International Journal of Probiotics and Prebiotics Vol. 9, No. 3, pp. 69-76, 2014
ISSN 1555-1431 print, Copyright © 2014 by New Century Health Publishers, LLC
www.newcenturyhealthpublishers.com

IN VITRO PREBIOTIC ACTIVITY OF FRUCTANS WITH DIFFERENT FRUCTOSYL LINKAGE FOR SYMBIOTICS ELABORATION

[1]J. Arrizon, [2,4]A. Hernández-Moedano, [3]E. Toksoy Oner and [2]M. González-Avila

[1]CIATEJ, Unidad de Biotecnología Industrial, Guadalajara, Jal. México; [2]CIATEJ, Unidad de Biotecnología Médica y Farmacéutica, Guadalajara, Jal. México; [3]Marmara University, Department of Bio-Engineering, Istanbul, Turkey; and [4]Universidad de Guadalajara, CUCI, Departamento de Ciencias Médicas y de la Vida Ocotlán, Jal, México

[Received February 1, 2014; Accepted July 2, 2014]

ABSTRACT: *The ever growing interest in functional foods generated a drive towards developing new and effective symbiotic formulations. Considering the leading role of fructans in this market, three fructans with different fructosyl linkages were investigated for their potential use as prebiotics. For this, growth capacity of anaerobic and aerobic probiotics and pathogenic intestinal bacteria in the presence of these fructans were tested. . Enhanced growth of aerobic probiotics was observed with Agave tequilana fructans (ATF), while levan or inulin increased the growth of anaerobic probiotics. ATF and aerobic probiotics were selected for antibiotic activity. The extracellular metabolites were more effective for antibiotic activity than the direct contact probiotic-pathogenic bacteria. The in vitro effects of prebiotics were found to depend on the type of probiotic. The probiotic extracellular metabolites induced by ATF showed the highest antibiotic activity. This in vitro study clearly showed the importance of selecting the appropriate prebiotics and probiotics for the effective formulation of symbiotics with antibiotic activity.*

KEY WORDS: Antibiotic Activity, Fructosyl Linkage, Prebiotic, Probiotic, Symbiotic

Corresponding Author: Dr. Marisela González Avila, CIATEJ, Unidad de Biotecnología Médica y Farmacéutica, Guadalajara, Jal. México; FAX: +52 33 33455245; E-mail: mgavila@ciatej.mx

INTRODUCTION

Human gut microbiota is known to play an important role in maintaining intestinal and immune system homeostasis (for a recent review, Xu et al., 2013). Since abnormalities in its composition (also known as dysbiosis) are associated with diseases such as inflammatory bowel disease, colon cancer, colitis, obesity and cardiovascular diseases, significant effort is put into preventing dysbiosis by understanding the main causes for it and by developing strategies for better health (Koropatkin et al., 2012). One such strategy is the rational design of symbiotics by combining beneficiary non-digestible food ingredients (prebiotics) with appropriate probiotics that are health-promoting microorganisms (Rastall and Maitin, 2002; Gibson et al. 2004). With the increased consumer awareness, the market for functional foods and symbiotics is growing at a fast pace and fructans are playing the leading role due to their long tradition as prebiotics (Van den Ende et al., 2013; Van den Ende et al., 2011).

Chicory fructan is one of the most popular prebiotic, which is a β (2-1) fructosyl linkages linear fructan with a terminal glucose unit and when it reaches the caeco-colon intact, it is degraded by select groups of bacteria. Some of these microorganisms form part of the healthier microbiota composition (probiotics), obtained when population of *Firmicutes*, *Lactobacilli* and *Bacilli* increase in the gut and their metabolism can positively affect the host biochemically, physiolocally or immunologically, minimizing the risk of many diseases (Urías-Silvas et al. 2008; Angelakis and Raoult 2010).

Recent reports indicate that fructans from other sources like microbial levan and agave have potential prebiotic effect (Bello et al., 2001; Kang et al., 2009; Gomez et al. 2010). Microbial levan is a high molecular weight linear fructan with β (2-6) fructosyl linkages and occasional β (1-2) branches and it is produced as an exopolysaccharide (EPS) by a wide variety of microorganisms. Biological activity of levan is known to change with the size and degree of branching which in turn is largely determined by the microbial system and the production conditions (Kang et al., 2009). Bello et al. (2001) studied the *in vitro* fermentation properties of commercial levan produced by *Erwinia herbicola* and the levan-type EPSs of *Lactobacillus sanfranciscensis* and only the latter favored the growth of the bifidobacteria. The lack of bifidogenic activity of levan

by *Erwinia* is attributed to the differences in their chemical structure. *In vivo* hypolipidemic effects including anti-obesity and lipid-lowering activities were also reported for the dietary levan from *Zymomonas mobilis* (Kang et al., 2006). Absence of levan in the faeces suggested its partial hydrolysis by the gastric juice followed by its complete fermentation by lumen bacteria in the ceacum and colon (Kang et al., 2009).

Despite the vast literature on the prebiotic activity of fructooligosaccharides (FOS) and inulin-type fructans, there are only a very limited number of reports on microbial levans and the identity of levan-fermenting bacteria. In the case of *A. tequilana* fructans (ATF), they are extracted from *A. tequilana* plants harvested before flowering, when the highest fructan content is reached and concentrated in the heart or stem of the plant, normally called "piña" (Wang and Nobel 1998; López et al. 2003; Waleckx et al. 2008; Arrizon et al. 2010). ATF is composed of a mixture of branched complex molecules with β (2-1) and β (2-6) linkages with an internal glucose unit (Lopez et al 2003). The proportion of both linkages in ATF varies with the *Agave* species and region of cultivation (Manicilla-Margalli and Lopez 2006) as well as maturation of the plant (Arrizon et al. 2010). ATF is principally used in Mexico as raw material for the production of tequila, which is produced in selected regions according with the Mexican Official Norm NOM-006-SCFI-1994. Nevertheless, a huge quantity of *A. tequilana* is cultivated out of the protected region that is a problem for agave producers as they can't be used for the tequila industry (Pinal et al. 2009). Therefore, the diversification of ATF utilization is urgent in Mexico for social and economical concerns. In fact, it has been found that ATF have potential in dietary products as well as in health prophylactic treatment against steatosis, glycaemia and enteric infections due to their antiobesity effect and technological properties as fat substitute or as texture improver (Urías-Silvas et al. 2008; Marquez-Aguirre et al. 2013), as well as their prebiotic effect comparable with inulin (Gomez et al. 2010). Thus, ATF could be utilized in the symbiotic formulations at the functional food industry. For this, the antibiotic activity of probiotics against pathogen bacteria have to be evaluated, which also depends on the type of probiotic (Saulnier et al. 2009). Many species have been reported as probiotics. Some lactic acid bacteria like *Lactobacillus delbrueckii* ssp *bulgaricus* have clearly demonstrated improved host digestion and enhanced host immune response (Guglielmotti et al. 2007).

Besides lactic acid bacteria, *Bacillus* species have been used as probiotics for at least 50 years in commercial products like Enterogermina®, therefore scientific interest in this genus of probiotics has occurred in the last 15 years and the species that have been most extensively examined are *Bacillus subtilis, Bacillus clause, Bacillus cereus, Bacillus coagulans* and *Bacillus licheniformis. Bacillus* species are advantageous over non-spore forming bacteria such as *Lactobacillus spp.* since the spore is capable of surviving the low pH of the gastric barrier (Cutting 2011). As can be seen, for the elaboration of symbiotics, the type of prebiotic and probiotic is important. Since ATF contains β (2-1) and β (2-6) linkages, and it is a mixture of branched molecules, it could be interesting to compare the prebiotic effect of ATF to those of linear fructans with only one type of fructosyl linkage, like inulin and levan with β (2-1) or β (2-6) fructosyl linkages, respectively. Thus, in this work, an evaluation of prebiotic effect of fructans with different fructosyl linkages was carried out, as well as the antibiotic activity of different probiotics in order to select potential combinations for their use in symbiotics formulations.

MATERIAL AND METHODS

Microorganisms

Six strains of probiotic microorganisms (*Lactobacillus acidophilus* LAFTI® L10, *L. rhamnosus* ATCC SD5675, *Lactobacillus casei, Bifidobacterium lactis, B. adolescentis* and *Saccharomyces boulardii*) and three pathogenic microorganisms (*Clostridium spp, Salmonella typhimurium* and *Listeria monocytogenes*) from CIATEJ collection were used.

Carbon sources

Microbial levan was produced by halophilic *Halomonas smyrnensis* sp. AAD6^T cultures (Poli *et al.*, 2013) under optimized conditions and purified as described in Poli *et al* (2009). Inulin and sucrose were acquired from SIGMA Aldrich, and *Agave tequilana* fructans were extracted in CIATEJ according to Arrizon et al (2010).

Maintenance culture media

MRS Medium (Man-Rogosa-Sharpe) BD (BIOXON^R) was used for acidolactic microorganism from genus *Lactobacillus* and *Saccharomyces boulardii.* For bacteria of the genus *Bifidobacterium*, MRS medium was supplemented with cysteine. From the pathogenic microorganisms, whereas *Salmonella typhimurium* and *Listeria monocytogenes* were cultivated in nutritive medium BD (BIOXON^R), *Clostridium spp* bacteria were cultivated in TSC (Tryptose Sulphite Cycloserine) medium (SIGMA Aldrich).

Prebiotic assay

Precultures were prepared by inoculating an aliquot of 5 mL containing 1 x 10^6 cells of each bacterium stock into 50 mL of their respective maintenance culture media and incubating at 37°C for 24 h. An appropriate modified medium was prepared for each bacterium, substituting glucose with other carbon sources (levan, ATF or inulin) as well as with 20 g/L sucrose as control. 200 µL of each modified medium and the control were transferred to a 96-well micro-plate in quadruplicate, and then inoculated in triplicates with 1.2 x10^6 CFU of precultures, leaving one blank per assessed sample. Optical densities (OD) were measured every hour up to 12 h using a micro-plate reader (X-Mark Microplate Absorbance Spectrophotometer,

Bio-rad, California, USA) at 490 nm, 37°C and shaken in the micro-plate before every measurement for 10 s at high speed in the reader, depending of the microorganism aerobic or anaerobic conditions (growth under a CO_2 atmosphere) were used. Bacterial growth experimental data were adjusted to an exponential model (Equation 1), using Origin 7.0 SR0 (Microsoft):

$$y(t) = a \exp^{(bt)}$$

Where y is the number of cells (N_t in CFU/mL), a is the initial value (N_0 in CFU/mL), b is the growth rate (in h^{-1}), and t is the time (in h).

Growth inhibition test

The growth inhibition test against pathogenic bacteria was carried out with four selected probiotics according to Kirby-Bauer (1960) where each probiotic and pathogenic bacterium was grown in its respective maintenance culture media at 37 °C for 16 hours under aerobic or anaerobic conditions depending on the microorganism. Then, 1 x 10^6 CFU in 100 µL of each pathogenic bacteria was homogeneously spread on nutritive agar in Petri dishes that were previously marked in 8 sections with the same area. After drying the agar under sterile air for 5 minutes, a hole with diameter of 6 mm was made in each section then inoculated with 1 x 10^9 cells that were grown with ATF as carbon source. In this way, in each Petri dish, the four probiotics were tested in duplicate. For each pathogenic bacterium, three Petri dishes were evaluated and all of them were incubated (37 ° C, 24 h). Then, the growth inhibition halo was recorded.

Antibiotic activity of probiotic extracellular metabolites

The two bacteria *L. casei* and *L. rhamnosus* were selected for this test. Two mL of stock culture were inoculated in 250 mL of MRS media with ATF as carbon source and it was incubated at 37 °C for 16 h and then kept at 4 °C. For the recovery of the probiotic extracellular metabolites (PEM), one mL culture was centrifuged (5000 rpm, 5 min, 4 °C), the supernatant was filtered with a 0,45 µm membrane , and then dried in a speedvac device for 8 hours. An inoculum of 100 µL containing 1 x 10^6 CFU of each pathogenic bacteria was spread on Petri dishes previously marked in 8 sections, they were dried under sterile air during 5 minutes. In each Petri dish section, a hole with 6 mm in diameter was made for the addition of 30 µL of a PEM solution at different concentrations (33.3, 166.5, 333 mg/mL) for each probiotic and streptomycin was used as a control (1 mg/mL). All the experiments were carried out in triplicates for each pathogenic bacteria. All the Petri dishes were incubated at 37 ° C during 24 h. Then, the growth inhibition halo was recorded.

Statistical analysis

The program Graph Pad Prism version 5 (GraphPad Software Inc., USA) was used for ANOVA tests with a significance level (p-value) of 0.05.

RESULTS

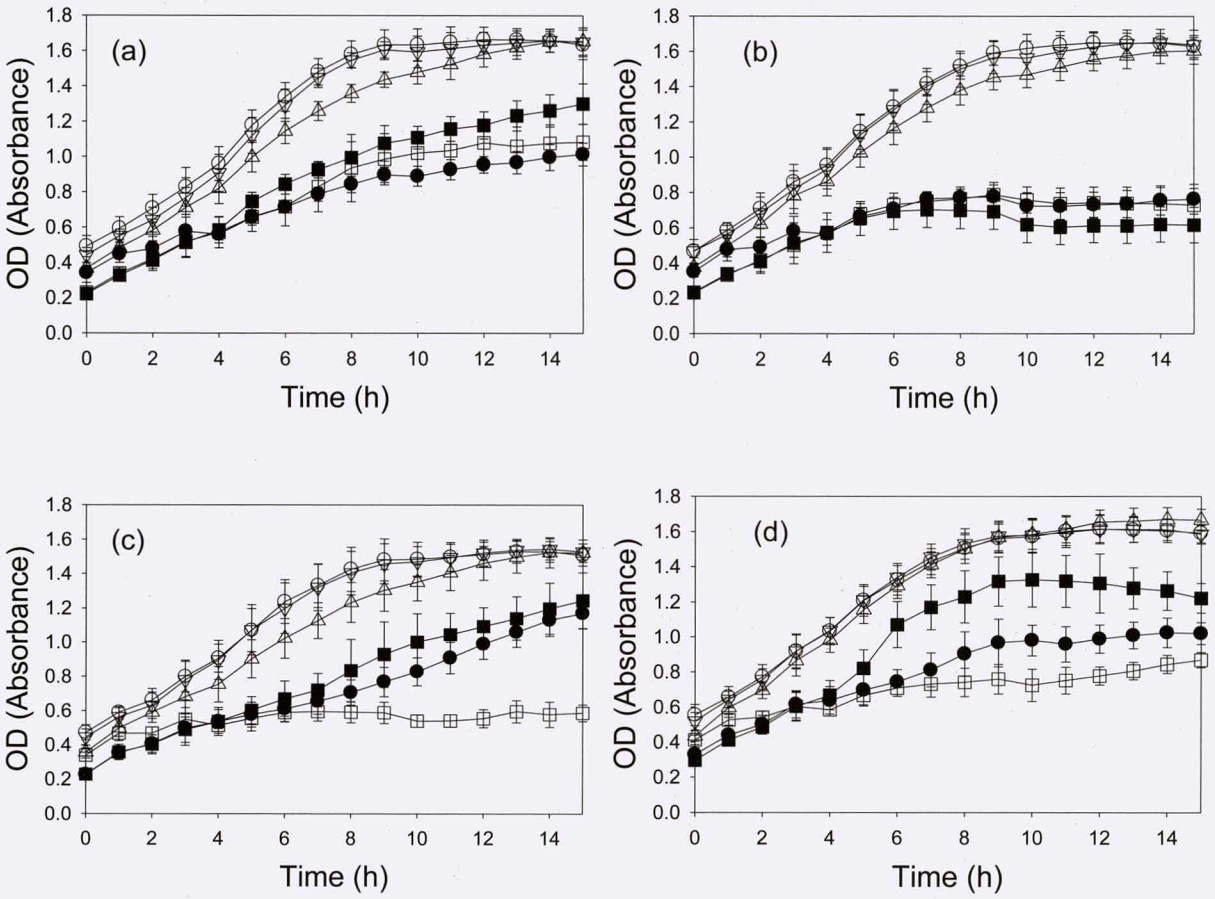
In vitro evaluation of different prebiotics on the growth capacity of probiotics and intestinal pathogenic bacteria.

The growth capacity of probiotic and intestinal pathogenic microorganisms varied as a function of the fructan type evaluated, the differences observed depended also if they were aerobic or anaerobic microorganisms. In general for sucrose and all the fructans tested, the three aerobic probiotics; *Saccharomyces boulardii*, *Lactobacillus rhamnosus* and *Lactobacillus casei,* showed the highest growth (Figure 1), while two anaerobic probiotics (*Bifidobacterium lactis* and *Bifidobacterium adolescentis*) and one anaerobic pathogenic bacteria (*Clostridium* spp) exhibited the lowest growth capacity in all the substrates (Figure 2). Analyzing the effect of each fructan in aerobic and anaerobic microorganisms, it was observed that *Agave tequilana* fructans (ATF) promoted a higher growth of the aerobic probiotic microorganisms *S. boulardii*, *L. rhamnosus*, and *L. casei* than the intestinal pathogenic microorganisms *Salmonella typhimurium* and *Listeria monocytogenes*, *Lactobacillus acidophilus* was the exception as it showed the lowest growth capacity (Figure 1 b). In the case of anaerobic microorganisms, ATF increased the growth of the pathogenic bacteria *Clostridium* spp but not for the probiotics *B. lactis* and *B. adolescentis* (Figure 2b). Inulin was also effective for the growth of the aerobic probiotics *S. boulardii*, *L. rhamnosus* and *L. casei*, nevertheless the growth of aerobic pathogenic bacteria like *S. typhimurium* and *L. monocytogenes* was higher than ATF (Figure 1c). Contrary to the behavior observed for the anaerobic microorganisms in ATF, inulin suppressed the growth of *Clostridium* spp during the first 8 hours (OD less than 0.2), while a slighter increase of *B. adolescentis* was observed, *Clostridium* spp and *B. lactis* started to grow after 10 hours (Figure 2c). For aerobic microorganisms using levan as prebiotic, the probiotics *S. boulardii*, *L. rhamnosus*, and *L. casei* showed the highest growth followed by the pathogenic bacteria *S. typhimurium*, while a lower growth was observed for *L. monocytogenes*. In the case of aerobic probiotics, for all the substrates tested, *Lactobacillus acidophilus* showed the lowest growth capacity (Figure 1). In the case of the anaerobic microorganism, levan caused a slighter growth of *B. adolescentis*, while *Clostridium* spp and *B. lactis* were suppressed during the first 8-10 hours (Figure 2d). It can be noticed that for all the prebiotics tested including sucrose as control, the aerobic microorganisms reached higher growth values than the anaerobic microorganisms. From the three prebiotics tested, it can be observed that for the growth capacity of aerobic probiotics ATF was the most effective and suppressed the growth of the two pathogenic aerobic bacteria while inulin and levan were not effective. In the case of anaerobic probiotics, inulin and levan were more effective than ATF, in particular with the probiotic *B. adolescentis.* Therefore, in order to evaluate the growth inhibition on pathogenic bacteria only aerobic probiotics were selected using ATF as the prebiotic.

TABLE 1. Growth inhibition halo (mm) on pathogenic bacteria by cells and extracellular metabolites of probiotics. *PEM: probiotic extracellular metabolites, §PC: probiotic cells.

Probiotics	Pathogen					
	Listeria monocytogenes		*Salmonella typhimurium*		*Staphylococcus aureus*	
	PEM*	PC§	PEM*	PC§	PEM*	PC§
Lactobacillus rhamnosus	12.2 ± 1.0	11.7 ± 1.8	15.3 ± 1.5	16.3 ± 5.5	13.7 ± 1.2	12.0 ± 2.3
Lactobacillus casei	11.9 ± 0.1	10.9 ± 0.5	15.2 ± 0.5	14.7 ± 2.0	12.3 ± 0.6	-
Lactobacillus Acidophilus	10.5 ± 1.6	-	12.0 ± 3.0	9.5 ± 1.5	12.0 ± 1.4	-
Saccharomyces boulardii	12.5 ± 4.2	10.5 ± 4.0	-	10 ± 3.2	-	-

FIGURE 1. Effect of carbon source on growth of aerobic probiotics. (a) sucrose as a control, (b) *Agave tequilana* fructans, (c) inulin and (d) levan. *Lactobacillus acidophilus* (□), *Lactobacillus casei*(○), *Lactobacillus rhamnosus* (▽), *Saccharomyces boulardii* (Δ) and aerobic pathogenic bacteria; *Salmonella typhimurium* (■) and *Lysteria monocytogenes*(●).



Growth inhibition and antibiotic activity against pathogenic bacteria by probiotics

Two methods were tested to measure growth inhibition of probiotics against pathogenic bacteria, namely, the probiotic extracellular metabolites (PEM) and the direct contact with probiotic cells (PC). There was a difference in function of the method tested, in general the PEM were more effective than the PC (Table 1), which could be caused by the quantity of active metabolites secreted by each bacterial strain. The two probiotics *L. rhamnosus* and *L. casei* showed the highest growth inhibition on three pathogenic bacteria (*L. monocytogenes*, *S. typhimurium* and *S. aurous*), while *L. acidophilus* and *S. boulardii* were less effective for growth inhibition of pathogenic bacteria (Table 1). According with these results, the extracellular metabolites produced by *L. rhamnosus* and *L. casei* with ATF as carbon source were selected to evaluate their antibiotic

activity compared to the streptomycin (antibiotic of wide spectrum). From the three PEM concentrations tested, at 33.3 mg/ml there wasn't any antibiotic effect for the two probiotics *L. rhamnosus* and *L. casei* (Figure 3a and b, respectively), only at the PEM concentrations of 166.5 and 333.3 mg/ml, there was a similar antibiotic activity against the pathogenic bacteria for both probiotics, this activity was comparable to streptomycin (Figure 3). In the case of *L. rhamnosus* (Figure 3a), the antibiotic activity was closer to streptomycin than *L. casei* (Figure 3b). Thus, this microorganism is the best probiotic against pathogenic bacteria with ATF as prebiotic.

DISCUSSION

The effectiveness of the different prebiotics tested was dependent on the oxygen and growth requirements of the

FIGURE 2. Effect of carbon source on growth of anaerobic probiotics. (a) sucrose as a control, (b) *Agave tequilana* fructans, (c) inulin and (d) levan. *Bifidobacterium lactis* (○) and *Bifidobacterium adolescentis* (□) and one anaerobic pathogenic bacteria, *Clostridium* spp(▼).

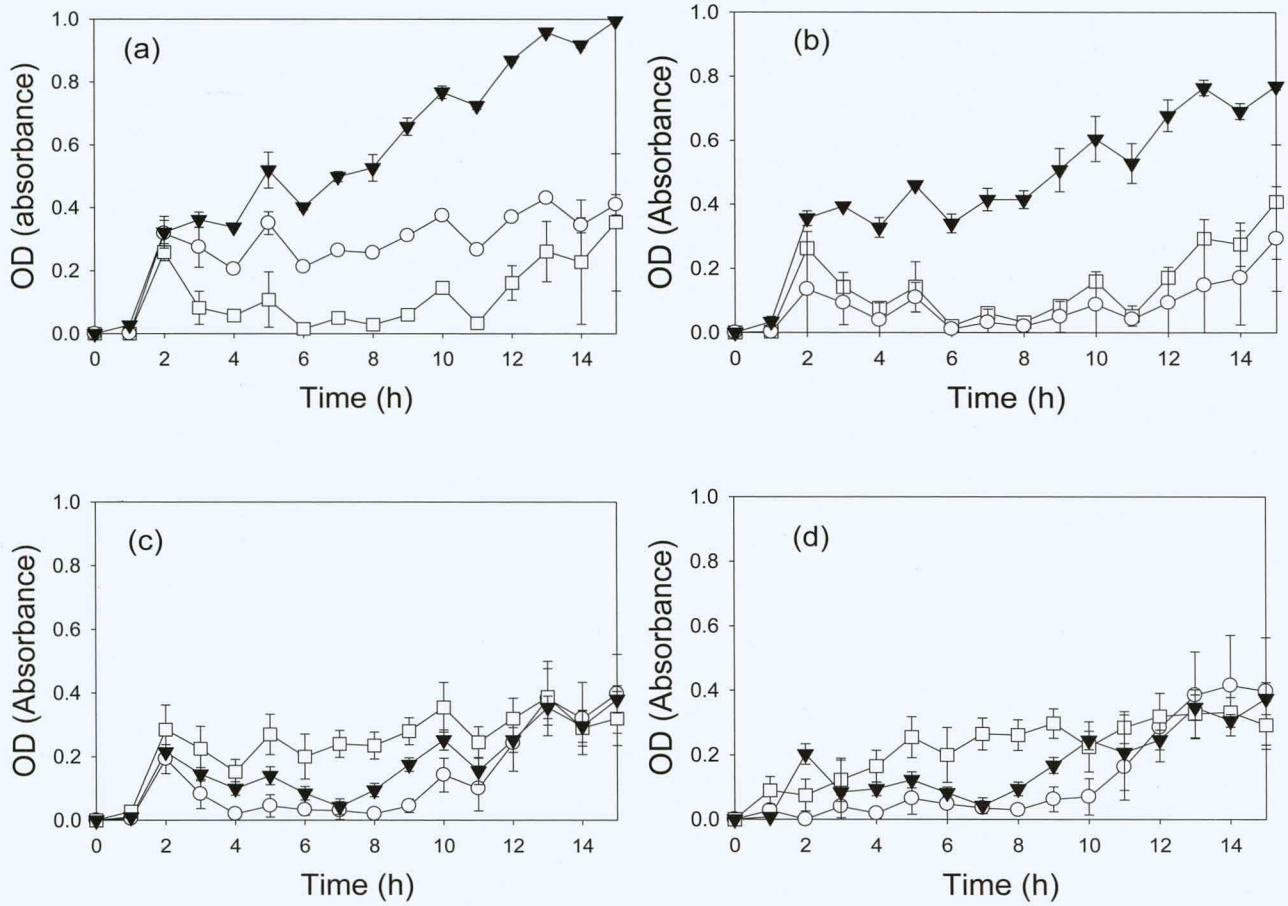
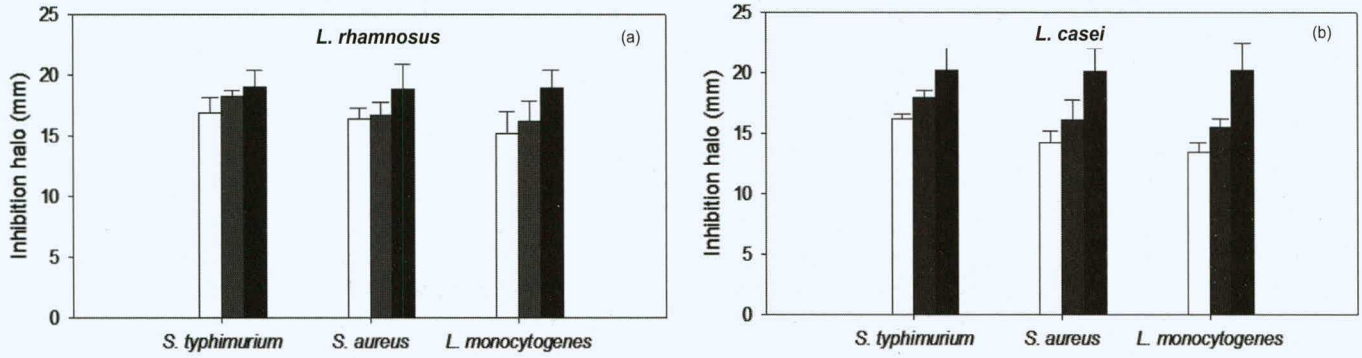


FIGURE 3. Evaluation of antibiotic activity of probiotic extracellular metabolites (PEM) from *Lactobacillus rhamnosus* (a) and *Lactobacillus casei* (b) against *Salmonella typhimurium*, *Staphylococcus aureus* and *Lysteria monocytogenes* at different concentrations of PEM: 166.5 (□) and 333 (■) mg/ml versus streptomycin 1 mg/ml as a control (■). There was no antibiotic activity at PEM concentration of 33.3 mg/ml.



probiotics, which in turn is directly related with the enzymatic capacity as well as metabolism of probiotics, in particular with the secretion of fructanhydrolases. ATF is known to be hydrolyzed better by exo-fructanhydrolases than endo-fructanhydrolases because of their branched structure (Lopez et al. 2003; Arrizon et al. 2010; Muñoz-Gutierrez et al. 2009; Arrizon et al. 2011). Therefore, the aerobic probiotics *L. rhamnosus*, *L. casei* and *S. boulardii* could be producing a higher quantity of exo-fructanhydrolases while the anaerobic probiotics such as *B. adolescentis* could be producing a mixture of exo and endo-inulinases as well as exo and endo-levanases for the efficient hydrolysis of inulin and levan, respectively. In the case of pathogenic bacteria, it could be possible that *S. typhimurium* and *L. monocytogenes* secreted in aerobic conditions exo and endo-inulinases with inulin as substrate, while levan could induce the production of exo and endo-levanases for the hydrolysis of levan. In the case of the anaerobic pathogen *Clostridium,* exo-fructanases could be excreted to the medium under anaerobic conditions with ATF as substrate.

The behavior of *L. acidophilus* was completely different than *L. rhamnosus* and *L. casei*, as this facultative bacteria can grow in strict anaerobic conditions (Saad et al. 2013) and most probably, under aerobic conditions, it could not excrete fructanases effectively. The growth of probiotics was more efficient in aerobic conditions than in anaerobic conditions, this could be related to the energy produced in the presence of oxygen with high ATP and NAD^+ levels (Stryer 1995). In aerobic conditions, ATF was the most effective prebiotic, which is a short branched fructan with β (2→1) andβ (2→6) fructosyl linkages (Lopez et al. 2003; Mancilla-Margalli and Lopez 2006, Arrizon et al. 2010). This substrate was compared with inulin and levan, which are linear and longer fructans with only β (2→1) andβ (2→6) fructosyl linkages, respectively (Saad et al. 2013; Poli et al. 2009). Thus fructosyl linkage type and molecular size of fructans affect their effectiveness, as has been observed in other works with linear β (2→1) fructans and branched fructans with different size (Coudray et al. 2003; van de Wiele et al. 2006; Gomez et al. 2010).

This is the first time that levan produced by an extremophilic halophile Halomonas sp is evaluated as prebiotic in comparison with ATF and inulin. As the digestive system at the colon functions in semiaerobic microaerobic conditions (Saulnier et al. 2009), the effect of prebiotics in anaerobic conditions has to be considered for applications with the evaluated probiotics. Thus, inulin or levan can be used, as these fructans were more effective in anaerobic conditions than ATF. Therefore, effective formulations could contain ATF in combination with inulin or levan. For the inhibitory effect of the different probiotics against pathogenic bacteria with ATF as the prebiotic, three pathogenic bacteria were used, *L. monocytogenes*, *S. typhimurium*, *S. aureus*, which cause common intestinal infections in Mexico and worldwide. It was observed that the probiotic extracellular metabolites were more effective than the direct contact probiotic-pathogen, at the experimental conditions tested (*in vitro* evaluation).

It is known that probiotics have different mechanism of action against pathogenic bacteria, most of them require the interaction with epithelial cells of the host intestine such as i) promoting mucin production to avoid permeability and penetration of pathogenic microorganisms as well as toxins ii) adhesion to host cells by surface proteins of probiotics to obstruct the pathogen adherence, iii) stimulating the immune response by increasing the mucosal antibody production, by boosting pro-inflammatory cytokine expression and enhancing host defensin production (Saulnier et al. 2009). Other mechanisms of probiosis doesn´t involve the interaction with host intestine cells, such as the secretion of extracellular metabolites like short organic acids (butyric, acetic and lactic acids) and inhibitory peptides (lantibiotics, bacteriocins and bacteriolysins) as well as H_2O_2production (Saulnier et al. 2009; Saad et al. 2013). This could explain why PEM was more effective than PC in Table 1, as some mechanisms of probiotic action need the interaction with the host intestine cells. In this work, only *in vitro* experiments were carried out. Therefore, future *in vivo* evaluation or clinical studies have to be performed. From the probiotics evaluated *L. rhamnosus* and *L. casei* showed the highest inhibitory effect against the three pathogenic bacteria tested with ATF as the prebiotic used.

It has been found that, in general lactic acid bacteria produce principally lactic and acetic acids, some of them have surface proteins to obstruct the adhesion of pathogens (*Lactobacillus crispatus* and *Lactobacillus helveticus* for example) and other secreted peptides such as reuterin by *Lactobacillus reuteri* (Saulnier et al. 2009). Thus, it could be possible that some of these compounds have been secreted by *L. rhamnosus* and *L. casei* and inhibited the pathogenic bacteria. In addition, *L. rhamnosus* and *L. caseishirota* increased the cytotoxic activity of NK cells, which is related with anti-infectious or anticancer properties of probiotics (Gill et al. 2001; Takeda et al. 2006), these two probiotics have a wide spectrum of health benefits, in particular *L. rhamnosus* (Saad et al. 2013). Therefore, it could be interesting to go into a more in dept characterization of health properties of *L. rhamnosus* with ATF as prebiotic *in vivo*. In order to test the effective concentration range of PEM of *L. rhamnosus* and *L. casei*, different PEM concentrations were tested on pathogenic bacteria and compared against a conventional wide spectrum antibiotic (Figure 3). It can be observed that PEM of *L. rhamnosus* (Figure 3a) showed a stronger effect than *L. casei* (Figure 3b), for both probiotics the PEM concentrations of 166 and 333.3 were closer to the effect of streptomycin, thus 166 mg/ml can be used for *L. rhamnosus* formulations as a natural antibiotic.

These results confirm the inhibitory capacity of *L. rhamnosus* with ATF as prebiotic, which can be used against common intestinal pathogen bacteria in Mexico. It is also important to investigate the effect of PEM of anaerobic probiotics like *Bifidobacterium adolescentis* with levan or inulin as substrates. Therefore, to know the effectiveness of *L. rhamnosus* against

intestinal illnesses, clinical studies have to be performed with ATF as prebiotic, which could be more effective in combination with other probiotics such as *B. adolescentis* and other prebiotics (inulin or levan).

ACKNOWLEDGEMENTS

The financial support of The Scientific and Technological Research Council of Turkey (TUBITAK, Project number: 111M232) is gratefully acknowledged. We also want to thanks to SAGARPA-CONACYT project 7084 and to Nereyda Bautista for technical support in some experiments.

REFERENCES

Angelakis, E. and Raoult, D. (2010) Q fever. *Veterinary Microbiology* **140**: 297-309.

Arrizon, J., Morel, S., Gschaedler, A. and Monsan, P. (2010) Comparison of the water soluble carbohydrate composition and fructan structures of *Agave tequilana* plants of different ages. *Food Chemistry* **122**: 123–130.

Arrizon, J., Morel, S., Gschaedler, A. and Monsan, P. (2011) Purification and substrate specificities of a fructanase from *Kluyveromyces marxianus* isolated from the fermentation process of Mezcal. *Bioresource Technology* **102**: 3298–3303.

Bello, F.D., Walter, J, Hertel, C. and Hammes, W.P. (2001) *In vitro* study of probiotic properties of levan-type exopolysaccharides from Lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. *Systematic and Applied. Microbiology 24*: 232–237.

Coudray, C., Tressol, J.C., Gueux, E. and Rayssiguier, Y. (2003) Effects of inulin-type fructans of different chain length and type of branching on intestinal absorption and balance of calcium and magnesium in rats. *European Journal of Nutrition* **42**: 91-98.

Cutting, S.M. (2011) *Bacillus* probiotics. *Food Microbiology* **28**: 214-220.

Gibson, G.R., Probert, H.M., Van Loo, J.A.E. and Roberfroid, M.B. (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Review* **17**: 257-259.

Gill, H.S., Rutherfurd, K.J. and Cross, M.L. (2001) Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. *Journal of Clinical Immunology* **21**: 264-271.

Gomez, E., Tuohy, K. M., Gibson, G. R., Klinder, A. and Costabile, A. (2010) In vitro evaluation of the fermentation properties and potential prebiotic activity of Agave fructans. *Journal of Applied Microbiology* **108**: 2114-2121.

Guglielmetti, S., Mora, D., Gschwender, M. and Poop, K. (2011) Randomised clinical trial: bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life-a double-blind, placebo-controlled study. *Alimentary Pharmacology & Therapeutics* **33**: 1123-1132.

Kang, S. A., Jang, K. H., Seo, J. W., Kim, K. H., Kim, Y. H., Rairakhwada, D., Seo, M., Lee, J. O., Ha, S. D., Kim, C. H. and Rhee, S. K. (2009) Levan: applications and perspectives. In: Bernd H. A. Rehm (ed) Microbial production of biopolymers and polymer precursors, Caister Academic Press, 145-161.

Kang S.A., Hong K., Jang K.-H., Kim Y.-Y., Choue R. and Lim Y. (2006) Altered mRNA expression of hepatic lipogenic enzyme and PPARα in rats fed dietary levan from *Zymomonas mobilis*. *Journal of Nutritional Biochemistry* **17**: 419-426.

Koropatkin N. M., Cameron E. A. and Martens E. C. (2012) How glycan metabolism shapes the human gut microbiota. *Nature Reviews Microbiology* **10**: 323-335.

López, M., Mancilla-Margalli, N., Mendoza-Díaz, G. (2003) Molecular structures of fructans from *Agave tequilana* Weber var. *azul*. *Journal of Agricultural and Food Chemistry* **51**: 7835-7840.

Mancilla-Margalli, N.A. and López, M.G. (2006) Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. *Journal of Agricultural and Food Chemistry* **54**: 7832-7839.

Márquez-Aguirre, A. L., Camacho-Ruiz, R. M., Arriaga-Alba, M., Padilla-Camberos E., Kirchmayr, M. R., Blasco, J. L. and González-Avila, M. (2013) Effects of *Agave tequilana* fructans with different degree of polymerization profiles on the body weight, blood lipids and count of fecal *Lacto bacilli/ Bifidobacteria* in obese mice. *Food & Function* **4**: 1237-1244.

Muñoz-Gutierrez, I., Rodríguez-Alegría, M.E. and López-Munguía, A. (2009) Kinetic behavior and specificity of b-fructosidases in the hydrolysis of plant and microbial fructans. *Process Biochemistry* **44**: 891-898.

NOM-006-SCFI-1994, 1994. Norma Oficial Mexicana. Bebidas Alcohólicas. Tequila. Especificaciones. México, D.F.: Gobierno Mexicano.

Pinal, L., Cornejo, E., Arellano, M., Herrera, E., Nuñez, L., Arrizon, J. and Gschaedler, A. (2009) Effect of *Agave tequilana* age, cultivation field location and yeast strain on tequila fermentation process. *Journal of Industrial Microbiology & Biotechnology* **36**: 655-661.

Poli, A., Nicolaus, B., Denizci, A. K., Yavuzturk, B. and Kazan, D. (2013) *Halomonas smyrnensis* sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium. *International Journal of Systematic and Evolutionary Microbiology* **63**: 10-18.

Poli, A., Kazak, H., Gurleyendag, B., Tommonaro, G., Pieretti, G., Toksoy Oner, E. and Nicolaus, B., 2009. High level synthesis of levan by a novel *Halomonas* species growing on defined media. *Carbohydrate Polymers* **78**: 651-657.

Rastall, RA and Maitin V. (2002) Prebiotics and synbiotics: towards the next generation. *Current Opinion in Biotechnology.* **13**:490-496.

Saad, N., Delattre, C., Urdaci, M., Schmitter, J.M. and Bressollier, P. (2013) An overview of the last advances in probiotic and prebiotic field. *Food Science and Technology* **50**: 1-16.

Saulnier, D.M.A., Spinler, J.K., Gibson, G.R., Versalovic, J. (2009) Mechanisms of probiosis and prebiosis : considerations for enhanced functional foods. *Current Opinion in Biotechnology* **20**: 135-141.

Stryer, L. (1995) Glicolisis. In Bioquimica ed Reverte, pp. 483-508. Madrid.

Takeda, K., Suzuky, T., Shimada, S.I., Shida, K., Nanno, M., Okumura, K. (2006) Interleukin-12 is involved in the enhancenment of human natural killer cell activity by *Lactobacillus casei Shirota. Clinical &Experimental Immunology* **146** : 109-115.

Urías-Silvas, J. E., Cani, P. D., Delmée, E., Neyrinck, A., López, M.G., Delzenne, N. M. (2008) Physiological effects of dietary fructans extracted from *Agave tequilana Gto.* and *Dasylirion spp. British Journal of Nutrition* **99**: 254-261.

Van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H., Verstraete, W. (2007) Inulin-type fructans of longer degree of polymerization exert more pronounced *in vitro* prebiotic effects. *Journal of Applied Microbiology* **102**: 452-460.

Van den Ende,W., Peshev,D., and De Gara, L. (2011) Disease prevention by natural antioxidants and prebiotics acting as ROS scavengers in the gastrointestinal tract. *Trends in Food Science & Technology* **22**: 689–697.

Van den Ende,W. (2013) Multifunctional fructans and raffinose family oligosaccharides. *Frontiers in Plant Science* **4**: 1-11.

Waleckx, E., Gschaedler, A., Colonna-Ceccaldi, B., Monsan, P. (2008) Hydrolisis of fructans from *Agave tequilana* Weber var. *azul* during the cooking step in a traditional elaboration process. *Food Chemistry* **108**: 40-48.

Wang, N. and Nobel, P.S. (1998) Phloem transport of fructans in the crassulacean acid metabolism species *Agave deserti. Plant Phisiology* **116**: 709-714.

Xu X, Xu P, Ma C, Tang J, Zhang X. (2013) Gut microbiota, host health, and polysaccharides. *Biotechnology Advances.* **31**:318-37.