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RESEARCH ARTICLE

Prebiotic Effect of Mucilage and Pectic-derived Oligosaccharides from Nopal (*Opuntia ficus-indica*)

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Abstract Prebiotic effect of mucilage (MO) and pectic-derived (PO) oligosaccharides from prickly pear cactus stems was assessed on both cultures and metabolic activity of microbial communities from the human colon. The MO treatment enhanced lactobacilli growth up to 23.8%, while PO increased the bifidobacteria population by 25%. Furthermore, the addition of MO produced a slight decrease in enterococci, enterobacteria, staphylococci, and clostridia of about 4%. Increased levels of the short-chain fatty acids (SCFA) were attained in the cultures at rates of 35 and 16% in response to MO and PO treatments, respectively. Propionic acid (propionate) and butanoic acid (butyrate) production increased at least 50% throughout MO and PO treatments. A decrease in the ammonium level of 11.5% was produced by MO treatment. This research indicates that a mixture of MO and PO oligosaccharides from nopal (*Opuntia ficus-indica*) could act as prebiotic.

Keywords: *Bifidobacterium*, *Lactobacillus*, prebiotic, short fatty acid, soluble dietary fiber

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Introduction

Mucilage and pectin are considered soluble dietary fibers and exert physiological effects like hypocholesterolemic and hypoglycemic in humans during short-term trials. Mucilage obtained from nopal (*Opuntia ficus-indica*) was reported as a therapeutic agent against topical inflammation, skin ulcerations, gastritis, and also showed anti-ulcer activity. Other benefits that have been attributed to the ingestion of mucilage and pectin are interactions with drugs and intestinal ion homeostasis (1).

As other dietary fibers, pectin reaches the large intestine intact where it is extensively fermented by the gut microflora, creating a prebiotic effect. Highly esterified pectins are more slowly degraded than less esterified forms (2). *In vitro* fermentations of other plant polysaccharides such as xyloglucan showed that some intestinal bacteria that did not grow on the polysaccharide were capable of completely fermenting the oligosaccharides. This difference in fermentation patterns among some polysaccharides and oligosaccharides has also been reported for dextran, oligodextrans (3), and various glucose oligosaccharides with different degrees of polymerization (4).

Mucilages from nopal are complex polysaccharides with extremely branched structures (5) that contain various proportions of L-arabinose, pyranose, and furanose forms, D-galactose, L-rhamnose, and oxane-2,3,4,5-tetrol D-xylose, as the major neutral sugar units as well as galacturonic acid in different proportions. The suggested primary structure describes the molecule as a linear repeating core chain of 1,4-β-D-galacturonic acid, and α-1,2-L-rhamnose with trisaccharide side chain of β-1,6-D-glucose attached to *O*-4-L-rhamnose residues (6).

The other mucilage's nopal structure proposed takes into consideration 2 distinctive water-soluble fractions. One

fraction is a pectin with Ca^{2+} dependent gelling properties and the other fraction is a non-gelling mucilage (5). In nopal the water-soluble polysaccharide fraction with thickening properties represents less than 10% of the water-soluble material (7).

The nopal pectin composition varies depending on the source and conditions of extraction, location, and other environmental factors. The main component in pectin is a backbone chain structure of α-(1→4)-linked D-galacturonic acid units interrupted by the insertion of (1→2) linked L-rhamnopyranosyl residues in adjacent or alternate positions. The linear segments consisting predominantly of galacturonan are called homogalacturonans. The nopal pectin sugar composition extracted by alkaline process is uronic acid 56.3%, rhamnosa 0.5%, arabinosa 5.6%, galactosa 6.5%, and xylosa 0.9% while in acid process the sugar composition is uronic acid 64.0%, arabinosa 6.0%, galactosa 22.0%, glucosa 2.6%, and xylosa 2.1%. Other characteristic from nopal pectin is the low-methoxyl degree (<50). The yield of soluble pectin in various *Opuntia* species from Mexico is within a wide range of 0.13 to 2.64% in wet basis (1.0 to 23.87% in dry weight basis) (5).

In prehispanic times, the Aztecs reported that nopal was used to treat colon cancer (8). Nowadays, we might relate this effect with the soluble fiber composition (mucilage and pectin) and its content (1-2% wet weight) in prickly pear cactus stems (1). Therefore, it is likely that mucilage (MO) and pectic-derived (PO) oligosaccharides have a prebiotic effect. In terms of the rapidly growing bio-functional food industry, the use of cactus steam PO and MO as prebiotics has not yet been investigated. The objective of this research was to study the bifidogenic properties of MO and PO via their *in vitro* fermentation by human gut bacteria population.

Materials and Methods

Chemicals and solvents Reagents and media were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. All solvents were purchased from J.T. Baker (Baker Mallinckrodt, Mexico). HPLC-grade water was prepared by a Milli-Qplus purification system (Millipore Corp., Bedford, MA, USA).

Mucilage extraction Prickly pear cactus stems (*Opuntia ficus-indica*, cv. Milpa Alta) (about 15 cm long) were obtained from a commercial plantation in Milpa Alta, D.F., Mexico. Mucilage's extraction was carried out according to Sepulveda *et al.* (9). Nopal cladodes were crushed in a food processor (9110; Sunbeam Products Inc., Miami, FL, USA), homogenized with water (1:1 w/w), and thermally treated for 20 min at 85°C. The mixture was filtered through a fine cloth and centrifuged at 4,000×*g* for 10 min at 25°C (Z-383; Hermle-Labortechnik GmbH, Wehingen, Germany). Supernatant was then recovered and mucilage was precipitated with 65% ethanol for 20 h at 4°C. Then, samples were washed twice in ethanol (95%) and freeze-dried (Cryodos-80, freeze-drier; Telestar Industrial, S.L., Terrassa, Spain).

Pectin extraction The starting material to carry out pectin extraction was nopal flour obtained by freeze-drying. Twenty g of flour were mixed with 100 mL ethanol and stirred during 20 min. Then the mixture was centrifuged (4,000×*g*, 10 min, at 25°C), the solids were dissolved in 100 mL of acetone, shaken for 20 min, and finally a second centrifugation was carried out under the same conditions. The colorless flour was dissolved in 1 L of distilled water and heated to 85°C for 20 min. The mixture was cooled at room temperature and centrifuged. The solids were resuspended in 1 L of distilled water, the pH of the mixture was adjusted to 2.0 with 4 N HCl, heated at 90°C for 1 h, cooled at room temperature, and the pH was adjusted to 7 with 0.4 N NaOH. Solution was clarified by centrifugation; its pH was adjusted to 2 with 4 M HCl and clarified again. The pellet obtained was resuspended in 100 mL of distilled water and the pH was adjusted to 8 with 0.1 N NaOH. Finally pectin was precipitated by adding absolute ethanol (1:1). After centrifugation, the gel obtained was sequentially washed with absolute ethanol (pH 8). Finally the pectin collected from several runs (extractions) was freeze-dried, ground in a mortar, and stored a −20°C. The yield of pectin was determined gravimetrically.

Partial hydrolysis of the mucilage and pectin Mucilage-oligosaccharides (MO) and pectic-oligosaccharides (PO) used in the experiments were obtained from the controlled hydrolysis of natural mucilage with 1.0 N HCl, 5 min and pectin according to Olano-Martin *et al.* (10).

Molecular weights of the mucilage and pectin fractions were determined by size exclusion HPLC (SEC-HPLC) using a HPLC system (1290, LC system; Varian Inc., Walnut Creek, CA, USA) composed by a ternary pump (9012, Solvent Delivery System; Varian Inc.) and a detector UV/Vis (9050, Prostar 325; Varian Inc.). A column (TSK-Gel GPW_{XL}, 7.8×300 nm; Tosoh Bioscience, Tokyo, Japan) kept a 40°C and as mobile phase 0.2 M phosphate buffer at flow rate of 1 mL/min were used. A standard curve made with dextran solutions of known molecular weights (1,000, 5,000, 12,000, 25,000, 50,000, and 80,000 Da), which were prepared from a 1 mg/mL stock solution, was used to determine the molecular weight of oligosaccharides.

Microbial culture The culture medium for the microbial inoculum consisted of the following components (g/L): 4.2

starch, 2.0 peptone water, 2.0 yeast extract, 0.1 NaCl, 0.04 K_2HPO_4, 0.04 KH_2PO_4, 0.1 $MgSO_4 \cdot 7H_2O$, 0.01 $CaCl_2 \cdot 2H_2O$, 2.0 $NaHCO_3$, 0.5 Cysteine HCl, 0.5 bile salts, 0.05 vitamin K1, and 0.5 hemin, plus 2.0 mL/L (Tween 80).

Culture system The continuous culture system consisted of a 2-L double-jacketed vessel (triple wall double jacketed reactor; Asahi Glass Process System Ltd., Gujurat, India) maintained at 37°C and flushed twice a day for 15 min with N_2 to ensure anaerobic conditions. The vessel contained a mixed microbial community and the pH was maintained in the range 5.6-6.8 using a semi-automatic controller throughout continuous operation dosing 10 M NaOH with a peristaltic pump (950-1050; Masterflex, Barnant, IL, USA). Mixing at 250 rpm was performed with 2 Rushton turbins. Operation started in batch mode and continuous regimen began 12 h after.

The source of colon microbiota was a fecal sample from a healthy adult volunteer (aged 25 years) who did not receive antibiotic therapy during the past 6 months prior to sample collection for this study. Whole stool was collected and 20 g of freshly voided feces previously suspended in 20 mL of 50 mM phosphate buffer (pH 6.8) containing 0.05% cysteine was used as the inoculum of the vessel (2%, w/v). Two reactor runs were carried out separately using the same inoculum.

Experimental set-up During the pre-treatment period, the standard nutritional medium was fed to the reactor vessel. This medium enabled the adaptation of the microbial communities to the nutritional and physicochemical conditions of the reactor. After 2 weeks, the treatment period was initiated, which was extended over a 3 week period. Nutritional medium for this period consisted of the standard formulation lacking of starch, which was replaced by MO for the 1st run and by PO for the 2nd run. The dose of oligosaccharides applied to each reactor was 2.5 g/day L, which was equivalent to a human daily intake of 5 g. As the oligosaccharides replaced the starch concentration in the medium, the amount of available carbohydrates for the microorganisms was the same throughout the entire run. After the treatment period, a final post-treatment concluded the run, in order to verify whether the metabolic parameters and/or microbial concentrations evolved towards their initial values from the pre-treatment period. This period lasted for another 2 weeks, in which the oligosaccharide compound was replaced by starch in the medium.

Metabolic activity analysis To study the microbial fermentation of MO and PO, the short-chain fatty acids (SCFA) and ammonium production as the composition of the microbial community were monitored continuously throughout the 2 reactor runs.

SCFA analysis Culture samples were collected and frozen at –20°C for subsequent analysis. The SCFA were extracted from the samples with diethyl ether and determined using a GC (HP 6890; Variant Inc.). The GC system was equipped with a capillary free fatty acid packed column (30 m×0.25 mm i.d., 0.25 μm; Nukol, Sigma-Aldrich, St. Louis, MO, USA), a flame ionization detector, and a HP-3395 integrator. Helium (Praxair Mexico, Praxair, Mexico) was used as the carrier gas at a flow rate of 20 mL/min. The oven temperature was set at 180°C.

Ammonium Using a Kjeldahl digester (2117600; Labconco Corp., Kansan City, MO, USA), ammonium in the sample was obtained in the form of ammonia by the addition of an alkali (MgO). The released ammonia was distilled (2127600, Distillation unit; Labconco Corp.) from the sample into a boric acid solution. For the colorimetric assay, absorbance measurements were made with a single-beam spectrophotometer (Carry 50; Varian Inc.) at 460 nm with a 2.5 cm path length flow-through cell (11).

Microbial community analysis (plate counting) The following bacterial groups were quantified by growth on specific media: lactobacilli (rogosa agar), bifidobacteria (raffinose *Bifidobacterium* agar), enterococci (*Enterococcus* agar), enterobacteria (MacConkey agar), staphylococci (mannitol salt agar), and clostridia (tryptose sulfite cycloserin agar). Liquid samples were withdrawn from the culture system and serially diluted in saline solution (8.5 g/L of NaCl). Three plates were inoculated with a 0.1 mL sample of the 3 dilutions, and incubated at 37°C (43°C for enterobacteria) for 48 h under aerobic or anaerobic conditions, where necessary. The anaerobic incubation of plates was performed in jars with a defined gas atmosphere (84% N_2, 8% CO_2, and 8% H_2) (28029; Fluka-Sigma-Aldrich). After this incubation period, the counting of probiotic viable cells was carried out, and expressed as colony-forming units (CFU)/mL.

The choice of microbial groups was based on taking in consideration the most abundant groups of bacteria to the least abundant ones. For that reason, we selected bifidobacteria (10^9-10^{11} CFU/g) and clostridia (10^6-10^9 CFU/g) like principal groups of bacteria; enterobacteria (10^5-10^8 CFU/g), enterococci (10^5-10^7 CFU/g), and lactobacilli (10^5-10^8 CFU/g) like secondary groups of bacteria, and finally staphylococci with a concentration in fecal samples of 0-10^4 (CFU/g) approximately. We did not take in consideration the bacteroides because the concentration in the gut of the host is constant from teenager to senior citizen (12).

Statistical analysis A 2 sample student's *t*-test was carried between treatment and post-treatment versus pre-

treatment condition. All determinations were performed 3 times. The results displayed are the mean±standard deviation (SD) of each treatment (n=3). The differences were considered significant when p<0.05.

Results and Discussion

To evaluate prebiotic effect of PO and MO, 2 cultures were initially inoculated in the corresponding vessels with the same microbial inoculums in order to have identical initial conditions. This allowed evaluating specific effects generated as consequence of the presence of PO and MO. We validated initial conditions by measuring the growth and metabolic parameters during the pre-treatment period in both cultures; no significant differences in microbial community or metabolic activity were observed (data not shown).

Effect of MO or PO uptake on SCFA production by gut microorganisms An analysis of the metabolic activity during both cultures showed that the SCFA production increased 35% during MO treatment (71.05 mmol/L) and by 16% during PO treatment (62.57 mmol/ L; p<0.05) (Fig. 1). Both MO and PO produced an increase in SCFA production during both treatments, effect primarily attributed to an increase in the propionate and butyrate production. Propionate production doubled during MO treatment (21.57±1.94 mmol/L) while a 50% increase was observed with PO treatment (15.52±0.99 mmol/L). Butyrate was increased 50% throughout MO (19.06±0.98 mmol/L) and PO (12.72±1.98 mmol/L) treatments. The increase on propionate and butyrate production was not sustained after the treatment period. Moreover, branched chain fatty acid production was doubled during MO treatment (5.16±1.34 mmol/L) while this parameter increased by 50% during PO treatment (9.16±2.56 mmol/L; p<0.05). Both treatments showed a light residual effect (not significantly different, p<0.05) on branched fatty acids production.

The overall beneficial effects produced by MO were higher than those induced by PO. Administration of MO and PO to the cultures beneficially influenced the fermentation patterns of the colon microbiota reflected as higher SCFA production and remarkable higher propionate and butyrate levels. This can be considered as positive beneficial effects on human health since propionate is largely metabolized in the liver, it is gluconeogenic, and can inhibit *de novo* lipogenesis (13). Butyrate is the major energy source for the colonocytes and it has been implicated in the prevention of colitis and colorectal cancer. Both butyrate and propionate stimulate apoptosis of colorectal carcinoma and gastrointestinal tract cells (14).

Moreover, microbial propionate also contributes to decrease plasma cholesterol levels by inhibiting the synthesis of hepatic cholesterol (15). This shift on SCFA leading to higher propionate and butyrate production was previously noted during supplementation with fructo-oligosaccharides to rats (16) and with different oligosaccharides on both *in vitro* (17) and *in vivo* (16) systems.

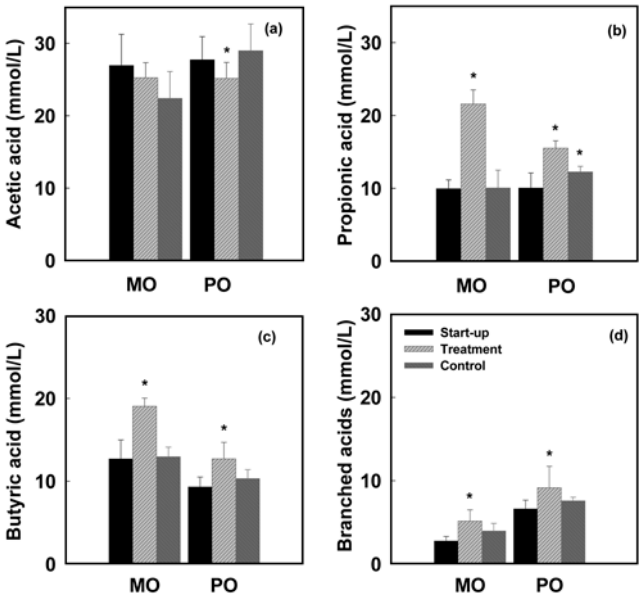

Fig. 1. Short-chain fatty acid concentration of acetic acid (a), propanoic acid (b), butanoic acid (c), and branched acids (d) concentrations (mmol/L). *Indicates a significant differences compared to pre-treatment at p<0.05. MO, mucilage-oligosaccharides; PO, pectic-oligosaccharides

Nevertheless, higher propionate and butyrate production levels were not accompanied to an increased growth for bifidobacteria or lactobacilli in the same proportion (Fig. 3), as these bacteria primarily produce acetic acid and 2-hydroxypropanoic acid (lactate). The increased SCFA production can be explained by the additional bifidobacterial biomass, created by the prebiotic effect from nopal-derived oligosaccharides.

Additionally, other microbial groups from the colon microflora might alternatively ferment carbon sources from the medium to produce SCFA. Other microbial groups have been implicated in the conversion of lactate or acetic acid into butyrate (18). Belenguer *et al.* (19) have described that butyrate-producing species such as *Anaerostipes caccae* and *Eubacterium halli* can be cross-fed with lactate produced by *Bifidobacterium adolescentis* grown in the presence of fructo-oligosaccharides, while a nonlactate-utilizing, butyrate-forming *Roseburia* sp. could assimilate products of the oligosaccharide hydrolysis performed by *Bifidobacterium*.

Duncan *et al.* (20) showed that a possible explanation for this behavior is the conversion of acetic acid to

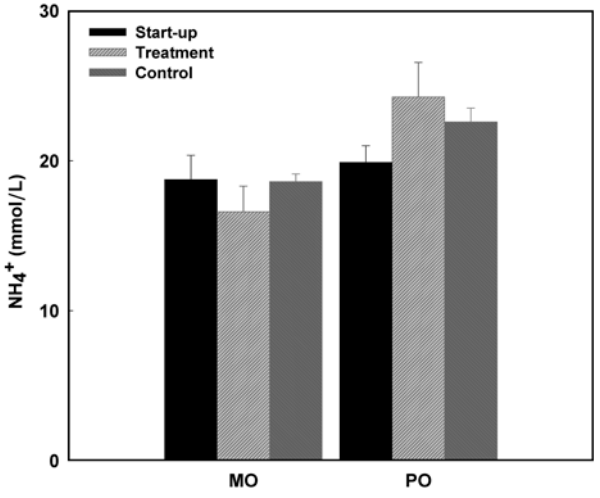

Fig. 2. Concentration of ammonium (mmol NH_4^+/L). Ammonium production at the end of the pre-treatment, treatment, and post-treatment. *Indicates a significant differences compared to pre-treatment at $p<0.05$. MO, mucilage-oligosaccharides; PO, pectic-oligosaccharides

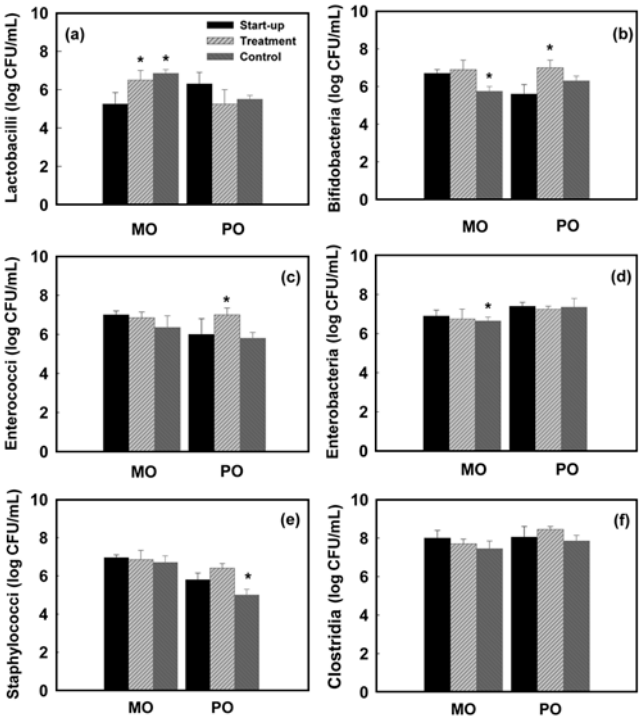

Fig. 3. Microbial growth counts of lactobacilli (a), bifidobacteria (b), enterococci (c), enterobacteria (d), staphylococci (e), and clostridia (f). *Indicates a significant differences compared to pre-treatment at $p<0.05$. MO, mucilage-oligosaccharides; PO, pectic-oligosaccharides

butanoic acid by species like *Megasphaera elsdenni* or *Roseburia* sp. Similar processes could explain the relative constant acetic acid concentrations during MO and PO treatments, while bifidobacterial biomass is increased.

Influence of MO or PO on gut microorganisms metabolism A second effect of oligosaccharides on the microbial metabolism was deducted by the ammonium production. The ammonium levels were not significantly different between both cultures during the pre-treatment period (MO 18.75±1.62 mmol/L, PO 19.91±1.17 mmol/L). Similarly the presence of MO or PO throughout the treatment period did not show differential effects on the ammonium levels. Addition of MO resulted in a not significant decrease in ammonium levels (16.6±1.73 mmol/L) which corresponds to 11.5% ($p<0.05$, Fig. 2). In contrast, supplementation of PO resulted in a not significant general increase in ammonium production (24.25±2.31 mmol/L) which corresponds to 21.8% ($p<0.05$). During the post-treatment, PO treatment showed a not significant slightly increase in ammonium production (residual effect).

Ammonia is produced in the colon by bacterial hydrolysis of urea as well as by bacterial deamination of amino acids, peptides, and proteins. Unlike carbohydrate fermentation, some of the protein degradation end products can be toxic to the host. High concentrations of ammonia in the colon have been related to increased DNA synthesis and neoplastic proliferation (21). Different indoles, amines, and phenols which are products from amino acid fermentation have been linked to a range of pathologies including schizophrenia, migraine, and hypertension (22). Lower proteolytic activities are therefore related to health-promoting effects.

The inhibition or not stimulation by MO or PO of these proteolytic end products and a shift towards a more saccharolytic environment may lie at the origin of its chemopreventive activity towards microbial bioactivation of cancer precursors. Ammonium production can specifically be linked to proteolytic conditions in the gut that could promote tumorigenesis and induce toxicity (23), thus mucilage and pectin can be considered beneficial in this aspect.

Effect of MO or PO uptake on gut microbial community growth Bacterial populations showed differences during the pre-treatment, except for clostridia. Changes in microbial community could be noted when the oligosaccharides compounds were supplemented to the reactor (Fig. 3).

Concentration of the beneficial microbial group increased in vessels throughout the MO treatment, lactobacilli (1.25 log CFU/mL) and bifidobacteria in lesser proportion (0.2 log CFU/mL, not significantly different) showing a slight bifidogenic effect ($p<0.05$). Addition of MO also resulted in a slight decrease (not significantly different) in enterococci (0.15 log CFU/mL), enterobacteria (0.15 log CFU/mL), and clostridia (0.3 log CFU/mL). MO addition also showed

a residual effect (not significantly different) throughout the post-treatment on these groups of bacteria. A higher concentration of bifidobacteria (7 log CFU/mL) was observed during the addition of PO ($p<0.05$), unlike lactobacilli that decreased 1.05 log CFU/mL. However, PO showed no inhibition of opportunistic pathogens during supplementation.

During the post-treatment, starch replaced the oligosaccharides in the nutrition of vessels; minor changes in the microbial community were observed. The increase in the number of lactobacillus (6.85 log CFU/mL) and bifidobacteria (6.3 log CFU/mL) were maintained for MO (significantly different, $p<0.05$) and PO (not significantly different), respectively. The other group of bacteria showed a slight decrease in this period (not significantly different). In regard to the effects on the microbial community, bifidobacteria have a competitive advantage over other intestinal microorganisms in a mixed culture environment, due to their β-fructofuranosidase and similar enzymes, allowing them to break down and utilize inulin-type fructans and other oligosaccharides. Besides their nutritional advantage, bifidobacteria have been suggested to inhibit excessive growth of pathogenic bacteria, modulate the immune system, suppress the activities of rotaviruses, and restore microbial integrity in the gut microbiota after antibiotic therapy (24).

Plate count analysis revealed that the addition of MO primarily increased lactobacillus, whereas PO resulted in an increase of the bifidobacteria population. Both bacterial groups are part of the lactate-producing bacteria that can produce an acidic environment leading to detrimental conditions for opportunistic pathogens.

The pathogen inhibitory effects from lactic acid bacteria can explain the unchanged concentrations of enterococci, enterobacteria, staphylococci, and clostridia during both the MO treatment and the post-tretment. This result is supported by other findings showing a negative influence of MO on the growth of *Clostridium difficile in vitro* (25). Several authors have reported a significant increase in bifidobacteria and a concomitant decrease in *Enterococcus* spp. upon fructooligosaccharides supplementation in humans (26) and rats (27).

Since long chain oligosaccharides are typically digested slowly in comparison to the shorter ones, a relatively long supplementation period is required for long chain oligosaccharides to exert a prebiotic effect *in vitro* and *in vivo* (28). It was observed that a combination of short-chain and long-chain oligosaccharides is physiologically more active than the individual fractions (18). However, complaints of flatulence, abdominal pain, and bloating have been reported in human feeding studies involving prebiotics (13). Evidence suggests that a rational dose of up to 20 g/day can produce gas distension (24).

Our *in vitro* system showed that MO and PO from nopal act as prebiotics which implies specific changes in the composition and/or metabolism of the colon microbiota, which could provide benefits on host's health. These beneficial effects were observed on the metabolic activity and the microbial community composition at doses of 2.5 g/day, which falls within the range of previous *in vivo* and *in vitro* studies on prebiotics like pectic-oligosaccharides and inulin (29).

In the present study, we have shown that MO produce significant changes on both metabolic and growth on cultured colon microbiota, these effects could be associated with the health benefits reported after nopal intake. Interestingly, these effects were higher than those produced by PO. It was also observed that long lasting treatment is required to effectively induce and maintain the observed effects. Furthermore, we found that higher supplementation levels of MO are needed in comparison to PO in order to further reduce putrefactive ammonium production, to increase SCFA production and to sustain more pronounced bifidogenic effects over longer periods of time. The positive changes induced in the metabolism and microbial community indicate that the nopal-derived oligosaccharides used in this study can act as prebiotics making them candidates as components of new functional foods.

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