**Emerging prebiotics obtained from lemon and sugar beet byproducts:
evaluation of their *in vitro* fermentability by probiotic bacteria**

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Abstract

Pectic oligosaccharides (POS) derived from industrial byproducts, such as lemon peels (LP) and sugar beet pulp (SBP), have been reported to be non-digestible oligosaccharides and catalogued as promising candidates as prebiotics. This work has evaluated the selective fermentation of POS by potential probiotic *Lactobacillus* and *Bifidobacterium* strains. POS were fully characterized based on molecular weight and proportion of neutral and acidic oligosaccharides. All the tested probiotic strains reached better growth results when SBP POS were added to the growth media than LP POS. On the other hand, while *Lactobacillus rhamnosus* GG showed poor growth with LP POS-2, *Bifidobacterium lactis* Bb-12 was the only strain able to ferment these compounds. Lactic, acetic and formic acids were the main organic acids derived from POS fermentation by the assayed strains. The cocultures with *Bacteroides fragilis* did not modify the fermentation capability of the probiotic bacteria. This study has demonstrated that complex POS derived from lemon and sugar beet byproducts could be used as carbohydrate source by lactobacilli and bifidobacteria, showing the low-molecular-weight LP POS-2 a specific bifidogenic effect towards *B. lactis*. Results are promising for the development of synbiotic supplements.

Keywords: byproducts, pectic oligosaccharides, prebiotics, *Lactobacillus*, *Bifidobacterium*, organic acids

1. Introduction

Nowadays, different natural products have been explored due to their functional and/or health benefits, among which agroindustrial wastes could have higher economical and environmental attraction within the biorefinery concept. Particularly, both lemon and sugar beet are products widely consumed worldwide, in whose industrial processes (lemon juice and sugar manufacture, respectively) large amounts of pulp and peels with high pectin contents are generated (Martínez, Gullón, Yáñez, Alonso & Parajó, 2009; Gómez, Gullón, Yáñez, Parajó & Alonso, 2013). In this context, pectin was described as a complex heteropolymer, mainly composed of a backbone of galacturonic acid units connected by $\beta(1-4)$ links, which may be randomly acetylated and/or methylated, and that is occasionally interrupted by side chains formed by neutral sugars (Ele-Ekouna, Pau-Roblot, Courtois & Coutois, 2011). This polysaccharide could be hydrolyzed in different types of oligomers (with neutral or acidic nature) that have attracted the researcher and consumer's interest due to their potential properties on the gastrointestinal microbial ecosystem (Gullón *et al.*, 2013). In this field, pectic oligosaccharides (POS) are considered undigested compounds that can reach intact the colon and, recently, they have been catalogued as emerging prebiotics for which more studies are needed in order to prove their potential (Corzo *et al.*, 2015; Míguez, Gómez, Gullón, Gullón & Alonso, 2016).

Prebiotics are englobed in the functional foods concept, and are non-digestible compounds, which, through their fermentation by gut microbiota, promote the growth and/or activity of specific beneficial bacteria, as bifidobacteria and lactobacilli, thus conferring physiological positive effects on the host health (Bindels, Delzenne, Cani & Walter, 2015). Fructooligosaccharides and galactooligosaccharides are already commercially available prebiotics, whereas POS are still considered under-exploited and could contribute to health as new ingredients able to act on the more

distal colonic regions (where colonic diseases, such as ulcerative colitis or tumors, often prevail). Based on previous studies, the metabolism of POS results in the generation of organic acids (Gulfi, Arrigoni & Amadò, 2007; Gullón, Gullón, Sanz, Alonso & Parajó, 2011; Gómez *et al.*, 2014), called short chain fatty acids (SCFA). These SCFA can exert a number of healthy effects, such as the ability to reduce the growth of potentially pathogenic bacteria (Brandt, 2001; Jun, Lee, Song & Kim, 2006).

Gut microbiota needs collectivity and biodiversity for normal functioning (Guarner & Malagelada, 2003; Scott, Martin, Duncan & Flint, 2014). Anaerobic organisms prevail in the microbiota of adult humans in which *Bacteroides* and *Bifidobacterium* are among the predominant bacterial genera, while *Lactobacillus* populations are present in lower concentrations (Salminen *et al.*, 1998; Delgado, 2005). These microbial communities colonizing the intestine, in conjunction with the structures of the digestive tract, have important and specific metabolic, trophic and protective functions (Guarner & Malagelada, 2003; Gotteland, 2013). For instance, both bifidobacteria and lactobacilli do not produce toxins, reduce cholesterol levels and enhance the functions of the immune system (Steer, Carpenter, Tuohy & Gibson, 2000; Picard *et al.*, 2005), whilst *Bacteroides* have been reported to have both beneficial and harmful effects on host health by aiding the digestion or absorption of ingredients and, at the same time, being related to intestinal putrefaction and the generation of undesirable metabolites (Ladirat, 2014).

The functional activity of POS has been tested in some studies that used different microbial mixtures and especially fecal samples (Manderson *et al.*, 2005; Onumpai, Kolida, Bonnin & Rastall, 2011; Chen *et al.*, 2013; Gómez *et al.*, 2014; Leijdekkers *et al.*, 2014; Gómez, Yáñez, Parajó & Alonso, 2016), but it continues to be difficult to ascertain which POS are selectively fermented and which support the growth of microorganisms of interest. Therefore, selective hydrolysis, purification and characterization of POS are

essential to assign them specific biological effects. In the present work, we examine the utilization of purified POS from lemon peels and sugar beet pulp, as the sole carbohydrate source, by potential probiotic *Lactobacillus* and *Bifidobacterium* strains. We aimed to obtain information about the structure-function relationship of certain POS in order to design novel functional ingredients from natural sources to be included in effective dietary strategies for human health promotion.

2. Materials and methods

2.1. Production, purification and characterization of the studied POS.

Lemon peel wastes and sugar beet pulp samples were kindly supplied by Indulleida (Lleida, Spain) and Azucarera Ebro (La Bañeza, Spain), respectively. Samples were mixed in a single lot (one for each kind of raw material) to avoid compositional oscillations.

POS from lemon peels (denoted here as LP POS-1 and LP POS-2) were obtained by enzymatic treatment under conditions reported as optimal: previous sterilization, addition of commercial concentrate Viscozyme L in a polygalacturonase to solid ratio of 3 U/g, and reaction time of 7.5 h (for LP POS-1) or 24 h (LP POS-2). Then, both types of POS were refined by a two-step membrane process (consecutive steps of discontinuous diafiltration and concentration) using a 1 kDa regenerated cellulose membrane, following the scheme previously indicated by Gómez *et al.* (2016). Moreover, a thorough chemical characterization of these refined products was carried out by HPSEC, HPAEC, HILIC and MALDI-TOF analysis (Gómez *et al.*, 2016).

On the other hand, the production and purification of oligosaccharides from sugar beet pulp (denoted here as SBP POS) was carried out following the methodologies described by both Martínez *et al.* (2009) and Gullón, Martínez, Sanz, Alonso & Parajó (2011b). Briefly, the optimal operational conditions were polygalacturonase to solid ratio= 10 U/g, cellulase

activity to polygalacturonase activity ratio= 0.725 FPU/U, and $t = 12.82$ h. Regarding the purification, liquors from the enzymatic hydrolysis were subjected to a concentration-diafiltration process carried out at a linear velocity of 2.4 m/s and at a transmembrane pressure of 8 bar with a polymeric spiral membrane Osmonics GE2540 DESALTM (molecular weight cut-off 1 kDa).

Final purified POS were freeze-dried prior to their further utilization in the fermentation experiments. Detailed information on the experimental conditions and on the chemical composition of all the hydrolysis media are provided in the mentioned previous studies (Martínez *et al.*, 2009; Gullón *et al.*, 2011b; Gómez *et al.*, 2016).

The structural characterization of the SBP POS was performed for first time in the present work through different analytical methods:

2.1.1. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Both neutral and acidic oligomers are present in the hydrolysate and they were analysed by HPAEC-PAD using a Dionex (Sunnyvale, CA, USA) instrument equipped with a CarboPac PA-1 (4 mm × 250 mm) in combination with a CarboPac PA-1 guard column (4 mm × 50 mm) maintained at 30 °C. Standard linear arabinooligosaccharides (degrees of polymerization 2–8) and oligomers from the hydrolysis of 1% w/w pure polygalacturonic acid solution (121 °C for 40 min in media of pH adjusted to 4.4) were used to identify the retention times of the same compounds in the SBP POS sample.

The chromatogram A of the Figure 1 (containing mainly arabinooligosaccharides, AraOS) was obtained using a gradient prepared from 200 mM sodium hydroxide (eluent A) and 2 M sodium acetate in 200 mM sodium hydroxide (eluent B) according to the following sequence: 0-36 min, from 0 to 21% B; 36-42 min, 50% B; and 42-57 min, 0% B. Oligogalacturonides (or oligomers of galacturonic acid, OGaA) present in the same

stream are shown in the section B of the Figure 1 and they were detected using 1 M sodium acetate in 100 mM sodium hydroxide (A) and 100 mM sodium hydroxide (B) as mobile phases. The elution profile in this case was as follows: 0-15 min, from 0 to 5% A and from 100 to 95% B; 15-60 min, 70% A and 30% B; 60-70 min, 100% A; and 70-85 min, 100% B.

The samples were previously adjusted to a saccharide concentration of ≤ 0.1 g/L and filtered using 0.45 μ m cellulose acetate filters.

2.1.2. *High-performance size exclusion chromatography (HPSEC).*

The molecular mass distribution was determined by HPSEC using two 300 $\times$ 7.8 mm TSKGel G3000PWXL columns in series (Tosoh Bioscience, Stuttgart, Germany), in combination with a 40 $\times$ 6 mm PWX-guard column, operating at 55 °C with a Refractive Index Detector. The mobile phase was distilled water (flow rate 0.6 mL/min). Dextrans with different molecular weight (MW: 100, 50, 25, 12, 5 and 1 kDa) from Fluka (Sigma-Aldrich, Barcelona, Spain) were used as standards.

2.1.3. *MALDI-TOF/MS.*

MALDI-TOF MS analysis of the sample was performed using an Autoflex III Smartbeam workstation (Bruker Daltonics, Bremen, Germany). Samples were diluted with Millipore water at 1 mg/mL. After centrifugation for 10 min, 1 μ L of the sample solution was mixed on a MALDI plate with 1 μ L of matrix solution (25 mg/mL 2,5-dihydroxy benzoic acid, Bruker Daltonics) in 50% (v/v) acetonitrile and dried under air stream. The following conditions were used: nitrogen laser of 337 nm, positive mode and a laser power intensity of 60%. After a delayed extraction time of 350 ns, the ions were accelerated to a kinetic energy of 22 kV before detection.

2.2. *Microorganisms and culture media.*

Bacterial strains tested in this study belong to *Lactobacillus* and *Bifidobacterium* genera and are listed in Tables 1 and 2. The strains were routinely cultured in MRS broth (Pronadisa ref. 1215.00, Laboratorios Conda, Madrid, Spain) and incubated at 37 °C in a 5% CO₂ atmosphere incubator (Binder CB series, Tuttlingen, Germany) or under anaerobic conditions (GasPak EZ sachets, BD Biosciences, San Jose, CA, USA) when required. *Bacteroides fragilis* DSM2151 was grown anaerobically at 37 °C on Anaerobe basal broth (Oxoid, Basingstoke, United Kingdom).

Basic media for experiments were MRS fermentation broth (Pronadisa ref. 1288.00), which does not contain either glucose or meat extract, enriched with 8 g/L casein acid hydrolysate and 0.5 g/L L-cysteine (Broth 1). Broth 1 fortified with sodium thioglycolate (0.5 g/L), ferric pyrophosphate (0.5 g/L), haemin (0.005 g/L), vitamin K (0.001 g/L), vitamin B12 (0.01 g/L), vitamin B2 (0.001 g/L), biotin (0.001 g/L) and folic acid (0.001 g/L), and pH adjusted to 6.3, was named Broth 2. Broths 1 and 2 were supplemented with the tested oligosaccharides at a final concentration of 10 g/L. Each freeze-dried substrate was weighed and then added to the corresponding autoclaved basic media, and the mixtures were filter sterilized with 0.22-μm-pore-size sterile filters. Glucose was used as control of growth.

2.3. Evaluation of bacterial growth.

The bacterial strains were cultured overnight at 1% in MRS or Anaerobe broth, depending on the strains. The cells were harvested by centrifugation (10,000 × g for 10 min at 4 °C), washed twice with 0.85% NaCl solution, and inoculated (1%) into the basic media plus a freeze-dried sample of the refined carbohydrate. Bacterial growth was monitored by three or six replicates (depending on whether bacteria were incubated under aerobic or anaerobic conditions, respectively) in 300 μL-wells of sterile 96-well microplates with lid (Sarstedt, La Roca del Vallès, Spain). All strains were grown in

aerobic conditions at 37 °C for 48 h, with the exception of the *Bifidobacterium* strains and the combinations with *B. fragilis* that were incubated anaerobically at 37 °C. The optical densities at 600 nm (OD₆₀₀) of the strains growing aerobically were recorded at 60 min intervals with an automated microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA). Maximum growth rates ($\mu_{\max}$), maximum OD₆₀₀ (OD_{max}) and lag parameter (lag) of strains growing under aerobic conditions were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v.3.5 (available at <http://www.ifr.ac.uk/safety/DMfit/default.html>). In the case of the anaerobic strains, *B. breve* 26M2, *B. lactis* BB-12 and the combinations with *B. fragilis* DSM2151, microplates were kept at 37 °C for 48 h under anaerobic conditions. The OD₆₀₀ was recorded at the microplate reader at 0 h, 24 h and 48 h.

2.4. Measurement of organic acids in the fermented samples.

Cell-free supernatants from the fermentation assays were filtered through 0.20 μ m cellulose acetate membranes (Sartorius stedim biotech, Germany) and analysed in duplicate for organic acids (succinic, lactic, formic, acetic, propionic, and butyric) using an Agilent Technologies 1100 Series instrument with Refractive Index Detector (RID) and BioRad Aminex HPX-87H column. The separation of compounds was performed at a column temperature of 50 °C and flow rate of 0.6 mL/min.

2.5. Statistical analysis.

The statistical analysis was performed using the IBM SPSS Statistics 19 software (IBM, Chicago, IL, USA). Significant differences were determined using one-way analysis of variance (ANOVA). Duncan's test was performed to compare the mean values at a significance level of 95% ($P < 0.05$).

3. Results and discussion

208 3.1. Description of the POS enriched samples used as substrates in the fermentation
209 assays.

210 Based on previous studies (Martínez *et al.* 2009; Gullón *et al.* 2011b; Gómez *et al.*,
211 2016), mixtures of POS were obtained by enzymatic hydrolysis of natural byproducts and
212 purified by membrane filtration. Particularly, two streams enriched in POS with different
213 size were produced from lemon peels. Oligomers with molecular weight greater than 5900
214 Da were the most abundant in samples obtained at t= 7.5 h (LP POS-1), whereas longer
215 enzymatic treatment (24 h) led to hydrolysates mainly containing POS with a molecular
216 weight in the range 5900 to 738 Da (LP POS-2). Both products presented high contents of
217 oligosaccharides (83.4% and 87.5% for LP POS-1 and LP POS-2, respectively) and limited
218 contents in impurities (11.8 and 5.8% for LP POS-1 and LP POS-2, respectively). These
219 percentages are comparable with commercially available prebiotics. Moreover, the refined
220 fractions obtained from LP were characterized for a high OGalA content (> 50 kg/100 kg
221 NVC), significant amounts of AraOS (17-23 kg/100 kg NVC) and high degrees of
222 methylation (43.4% and 37.4% for LP POS-1 and LP POS-2, respectively). Meanwhile, an
223 increased degree of acetylation was observed for POS obtained in the experiment lasting
224 24 h (5.5% and 8.6% for LP POS-1 and LP POS-2, respectively).

225 HPAEC analysis revealed the presence of higher amounts of low/intermediate
226 molecular weight OGalA in LP POS-1. The opposite situation was observed for LP POS-2,
227 with larger peaks in compounds eluting after 55 min, corresponding to high molecular
228 weight OGalA. Gómez *et al.* (2016) showed in detail, in the Table 7, the product
229 distribution with saturated and unsaturated OGalA. In addition, HILIC-ELSD-MS allowed
230 to detect, as main products, partially methylesterified saturated OGalA with different
231 degrees of polymerization (DP), at least from 3 to 8. Furthermore, unsubstituted OGalA,
232 partially acetylated RG-I-derived oligomer ((Rha-GalA)₂Ac), as well as a partially

233 methylated unsaturated pentamer of GalA were also identified. The methylesterified
234 OGaLA generated from homogalacturonan fragments were also the most intense signals in
235 MALDI-TOF analysis, which agreed with the degree of methylation values. Other
236 oligosaccharides (combinations of neutral sugars and GalA containing methyl and acetyl
237 groups) were detected at lower intensities, especially in the LP POS-1.

238 On the other hand, POS from sugar beet pulp (SBP POS) showed higher AraOS content
239 (31 kg/100 kg NVC) and lower proportion of OGaLA (31 kg/ 100 kg NVC) than LP POS-1
240 and LP POS-2. Regarding the other non-volatile compounds or impurities, this product
241 also compared favourably with the commercial prebiotics with a percentage of 15%.

242 The structural features of the purified SBP POS were evaluated for the first time in this
243 study. As we expected, the HPAEC elution profile of neutral oligomers shows seven clear
244 peaks between the retention times of 11.7 and 30.9 min, which corresponds to the same
245 retention times of the standard linear AraOS with DP from 2 to 8 (Fig. 1A). This fact is in
246 accordance with the compositional data of the sample, in which AraOS represented a
247 percentage higher than 69.5% of the total neutral oligosaccharides content (galacto- and
248 glucooligosaccharides accounted for the remaining 30.3%). Among these AraOS, it is
249 remarkable the area corresponding to DP 3, whose value overcomes the sum of the other
250 six identified peaks. On the other hand, Fig. 1B shows the OGaLA that were identified
251 using a hydrolysate from pure polygalacturonic acid. Taking into account the chemical
252 composition of the sample, the amount of acidic oligomers was similar to the content in
253 neutral compounds, but the OGaLA were found in higher DP, specifically, up to DP 13. In
254 this case, the highest area corresponded to DP 2, but we have to consider that using this
255 technique the higher DP oligomers show minor response areas (the larger is the compound,
256 the peak is less appreciable). Meanwhile, MALDI-TOF MS spectra of the sample (data not
257 shown) presented higher m/z values for neutral structures made up of pentoses and hexoses

(which must be arabinose and glucose or galactose according to its composition). The longest identified arabinose-containing chain could correspond to 7 pentose and 3 hexose units with an acetyl group. In a previous research, the POS obtained by autohydrolysis of SBP showed a remarkably high degree of acetylation (37.4%), in contrast to the lower values found for LP POS (Gómez, Gullón, Yáñez, Schols & Alonso, 2016b).

Additional information about molecular weight distribution was obtained by the HPSEC response for SBP POS sample (Fig. 2), which presented a wide DP range, with saccharides greater than 100 kDa and also lower than 5 kDa.

3.2. Bacterial growth on the different media.

The three obtained POS substrates were evaluated for their fermentability by probiotic bacteria in different *in vitro* assays. To the best of our knowledge, no much data on the prebiotic properties of these kinds of POS are available in the literature.

Representative species from the genera *Lactobacillus* and *Bifidobacterium* were chosen to compare the selectivity of the novel carbohydrates. Maximum optical densities at 600 nm (OD_{max}), maximum growth rates (μ_{max}) and lag times (h) during growth of the strains under aerobic conditions on the assayed oligosaccharides are shown in Table 1. No growth was observed for any of the tested strains in the culture controls without carbohydrate source (data not shown). As we can see in Table 1, although growth parameters were highest with glucose, all the tested strains reached better significant growth results when SBP POS was added to MRS fermentation broth (Broth 1) than with LP POS-1 and LP POS-2. In particular, *L. reuteri* and *L. rhamnosus* reached the maximum optical density values (OD_{max} = 0.72 and 0.69, respectively). However, the fastest growth rate (μ_{max}) with SBP POS occurred with *L. plantarum*, reaching a value of $0.12\ h^{-1}$. In the case of the more acid substrates (LP POS-1 and LP POS-2), also the highest μ_{max} was observed with *L.*

plantarum (0.07 h^{-1}), whereas the highest OD_{max} was detected with *L. reuteri* and *L. brevis* for LP POS-1 and LP POS-2, respectively. The main differences among strains when growing on LP POS were observed for lag times, with the shortest time registered for *L. brevis* (0.82 and 1.97 h) and longer lag times (5.21 h and 6.20 h) for *L. casei*, whereas no growth was observed for *L. rhamnosus* GG on LP POS-2 (Table 1).

On the other hand, with the idea of testing combinations of bacteria that could improve POS fermentation by the probiotic strains, a richer medium (Broth 2) suitable to satisfy the requirements of anaerobic species (*Bacteroides* and *Bifidobacterium*) was prepared. The medium was based on MRS fermentation broth supplemented with vitamin K, vitamin B12, folic acid, hemin and sodium thioglycolate, among other products. Moreover, the pH was adjusted to 6.3, particularly to improve growth with LP POS. Table 1 shows growth parameters of *L. plantarum* IFPL935, *L. reuteri* DSM17938 and *L. rhamnosus* GG in this Broth 2. When compared these results with those obtained with Broth 1, there were practically no differences for the tested strains. Again, the best growth parameters were observed with SBP POS (Table 1). SBP POS have been described to stimulate *Lactobacillus* growth in fermentors inoculated with human faecal microbiota (Onumpai *et al.*, 2011) and in the cecal content of SBP fed Wistar rats (Tian *et al.*, 2016). In spite of the neutralization of pH and enrichment of the medium with vitamins, *L. rhamnosus* GG again showed not growth in lemon POS 2 and *L. reuteri* DSM17938 maintained the lowest growth parameters with this POS as carbohydrate source.

It is noteworthy that even though LP POS-2 was characterized for a smaller size than LP POS-1 (the 73% of the total galacturonic acid in LP POS-2 was present in form of OGalA with DP 2-10), and that in both media the pH was adjusted to 6.3, the probiotic strains *L. rhamnosus* GG and *L. reuteri* DSM17938 showed a better growth with LP POS-1.

Additional experiments were performed under anaerobic conditions with Broth 2 supplemented with the tested POS as sole carbohydrate source. Under these conditions, instead of creating growth curves by taking absorbance measurements at intervals of 1 h, two punctual OD₆₀₀ readings were performed at 24 h and 48 h (Table 2). The results reflect that *Bifidobacterium* strains were able to grow better in Broth 2 than the *Lactobacillus* ones, showing significant differences at the two recorded times. This was particularly more evident in the cultures of *B. lactis* Bb-12 with the LP and SBP POS samples. Furthermore, *B. lactis* Bb-12 grew in the same way or even more with LP POS-2 than with LP POS-1, that is, with the substrate with a smaller size (lower DP). However, the highest bacterial growth was observed with *B. lactis* at 24 h of incubation when the more neutral oligosaccharides (SBP POS) were used as substrate (OD₆₀₀= 0.95). This absorbance value was similar to the data obtained with the positive control of glucose. Onumpai *et al.* (2011) have described that pectins rich in arabinan, such as SBP, are bifidogenic. Arabinose content has been related with the bifidogenic effect described for POS from orange peels (Di *et al.*, 2017), that would explain the good growth of *B. lactis* with LP POS (Table 2).

Regarding the results obtained with the *Lactobacillus* strains incubated anaerobically in Broth 2, OD₆₀₀ were similar to those obtained with Broth 1, being LP POS-1 and SBP POS the preferred carbohydrates (Table 2). *L. rhamnosus* GG confirmed its inability to fully grow with LP POS-2 as the sole carbohydrate source. Antimicrobial activity of POS has been correlated with their content in GalA and low DP compounds (Li, Xia, Nie & Shan, 2016).

In addition, assays of probiotic strains growth in combination with *B. fragilis* DSM2151 were also carried out (Table 2). In general, *Bacteroides* are considered appropriate to ferment and degrade all types of substrates, including polysaccharides with high DP and/or highly branched (Rios-Covian, Salazar, Gueimonde & Reyes-Gavilan, 2017). Comparing

data of lactobacilli and bifidobacteria growth in mono and coculture, it was observed that there were increases in the OD₆₀₀ for SBS POS cultures, except for *L. plantarum*, reaching values higher than with glucose. These differences were especially evident with *L. reuteri*, *L. rhamnosus* and *B. breve* (Table 2). *Bacteroides* have revealed important findings regarding gut microbiota ecology involving their capability to break down colonic food residues (Wexler & Goodman, 2017). In this regard, cocultures of *B. fragilis* with *Escherichia coli* have demonstrated that *B. fragilis* is a primary metabolizer of oligosaccharides whereas *E. coli* is a secondary consumer of the released monosaccharides (Charbonneau et al., 2016).

3.3. Organic acid production during probiotic growth with POS.

As it is well-known, a variety of the beneficial effects of prebiotics are related to the production of organic and short chain fatty acids, as well as to the pH reduction in the gut. As shown in Fig. 3, the POS fermentation resulted in formation of lactate and other organic acids (basically acetic acid >> formic acid > succinic acid). Similar concentrations of total acids (40-45 mM) were noticed in all the incubations with glucose. Fig. 3 shows the most representative results obtained during growth of the probiotic strains incubated in Broth 2 with glucose and POS. Amounts of the produced organic acids were associated with the growth ability of the strains in the different carbohydrates (Tables 1 and 2). Therefore, incubations with glucose and SBP POS yielded the highest concentrations of acids (Fig. 3).

Moreover, incubations with glucose in anaerobic conditions switched the homofermentative character of the lactobacilli strains towards more acetic and formic acid production at the expense of lactic acid production. This shift in fermentation profiles was also observed during growth of the lactobacilli strains with POS under both aerobic and anaerobic conditions. This metabolic shift has been also described in *Lactobacillus* and

Bifidobacterium species grown on fructooligosaccharides (Tabasco, Palencia, Fontecha, Peláez & Requena, 2014) and in conditions of slow growth (Rios-Cobian *et al.*, 2013). Under these circumstances, more ATP is formed per mole of sugar consumed resulting in a more efficient use of the available energy source (Van der Meulen, Avonts & De Vuyst, 2004).

As shown in Fig. 3, *B. lactis* Bb-12 was the only strain able to ferment LP POS-2 with an equimolar formation of acetic and lactic acids. These were the only acids produced by this strain, whereas *B. breve* was able to produce formic acid. Rios-Cobian *et al.* (2013) have described that some *B. breve* strains are characterized to produce higher amount of formic acid than lactic acid. Similarly, *L. rhamnosus* GG reached the highest production of formic acid (7.6 mM) in the anaerobic incubation with SBP POS (Fig. 3B). On the other hand, production of succinic acid was scarcely observed and only under anaerobic conditions, with maximal concentrations (in the range of 1.9-2.2 mM) that were achieved with lactobacilli and *B. lactis* growing with glucose. Changes in the production of succinic acid or the formation of propionic acid in the co-cultures with *B. fragilis* were not detected (results not shown) in spite that *Bacteroides* are characterized by being the largest propionate producers among the gut microbiota through the succinate pathway (Rios-Cobian *et al.*, 2017).

4. Conclusions

This study demonstrates the fermentation of complex refined POS derived from lemon and sugar beet byproducts by lactobacilli and bifidobacteria. In this regard, POS from the sugar beet pulp might have great potential as a prebiotic. Meanwhile, the low-molecular-weight LP POS-2 has shown to favour growth of *B. lactis* in comparison with *B. breve* and lactobacilli. Although these pure culture models do not reproduce the bacterial interactions

in the gut microbiota, they could be adequate for screening studies aimed at the initial selection of adequate combinations to obtain synbiotics.

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Legends to figures:

Figure 1. HPAEC-PAD chromatograms of oligomers detected in the SBP POS sample: (A) neutral oligosaccharides with indication of standard arabinooligosaccharides (degrees of polymerization, DP=2–8), and (B) oligogalacturonides whose retention times were identified from a hydrolysate of pure polygalacturonic acid.

Figure 2. HPSEC chromatogram of SBP POS sample (with indication of dextran standards).

Figure 3. Organic acids produced during incubation of single strains in Broth 2 under aerobic (A) and anaerobic conditions (B). The results are the average of two replicates.

TABLES

Table 1. Maximum growth rates ($\mu_{\max}$, h^{-1}), optical densities ($\text{OD}_{\max}$ at 600 nm) and lag (h) parameters of bacteria growing under aerobic conditions and using both different media and carbohydrate sources.

Species	Parameters	Glucose	LP POS-1	LP POS-2	SBP POS
		Broth 1			
<i>L. plantarum</i> IFPL935	$\mu_{\max}$	0.30 (0.01) ^c	0.07 (0.00) ^{a4}	0.07 (0.00) ^{a3}	0.12 (0.01) ^{b4}
	$\text{OD}_{\max}$	1.73 (0.01) ^{c4}	0.36 (0.00) ^{a1}	0.35 (0.02) ^{a2}	0.51 (0.04) ^{b1}
	lag	4.34 (0.05) ^{c2}	2.20 (0.06) ^{a2}	2.54 (0.16) ^{b2}	2.70 (0.02) ^{b4}
<i>L. reuteri</i> DSM17938	$\mu_{\max}$	0.31 (0.01) ^c	0.03 (0.00) ^{a2}	0.02 (0.00) ^{a1}	0.06 (0.00) ^{b2}
	$\text{OD}_{\max}$	1.61 (0.02) ^{d2}	0.54 (0.00) ^{b3}	0.30 (0.00) ^{a1}	0.72 (0.02) ^{c4}
	lag	4.42 (0.22) ^{b2}	1.71 (0.27) ^{a2}	5.14 (0.18) ^{c3}	1.74 (0.21) ^{a2}
<i>L. rhamnosus</i> GG	$\mu_{\max}$	0.30 (0.01) ^c	0.03 (0.00) ^{a2}	NG	0.05 (0.00) ^{b1}
	$\text{OD}_{\max}$	1.56 (0.02) ^{c1}	0.52 (0.01) ^{a3}	NG	0.69 (0.03) ^{b4}
	lag	3.40 (0.88) ^{b1}	2.23 (0.21) ^{a2}	NG	1.37 (0.13) ^{a1}
<i>L. brevis</i> IFPL737	$\mu_{\max}$	0.31 (0.00) ^d	0.06 (0.00) ^{b3}	0.03 (0.00) ^{a2}	0.08 (0.00) ^{c3}
	$\text{OD}_{\max}$	1.68 (0.01) ^{d3}	0.53 (0.01) ^{b3}	0.47 (0.01) ^{a3}	0.65 (0.00) ^{c2, 3}
	lag	4.44 (0.04) ^{c2}	0.82 (0.03) ^{a1}	1.97 (0.23) ^{b1}	1.73 (0.10) ^{b2}
<i>L. casei</i> IFPL710	$\mu_{\max}$	0.30 (0.00) ^c	0.02 (0.00) ^{a1}	0.02 (0.00) ^{a1}	0.05 (0.00) ^{b1}
	$\text{OD}_{\max}$	1.76 (0.01) ^{c5}	0.46 (0.04) ^{a2}	0.45 (0.03) ^{a3}	0.62 (0.02) ^{b2}
	lag	3.26 (0.03) ^{b1}	5.21 (0.69) ^{c3}	6.20 (0.48) ^{d3}	2.00 (0.00) ^{a3}
Broth 2					
<i>L. plantarum</i> IFPL935	$\mu_{\max}$	0.29 (0.01) ^c	0.06 (0.00) ^{d2}	0.06 (0.00) ^{a2}	0.12 (0.00) ^{b2}
	$\text{OD}_{\max}$	1.32 (0.02) ^c	0.38 (0.01) ^{a1}	0.36 (0.01) ^a	0.63 (0.01) ^{b1}
	lag	3.91 (0.01) ^{c1}	1.38 (0.04) ^{a1}	2.02 (0.06) ^{b1}	2.48 (0.56) ^{b2}
<i>L. reuteri</i> DSM17938	$\mu_{\max}$	0.21 (0.09) ^b	0.04 (0.01) ^{a1}	0.02 (0.01) ^{a1}	0.06 (0.00) ^{a1}
	$\text{OD}_{\max}$	1.35 (0.04) ^a	0.60 (0.01) ^{b2}	0.45 (0.09) ^a	0.75 (0.01) ^{c2}
	lag	5.55 (0.47) ^{b2}	1.45 (0.22) ^{a1}	7.36 (0.61) ^{c2}	1.64 (0.13) ^{a1}
<i>L. rhamnosus</i> GG	$\mu_{\max}$	0.19 (0.10) ^b	0.03 (0.00) ^{a1}	NG	0.03 (0.00) ^{a1}
	$\text{OD}_{\max}$	1.25 (0.10) ^b	0.58 (0.05) ^{a2}	NG	0.62 (0.02) ^{a1}
	lag	4.99 (0.65) ²	3.78 (0.77) ²	NG	3.43 (0.00) ³

Broth 1: MRS fermentation broth.

Broth 2: MRS fermentation broth enriched with vitamins and minerals and pH adjusted to 6.3.

LP POS-1 and LP POS-2: Pectooligosaccharides from the enzymatic hydrolysis of lemon peels during 7.5 and 24 h, respectively.

SBP POS: Pectooligosaccharides from the enzymatic hydrolysis of sugar beet pulp.

^{a-d} Means in the same row not followed by a common superscript letter are significantly different ($P < 0.05$; Duncan test). ¹⁻⁵ Means of each parameter in the same column not followed by a common superscript number are significantly different ($P < 0.05$; Duncan test). Standard deviations are shown in parentheses ($n = 3$). NG, no growth.

Table 2. Values of OD₆₀₀ after 24 and 48 h of strains incubated under anaerobic conditions in Broth 2 (MRS fermentation broth enriched with vitamins and minerals and pH adjusted to 6.3).

Species	Time (h)	Single strain cultures			
		Glucose	LP POS-1	LP POS-2	SBP POS
<i>L. plantarum</i> IFPL935	24	0.54 (0.03) ^{c1}	0.42 (0.03) ^{b1}	0.35 (0.03) ^{a2}	0.57 (0.03) ^{c1}
	48	0.58 (0.02) ^{c1}	0.45 (0.04) ^{b1}	0.41 (0.02) ^{a1}	0.58 (0.02) ^{c1}
<i>L. reuteri</i> DSM17938	24	0.77 (0.02) ^{c3}	0.57 (0.02) ^{b3}	0.35 (0.02) ^{a2}	0.72 (0.02) ^{c2}
	48	0.77 (0.01) ^{d3}	0.52 (0.01) ^{b2}	0.43 (0.02) ^{a1}	0.71 (0.01) ^{c2}
<i>L. rhamnosus</i> GG	24	0.72 (0.01) ^{c2}	0.59 (0.03) ^{b3}	0.19 (0.01) ^{a1}	0.73 (0.02) ^{c2}
	48	0.72 (0.01) ^{c2}	0.61 (0.02) ^{b3}	0.37 (0.10) ^{a1}	0.74 (0.01) ^{c3}
<i>B. lactis</i> Bb-12	24	1.09 (0.02) ^{d4}	0.72 (0.01) ^{a4}	0.78 (0.03) ^{b4}	0.95 (0.06) ^{c3}
	48	1.12 (0.03) ^{d5}	0.66 (0.01) ^{a4}	0.72 (0.03) ^{b3}	0.92 (0.03) ^{c4}
<i>B. breve</i> 26M2	24	1.11 (0.03) ^{d4}	0.47 (0.03) ^{b2}	0.41 (0.03) ^{a3}	0.59 (0.06) ^{c1}
	48	1.00 (0.02) ^{c4}	0.61 (0.02) ^{a3}	0.61 (0.03) ^{a2}	0.77 (0.03) ^{b3}
Combinations with <i>B. fragilis</i> DSM2151					
		LP POS-1	LP POS-2	SBP POS	
<i>L. plantarum</i> IFPL935	24	0.50 (0.02) ^{b1}	0.48 (0.01) ^{a2}	0.59 (0.01) ^{c1}	
	48	0.52 (0.04) ^{b1}	0.46 (0.02) ^{a1}	0.61 (0.01) ^{c1}	
<i>L. reuteri</i> DSM17938	24	0.72 (0.01) ^{b2}	0.57 (0.00) ^{a3}	0.93 (0.01) ^{c3}	
	48	0.60 (0.01) ^{a2}	0.61 (0.02) ^{a2}	0.80 (0.00) ^{b2}	
<i>L. rhamnosus</i> GG	24	0.76 (0.01) ^{b3}	0.39 (0.01) ^{a1}	0.93 (0.03) ^{c3}	
	48	0.68 (0.02) ^{b3}	0.50 (0.14) ^{a1}	0.84 (0.02) ^{c2, 3}	
<i>B. lactis</i> Bb-12	24	0.79 (0.01) ^{a4}	0.86 (0.02) ^{b5}	0.99 (0.06) ^{c4}	
	48	0.72 (0.02) ^{a4}	0.80 (0.02) ^{b4}	0.95 (0.04) ^{c4}	
<i>B. breve</i> 26M2	24	0.73 (0.00) ^{b2}	0.65 (0.04) ^{a4}	0.80 (0.04) ^{c2}	
	48	0.69 (0.02) ^{a3, 4}	0.70 (0.01) ^{a3}	0.83 (0.05) ^{b2}	

LP POS-1 and LP POS-2: Pectooligosaccharides from the enzymatic hydrolysis of lemon peels during 7.5 and 24 h, respectively.

SBP POS: Pectooligosaccharides from the enzymatic hydrolysis of sugar beet pulp.

^{a-c} Means in the same row not followed by a common superscript letter are significantly different ($P < 0.05$; Duncan test). ¹⁻⁵ Means of each time in the same column not followed by a common superscript number are significantly different ($P < 0.05$; Duncan test). Standard deviations are shown in parentheses ($n = 6$).

Figure 1

Figure 2

Figure 3