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Prebiotic effect of commercial saccharides on probiotic bacteria isolated from commercial products

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Abstract

This study deals with the ability of probiotic bacteria to ferment prebiotics *in vitro* to estimate the prebiotic activity score and prebiotic index based on specific substrates and strains. Five probiotic bacteria were isolated from commercial products and their fermentation capability of three commercial prebiotics was assessed. Growth kinetics of probiotics showed that all of them were able to use the prebiotics as carbon sources. Mostly bacteria grew faster on Frutafit and Oligomate 55 than on lactulose. The prebiotic activity score and prebiotic index concepts were introduced to evaluate the performance of every probiotic with each prebiotic; accordingly, the highest values were for *L. rhamnosus* on Oligomate 55, while the worst according to these parameters was *L. casei* Shirota on Frutafit. Prebiotic index and prebiotic activity score describe well the selective growth of probiotics supported by prebiotics; therefore, they could be useful tools to define adequate combinations in colonic foods development.

Keywords: prebiotic; probiotic; prebiotic activity score; prebiotic index; synbiotic.

Practical Application: Prebiotic index and prebiotic activity score can be useful and quick tools to define synbiotics foods

1 Introduction

Prebiotics are non-digestible dietary supplements, which selectively stimulate growth and/or activity of one or a group of beneficial bacteria in the colon, thereby improving the health of the host (Patel & Goyal, 2011). Prebiotics have become important commodities for the industry of functional foods as a part of the called "colonic foods" which can improve health through the colonic microbiota (Roberfroid et al., 2010). Prebiotics must be capable of promoting growth of bifidobacteria and lactobacilli in the colon, passing through the upper intestinal tract without being hydrolyzed (Kneifel et al., 2000).

Several carbohydrates function as prebiotics, including fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), and lactulose (Fuller & Gibson, 1998). FOS and inulin are oligomers and polymers of fructose respectively, with the generic structure: α-D-Glu-(1-2)-[β-D-Fru-1-2-]$_n$, where n is from 2 to 4 for FOS and up to 60 for inulin. The general structure for GOS is: α-D-Glu-(1-4)-[β-D-Gal-1-6-]$_n$ where n is from 2 to 5. Lactulose is the disaccharide with the structure: β-D-Gal-D-(1-4) D-Fru (Crittenden & Playne, 1996). Some strains of probiotics are added to yogurt and other fermented dairy foods; in some cases, prebiotics are also added, which may be metabolized by the probiotics in the intestinal tract; this combination of probiotic and prebiotic (synbiotic) may lead to an improvement of the gut microbiota. The resulting enrichment of gut microbiota may lead to an antagonistic effect against harmful intestinal bacteria improving host health in several ways (Wang & Gibson, 1993). Thus, prebiotics alone, or combined with probiotic bacteria as synbiotics, are recognized to have the ability to promote and improve gastrointestinal health of humans (Tuohy et al., 2003; Oliveira et al., 2009).

Several studies have shown that the ability of lactobacilli to ferment prebiotic carbohydrates is substrate specific (Schrezenmeir & de Vrese, 2001; Kaplan & Hutkins, 2003). In addition, it is not clear which prebiotic carbohydrates are the most suitable substrates for selective growth of specific strains. Several quantitative approaches have been devised to determine the functional activity of prebiotics under *in vitro* fermentation conditions (Palframan et al., 2003; Jiménez-Vera et al., 2008; Figueroa-González et al., 2010). In general, these methods provide rates that reflect the relative ability of a given prebiotic to produce specific effects, and they are based on the measurement of microbial populations, growth rates, substrate assimilation rates, and/or production of short-chain fatty acids. Rates then have been used to rank various carbohydrates for their potential to stimulate growth of specific members of a mixed microbiota. As defined by Huebner et al. (2007), prebiotic index reflects the ability of a given substrate to support the growth of an organism compared to that of other organisms, and the growth on a non-prebiotic substrate, such as glucose or any other sugar used as control. Therefore, carbohydrates have a positive activity score if they: 1) are metabolized as well as the control by probiotic

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strains; and 2) are selectively metabolized by probiotics but not by other intestinal bacteria.

A variety of probiotic strains and prebiotics have been incorporated in different combinations for the development of synbiotics. Because of this, it is important to consider the influence of specific prebiotics on the growth of specific strains of probiotics. Therefore, the objectives of this study were: 1) to investigate the ability of some commonly used strains of lactobacilli as probiotics to ferment commercial prebiotics and 2) to estimate the prebiotic activity score and prebiotic index based on specific substrates and strains.

2 Materials and methods

2.1 Bacterial strains isolation

Five probiotic strains isolated from commercial products were used in this study; commercial microorganisms reported by the manufacturer, and their identifications are shown in Table 1. They were isolated preparing dilutions from 10^{-1} to 10^{-4} in 1% peptoned water (Difco, Detroit, MI, USA), pH 7.2, and the dilutions were plated on Man-Rogosa-Sharpe (MRS) agar (Difco, Detroit, MI, USA) according to Tharmaraj & Shah (2003). All strains were stored at 4°C in MRS agar. *Escherichia coli* K-12 was maintained on Nutritive Agar (B. D. Bioxon, Mexico City) at 4 °C.

2.2 Identification of the isolated bacteria

Isolated bacteria were examined microscopically for cellular morphology and Gram stain phenotype. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene of the isolated bacteria. Total DNA was isolated by Wizard® Genomic DNA purification kit (Promega, USA). PCR amplification was carried out using 16S rDNA bacterial primers E9F (5'-GAGTTTGATCCTGGCTCAG-3') and E939R (5'-CTTGTGCGGGCCCCCGTCAATTC-3') according to Forney et al. (2004). Nucleotide sequence was compared with published bacterial 16S rDNA sequences through a standard nucleotide–nucleotide BLAST (http://www.ncbi.nlm. nih.gov/blast) homology search. The GenBank accession numbers for the sequence are shown in Table 1.

2.3 Prebiotics

Three commercial prebiotics were tested; they were Frutafit HD (mainly inulin) (Sensus, Roosendaal, The Netherlands); Oligomate 55 (galactooligosaccharides 55%, lactose 40% and monosaccharides 5%) (Yakult Honsha, Tokyo Japan), and Regulact (lactulose) (Laboratorios Servet, Mexico City, Mexico). Lactose (J. T. Baker, Phillipsburg NJ, USA) was used as reference carbohydrate.

2.4 Growth kinetics of isolated microorganisms

Microorganisms were cultivated in 100 mL serological bottles in batch cultures with 50 mL of the corresponding culture medium at 37°C for 24 h, in a G25 New Brunswick incubator (New Brunswick Scientific, Edison NJ, USA), with a shake rate of 50 rpm. Medium was inoculated with 10^6 colony-forming units (CFU) of each strain of lactobacilli (inoculated with 1% v/v of an overnight culture obtained from a single colony).The culture medium contained: 10 g/L, either lactose, used as control, or one of the studied prebiotics; 3 g/L, yeast extract (B. D. Bioxon, Mexico City); 5 g/L, casein peptone (B. D. Bioxon, Mexico City) according to that described by Hopkins et al. (1998) (17). Growth was measured each 4 h, by absorbance at 650 nm with a spectrophotometer (Shimadzu 160A, Japan) and the number of colony-forming units (CFU) was determined by counting them in MRS agar plates with the Miles and Misra technique (Weng et al., 2004) incubating at 37°C, 48 h.

2.5 Carbohydrate analysis

The consumption of carbohydrates throughout fermentations was determined collecting samples at 4 h intervals for 24 h. The samples were centrifuged at 20,000x*g* (Beckman J2-MI, Beckman Instruments, Palo Alto CA, USA), 4 °C, 10 minutes, to remove microbial cells, and they were analyzed by HPLC (LabAlliance, State Collage PA, USA) using a Rezex, RHM-7.8x300 mm column (Phenomenex, Torrance CA, USA) of monosaccharides and a light-scattering detector (Polymer Laboratories, Amherst MA, USA). They were eluted with deionized water at a flow rate of 0.3 mL/min. The column temperature was kept at 75°C. Concentrations of carbohydrates were calculated using standard curves for each one.

Table 1. Identification of probiotic strains isolated from commercial products.

Microorganism reported by the manufacturer	Type of product	Microorganism identified	Sequence length (bp)	Similarity (%)	GenBank accession number
Lactobacillus casei	Fermented milk	*Lactobacillus casei* 1	561	99	*GU550098*
Lactobacillus casei Shirota	Fermented milk	*Lactobacillus casei* Shirota	851	99	*GU550103*
Lactobacillus johnsonii	Fermented milk	*Lactobacillus casei* 2	703	99	*GU550101*
Lactobacillus rhamnosus	Power milk formula	*Lactobacillus rhamnosus*	863	99	*GU550102*
Lactobacillus rhamnosus GG	Capsules	*Lactobacillus rhamnosus* GG	851	99	*GU550100*

2.6 Prebiotic index

Prebiotic index (I_{preb}) was calculated according to Palframan et al. (2003); it is the ratio of probiotic growth in the prebiotic to probiotic growth in a control carbohydrate. A prebiotic index higher than 1 means that the carbohydrate has a positive effect on the probiotic growth. If the prebiotic index is near to 1, indicates a low effectiveness of the evaluated carbohydrate. The prebiotic index was calculated according to equation 1:

$$I_{preb} = \frac{CFU\ of\ probiotics\ in\ prebiotic\ carbohydrate}{CFU\ of\ probiotics\ in\ control\ carbohydrate} \quad (1)$$

2.7 Prebiotic activity score

Prebiotic activity scores (A_{preb}) were determined using the equation 2, previously reported by Huebner et al. (2007):

$$A_{preb} = \frac{\left(LogP_{24} - LogP_0\right)_{prebiotic}}{\left(LogP_{24} - LogP_0\right)_{lactose}} - \frac{\left(LogE_{24} - LogE_0\right)_{prebiotic}}{\left(LogE_{24} - LogE_0\right)_{lactose}} \quad (2)$$

where A_{preb} is the prebiotic activity score; Log P are the log of growth (CFU/mL) of the probiotic bacteria at 24 h (P_{24}) and 0 h (P_0) of culture on prebiotic and lactose; Log E are the log of growth (CFU/mL) of *E. coli* K12 at 24 h (E_{24}) and 0 h (E_0) of culture on prebiotic and lactose.

By definition, substrates with a high prebiotic activity score support good growth of probiotic bacteria, with cell counts (CFU/mL) comparable with that when grown on lactose. However, the development of *E. coli* K12 grown on the prebiotics should, in theory, be very low compared to that on lactose. Therefore, using equation 2, the prebiotic activity score of a oligosaccharide can be determined relative to any given strain.

2.8 Statistical analysis

All experiments were carried out at least in triplicate, and results are expressed as mean values with standard deviations. To determine if there were differences in the growth of microorganisms between the different sources of carbon used, an analysis of variance (ANOVA) and a Tukey-Kramer test were performed using the statistical software NCSS, with $\alpha < 0.05$ used as a threshold of statistical significance.

3 Results and discussion

3.1 Identification of probiotics

Probiotics isolated were Gram positive rods. The determined 16S rDNA sequences of the five microorganisms were compared directly with the BLAST database and were registered in GenBank database. A high level of similarity of 16S ribosomal DNA nucleotide sequences (99% of matches) were obtained for the five isolated strains as it is shown in Table 1, where the length of the compared sequences is also shown. As it can be observed, in one of the products, the identified microorganism was not the same that the reported by manufacturer. According to the cluster analysis made by Watson et al. (2012) the strain *L. johnsonii* LA1 is between the strains of *L. casei* and *L. paracasei,* which explains the difference in the identity of the strain reported by the manufacturer as *L. johnsonii*.

3.2 Growth behavior of probiotic in media containing prebiotics

The growth of the five probiotics in the commercial prebiotic carbohydrates is shown in Figure 1. All probiotics grew in media containing the tested prebiotics. We observed that the growth of *L. casei* Shirota in prebiotics showed the same growth as in lactose, except in Frutafit in which the final growth was significantly lower (α=0.0001). The other strain, *L. casei* 1, showed no significant difference in its final growth (α=0.0196) between most of the prebiotics and lactose; however, it grew faster in Frutafit and Oligomate 55 (μ=0.29 1/h and 0.44 1/h, respectively) than in lactose and lactulose (μ=0.19 1/h for both) (α=0.0006). Neither the final growth nor the growth rate of *L. casei* 1, *L. casei* 2, *L. rhamnosus* GG and *L. rhamnosus* on lactulose were significantly different (α=0.0568) from those on lactose (μ=0.19 1/h in all cases), whereas Frutafit and Oligomate 55 led to higher final growth and growth rate (μ = 0.33 1/h and 0.44 1/h, respectively for the four bacteria). In general, a slower growth of the microorganisms on lactulose (except *L. casei* Shirota) was observed, resulting like that of lactose.

The results obtained in this study showed that the probiotics were able to grow using the prebiotics (galacto- and fructooligosaccharides) as a carbon source, although growth rates were different for each substrate. Lactose was used as the control sugar since it is the natural substrate in dairy foods, and lactobacilli are usually well adapted to ferment this carbohydrate. Similar results have been observed by other authors; as reported by Kneifel et al. (2000), lactulose generates good growth of several strains of lactobacilli (*L. rhamnosus* and *L. casei*); during this study the above-mentioned effect was observed.

In a previous report of the working group it was demonstrated that some of these probiotics were able to grow on all the prebiotic carbohydrates and were also able to produce pathogen inhibiting compounds such as SCFA and H_2O_2 (Cruz-Guerrero et al., 2014); in this way, Oligomate 55 was particularly suitable for stimulating the production of these antimicrobials. Even though other non-identified compounds could be present leading to growth inhibition on the pathogens.

3.3 Prebiotic consumption

In order to assess the prebiotics consumption, the carbohydrates concentration was measured at the beginning and end of the fermentation and then the consumed proportion was calculated (Figure 2). All prebiotics were used as a carbon source by all bacteria. The highest prebiotic consumption was for Frutafit by *L. rhamnosus* (50%) and *L. casei* 2 (46%) (α=0.0002), while the lowest was for Oligomate 55 by *L. casei* 1 (23%) and *L. rhamnosus* (23%) (α=0.0023).

The consumption of Oligomate 55 in the fermentation media for the cases of *L. casei* 2 and *L. rhamnosus* is shown in Figure 3 (data from other microorganisms are not shown). In the kinetics on Oligomate 55 consumption, it can be observed

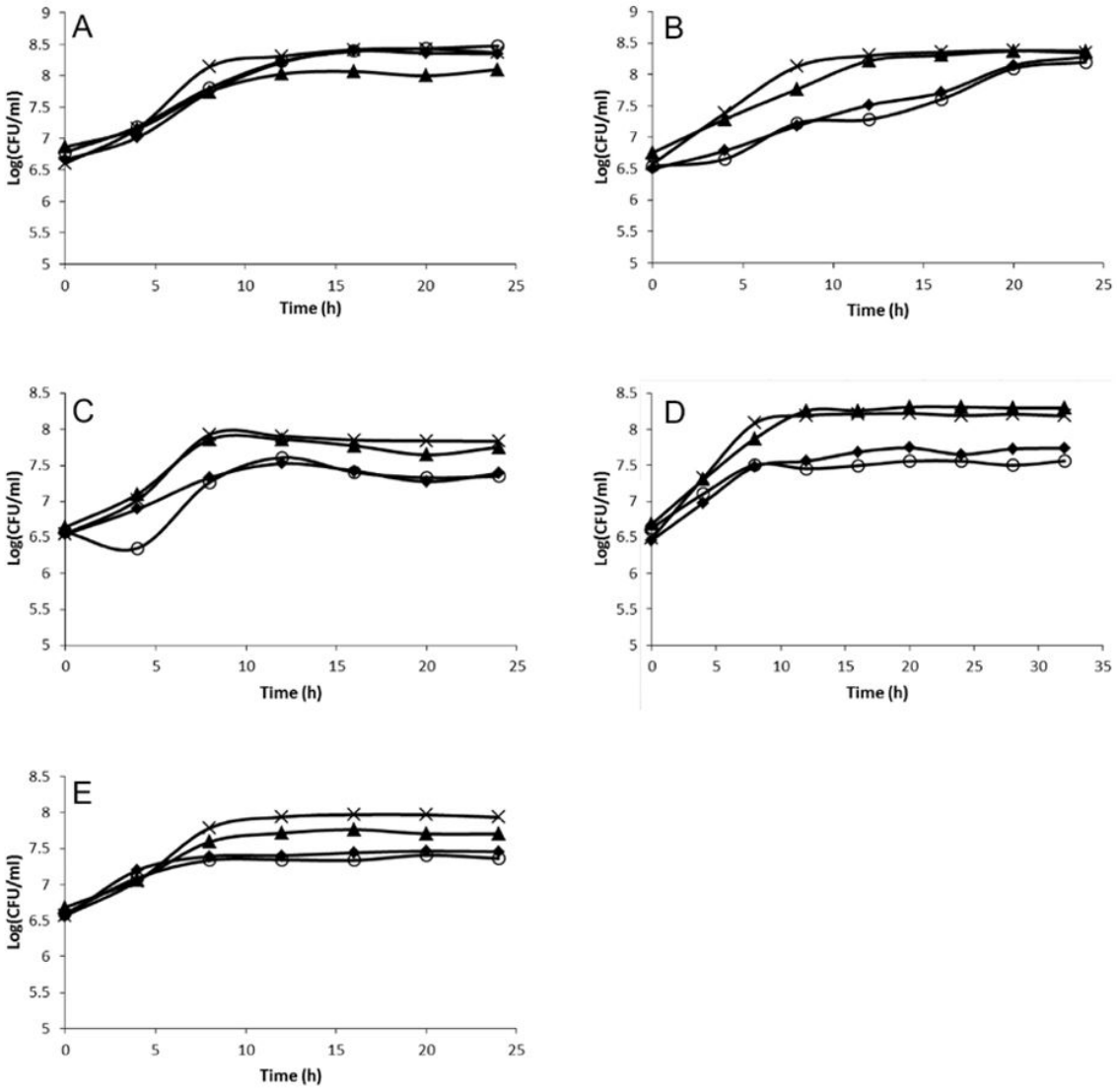

Figure 1. Growth of *L. casei* Shirota (A), *L. casei* 1(B), *L. casei* 2 (C), *L. rhamnosus* GG (D), and *L. rhamnosus* (E). Lactose (○) used as a control, Lactulose (◆), Frutafit (▲) and Oligomate 55 (×).

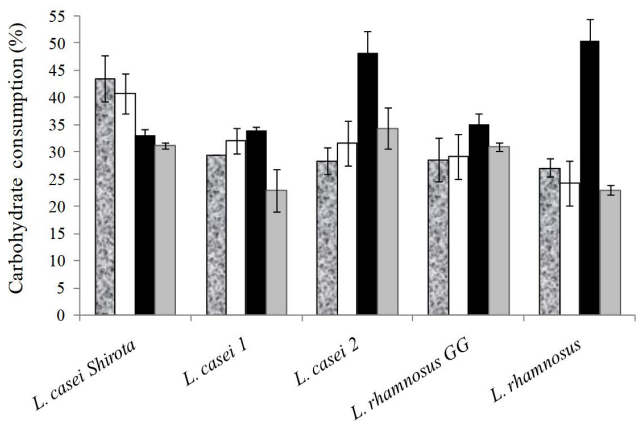

Figure 2. Consumption of commercial prebiotics and lactose by probiotics. Lactose (▩), Lactulose (□), Frutafit (■) and Oligomate 55 (■).

that microorganisms consumed both oligosaccharides (tri- and disaccharides) and glucose.

All the probiotics under study consumed Frutafit at different rates, even being of the same genus, so we can assume that the metabolism of these oligosaccharides is different in each strain. Kaplan & Hutkins (2003) reported that *L. rhamnosus* GG, a widely used probiotic strain, was unable to use FOS as an energy source. However, this strain is able to ferment fructose, indicating the presence of at least one fructose transport system. In our work all tested microorganisms, including *L. rhamnosus* GG, consumed Frutafit, which is a remarkable difference with the results previously referred. These authors reported that exist different pathway for FOS metabolism; for example, in *L. paracasei* 1195, it is suggested that FOS uptake and hydrolysis were mediated by an ATP-dependent binding cassette transport system and a cytoplasmic beta-fructofuranosidase, respectively.

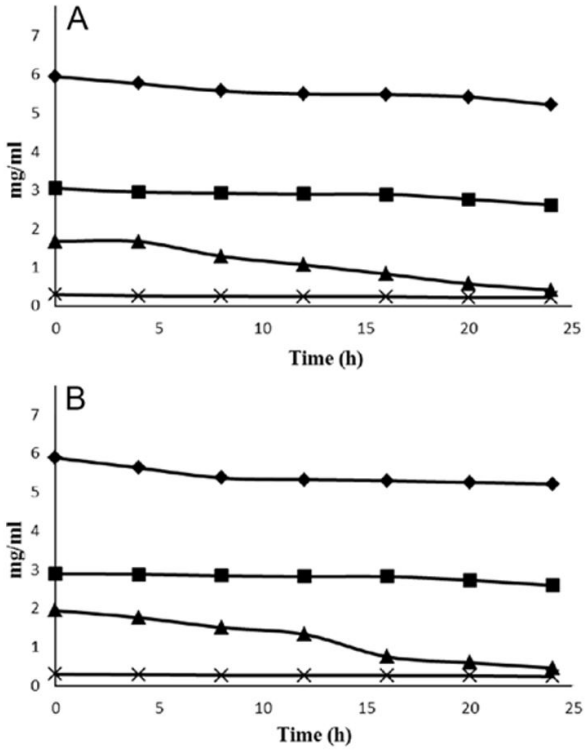

Figure 3. Consumption of Oligomate 55 by *L. casei* 2 (A) and *L. rhamnosus* (B). Galactooligosaccharides (◆), Glucose (▲), Galactose (×) and disaccharides (□).

This could explain that in the present work, all *L. casei* strains were able to consume Frutafit. Watson et al. (2012) grouped *Lactobacillus* strains in clusters based on their ability to utilize carbohydrates: *L. casei* and *L. casei* Shirota, and even *L. johnsonii* LA1, are in the same cluster because their ability to utilize GOS; while *L. rhamnosus* GG is in a different cluster due to its ability to utilize FOS.

Gopal et al. (2001) also reported that *L. rhamnosus* DR20 consumed galactooligosaccharides from Oligomate 55, preferring monosaccharides and disaccharides. Kneifel et al. (2000) reported that Elix'or syrup, representing an industrially manufactured galactooligosaccharide, yielded marked utilization patterns with all lactobacillus strains studied (*L. rhamnosus, L. casei* and *L. paracasei*), except for *L. rhamnosus* GG; while Oligomate 55 powder rendered a lower growth of two *L. casei* (01 and CRL431). In this study *L. rhamnosus* GG in Oligomate 55 consumed both galactooligosaccharides and glucose, and possibly galactose since accumulation of this monosaccharide was not observed.

3.4 Prebiotic index

Prebiotic index obtained with the different carbohydrate is shown in Figure 4, the highest value was for *L. rhamnosus* in Oligomate 55 (7.22), being significantly different from the other prebiotics (α=0.0002). The major prebiotic index for all probiotic strains was found for Oligomate 55 except for *L. casei* 1, which did not show significant differences in prebiotic index values

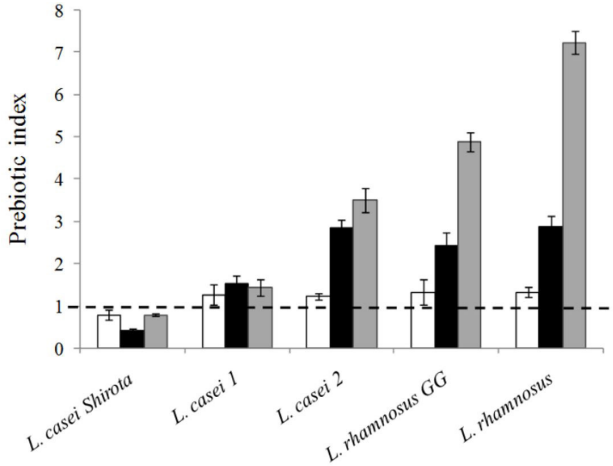

Figure 4. Prebiotic index values of probiotics grown on commercial prebiotics. Lactulose (□), Frutafit (■) and Oligomate 55 (■).

for all prebiotics (α=0.5877). None of the tested carbohydrates showed a prebiotic effect on *L. casei* Shirota.

Generally, prebiotic effects described in the literature are expressed in a qualitative form. Prebiotic index expresses a quantitative value for comparison of different prebiotic carbohydrates; if the ratio expressed in Equation 1 is higher than 1, indicates that the growth of the microorganism is stimulated by the tested prebiotic in comparison to the control carbohydrate (Palframan et al., 2003). According to the same authors, the highest prebiotic indexes are obtained with galactooligosaccharides and then are more effective prebiotics compared to inulin; these results agree with those obtained in this study for Oligomate 55 for all microorganisms since prebiotic indexes were higher than Frutafit, with exception of *L. casei* 1. In addition, all prebiotic indexes obtained in this study showed a beneficial effect of the tested carbohydrates over the growth of all probiotic strains, except for *L. casei* Shirota in which none of the evaluated prebiotics showed a stimulating effect on the growth different to lactose (control carbohydrate).

3.5 Prebiotic activity score

The prebiotic activity scores are shown in Figure 5. The highest prebiotic activity scores were for *L. rhamnosus* (6.68) and *L. rhamnosus* GG (5.46), both on Oligomate 55, which were significantly different from that of the other sugars (α= 0.00002) and the other bacteria (α= 0.00005). Conversely, the lowest score was for *L. casei* Shirota on Frutafit (-0.3) (α=0.0049). In general, the three strains of *L. casei* showed the lowest prebiotic activity scores in the evaluated prebiotics without significant differences between lactulose and Frutafit (α=0.1170). According to results, the lowest prebiotic activity scores were obtained in lactulose for all probiotic microorganisms, without significant difference (α=0.5067).

Regarding to the prebiotic activity score, Huebner et al. (2007) determined that, if the prebiotic activity score is lower than 1 or negative, it means that the growth of the tested strain is lower on a specific prebiotic compared to the control carbohydrate, and/or its growth is lower than the reference bacteria (*E. coli* K12) on

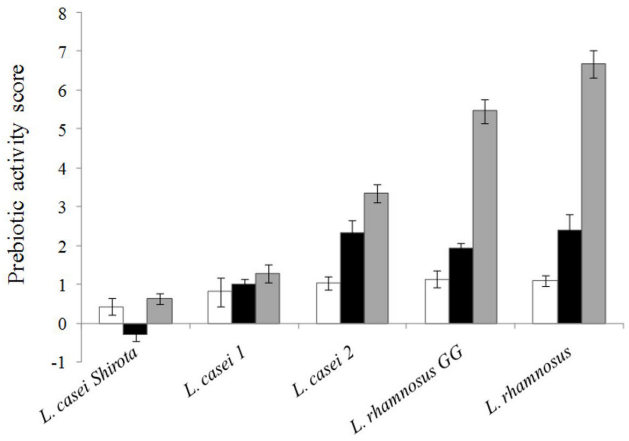

Figure 5. Prebiotic activity scores of probiotics grown on commercial prebiotics. Lactulose (□), Frutafit (■) and Oligomate 55 (■).

the prebiotic carbohydrates. All studied microorganisms had the highest prebiotic activity score in Oligomate 55 compared to the other prebiotics.

Given the known metabolic diversity of the lactobacilli, it might be expected that there was considerable variation in prebiotic activity scores for the different prebiotics used by a single probiotic strain. For example, *L. rhamnosus* had significantly higher scores (α=0.0002) on Oligomate 55 compared to Frutafit and lactulose. Besides, it was observed that even strains within a single species (e.g., *L. casei* Shirota and *L. casei* 1) had significantly different prebiotic activity scores, indicating that differences in their metabolic profile apparently exist. Utilization of particular prebiotics by probiotics requires the presence of specific hydrolysis and transport systems (Kneifel et al., 2000; Kaplan & Hutkins, 2003; Gopal et al., 2001; Goh et al., 2007); therefore, gene coding for these metabolic systems may result in specific activities for the different strains, resulting in varied prebiotic activity scores.

4 Conclusion

The results of the present work indicated that the probiotic strains grew generally well in media containing prebiotics. However, the single observation of the utilization of a carbohydrate as a carbon source is not enough to reflect its prebiotic capacity; better indicative parameters of the stimulation of probiotics by prebiotic carbohydrates may be obtained using quantitative prebiotic parameters such as prebiotic index and prebiotic activity score, which describe selective growth support. In this study, it was demonstrated that the best synbiotic combination was *L. rhamnosus* with Oligomate 55 since it had the highest prebiotic activity score and the highest prebiotic index, while the less favorable synbiotic combination was for *L. casei* Shirota and Frutafit according to both parameters.

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