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

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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

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, physiologically or immunologically, minimizing the risk of many diseases (Utrías-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 caecum 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 (Urias-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 claus, 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 snyrensis sp. AAD6 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®) 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®), 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 10⁶ 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 x10⁶ 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 CO₂ 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 10⁶ 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 10⁶ 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 10⁶ 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 1b). 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>
<thead>
<tr>
<th>Probiotics</th>
<th>Pathogen</th>
<th>Listeria monocytogenes</th>
<th>Salmonella typhimurium</th>
<th>Staphylococcus aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEM*</td>
<td>PC*</td>
<td>PEM*</td>
<td>PC*</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>12.2 ± 1.0</td>
<td>11.7 ± 1.8</td>
<td>15.3 ± 1.5</td>
<td>16.3 ± 5.5</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>11.9 ± 0.1</td>
<td>10.9 ± 0.5</td>
<td>15.2 ± 0.5</td>
<td>14.7 ± 2.0</td>
</tr>
<tr>
<td><em>Lactobacillus Acidophilus</em></td>
<td>10.5 ± 1.6</td>
<td>-</td>
<td>12.0 ± 3.0</td>
<td>9.5 ± 1.5</td>
</tr>
<tr>
<td><em>Saccharomyces boulardii</em></td>
<td>12.5 ± 4.2</td>
<td>10.5 ± 4.0</td>
<td>-</td>
<td>10 ± 3.2</td>
</tr>
</tbody>
</table>

**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. aureus*), 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 levans, 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 levans could induce the production of exo and endo-levanases for the hydrolysis of levans. In the case of the anaerobic pathogen Clostridium, exo-levanases 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) and β(2→6) fructosyl linkages (Lopez et al. 2003; Mancilla-Margalli and Lopez 2006, Arrizon et al. 2010). This substrate was compared with inulin and levans, which are linear and longer fructans with only β(2→1) and β(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 levans produced by an extremophlic 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 (Saaulnier 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 mucus 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 defenses production (Saaulnier 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 H2O2 production (Saaulnier 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 (Saaulnier 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. casei showed 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).

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