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FERMENTATION BY *Lactobacillus paracasei* OF GALACTOOLIGOSACCHARIDES AND LOW-MOLECULAR-WEIGHT CARBOHYDRATES EXTRACTED FROM SQUASH (*Curcubita maxima*) AND LUPIN (*Lupinus albus*) SEEDS

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ABSTRACT

The *in vitro* prebiotic activity of galactooligosaccharides (GOS) and low-molecular-weight carbohydrates (LMWC) extracted from lupin and squash seeds on the growth of *Lactobacillus paracasei* BGP1 was studied. To this end, the change in cell density after 24 h of *L. paracasei* growth on 1% (w/v) glucose, 1% (w/v) raffinose, 1% (w/v) commercial inulin GR, 1% (w/v) lupin extract, and 1% (w/v) squash extract relative to the change in cell density of a mixture of enteric strains under the same culture conditions were evaluated. Additionally, the principal components of GOS and LMWC in the extracts were identified using Thin Layer Chromatography. The highest prebiotic activity score was for *L. paracasei* grown on squash extract (0.55±0.03), followed by lupin extract (0.49±0.02), inulin (0.38±0.05) and raffinose (0.37±0.05). These results will contribute to selecting plant species as potential sources of prebiotic ingredients for the development of functional foods.

Keywords: Galactooligosaccharides (GOS), low-molecular-weight carbohydrates (LMWC), *Curcubita maxima*, *Lupinus albus*, *Lactobacillus paracasei*

INTRODUCTION

The growing awareness of consumers on the relationship between food and health has led to an increasing demand for functional foods (i.e. foods that provide health benefits further than the basic function of contributing nutrients). This had led to attempts to improve the microbiota in humans and animals through the use of probiotics, defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host **(FAO/WHO, 2002; Roberfroid, 2002)**. *Bifidobacteria* and *Lactobacilli* are examples of bacteria in the colon that have the potential to improve the host´s health **(Huebner *et al.*, 2007; Moongngarm *et al.*, 2011; Vulevic *et al.*, 2004)**. Another strategy that pursues the same purpose consists in including in diet prebiotics, defined as non-digestible food ingredients that beneficially affect the host´s health by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon **(Gibson *et al.*, 1995; Van Loo *et al.*, 1999)**. The most common prebiotics are inulin and frutooligosaccharides (FOS) **(Kaplan *et al.*, 2000)**, which are naturally found in various plants. Some other non-digestible oligosaccharides, such as galactooligosaccharides (GOS), are also currently considered as prebiotics **(Gopal *et al.*, 2001; Gulewicz *et al.*, 2000)**. Although legumes are the plant species that more GOS accumulate in their seeds, other plant families, such as Brassica, Composita, Curcubitacea and Malvacea, appear as good candidates as sources of these oligosaccharides **(Kuo *et al.*, 1988; Martínez-Villaluenga *et al.*, 2007)**. Since humans do not possess the enzyme called α-galactosidase necessary for hydrolyzing the linkage present in these oligosaccharides, they cannot digest them when consumed. Intact oligosaccharides reach the colon, where they are preferentially fermented by beneficial bifidogenic microorganisms. These microorganisms produce end products such as lactic acid and short-chain fatty acids that contribute to several positive effects on the host´s health **(Gibson *et al.*, 2000; Mandalari *et al.*, 2008; Wang *et al.*, 1993)**. Positive health effects include: decreased lactose intolerance, increased resistance to intestinal pathogenic bacterial species, enhanced immune system, increased bioavailability of minerals, positive action on lipid metabolism, and decreased risk of intestinal infectious diseases, cardiovascular diseases, non-insulin-dependent diabetes, obesity, osteoporosis and some cancers **(Salminen *et al.*, 1998; Schaafsma *et al.*, 1998; Zubillaga *et al.*, 2001)**.
The aim of this work was to evaluate the *in vitro* prebiotic activity of GOS and low-molecular-weight carbohydrates (LMWC) extracted from lupin and squash seeds on the growth of the probiotic strain *Lactobacillus paracasei* under different carbon sources. Finally, these results may be useful to identify combinations of a probiotic strain and prebiotics that could be added into functional foods.

MATERIAL AND METHODS

Plant material and carbohydrate extraction

Seeds of squash (*Curcubita maxima*) and lupin (*Lupinus albus*) were purchased from a local market (Tandil, Argentina) and stored in polyethylene bags until use. Seeds were ground in a coffee mill KSM2 (Braun, México) and sieved through a sieve (mesh 32). GOS and LMWC were extracted following the procedure described by **Gullewicz *et al.* (2000)**, slightly modified. Seeds were defatted with hexane for 4 h in a Soxhlet extractor and then air-dried. Defatted samples were extracted with 70% ethanol in a screw capped tube by orbital shaking at room temperature overnight. After paper filtration, the filtrate was discolored by activated carbon 1% w/v, evaporated on a rotavapor (Büchi, Vaccum-System B-169, Switzerland) and finally dried in an oven at 40 °C under vacuum to constant weight. The GOS and LMWC thus obtained were resuspended in distilled water (1:1) and subjected to ultrasound for 5 minutes. The resulting suspension was filtered through a 0.22-micron membrane filter and the filtrate kept in sterile containers at -18 °C until use.

Reagents

The reagents used were Agar agar, Tripticase-Soy-Agar (TSA), Man-Rogosa-Sharpe broth (MRS), Luria-Bertani broth (LB), MRS agar, meat and yeast extract (Britania, Argentina), glycerol (Biopack, Argentina), glucose, sucrose and cellulose-G (Merck, Germany), commercial prebiotic inulin GR (Beneo-Orafti, Belgium), and raffinose and stachyose (Sigma-Aldrich, Germany). All other reagents used were of p.a. quality.

Determination of GOS and LMWC by Thin Layer Chromatography (TLC)

GOS and LMWC contents were determined by TLC, following the procedure described by **Kotiguda *et al.* (2006)**. TLC of carbohydrates was performed on

cellulose-G plates with 2-PrOH/EtAc/H_2O (6:1:3 v/v/v). Carbohydrates were visualized by naphthoresorcinol (1% α-napthol in 95% ethanol containing 10% orthophosphoric acid).

***Lactobacillus paracasei* and growth conditions**

A lyophilized strain of *L. paracasei* BGP1 (Clericci-Sacco, Italy) was kindly donated by Tuteur Laboratory (Argentina). This strain has been reported as a probiotic in different previous studies **(Huebner *et al.*, 2007; Reid, 2008)**. This strain was selected using raffinose as positive control and then grown in MRS broth at 37 °C for 24 h under anaerobic conditions and maintained as frozen stock cultures at -70 °C with 20% glycerol, until required. Frozen cultures were sub-cultured overnight in MRS broth with or without sugars (basal medium). One percent of the overnight culture was inoculated on fresh culture medium and incubated under the same conditions. Samples were drawn at different times of incubation (0, 2, 3, 5, 6, 7, 9 and 24 h) for measurement of cell density, pH, titratable acidity and plate count.

Basal growth medium and carbohydrate substrates

To study the growth of *L. paracasei* on various oligosaccharides, carbohydrate-free MRS containing: 10 g L^{-1} protease peptone, 8 g L^{-1} meat extract, 4 g L^{-1} yeast extract, 1 mL Tween 80, 2 g L^{-1} dipotassium phosphate, 5 g L^{-1} sodium acetate, 2 g L^{-1} ammonium citrate, 0.02 g L^{-1} magnesium sulfate, and 0.05 g L^{-1} manganese sulfate, was used as basal growth medium. A volume of 0.05% of L-cysteine was added to improve anaerobic conditions and stimulate the growth of the probiotic strain. Solid medium was prepared using 1.5% agar, and 30 mg/L of bromocresol purple only for the 24 h plate culture, as an indicator of acidification activity. The medium was adjusted to pH 6.5±0.2 and finally sterilized at 121 ºC, 1 atmosphere for 20 minutes.
Glucose, inulin GR, raffinose, and squash and lupin extracts, were sterilized by membrane filtration and added to the MRS basal medium to a final concentration of 1% w/v.

Optical density, pH and titratable acidity

MRS broths with different carbohydrates and without carbon source were used to evaluate the growth of *L. paracasei* for 24 h. Overnight cultures obtained with the same medium to be tested were used as inoculum, and then incubated at 37 °C for 24 h under anaerobic conditions. Cell density, pH and titratable acidity were evaluated at different times of incubation. Cell density was determined by monitoring the optical density at 600 nm (OD_{600}) with a SPECTRO-16/18 Series spectrophotometer (Shangai, China). Physiological solution (9 g/L) was used as blank. Samples were drawn to measure pH with a pHmeter (HANNA, USA) and, subsequently mixed with the same volume of distilled water and titrated with 0.11 M NaOH solution using phenolphthalein as indicator. The titratable acidity as percentage of lactic acid (%LA) was calculated according to the following equation **(Martínez-Villaluenga *et al.*, 2007)**:

%LA = {volume NaOH (mL) x 0.11 x 90 x 100} / {3 x 1000}
where 90 is the molecular weight of lactic acid (g mol^{-1}).

Plate count as Colony Forming Units (CFU) mL^{-1} of *L. paracasei*

Cultures obtained at different times were diluted with physiological solution 0.9% and then spread onto the corresponding MRS agar in duplicate. The plates were incubated anaerobically at 37 °C for 48 h. Each assay was performed in triplicate.

Enteric mixture and growth conditions

To study the growth of enteric mixture in various oligosaccharides, M9 minimal broth containing: 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl, 1 mL $MgSO_4.7H_20$ 1 M, 10 mL $CaCl_2$ anhydrous 0.01 M, was used as basal growth medium. The medium was adjusted to pH 6.8±0.2 and finally sterilized at 121 ºC, 1 atmosphere for 20 minutes. The assay was performed by adding 1% of an overnight culture of three Shiga toxin-producing *Escherichia coli* (STEC) strains grown on LB broth. *E. coli* O157:H7 + *E. coli* O91:H21 + *E. coli* O171:H2 were mixed in a 1:1:1 ratio (enteric mixture) and grown in LB broth at 37 °C for 18 h. One percent of the overnight enteric mixture was inoculated on separate tubes containing M9 minimal broth with 1% (w/v) glucose, 1% (w/v) prebiotic or 1% (w/v) plant extracts. The cultures were incubated under the same conditions and counted on TSA at 0 and 24 h of incubation. Each assay was performed in triplicate.

Prebiotic activity score

According to **Huebner *et al.* (2007)**, prebiotic activity reflects the ability of a given substrate to encourage the growth of a probiotic strain, such as *L. paracasei*, compared with that of other organisms (*E. coli* in this study) and relative to growth on a non-prebiotic substrate, such as glucose. Thus, carbohydrates have a positive prebiotic activity if they are metabolized similar to or as well as glucose by a test strain, but not by other intestinal bacteria. As a measure of prebiotic activity, the prebiotic score was calculated according to the following equation **(Huebner *et al.*, 2007)**:

Prebiotic activity score ={(probiotic log CFU mL^{-1} on the prebiotic at 24 h – probiotic log CFU mL^{-1} on the prebiotic at 0 h) / (probiotic log CFU mL^{-1} on glucose at 24 h – probiotic log CFU mL^{-1} on the glucose at 0 h)} – {(enteric log CFU mL^{-1} on the prebiotic at 24 h – enteric log CFU mL^{-1} on the prebiotic at 0 h) / (enteric log CFU mL^{-1} on glucose at 24 h – enteric log CFU mL^{-1} on glucose at 0 h)}.

Statistical analysis

Final growth (log CFU mL^{-1}), pH values and prebiotic activity scores, were used to analyze the degree in which different carbon sources present in the culture medium were used by the probiotic strain. All data are expressed as the mean and standard deviation of three replicates. Results were interpreted using one-way analysis of variance (ANOVA) followed by LSD Fisher Test to determine significant differences ($p<0.05$) using Infostat version 2011.

RESULTS AND DISCUSSION

Thin Layer Chromatography (TLC) of GOS and LMWC

Components of the GOS family and LMWC present in lupin and squash seed extracts were separated using TLC under the chromatographic conditions described above. We were able to identify sucrose, raffinose and stachyose as the principal sugars in such extracts (**Figure 1**). Under different chromatographic conditions, monosaccharides like glucose and galactose were also visualized when plates were sprayed with naphtoresorcinol (results not shown).

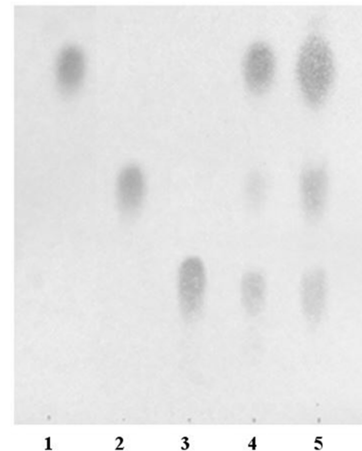

Figure 1 Thin layer chromatogram of oligosaccharides from lupin and squash seeds: (1) sucrose standard; (2) raffinose standard; (3) stachyose standard; (4) lupin extract; (5) squash extract

OD_{600}, pH and titratable activity

Although the composition of the MRS basal medium was enough to promote colony formation, a positive (+) growth of the strain was considered only in the case that carbohydrate fermentation occurred and the acid produced caused a notable change in the color of the medium. **Table 1** shows the results of carbohydrate utilization by *L. paracasei.*

Table 1 Carbohydrate utilization by *Lactobacillus paracasei*

Carbohydrate source	Growth[a]	△OD[b]	△%TA[c]	△pH[d]
Without carbohydrate (basal medium)	-	0.481	0.1	-0.51
Raffinose	+	3.095	0.65	-2.82
Inulin GR	+	5.65	1.35	-3.17
Glucose	+	5.93	1.49	-3.05
Squash extract	+	2.33	0.55	-2.46
Lupin extract	+	2.18	0.52	-2.66

[a]Disappearance of the purple color on the modified MRS agar was ascribed to the fermentative metabolism of *Lactobacillus paracasei* with acid production; (+) if colonies were surrounded by a yellow zone and (-) if no disappearance of purple color was observed. [b]Optical density (OD_{24h} – OD_{0h}) at 600 nm after 24 h in modified MRS broth. [c]Percentage of titratable acidity ($\%TA_{24h}$ – $\%TA_{0h}$) after 24 h in modified MRS broth. [d](pH_{24h} – pH_{0h}) after 24 h in modified MRS broth.

The analysis of the data allowed us to conclude that commercial inulin, raffinose and all the carbohydrates extracted from plant sources were fermented by *L. paracasei*.

Growth of *L. paracasei* with different carbohydrate sources

The growth of *L. paracasei* (log CFU mL^{-1}) for 24 h on 1% (w/v) glucose, 1% (w/v) inulin, 1% (w/v) raffinose, 1% (w/v) plant extracts and without added carbohydrate is shown in **figure 2**. Since a given carbohydrate with prebiotic activity should be metabolized by a test strain as well (or nearly as well) as glucose **(Huebner *et al.*, 2007)**, glucose was included in this study for comparative purposes (control). **Figure 3** shows *L. paracasei* growth reached at 24 h with the different carbon sources. The absence of carbon source in the medium (basal) significantly decreased the growth of this probiotic compared with the control ($p<0.05$) (**Figures 2** and **3**). In contrast, *L. paracasei* growth on MRS medium supplemented with raffinose, commercial inulin, lupin and squash extracts was not significantly different from that obtained using glucose ($p<0.05$). In particular, MRS added with squash extract resulted in a significantly higher growth than that obtained using raffinose ($p<0.05$).

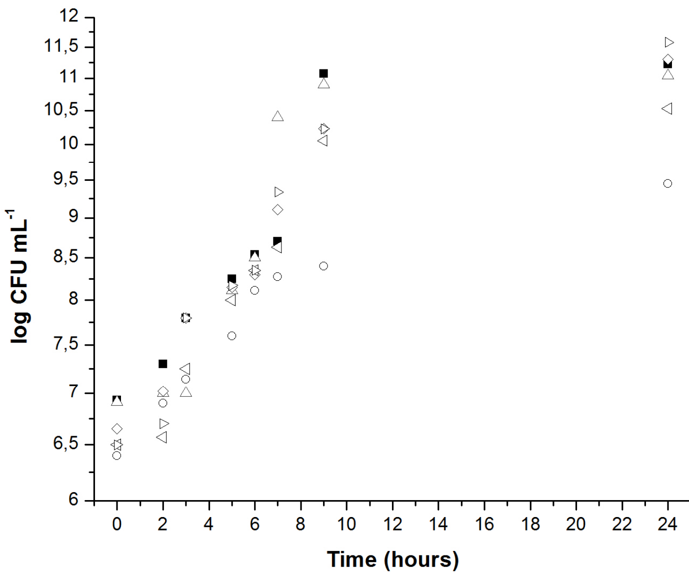

Figure 2 Growth of *Lactobacillus paracasei* in MRS broth containing either 1% glucose (■), 1% commercial inulin GR (△), 1% commercial raffinose (◁), 1% squash extract (▷), 1% lupin extract (◇), and no added sugar (○) ($p<0.05$).

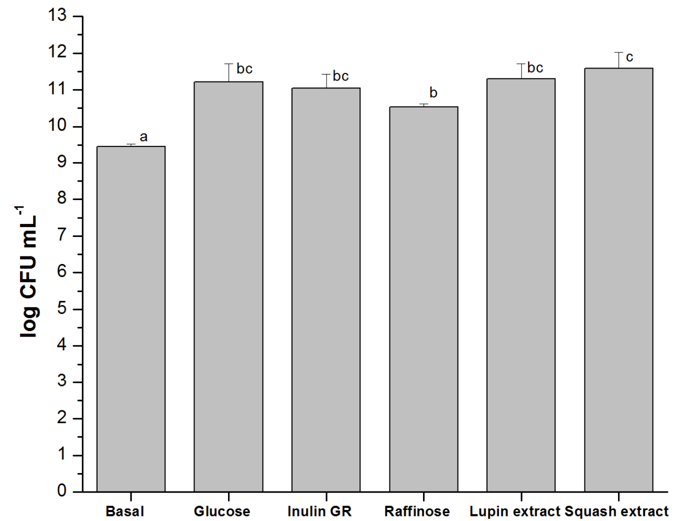

Figure 3 Effect of the presence or absence (basal) of a carbohydrate source on MRS medium at 24 hours of the growth of *Lactobacillus paracasei*. Error bars are ± SD. Results with the same superscript are not significantly ($p<0.05$) different.

Growth of *L. paracasei* and enteric mixture on prebiotics and plant extracts

The other characteristic property of a prebiotic substrate is that it should be selective and not fermented by commensal organisms **(Huebner *et al.*, 2007)**. Therefore, growth on each carbohydrate was also determined for a mixture of *E. coli* O157:H7, *E. coli* O91:H21 and *E. coli* O171:H2, corresponding to three enteric bacteria. The increases in cell densities of *L. paracasei* following 24 h growth on different carbohydrate sources are shown in **Table 2**. The growth of enteric mixture on all the carbohydrate sources was significantly lower ($p<0.05$) than that on glucose (**Table 2**).

Table 2 Cell density (log CFU mL^{-1}) at 0 h and 24 h, for bacterial cultures grown with different carbohydrate sources. All data are expressed as the mean ± SD of three replicates. Results with the same superscript are not significantly ($p<0.05$) different

Carbohydrate source	*Enteric mixture* (log CFU mL^{-1})			*Lactobacillus paracasei* (log CFU mL^{-1})		
	0 h	24 h	Difference	0 h	24 h	Difference
Without carbohydrate	6.07±0.02 a	7.81±0.30 a	1.74	6.45±0.19 a	9.44±0.07 a	2.99
Raffinose	7.36±0.04 d	8.73±0.01 c	1.37	6.51±0.05 a	10.54±0.09 b	4.03
Inulin GR	7.13±0.03 b	8.52±0.05 b	1.39	6.91±0.09 b	11.04±0.93 bc	4.13
Glucose	7.24±0.01 c	9.62±0.16 e	2.38	6.93±0.10 b	11.22±0.48 bc	4.29
Squash extract	7.35±0.01 d	8.85±0.03 d	1.5	6.49±0.07 a	11.57±0.43 c	5.08
Lupin extract	7.33±0.09 d	8.75±0.03 c	1.42	6.65±0.27 a	11.30±0.40 bc	4.65

The prebiotic score represents the difference between the relative growth of the probiotic at 24 h using prebiotic and glucose as carbon source, respectively, and the relative growth of the pathogen at 24 h using prebiotic and glucose as carbon source, respectively. The higher the score, the higher the relative growth of the probiotic and/or the lower the relative growth of the pathogen, which indicates a better and selective use of prebiotic in relation to glucose by the probiotic microorganism and/or a limited use of the prebiotic in relation to glucose by the pathogenic microorganism. Although growth of *L. paracasei* using lupin and squash seed extracts in the medium as the only carbon source may be attributed to the presence of sugars different from the GOS family, such as sucrose or glucose, the prebiotic scores obtained were indicative of a selective use of GOS by the microorganism respect to pathogenic strains.

Figure 4 Prebiotic activity scores of raffinose, inulin and carbohydrates extracted from food plants. Error bars are ± SD. Results with the same superscript are not significantly ($p<0.05$) different.

Figure 4 shows the prebiotic activity score derived from cell density values from Table 2. The highest prebiotic activity score was for *L. paracasei* grown on squash extract (0.55±0.03), followed by that on lupin extract (0.49±0.02), inulin GR (0.38±0.05) and raffinose (0.37±0.05). A negative prebiotic activity score was obtained for the medium without carbon source (M9 medium) (-0.02±0.01). We include this value as negative control. In this case, the probiotic strain grew less well on the medium without carbohydrate source than on that with glucose and/or had less growth on the medium without carbohydrate source than the growth of the enteric mixture on the prebiotic carbohydrate.

CONCLUSION

Here we investigated the capacity of *L. paracasei* to ferment different carbohydrate. The highest prebiotic activity score was obtained for *L. paracasei* grown on squash seed extract, followed that on lupin seed extract, whereas the lowest score was found for cultures grown on commercial raffinose and inulin. The results obtained allow us to consider GOS and LMWC extracted from seeds of squash and lupin as potential prebiotic substances. This study will contribute to selecting plant species as possible sources of new bioactive ingredients for the development of functional foods. However, considering that the ability of a substance to be considered as a prebiotic relies on its ability to selectively stimulate the growth and proliferation of beneficial intestinal microflora, this condition should be confirmed using additional *in vitro* and *in vivo* studies.

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