Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

*Available online at*www.jcbsc.org

Section A: Food Biotechnology

CODEN (USA): JCBPAT ResearchArticle

Changes in Intestinal Microorganisms Influenced By Agave Fructansin A Digestive Tract Simulator.

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Abstract : *Agave tequilana* Weber *var azul* fructans have potential use in dietary products and in drug delivery systems. They also have a potential use as dietary supplements because of their prebiotic properties, since them are resistant to hydrolysis by human digestive enzymes, the fructans can be used by intestinal microorganisms as a feed source by colonic microbiota. The prebiotic effect of an ingredient can be evaluated in terms of its selective effect on the growth of different bacterial groups in the human digestive tract.

The purpose of this study was to evaluate the prebiotic effect of four different fructan fractions by studying the increase of bacterial growth with beneficial effects on human health such as *Lactobacillus* and *Bifidobacterium*, and the fructan's effect in decreasing the growth of undesirable bacteria such as Clostridium. All results were compared with commercial chicory fructans (*Orafti Synergy1*), which is known to be a bifidogenic product. All experiments were done under human like physiological conditions using a human digestive tract simulator (HDTS). The four fructan fractions evaluated, hTF (DP>10), FOS (DP<10), dTF (Agave extract without minerals) and TF (total Agave extract), were set together with microbiota in HDTS, and samples were collected after day 4 and 9, the colony forming units (CFU) were measured in all samples from the different reactors that mimic the ascending colon (AC), transverse colon (TC) and descending colon (DC). The results showed, of the four fractions evaluated, the dTF fructan fraction represents the best option for use as a prebiotic because it increased the probiotic effect observed by bacteria growth (lactobacilli and bifidobacteria) and reduced the growth of undesirable bacteria (clostridia). By promoting the growth of good bacteria and decreasing the growth of undesirable bacteria, these results suggest that dTF could offer health benefits to consumers.

Keywords: fructans, probiotic, prebiotic, Lactobacillus, Bifidobacterium

INTRODUCTION

Fructans are polymers of fructose generally linked to a moiety terminal glucose. Due to the β-configuration of the anomeric C2 in their fructose monomers, fructans are resistant tohydrolysis by human digestive enzymes and can be fermented by colonic microbiota producing short chain fatty acids. In the case of agave, this produces a molecular structure composed of a complex mixture of fructans consisting of principally β(2-1) linkages but also some β(2-6) linkages. Inulin is a carbohydrate material consisting of β(2 ->1) fructosyl. An inulin molecule has a different degree of polymerization and can be degraded enzymatically or chemically down to a mixture of oligosaccharides. When inulin is orally ingested, it enters into the colon almost in complete form (>90%) and subsequently is used as a feed source by the colonic microbiota (4). Through their presence and subsequent fermentation in the large bowel, both agave fructans and inulin influence the colonic metabolism in its lumen and the integrity and function of the epithelial cell lining. Intestinal microbiota is considered to be a metabolically adaptable and rapidly renewable organ in the body. In the colon, inulin-type fructans are completely converted by the microbiota into bacterial biomass, organic acids (lactic acid), short-chain fatty acids (SCFA: acetic, propionic and butyric acid) and gasses (CO_2, H_2, CH4). SCFA and lactate contribute to the host's energy metabolism.

Prebiotics are non-digestible food ingredients that selectively stimulate growth and/or activity of a limited number of bacteria in the colon, thereby improving the host´s health.

Prebiotic properties have been demonstrated for inulin-type fructans, galactoolicosaccharides and lactulose. Fructooligosaccharides (FOS) and agave fructans represent an important source of prebiotic compounds that are widely used as ingredients in functional foods. Exploratory *in vitro* work with fecal slurries, starting in the early nineties, indicated that inulin and oligofructose are completely fermented by the colonic microbiota and selectively stimulates growth of intestinal bifidobacteria and lactobacilli. The administration of inulin and oligofructose alone or as a symbiotic product has demonstrated a selective increase in the numbers of bifidobacteria in the luminal epithelium as well as the mucosa-associated microbiota, representing a prebiotic effect[1-7].

Some studies had shown that fructans from agave stimulate the growth of *Bifidobacterium breve* and *Lactobacillus casei*. Therefore, they have a prebiotic activity[8].

As research progressed, three criteria were established and accepted as to what a food ingredient should fulfill before it can be classified as a prebiotic. First, it should be non-digestible and resistant to gastric acidity. Second, probiotics should be fermentable, and thirdly, they should, in a selective way, stimulate growth of bacteria and/or improve the metabolic activity of intestine associated with health and wellbeing[9]. Is well established prebiotic compounds currently are inulin, oligofructose (or fructo-oligosaccharides), galacto-oligosaccharides and lactulose. However, extensive research is ongoing to strengthen the scientific basis of promising new candidates.

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit onto the host[10, 11]. The normal human digestive tract contains about 400 types of probiotic bacteria, which reduce the growth of harmful bacteria and promote a healthy digestive system. Popular probiotic species that are used commercially include: *Lactobacillus paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. johnsonii*, *L.*

fermentum, *L. reuteri*, *L. plantarum*, *Bifidobacterium longum* and *B. animals* besides other bacteria. The phylogenetic differences are extremely wide between *Lactobacillus* and *Bifidobacterium* as they belong to different *phyla*, however there are also great differences between *Lactobacillus* species, since L. *acidophilus*, *L. fermentum*, *L. reuteri* and *L. plantarum* have differences in their growth patterns, affinity to different substrates and have different metabolic requirements[12].

In humans, there have been several clinical trials using treatment with probiotic of different strains/species, and has demonstrated a beneficial effect of the *Lactobacillus* and *Bifidobacterium* bacteria on the health of the host, particularly at the composition of colon[13].

Unlike the above, *Clostridium* genus bacteria are a major enteric pathogen causing a wide range of diseases from mild antibiotic-associated diarrhea (AAD) to severe pseudomembranous colitis (PMC)[14]. *C. perfringens* is one of the most common causes of foodborne illness in the United States. It is estimated that it causes nearly a million cases of foodborne illness each year. The severity of the disease depends on the strain virulence and the host response regarding the resilience of the microbiota and the immune response as well [15]. The composition of the human gut microbial community is host specific. The microbial community is continually evolving due to exogenous and endogenous modifications. The microbiota can be at the center of the cause of many diseases, which can have serious effects on all organ systems in the body. The compositional make-up of the microorganisms in the human gut is an important determinant to maintain homeostasis. However, maintaining the balance between the microorganisms that compose microbiota is very important and an unbalance of these bacteria could cause many diseases.

The most well-known food-based strategy to modulate the composition of the intestinal microbiota includes dietary prebiotics and a mixture of different probiotics taken together because their combination promotes a symbiotic effect.

Using a human digestive tract simulator (HDTS), based off of the human intestinal microbial ecosystem, allows us to study the microbial colonization process. *In vitro* multicompartment gastrointestinal simulators are often used to monitor gut microbial dynamics and activity. These reactors need to harbor a microbial community that is stable upon inoculation, colon region specific, and run under *in vivo* conditions.

The aim of this study was to evaluate the prebiotic effect of different fructan fractions by increase of bacterial growth with benefic effect (*Lactobacillus* and *Bifidobacterium*), and their negative effect by decrease of growth of undesirable bacteria (*Clostridium*) all under physiological conditions in the HDTS.

METHODS

Count of *Lactobacillus, Bifidobacterium* and *Clostridium:* Colony forming units (CFU) from bacteria of genus *Lactobacillus, Bifidobacterium* (probiotics) and *Clostridium* (undesirable bacteria) were quantified by counting in plaque. Four fructan fractions: hTF, FOS, dTF and TF, were evaluated against a control OS-1, to compare the effect of bacterial growth. The fructan fractions or control (OS-1) were added into HDTS with all three bacteria. Samples were taken at different time intervals and evaluated from all three colon segments (AC, TC, and DC).

Agave fructans: Agave juice was obtained from agave head halves that were smashed and mixed with potable water (10:6 w/v) to obtain water soluble carbohydrates suspension (WSC). The mixture was blended in a stainless steel mechanical device and stirred at 70°C for 7 h, the WSC suspension was filtered through a

80-100 mesh until a final concentration of 10-15°Brix was met The WSC was then spray dried with an inlet temperature of 90°C and an outlet temperature of 170-190°C. The powder obtained was later processed to obtain fractions.

Agave fructan fractions: The WSC from 6 year old agave plants was passed through an ion exchange column, resulting in a product without color or minerals, named the dTF fraction. Next, the dTF fraction was processed by tangential flow filtration (TFF) through a 3kDa molecular weight cut off membrane (MWCO), which separates fructans with a degree of polymerization (DP) >10 in the retentate. The retentate was named hTF. The residual fraction had fructans with DP<10, containing fructooligosaccharides (FOS), which are rich in glucose and fructose. The FOS fraction rich solution was passed through a 1 kDa MWCO to obtain a retentate of FOS without monosaccharides. The agave fructan fractions tested in this study were: hTF (DP>10), FOS (DP<10), dTF (Agave extract without minerals) and TF (total Agave extract). A commercially available prebiotic fructan from chicory roots, which has been proven by to have a bifidogenic effect, was evaluated in this study as a control, Synergy1™ (Orafti® Synergy1™ Belgium mixture of oligosaccharides and inulin), which is referred to as OS-1.

Evaluation in the human digestive tract simulator (HDTS): The HDTS was run under controlled environmental conditions (pH, residence time, inoculum, and temperature) to resemble *in vivo* conditions as defined by Molly[16]. The system consists of five double-jacketed vessels, sequentially connected simulating the stomach, small intestine, ascending, transverse and descending sections of the colon. The system has a total retention time of 72 h. The reactor were setup and the composition of the liquid feed, which the system must be fed three times per day, were previously described by Possemiers[13]. In our study, the colon reactors were inoculated with bacteria from fecal samples of 5 healthy adult male volunteers, who had no history of antibiotic treatment for 6 months prior to the study or had recent consumption of fermented foods. Aliquots (10 g) of freshly voided fecal samples were diluted and homogenized with 100 mL of sterilized phosphate buffer (0.1 mol/L, pH 7). After removal of the particulate material by centrifugation, supernatants were pooled and 50 mL were introduced into each vessel of the colon simulation system. The microbial inoculum was stabilized over a period of 2 weeks on a stabilizer medium. This allowed the vessels to adapt and equilibrate to specific conditions that mimic different segments of the colon: the ascending colon (AS), transverse colon (TC) and descending colon (DC). pH range, retention time and available carbon sources were set according to Possemiers[13]. After the stabilization period, the system was subjected to two weeks of monitoring in order to establish that the system was ready and to quantify all steady state bacterial parameters which were used as a starting point to evaluate the effect of a specific treatment[16,17]. After the two weeks required for initial stabilization and two weeks more for a control period, agave fructan fractions (20 g/L) were added separately to the food feed and digested over consecutive 9 days in the HDTS.

Microbial analyses of the HDTS: The samples were collected at day 0, 4 and 9 from the three different colon segments (AC, TC and DC reactors) of the digestive system. The samples were diluted to make decimal dilutions in peptone water, plated and incubated at 37°C per 24 h. Bacterial counts were performed using MRS media (BIOXON) for lactobacilli (24 h, microaerophilic), BSM (Sigma Aldrich) for bifidobacteria (48 h, anaerobic), and for clostridia (24 h, anaerobic) TSC (Sigma Aldrich) was used.

RESULTS

The hTF fructan fraction addition to the HDTS showed an increased effect on *Bifidobacterium* CFU in TC and DC, compared to OS-1 where an increase in *Bifidobacterium* CFU was observed in all three colon

section. The *Lactobacillus* genus showed a slight decrease in CFU in AC, TC and DC segments similar to the effect produced by the control, OS-1. Different results were obtained with the *Clostridium* genus, which showed a decrease in CFU, around one log in all segments with hTF. This is contrary to the results observed with OS-1, which showed a slight increase in CFU with hTF. Results are located in Table 1.

When the FOS fructan fraction was added to the HDTS, *Bifidobacterium* genus was maintain in CFU for AC and TC, and a slight decrease in DC was observed. The results with OS-1 showed an increase in CFU of *Bifidobacterium* in AC and TC and a decrease in DC. Similar growth patterns were observed in *Lactobacillus* genus with FOS, which showed an increase around one log in all three colon section similar to the control, OS-1. The growth of *Clostridium* genus showed a significant increase in all three colon section, which was similar to the control, as seen in Table 1.

TF fructan fractions, when added to HDTS, showed a decrease in CFU in AC, an no change in TC and a slight increase in DC for *Bifidobacterium* genus, while OS-1 showed an increase in *Bifidobacterium* CFU in AC and TC and a slight decrease in DC. *Lactobacillus* genus showed a decrease in AC and TC and an increase in DC, compared with OS-1, which showed a slight decrease in *Lactobacillus* CFU in all the three colon sections (AC, TC and DC). At the same time, *Clostridium* was decrease in all three colon sections with the TF fraction; this effect was less than with the dTF fraction, contrary to OS-1 which had an increase of CFU *Clostridium* in all three colon section (AC, TC and DC).

Table 1: CFU of*Lactobacillus*, *Bifidobacterium* and *Clostridium* with Fructans TF, dTF, FOS, hTF and OS1 in all HDTS sections and at 0, 4 and 9 days

		Day 0 (control)			Day 4			Day 9		
		AC	TC	DC	AC	TC	DC	AC	TC	DC
OS1	Bifidobacterium	3.E+03	1.E+03	1.E+04	3.E+04	4.E+04	2.E+04	7.E+04	6.E+04	7.E+03
	Lactobacillus	2.E+07	3.E+07	5.E+07	1.E+08	1.E+08	7.E+07	3.E+07	2.E+07	2.E+07
	Clostridium	5.E+06	1.E+07	1.E+07	3.E+07	2.E+07	2.E+07	3.E+07	1.E+07	1.E+07
dTF	Bifidobacterium	2.E+03	2.E+04	8.E+03	1.E+04	1.E+04	9.E+03	**3.E+04**	**4.E+04**	3.E+04
	Lactobacillus	6.E+06	1.E+06	6.E+05	**9.E+06**	**2.E+06**	8.E+05	**8.E+06**	**3.E+06**	**4.E+06**
	Clostridium	4.E+03	1.E+04	2.E+04	4.E+02	1.E+03	**1.E+03**	**5.E+01**	**3.E+02**	4.E+02
TF	*Bifidobacterium*	6.E+04	9.E+02	2.E+04	9.E+04	2.E+03	3.E+03	2.E+03	2.E+03	1.E+03
	Lactobacillus	3.E+07	2.E+07	2.E+06	1.E+08	4.E+06	7.E+06	7.E+06	7.E+06	1.E+07
	Clostridium	6.E+03	2.E+05	4.E+05	8.E+02	2.E+04	4.E+04	4.E+02	4.E+04	4.E+04
FOS	*Bifidobacterium*	6.E+04	2.E+04	1.E+04	1.E+05	2.E+04	1.E+05	5.E+04	2.E+04	2.E+03
	Lactobacillus	3.E+06	1.E+06	2.E+06	1.E+08	2.E+07	5.E+07	1.E+08	6.E+07	5.E+07
	Clostridium	5.E+05	1.E+06	2.E+05	5.E+07	1.E+07	1.E+07	1.E+08	3.E+07	1.E+07
hTF	*Bifidobacterium*	7.E+03	7.E+03	8.E+03	5.E+03	8.E+04	8.E+04	6.E+03	3.E+04	1.E+05
	Lactobacillus	1.E+08	1.E+08	6.E+07	7.E+07	7.E+07	8.E+07	2.E+07	3.E+07	2.E+07
	Clostridium	8.E+07	7.E+07	1.E+08	2.E+07	1.E+07	1.E+07	1.E+07	8.E+06	5.E+06

The dTF fructan fraction showed an increase of CFU in *Bifidobacterium* in AC similar to OS-1 and showed, in general, an increased in TC and DC segments with a slight decrease during day 4 but recovered at day 9. These results proved that dTF has a bifidogenic effect, which is compatible with the bifidogenic effect of OS-1, which is the best commercially produced bifidogenic product available. In *Lactobacillus*, CFU was maintained in AC and TC and showed a slight increase in DC, which is different than the effect seen in OS-1. In OS-1, an increase in all colon sections was observed. In contrast with the other three fructan fractions that were evaluated, dTF showed the most significant beneficial results, since the CFU of *Clostridium* genus

were remarkably reduced in all the three sections (AC, TC and DC) either by day 4 or 9. This is contrary to what was seen in OS-1 wherein *Clostridium* genus bacteria was increased in AC, TC and DC. As show in Figure 1.

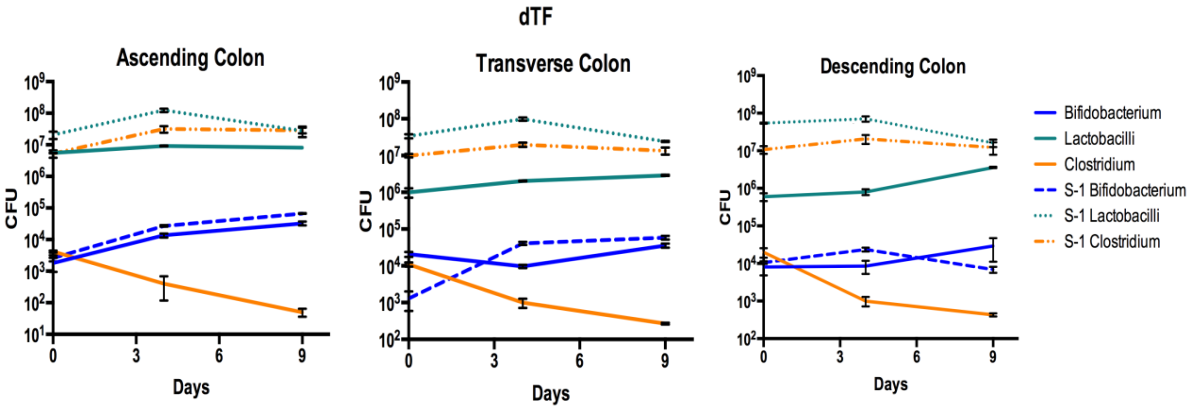

Fig.1: CFU of *Lactobacillus*, *Bifidobacterium* and *Clostridium* with the dTF fructan fraction in all HDTS sections, at 4, 7 and 9 days.

CONCLUSIONS

dTF fructan fraction represents a good option for use as a prebiotic because it increased the probiotic bacteria growth (lactobacilli and bifidobacteria) and reduced the growth of undesirable bacteria (clostridia). This combination of promoting growth of good bacteria and decreasing growth of undesirable bacteria offers as health benefits to consumers.

ACKNOWLEDGEMENTS

This work was supported by SAGARPA grant (109799). We would like to thank the Peace Corps volunteer Tina Kim Coop for reviewing the English version of this paper.

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