May 26, 2011