Intestinal Anti-inflammatory Effects of Oligosaccharides Derived from Lactulose in the Trinitrobenzenesulphonic Acid Model of Rat Colitis

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Short title: Lactulose-derived oligosaccharides in experimental rat colitis
ABSTRACT

Intestinal microbiota modulation is becoming an interesting approach to manage inflammatory bowel disease, and can be achieved by the administration of prebiotics. Previous studies showed the intestinal anti-inflammatory effects of the prebiotic lactulose. The aim of the present study was to test the preventative effects of oligosaccharides derived from lactulose with prebiotic properties (OsLu), in the trinitrobenzenesulphonic acid model of rat colitis, and compare them with lactulose. Both treatments modified bacterial profile in intestinal contents, increasing the bifidobacteria and lactobacilli counts, and up-regulating the production of short chain fatty acids, although OsLu generated a larger amount. OsLu also inhibited in greater extent different pro-inflammatory markers such as interleukins (IL) 1, 6, 12 and 23, and chemokines (MCP-1 and CINC-1). However both prebiotics equally restored colonic epithelial integrity, evaluated both with a histological score (OsLu 9.8±2.2 and lactulose 12.1±2.1, vs. colitic control 27.3±3.3) and measuring several key proteins of the mucosal barrier (MUC-2, MUC-3 and TTF-3). OsLu effect was also associated with an inhibition of iNOS expression and a reduction of Th17 cell activity in the inflamed tissue that facilitated the intestinal mucosa barrier recovery. In conclusion, OsLu showed a better anti-inflammatory profile than lactulose in this model of experimental colitis.

Keywords: intestinal anti-inflammatory effect; lactulose-derived oligosaccharides; prebiotic; TNBS rat colitis.
INTRODUCTION

Inflammatory bowel disease (IBD) usually refers to two related conditions, ulcerative colitis (UC) and Crohn's disease, characterized by chronic and spontaneously relapsing inflammation. Among the clinical symptoms of IBD are abdominal pain, diarrhea, rectal bleeding, malaise and weight loss. Although the exact etiology of IBD remains unknown, both inflammatory conditions have similar pathophysiology background, and several studies indicate that a combination of factors such as genetic susceptibility, intestinal microbiota, dietary factors, intestinal barrier dysfunction, and an abnormal immune response to intestinal bacteria lead to chronic intestinal inflammation. In fact, IBD may be considered as a maladaptation of host commensal mutualism that promotes intestinal microbiota imbalance, termed as dysbiosis. It is typically characterized by a decline in bacterial diversity, with relative predominance of potential pathogenic bacteria and insufficient amount of protective species, that leads to chronic intestinal inflammation. In consequence, an interesting approach to IBD management could be the change of the microbiota, in a preventative and/or therapeutic manner, to protect intestine against inflammation, stimulate tissue repair and restore the homeostasis in genetically susceptible hosts. This could explain, at least partially, the remission that sometimes is achieved in intestinal inflammation after treatment with antibiotics such as metronidazole or ciprofloxacin. Unfortunately, the prolonged use of antibiotics in the management of these intestinal conditions is limited due to the risk of severe adverse effects. For this reason, it is very important to find other effective strategies focused on microbiota balance restoration, with a better safety profile. This could be the case of prebiotics, which are defined as a selectively fermented ingredient that allows specific
changes, both in the composition and/or activity in the gastrointestinal microbiota, that confers benefits upon host well being and health.\textsuperscript{4}

The efficacy of the prebiotics on intestinal inflammation has been most extensively studied in experimental colitis models, which, in addition, have provided insight into possible mechanisms involved in their intestine anti-inflammatory properties.\textsuperscript{5} One of these consists in the modification of the intestinal microbial composition by stimulating the growth of commensal protective bacteria and therefore enhancing resistance to disease-inducing bacteria colonization and contributing to colitis improvement. Besides, the promotion of protective bacteria growth can result in up-regulation of epithelial defense mechanisms that protect against intestinal inflammation. Prebiotics are fermented by anaerobic colonic microbiota and there is an increase of short chain fatty acids (SCFA), mainly acetate, butyrate, and propionate, that may also improve colitis condition; thus, butyrate is the major energy source for colonocytes, and has an essential role in the maturation of colonic epithelium, regeneration of mucosa and induction of cell differentiation.\textsuperscript{6} Moreover, the impact of prebiotics on the host immune system can also contribute to their beneficial effects. In this regard, it has been describe that prebiotics have the ability to modulate the production of pro- and anti-inflammatory cytokines, and thus lessen the intestinal inflammation.

Many different substances are claimed to be prebiotics, but mainly carbohydrates have proven to display prebiotic properties in humans.\textsuperscript{4,7} Lactulose is a synthetic disaccharide composed of fructose and galactose primarily used as a laxative and for the treatment of portal-systemic encephalopathy.\textsuperscript{8,9} Following oral administration, intact lactulose reaches the colon, where it is split by bacteria in distal
small intestine and colon, lowering fecal pH and promoting beneficial microbiota growth such as bifidobacteria and lactobacilli and inhibiting pathogenic bacteria expansion like *Salmonella*.\textsuperscript{11}

In fact, preclinical studies have demonstrated a protective effect of lactulose in rodent models of colitis.\textsuperscript{11-13} Up to date, there is only one pilot study reported in humans, in which oral lactulose was administered as adjuvant therapy to standard medication. The study concluded that prebiotic consumption did not ameliorate clinical activity, endoscopic score or immunohistochemical parameters, although it improved the quality of life of UC patients receiving the prebiotic.\textsuperscript{14} Furthermore, and although generally considered safe and innocuous, lactulose may be associated with several adverse events, including abdominal discomfort, abdominal distension, increased intestinal gas production, and flatulence, due to the fact that it is mainly consumed by the bacteria in the proximal colon.\textsuperscript{15} All these facts could justify the search for alternatives to lactulose with modified prebiotic properties, thus achieving a higher efficacy in intestine inflammation, with fewer side effects.

Oligosaccharides derived from lactulose (OsLu) are new bioactive carbohydrates synthesized with the aim of obtaining more slowly fermenting prebiotics able to reach distal colon unhydrolysed.\textsuperscript{16-18} Different *in vitro* fermentation assays using pure cultures as well as fecal inoculums have demonstrated that when these oligosaccharides were used as sole carbon source, there was a stimulation of bifidobacteria and lactobacilli growth. Thus, when long incubation periods were used in fermentative assays, OsLu showed higher prebiotic index values than the native disaccharide.\textsuperscript{19,20} These investigations proved that OsLu improved lactulose prebiotic properties and therefore, it is reasonable to suppose that OsLu could present a higher efficacy in intestine
inflammation. More recently, it has been evaluated the modulatory effects of OsLu on microbial composition in the cecum and colon of growing rats, with particular emphasis on their bifidogenic effect, showing that OsLu produced a significant and selective increase of *Bifidobacterium animalis* in these intestine locations. All these findings support a prebiotic role of galactosyl-fructoses in functional foods.

The aim of the present study was to test the preventative effects of OsLu in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation with some resemblance to human IBD, and compare its efficacy with its parent compound, lactulose. Special attention was paid to their effects on the expression of some of the inflammatory mediators, such as pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-12, IL-17 and IL-23), chemokines (cytokine-induced neutrophil chemoattractant (CINC)-1 and monocyte chemotactic protein (MCP)-1) and an inducible enzyme (inducible Nitric oxide synthase (iNOS)), as well as different markers of epithelial integrity in the mucosa, like the mucins MUC-2 and MUC-3, and the protein trefoil factor (TFF)-3. The prebiotic capacity was assessed by measuring the production of SCFA and the effects on the microbiota composition. In summary, OsLu therapeutic efficacy was greater than lactulose, maybe due to its better immunomodulatory properties together with a superior capacity of SCFA production.

**MATERIALS AND METHODS**

This study was carried out in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ as promulgated by the National Institute of Health. The experimental protocol was approved by the Commission of Ethics in Animal
Experimentation (Protocol number CEEA 2010-286) of the University of Granada (Spain).

Reagents and synthesis of oligosaccharides derived from lactulose (OsLu).

All chemicals were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. Glutathione reductase was provided by Boehringer Mannheim (Barcelona, Spain). A commercial preparation Duphalac® (Abbott Healthcare; Barcelona, Spain), which contains 670 g/L of lactulose, was used as control and to synthesize OsLu.

Enzymatic reactions have been performed at 50°C and pH 6.5 using a β-galactosidase from Aspergillus oryzae (16 Ud/mL) in an orbital shaker at 300 rpm for 24 h. Afterward, samples were immediately immersed in boiling water for 10 min to inactivate the enzyme. Then, the mixture of oligosaccharides was treated with yeasts to eliminate monosaccharides following the method of Sanz et al.,23 with some modifications. Briefly, oligosaccharide reaction mixture 20% (w/v) was treated with fresh Saccharomyces cerevisiae 1.5% (w/v) (Levital, Panibérica de Levadura, Valladolid, Spain) at 30°C for 48 h in an orbital shaker (300 rpm) and submitted to vacuum filtration to remove the yeasts. Sample was concentrated at 38-40°C in a rotary evaporator (Büchi Labortechnik AG. Flawil, Switzerland). Mono- and disaccharides as well as OsLu were analysed by GC-FID.24 Degree of polymerization (DP) of the oligosaccharides found in the purified fraction from the synthesis mixture was also determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDITOF-MS). Before yeast treatment, the transglycosylation mixtures contained 76% of sugars which corresponded to 40% of monosaccharides, 6% of lactulose and 30% of OsLu. After yeast treatment the composition of mixtures was 28% of monosaccharides, 12% of lactulose and 36% of OsLu (17% disaccharides, 13%
trisaccharides, 5% tetrasaccharides and 1% pentasaccharides. MALDI-TOF-MS analysis allowed to detect oligosaccharides with a higher DP of 6, 7 and 8 which could not be quantified by gas chromatography. Also, other analysis of OsLu showed the presence 5% of salts and 1% of nitrogen with a dry matter of 82%.

**Experimental design.** Female Wistar rats (180-200 g) obtained from Janvier (St Berthevin Cedex, France) were housed individually in makrolon cages, maintained in an air-conditioned atmosphere with a 12-h light-dark cycle, and provided with free access to tap water and food. The rats were randomly assigned to four groups (n=10); two of them (non-colitic and control groups) received tap water and the others (treated groups) were given lactulose or OsLu in the drinking water, daily prepared, at the concentration of 2.5% (w/v) for three weeks. The average rat intake was approximately of 0.25 g/day (the mean water intake was 10.2 ± 0.9 ml/rat and day, without showing differences among groups). This dose was equivalent to that used in a previous study with lactulose in the same experimental model of rat colitis.\textsuperscript{12}

Two weeks after starting the experiment, the rats were fasted overnight and rendered colitic as previously described.\textsuperscript{12} Briefly, they were anaesthetised with isofluorane and given 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. During and after TNBS administration, the rats were kept in a head-down position until they recovered from the anesthetic, and were then returned to their cages. Rats from the non-colitic group were administered intracolonically 0.25 ml of phosphate buffered saline instead of TNBS. All rats were killed with an overdose of halothane one week after colitis induction.

In another set of experiments, the efficacy of both lactulose and OsLu was evaluated in the same experimental model for a longer period of time, three weeks. In
this assay, a second dose of 10 mg of TNBS dissolved in 50% ethanol was administered two weeks after the initial TNBS colonic instillation, in an attempt to mimic the relapses common in human IBD. Ten animals from each colitic group (control and prebiotic treated) and five from the non-colitic group were sacrificed after 1, 2 and 3 weeks of colitis induction. So the efficacy of Oslu after one week was performed twice.

**Assessment of colonic damage.** The body weight, water and food intake were recorded daily throughout the experiment. Once the rats were sacrificed, the colon was removed aseptically and placed on an ice-cold plate, longitudinally opened and luminal contents were collected for the microbiological studies (see below). Afterwards, the colonic segment was cleaned of fat and mesentery, blotted on filter paper; each specimen was weighed and its length measured under a constant load (2 g). The colon was scored for macroscopically visible damage on a 0-10 scale by two observers unaware of the treatment, according to the criteria previously reported, which take into account the extent as well as the severity of colonic damage. Representative whole gut specimens (0.5 cm²) were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered formaldehyde for the histological studies. Equivalent colonic segments were also obtained from the non-colitic group. The colon was subsequently minced, aliquoted and kept frozen at -80°C until biochemical determinations and RNA extraction was performed.

For the histological studies, cross-sections were selected and embedded in paraffin. Full-thickness sections of 5 µm were obtained at different levels and stained with hematoxylin and eosin. The histological damage was evaluated by a pathologist.
observer, who was blinded to the experimental groups, according to the criteria previously described by Arribas et al.\textsuperscript{25}

Myeloperoxidase activity (MPO) was measured according to the technique previously described;\textsuperscript{26} and the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 µmol hydrogen peroxide per minute at 25ºC. Total glutathione content (GSH) was quantified with the recycling assay,\textsuperscript{27} and the results were expressed as nmol/g wet tissue.

The analysis of gene expression in the colonic samples was performed by real-time quantitative PCR (RT-qPCR). For this purpose total RNA from colonic samples was isolated using Trizol\textsuperscript{®} following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop\textsuperscript{TM} 2000 Spectrophotometer and 2 µg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). RT-qPCR amplification and detection was performed on optical-grade 48well plates in a EcoTM Real-Time PCR System (Illumina, San Diego, CA, USA) with 20 ng of cDNA, the KAPA SYBR\textsuperscript{®} FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primers at their annealing temperature (Ta) (Table 1). To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. The mRNA relative quantitation was calculated using the ΔΔCt method.

**Microbial analysis of the colonic contents.** For DNA extraction, samples from colonic contents were diluted 1:12 (w/v) in the lysis buffer ASL provided by the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The suspensions were transferred to a Lysing Matrix E tube that contained a mixture of ceramic and silica particles designed to efficiently lyse
microorganisms (Qiagen, Hilden, Germany). Tubes were shaken in a Precellys apparatus (VWR, Villeurbanne, France) at 5.5 rpm/min for 30 s twice. DNA was then extracted by using a QIAamp DNA stool minikit from Qiagen, as recommended by the manufacturer (protocol for isolation of DNA for pathogen detection), except that a supplemental mixture of enzymes (mutanolysin at 90 U and lysozyme at 9 mg/mL) was added to the lysis buffer. RT-qPCR was used to characterize the bacterial DNA present in the colonic content samples as reported previously.\textsuperscript{28} For this purpose, a series of genus-specific primer pairs were used (Table 2). PCR amplification and detection was performed on optical-grade 48-well plates in a Eco\textsuperscript{TM} Real-Time PCR System (Illumina, San Diego, CA, USA). In this case, each reaction mixture (10 µl) was composed of 5 µL of KAPA SYBR \textsuperscript{®} FAST qPCR Master Mix (Kapa Biosoftware, Inc., Wilmington, MA, USA), 0.25 µl of each specific primer at a concentration of 10 µM and 2 µl of DNA template. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from pure cultures with a bacterial population ranging from 2 to 9 log colony forming units (CFUs), as determined by plate counts. One strain belonging to each of the bacterial genera or groups targeted in this study was used to construct the standard curve. More specifically, the strains from which the DNA was extracted were the following: \textit{Bifidobacterium longum} CECT 4551, \textit{Clostridium coccoides} DSMZ 935, \textit{Bacteroides fragilis} DSMZ 2151, \textit{Lactobacillus salivarius} CECT 2197. All of them were obtained from the Spanish Collection of Type Cultures (CECT) or the German Collection of Microorganisms and Cell Cultures (DSMZ).

**Short chain fatty acid (SCFA) determinations.** To quantify the SCFA concentrations in the colonic luminal contents, the samples were homogenized with 150 mM NaHCO\textsubscript{3} (pH 7.8) (1:5 w/v) in an argon atmosphere. Samples were incubated for
24 h at 37 °C and stored at −80 °C until the extraction. To extract the SCFAs, 50 µL of
the internal standard 2-methylvaleric acid (100 mM), 10 µL of sulphuric acid and 0.3
mL of chloroform were added to 1 mL of the homogenate and, then, centrifuged at
10,000 × g for 5 min at 4 ºC. The organic layer was dehydrated with sodium anhydrous
sulfate and centrifuged at 10,000 × g for 5 min at 4 ºC. 1 µL of the supernatant was
injected into a gas chromatograph (Perkin-Elmer Autosystem GC-FID, Waltham, MA,
USA) equipped with a capillary column (CPWAX 52CB 60 m × 0.25 mm, 0,25 µm,
Varian, Middelburg, The Netherlands) and connected to a Star Chromatography
WorkStation program (version 6, Varian, Middelburg, The Netherlands) to quantify the
samples. Operating conditions were: injector 275ºC; detector 300 ºC; initial column
temperature 90ºC for 0.1 min then increasing 15ºC/min up to 245ºC and 4 min at 245ºC.
The flow of carrier gas (nitrogen) was 1 mL/min. The gas chromatography system had a
split ratio 1:33. Acetate, propionate and butyrate concentrations between 0.30-60 mM
were used to make the standard curve. A linear relationship was found between the peak
area ratio SCFA/IS using the same protocol than that for samples.

Statistics. All results are expressed as the mean ± SEM. Differences between
means were tested for statistical significance using a one-way analysis of variance
(ANOVA) with Tukey post-hoc test. Differences between proportions were analyzed
with the chi-squared test. All statistical analyses were carried out with the GraphPad
Prism version 5.0 (La Jolla, CA, USA), with statistical significance set at P<0.05.

RESULTS
Preventative effects of lactulose and OsLu on TNBS rat colitis. The administration of either lactulose or OsLu for two weeks did not induce any symptoms of diarrhea or significantly affect weight evolution. However, once the colitis was induced, both groups of treated rats showed an overall lower impact of TNBS-induced colonic damage compared to the TNBS control group. The anti-inflammatory effect was evidenced macroscopically by a significantly lower colonic damage score than that of control rats ($P<0.05$), with a significant reduction in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS/ethanol (Table 3). The weight/length ratio was increased significantly in colitic rats as a consequence of the inflammatory process when compared with non-colitic rats, and no significant differences were observed among the two treated groups and the corresponding colitic control (Table 3).

The histological evaluation of the colonic segments confirmed the intestinal anti-inflammatory effect of both lactulose and the OsLu (Figure 1). The colonic samples from the untreated colitic control group showed extensive ulceration of the mucosa that typically affected over 75% of the surface. Also, severe inflammation that involved all the intestinal layers was observed, and it was associated with diffuse leukocyte infiltration, mainly composed of neutrophils in the mucosa layer and, to a lesser extent, lymphocytes in the submucosa. In addition, the inflammatory process was associated with severe goblet cell depletion (Figure 1B). In this group of rats, the grade of lesion was considered as severe, with a score value (mean ± SEM) of 27.3 ± 3.3 (Figure 1E). On the contrary, the histological assessment of the colonic specimens from rats treated with either lactulose (Figure 1C) or OsLu (Figure 1D) showed an evident recovery of the colonic tissue, displaying a significantly reduced score in comparison with untreated...
colitic rats. Thus, most of the samples revealed a restoration of the epithelial cell layer, being only affected a maximum of 25% of the epithelium in contrast to the extensive ulceration observed in TNBS control group. In addition, goblet cells appeared replenished with their mucin content, and it was also observed a reduction in the inflammatory infiltrate, which was slight to moderate with a patchy distribution.

The intestine anti-inflammatory effect was confirmed biochemically, since both treated groups showed significantly reduced colonic MPO activity (Table 3), which agrees with the reduction of neutrophil infiltration observed in the histological study and thus demonstrates the intestine anti-inflammatory effect of the treatment. In addition, the administration of lactulose or OsLu partially restored colonic glutathione content, a tripeptide with antioxidant properties, which was depleted in colitic rats as a consequence of the colonic oxidative stress induced by the inflammatory process, similarly to that previously reported in this model of experimental colitis (Table 3).

The colonic inflammatory status in control colitic rats was also characterized by an increased expression of the different pro-inflammatory markers evaluated, including the cytokines IL-1β, IL-6, IL-12, IL-17 and IL-23, the chemokines CINC-1 and MCP-1, and the inducible enzyme iNOS, in comparison with the non-colitic group. The evaluation of the cytokines revealed that the pretreatment of colitic rats with OsLu was able to significantly reduce the expression of all of them, whereas lactulose administration only decreased the expression of IL-6 in the colonic tissue, without showing statistical differences with the other cytokines assayed in comparison with untreated colitic rats (Figure 2). These results suggest that the amelioration of the altered immune response that characterizes the colonic inflammatory process is involved in the beneficial effect observed in both treated groups, but OsLu showed a
higher efficacy than lactulose. Similarly, only the treatment of colitic rats with OsLu resulted in a significant reduction of the expression of colonic iNOS (Figure 2), and the chemokines CINC-1 and MCP-1 (Figure 3), that could support the lower infiltration of either neutrophils and macrophages in the inflamed tissue, that was evidenced both biochemically and histologically. Of note, both lactulose and OsLu managed to completely restore the expression of proteins involved in colonic epithelial integrity (MUC-2, MUC-3 and TFF-3), which were significantly reduced in colitic rats when compared to the healthy ones (Figure 3) and could promote the recovery of the epithelial layer observed in the histological studies.

**Effects of lactulose and OsLu on TNBS reactivated rat colitis.** In this assay the impact of a longer prebiotic treatment was evaluated. Also, and in order to simulate the ‘flare-ups’ that occur in human IBD, a second intracolonic dose of 10 mg of TNBS in ethanol was administered two weeks after the first administration, which resulted in a reactivation of the colonic inflammatory process. The results confirmed the intestine anti-inflammatory effects exerted by lactulose or OsLu in this experimental model of colitis, at the different time points evaluated. Thus macroscopically, both compounds were able to significantly reduce the colonic score and weight/length ratio, one and two weeks after the first colonic insult, as well as one week after the second TNBS administration (Table 4). These beneficial effects were confirmed biochemically by a reduction in the colonic MPO activity, thus reflecting a lower leukocyte infiltration, which was significant for all treated groups at the different time points evaluated, except for lactulose one week after reactivation of the inflammatory process (Figure 4). When the colonic oxidative status was assessed by glutathione content determination, both lactulose and OsLu were able to significantly reduce the depletion of this peptide one
week after the first administration of TNBS, whereas only the lactulose derivative OsLu was able to significantly restore its colonic content after the reactivation of the colonic damage (Figure 4).

The evaluation of the different biochemical markers by qPCR, confirmed the results described above, for both lactulose and OsLu, when the inflammatory process was evaluated one week after the first administration of TNBS. The evaluation after two weeks revealed that the expression of the proinflammatory cytokines and chemokines were reduced in the control colitic group in comparison with the data observed in the same group at the first week, although most of them were still significantly higher than in healthy rats. However, the evaluation of the proteins related with the epithelial integrity (MUC-2, MUC-3 and TFF-3) revealed that only MUC-2 showed a significant reduced expression in comparison with non-colitic rats (Figures 5 and 6). Since the amelioration of the colonic inflammatory process took place at this time due to its normal resolution, the beneficial effects exerted by the treatments were less evident than in the first week of treatment. In fact these were noticeable for both compounds when IL-6 or IL-17 were considered, and only for OsLu when IL-12 or the chemokines MCP-1 and CINC-1 or the mucin MUC-2 were evaluated (Figures 5 and 6).

The reactivation of the inflammatory process typically resulted in a new increment of the expression of most of the pro-inflammatory cytokines, including IL-1β, IL-6, IL-12 and IL-23, as well as for iNOS, together with a reduction of the expression of the mucins and TFF-3. At this time, OsLu also showed a higher beneficial effect than lactulose, since the derivative reduced the expression of all the cytokines whose expression was upregulated one week after the second administration of TNBS, whereas lactulose only reduced IL-6 (Figure 5). At this time, only MUC-2 expression was
restored after the treatment with both lactulose and OsLu, without showing a significant
effect in comparison with colitic control group, when MUC-3 or TFF-3 were considered
(Figure 6).

Effects of lactulose and OsLu on TNBS on microbiota composition and
SCFA production in colitic rats. The evaluation of the microbial content in the colonic
lumen one week after the first administration of TNBS evidenced a significant decrease
in the counts of both lactobacilli and bifidobacteria and, on the contrary, an increase in
clostridia and bacteroides in TNBS colitic rats in comparison with the healthy ones
(Figure 7). The treatment with lactulose or with OsLu resulted in a significant increase
in both lactobacilli and bifidobacteria counts when compared with the control colitic
group, although no significant modification was observed when clostridia or bacteroides
counts were considered. When the microbial content was evaluated after two weeks, a
restoration in both lactobacilli and bifidobacteria counts was observed in the rats from
the colitic control group, without showing statistical differences with the healthy group;
however, increased counts of clostridia and bacteroides were obtained in colitic rats in
comparison with non-colitic ones. No significant modification was obtained in any of
the bacterial groups studied with either lactulose or OsLu when compared with the
corresponding colitic control group (Figure 7).

The reactivation of the inflammatory process after the second dose of TNBS
resulted in a new reduction of lactobacilli and bifidobacteria after one week, while the
number of clostridia and bacteroides were increased. OsLu was again able to
significantly increase the counts of lactobacilli and bifidobacteria in comparison with
the control colitic rats, whereas lactulose was devoid of any significant effect. Also, no
significant modifications were observed among colitic groups when clostridia or
bacteroides were considered (Figure 7).

As a consequence, the colonic inflammatory process was associated, at week 1
and 3, with a significant reduction in the production of the different SCFA evaluated
(acetate, propionate and butyrate) (Figure 8), as it has been previously reported in the
same model of experimental colitis. The beneficial effects exerted by the treatments of
lactulose and OsLu in colitic rats were associated with an increased production of all the
SCFA one week after the first administration of TNBS. Moreover, OsLu was able to
restore these levels without showing statistical differences with non-colitic rats (Figure
8). However, one week after colitis reactivation, only OsLu was able to raise the
production of the SCFA in comparison with non-treated colitic rats (Figure 8).

DISCUSSION

A decrease in bacterial diversity has been reported to occur in IBD patients,
characterized by reduced levels of protective species with relative predominance of
pathobionts, thus promoting a deregulation of the immune response. In consequence, an
interesting approach to the treatment of intestine inflammation would be the restoration
of microbiota balance.

In the present study we described the beneficial effects exerted by
oligosaccharides derived from lactulose (OsLu) in the TNBS model of rat colitis,
comparing them with those achieved by its precursor, lactulose. OsLu has been
characterized by a slower fermentation by the colonic microbiota than lactulose, so the
oligosaccharides are able to reach distal colon without great alterations.
Theoretically, these properties could be of great interest for the treatment of human IBD, since distal areas of the large intestine are typically affected in UC and very frequently in Crohn’s disease. Furthermore, the delayed fermentation could account for a better patient tolerability than lactulose, since the effects derived from its fermentation in proximal colon would be avoided. Actually, both lactulose and OsLu were able to reduce the colonic damage observed one week after colonic induction with TNBS. Previous studies carried out with different oligosaccharides also displayed an improvement in the macroscopic scores, indicating changes in the colitic rats receiving the pretreatment with oligosaccharides. Thus, for example, colitic rats pretreated with goat milk oligosaccharides\textsuperscript{30} or FOS\textsuperscript{31} showed significant amelioration of the intestine inflammatory process in comparison with non-treated groups. It is interesting to note that this beneficial effect was also evidenced when the two prebiotics were administered for a longer period of time to colitic rats with a reactivation of the intestinal inflammation with a second instillation of TNBS, showing both prebiotics a similar efficacy. Unfortunately, a curative protocol with this type of products is not convenient since previous observations have shown a deleterious effect of lactulose or OsLu when the administration starts once the colitis is established. Other authors have reported similar observations with different treatments in experimental colitis in rodents.\textsuperscript{32}

As expected, the treatments were able to modify intestine bacteria composition that was altered due to the colitic process, mainly derived from their ability to increase the counts of either lactobacilli or bifidobacteria. In addition, the beneficial effect on microbiota composition exerted by both treatments was associated with an improvement of the defensive mechanisms of the intestine epithelial barrier, whose architecture appeared restored in the histological studies. The administration of lactulose or OsLu
normalized the expression of the mucins MUC-2 and MUC-3, primary constituents of the mucus layer in the colon, as well as of TFF-3, a bioactive peptide involved in epithelial protection and repair. This could be one of the mechanisms for preserving the mucus-secreting layer that covers the epithelium and acts as a physical barrier protecting its integrity. This effect may be key for the intestine anti-inflammatory capacity exerted by these prebiotics. Epithelial barrier function impairment is considered as one of the initial steps in intestinal inflammation that facilitates the access of antigens from the intestinal lumen, and hence generates an exacerbated immune response. Moreover, human IBD has been associated with a defective colonic mucus layer and a reduced number of goblet cells.

Furthermore, both lactulose and OsLu were able to increase the reduced production of SCFA observed in colitis rats, which may help to the regeneration of the inflamed mucosa, given the key role attributed to these acids in the intestine homeostasis and immune response. This may be responsible for the modifications in the biochemical markers of the immune response observed in the colonic tissue. It is interesting to note the differences observed between OsLu and lactulose on the Th17 pathway that could account for the higher anti-inflammatory efficacy of OsLu. IL-17 pathway is crucial in the pathogenesis of IBD. Thus, IL-17 contributes to neutrophil migration, expansion and function, and enhances dendritic cell maturation, T cell priming, and the production of inflammatory mediators from different cell types. Furthermore, IL-17 can synergize with other cytokines to promote the release of additional proinflammatory cytokines, thus being essential for maintaining the inflammatory response in the intestine. As confirmed in the present study, experimental colitis has also been reported to be associated with an increased IL-17 production; in
fact, in the TNBS colitis, the infiltration of CD4 lymphocytes and neutrophils, mostly
driven by a Th1/Th17 cytokine response, is one of the predominant features in the
initiation and perpetuation of the inflammatory process. Furthermore, the inhibition in
the expression of chemotactic mediators CINC-1 and MCP-1 in the inflamed tissue,
probably contributes to lower the leukocyte infiltration and activation that could also
account for the higher efficacy showed by OsLu since it is well described that, in the
first steps of the gut inflammation, margination and extravasation of circulating
leukocytes probably result in the perpetuation of the inflammatory process. Other
mechanisms may also explain the differences displayed by both prebiotics, and one of
these could be related to the ability of OsLu, but not lactulose, to significantly inhibit
colic iNOS expression in the inflamed intestine, thus avoiding the deleterious effect
that NO overproduction may exert on the colonic tissue in these intestinal conditions.

In conclusion, OsLu shows better anti-inflammatory properties than lactulose,
probably derived from the improvement of the luminal microbiota balance and a greater
SCFA production, which was associated with an inhibition of iNOS expression and a
reduction of Th17 cell activity in the inflamed tissue that, in turn, promote intestinal
membrane integrity and a faster recovery of the inflamed tissue. After this preclinical
study of OsLu, these oligosaccharides could be considered for a clinical study to test its
efficacy in maintenance of IBD remission and preventing relapses.

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Notes

The authors declare no competing financial interest

ABBREVIATIONS USED

CINC-1, cytokine-induced neutrophil chemoattractant-1; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; GSH, glutathione; IBD, inflammatory bowel disease; iNOS, nitric oxide synthase; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MPO, myeloperoxidase activity; OsLu, oligosaccharides derived from lactulose; SCFA, short chain fatty acids; TNBS, trinitrobenzenesulphonic acid; UC, ulcerative colitis.
REFERENCES


Differences in goblet cell differentiation between Crohn’s disease and ulcerative colitis. 


<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 3’-5’</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
</table>
| GAPDH  | FW: CCATCACCATCTTCCAGGAG  
         | RV: CCTGCTTCACCACCTTCTTG   | 60                         |
| IL-1β  | FW: GATCTTTGAAGAAGAGCCCG  
         | RV: AACTATGTCCCGACCATTGC    | 59                         |
| IL-6   | FW: CTTCCAGCCAGTTGCTTCTTG 
         | RV: TGGTCTGTGGTGGTGGTATCC   | 60                         |
| IL-12  | FW: ATCCAGTGTTGGTATGTTGTG  
         | RV: TGTCGAGTCCAGCCAGGTG     | 60                         |
| IL-17  | FW: TGGACTCTGAGCCGGAATGAGG  
         | RV: GACGCATGGCGGACAAATAGAGG | 60                         |
| IL-23  | FW: ATCCAGTGTTGGTATGTTGTG  
         | RV: TGTCGAGTCCAGCCAGGTG     | 60                         |
| iNOS   | FW: AAGAGACGCACAGGCAGAGG   
         | RV: AGCAGGGCAGACACAGATG     | 60                         |
| CINC-1 | FW: CCGAAGTCATAGCCACACTCAAG  
         | RV: TCACCAGACAGACGCCATCG    | 60                         |
| MCP-1  | FW: TCTTCCCTCACCACCTATGC  
         | RV: TCTCCAGCCGACTCFATTG     | 60                         |
| TFF-3  | FW: ATGGAGACCAGAGCCTTCTG   
         | RV: ACAGCTTTGTCTGACTGTA     | 59                         |
| MUC-2  | FW: ACCACCATTACCACACCCTCAG 
         | RV: CGATCACACCATTGCCCAGT    | 60                         |
| MUC-3  | FW: CACAAAGGCAAGAGTCCAGA   
         | RV: ACTGTCTTTGGTGCTGATG     | 60                         |
**Table 2.** Primer sequences used for microbiological analysis in real-time PCR (RT-qPCR) assays in the colonic contents.

<table>
<thead>
<tr>
<th>Target bacterial Group</th>
<th>Sequence (5’–3’)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides group</td>
<td>g-Bfra-F: ATAGCCTTTTCGAAAGRAAGAT</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>g-Bfra-R: CCAGTATCAACTGCAATTTTA</td>
<td></td>
</tr>
<tr>
<td>Clostridium XIVa–XIVb</td>
<td>g-Ccoc-F: AAATGACGGTGACTGACTAA</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>g-Ccoc-R: CTTTGAGTTTCATTCTTGACTAA</td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium group</td>
<td>g-Bifid-F: CTCCTGGAAACGGGTGG</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>g-Bifid-R: GGTGTTCTTCCCGATATCTACA</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus group</td>
<td>Lab 159: GGAAACAG(A/G)TGCTAATACCG</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Lab 677: CACCGCTACACATGGAG</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on colonic macroscopic damage score, weight/length ratio, myeloperoxidase (MPO) activity and glutathione (GSH) content in TNBS experimental rat colitis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Damage Score (0-10)</th>
<th>Weight/length (mg/cm)</th>
<th>MPO (mU/g tissue)</th>
<th>GSH (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-colitic</td>
<td>0</td>
<td>63.9 ± 1.9</td>
<td>14.5 ± 1.4</td>
<td>1878 ± 29</td>
</tr>
<tr>
<td>TNBS control</td>
<td>7.9 ± 0.2</td>
<td>175.8 ± 9.8</td>
<td>616.5 ± 67.6</td>
<td>603 ± 91</td>
</tr>
<tr>
<td>Lactulose</td>
<td>7.1 ± 0.2*</td>
<td>211.9 ± 26.7</td>
<td>355.5 ± 78.1*</td>
<td>871 ± 96*</td>
</tr>
<tr>
<td>OsLu</td>
<td>6.3 ± 0.3* ,#</td>
<td>156.8 ± 8.9*</td>
<td>335.7 ± 55.6*</td>
<td>878 ± 99*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *P<0.05 vs. TNBS control group; #P<0.05 vs. Lactulose-treated group. All colitic groups statistically differ from Non-colitic group (P<0.05).
Table 4. Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on colonic macroscopic damage score and weight/length (W/L) ratio in TNBS-reactivated experimental rat colitis.

<table>
<thead>
<tr>
<th>Group</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score (0-10)</td>
<td>W/L (mg/cm)</td>
<td>Score (0-10)</td>
</tr>
<tr>
<td>Non-colitic</td>
<td>0</td>
<td>68.7±3.1</td>
<td>0</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNBS control</td>
<td>7.6±0.5</td>
<td>148.4±3.0</td>
<td>5.3±0.3</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactulose</td>
<td>5.7±0.4*</td>
<td>124.1±13.9</td>
<td>3.6±0.6*</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OsLu</td>
<td>5.1±0.8*</td>
<td>104.8±9.1*</td>
<td>4.0±0.4*</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *P<0.05 vs. TNBS control group. All colitic groups statistically differ from Non-colitic group (P<0.05).
FIGURE LEGENDS

Figure 1. Histological sections of colonic tissue stained with hematoxylin and eosin showing the effects of lactulose and oligosaccharides derived from lactulose (OsLu) in TNBS rat colitis one week after damage induction: A) Non-colitic; B) TNBS-control; C) Lactulose; D) oligosaccharides derived from lactulose (OsLu); E) microscopic score assigned according the criteria previously described. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).

Figure 2. Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on the colonic expression of the cytokines IL-1β, IL-6, IL-12, IL-17 and IL-23, and inducible nitric oxide synthase (iNOS) analyzed by RT-PCR in TNBS rat colitis one week after damage induction. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).

Figure 3. Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on the colonic expression of the chemokines cytokine-induced neutrophil chemoattractant (CINC)-1 and monocyte chemotactic protein (MCP)-1, the mucins MUC-2 and MUC-3, and the trefoil factor (TFF)-3 analyzed by RT-PCR in TNBS rat colitis one week after damage induction. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).

Figure 4. Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on colonic myeloperoxidase (MPO) activity and glutathione content in reactivated TNBS-rat colitis. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).
**Figure 5.** Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on the colonic expression of the cytokines IL-1β, IL-6, IL-12, IL-17 and IL-23, and inducible nitric oxide synthase (iNOS) analyzed by RT-PCR in reactivated TNBS rat colitis. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).

**Figure 6.** Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on the colonic expression of the chemokines cytokine-induced neutrophil chemoattractant (CINC)-1 and monocyte chemotactic protein (MCP)-1, the mucins MUC-2 and MUC-3, and the trefoil factor (TFF)-3 analyzed by RT-PCR in reactivated TNBS rat colitis. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).

**Figure 7.** Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on the bacterial profile in the colonic luminal contents in reactivated TNBS rat colitis. The counts of lactobacilli, bifidobacteria, clostridia and bacteroides (expressed as log of colony forming units (CFU)) were analyzed by RT-PCR. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).

**Figure 8.** Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on short chain fatty acids (acetate, propionate and butyrate) production in the colonic luminal contents in reactivated TNBS rat colitis. Data (n=10 per group) are expressed as means ± SEM; groups with different letter statistically differ (P<0.05).
Figure 1

A

B

C

D

E

(0 - 59)

a

b

c

c

Non colitic
TNBS Control
Lactulose
OsLu
Figure 2

**IL-1β**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-6**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-12**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-17**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-23**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**iNOS**

- Non colitic
- TNBS Control
- Lactulose
- OsLu
Figure 3

**CINC-1**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**MCP-1**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**MUC-2**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**MUC-3**

- Non colitic
- TNBS Control
- Lactulose
- OsLu

**TFF-3**

- Non colitic
- TNBS Control
- Lactulose
- OsLu
Figure 4

**MPO**

![Graph showing MPO levels across different time points and conditions.]

**Glutathione**

![Graph showing Glutathione levels across different time points and conditions.]

Legend:
- Non colitic
- TNBS Control
- Lactulose
- OsLu
Figure 5

**IL-1β**

- 1 wk, 2 wks, 3 wks
- Fold Increase
- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-6**

- 1 wk, 2 wks, 3 wks
- Fold Increase
- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-12**

- 1 wk, 2 wks, 3 wks
- Fold Increase
- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-17**

- 1 wk, 2 wks, 3 wks
- Fold Increase
- Non colitic
- TNBS Control
- Lactulose
- OsLu

**IL-23**

- 1 wk, 2 wks, 3 wks
- Fold Increase
- Non colitic
- TNBS Control
- Lactulose
- OsLu

**iNOS**

- 1 wk, 2 wks, 3 wks
- Fold Increase
- Non colitic
- TNBS Control
- Lactulose
- OsLu
Figure 6

**MCP-1**

0 5 10 15
FOLD INCREASE

1 wk 2 wks 3 wks

**CINC-1**

0 5 10 15 20
FOLD INCREASE

1 wk 2 wks 3 wks

**MUC-2**

0.0 0.5 1.0 1.5
FOLD INCREASE

1 wk 2 wks 3 wks

**MUC-3**

0.0 0.5 1.0 1.5 2.0
FOLD INCREASE

1 wk 2 wks 3 wks

**TFF-3**

0.0 0.5 1.0 1.5
FOLD INCREASE

1 wk 2 wks 3 wks

Legend:
- Non colitic
- TNBS Control
- Lactulose
- OsLu
Figure 7

1 week

2 weeks

3 weeks

Log CFU


Non colitic  TNBS Control
Lactulose  OsLu
Figure 8

1 week

2 weeks

3 weeks

µmol / g luminal content

Acetate Propionate Butyrate

Acetate Propionate Butyrate

Acetate Propionate Butyrate

Non colitic TNBS Control Lactulose OsLu

ACS Paragon Plus Environment
Supplementary material

**Figure S1:** MALDI-TOF-MS profile of the purified fraction of oligosaccharides formed during enzymatic hydrolysis of Duphalac with β-galactosidase from *Aspergillus oryzae* has been included.
TOC graphic