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

1 **ABSTRACT**

2 Intestinal microbiota modulation is becoming an interesting approach to manage
3 inflammatory bowel disease, and can be achieved by the administration of prebiotics.
4 Previous studies showed the intestinal anti-inflammatory effects of the prebiotic
5 lactulose. The aim of the present study was to test the preventative effects of
6 oligosaccharides derived from lactulose with prebiotic properties (OsLu), in the
7 trinitrobenzenesulphonic acid model of rat colitis, and compare them with lactulose.
8 Both treatments modified bacterial profile in intestinal contents, increasing the
9 bifidobacteria and lactobacilli counts, and up-regulating the production of short chain
10 fatty acids, although OsLu generated a larger amount. OsLu also inhibited in greater
11 extent different pro-inflammatory markers such as interleukins (IL) 1, 6, 12 and 23, and
12 chemokines (MCP-1 and CINC-1). However both prebiotics equally restored colonic
13 epithelial integrity, evaluated both with a histological score (OsLu 9.8±2.2 and lactulose
14 12.1±2.1, vs. colitic control 27.3±3.3) and measuring several key proteins of the
15 mucosal barrier (MUC-2, MUC-3 and TTF-3). OsLu effect was also associated with an
16 inhibition of iNOS expression and a reduction of Th17 cell activity in the inflamed
17 tissue that facilitated the intestinal mucosa barrier recovery. In conclusion, OsLu
18 showed a better anti-inflammatory profile than lactulose in this model of experimental
19 colitis.

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

22

23 **INTRODUCTION**

24 Inflammatory bowel disease (IBD) usually refers to two related conditions, ulcerative
25 colitis (UC) and Crohn's disease, characterized by chronic and spontaneously relapsing
26 inflammation. Among the clinical symptoms of IBD are abdominal pain, diarrhea, rectal
27 bleeding, malaise and weight loss. Although the exact etiology of IBD remains
28 unknown, both inflammatory conditions have similar pathophysiology background, and
29 several studies indicate that a combination of factors such as genetic susceptibility,
30 intestinal microbiota, dietary factors, intestinal barrier dysfunction, and an abnormal
31 immune response to intestinal bacteria lead to chronic intestinal inflammation.[1] In fact,
32 IBD may be considered as a maladaptation of host commensal mutualism that promotes
33 intestinal microbiota imbalance, termed as dysbiosis. It is typically characterized by a
34 decline in bacterial diversity, with relative predominance of potential pathogenic
35 bacteria and insufficient amount of protective species, that leads to chronic intestinal
36 inflammation.[2] In consequence, an interesting approach to IBD management could be
37 the change of the microbiota, in a preventative and/or therapeutic manner, to protect
38 intestine against inflammation, stimulate tissue repair and restore the homeostasis in
39 genetically susceptible hosts. This could explain, at least partially, the remission that
40 sometimes is achieved in intestinal inflammation after treatment with antibiotics such as
41 metronidazole or ciprofloxacin.[3] Unfortunately, the prolonged use of antibiotics in the
42 management of these intestinal conditions is limited due to the risk of severe adverse
43 effects. For this reason, it is very important to find other effective strategies focused on
44 microbiota balance restoration, with a better safety profile. This could be the case of
45 prebiotics, which are defined as a selectively fermented ingredient that allows specific

46 changes, both in the composition and/or activity in the gastrointestinal microbiota, that
47 confers benefits upon host well being and health.[4]

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

65 Many different substances are claimed to be prebiotics, but mainly
66 carbohydrates have proven to display prebiotic properties in humans.[4,7] Lactulose is a
67 synthetic disaccharide composed of fructose and galactose primarily used as a laxative
68 and for the treatment of portal-systemic encephalopathy.[8,9] Following oral
69 administration, intact lactulose reaches the colon, where it is split by bacteria in distal

70 small intestine and colon, lowering fecal pH and promoting beneficial microbiota
71 growth such as bifidobacteria and lactobacilli and inhibiting pathogenic bacteria
72 expansion like *Salmonella*.[11]

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

85 Oligosaccharides derived from lactulose (OsLu) are new bioactive carbohydrates
86 synthesized with the aim of obtaining more slowly fermenting prebiotics able to reach
87 distal colon unhydrolysed.[16-18] Different *in vitro* fermentation assays using pure cultures
88 as well as fecal inoculums have demonstrated that when these oligosaccharides were
89 used as sole carbon source, there was a stimulation of bifidobacteria and lactobacilli
90 growth. Thus, when long incubation periods were used in fermentative assays, OsLu
91 showed higher prebiotic index values than the native disaccharide.[19,20] These
92 investigations proved that OsLu improved lactulose prebiotic properties and therefore, it
93 is reasonable to suppose that OsLu could present a higher efficacy in intestine

94 inflammation. More recently, it has been evaluated the modulatory effects of OsLu on
95 microbial composition in the cecum and colon of growing rats, with particular emphasis
96 on their bifidogenic effect, showing that OsLu produced a significant and selective
97 increase of *Bifidobacterium animalis* in these intestine locations.[21] All these findings
98 support a prebiotic role of galactosyl-fructoses in functional foods.

99 The aim of the present study was to test the preventative effects of OsLu in the
100 trinitrobenzenesulphonic acid (TNBS) model of rat colitis, a well-established model of
101 intestinal inflammation with some resemblance to human IBD,[22] and compare its
102 efficacy with its parent compound, lactulose. Special attention was paid to their effects
103 on the expression of some of the inflammatory mediators, such as pro-inflammatory
104 cytokines (interleukin (IL)-1β, IL-6, IL-12, IL-17 and IL-23), chemokines (cytokine-
105 induced neutrophil chemoattractant (CINC)-1 and monocyte chemotactic protein
106 (MCP)-1) and an inducible enzyme (inducible Nitric oxide synthase (iNOS)), as well as
107 different markers of epithelial integrity in the mucosa, like the mucins MUC-2 and
108 MUC-3, and the protein trefoil factor (TFF)-3. The prebiotic capacity was assessed by
109 measuring the production of SCFA and the effects on the microbiota composition. In
110 summary, OsLu therapeutic efficacy was greater than lactulose, maybe due to its better
111 immunomodulatory properties together with a superior capacity of SCFA production.

112

113 **MATERIALS AND METHODS**

114 This study was carried out in accordance with the 'Guide for the Care and Use of
115 Laboratory Animals' as promulgated by the National Institute of Health. The
116 experimental protocol was approved by the Commission of Ethics in Animal

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117 Experimentation (Protocol number CEEA 2010-286) of the University of Granada
118 (Spain).

119 **Reagents and synthesis of oligosaccharides derived from lactulose (OsLu).**
120 All chemicals were obtained from Sigma Chemical (Madrid, Spain), unless otherwise
121 stated. Glutathione reductase was provided by Boehringer Mannheim (Barcelona,
122 Spain). A commercial preparation Duphalac® (Abbott Healthcare; Barcelona, Spain),
123 which contains 670 g/L of lactulose, was used as control and to synthesize OsLu.
124 Enzymatic reactions have been performed at 50ºC and pH 6.5 using a β-galactosidase
125 from Aspergillus oryzae (16 Ud/mL) in an orbital shaker at 300 rpm for 24 h.
126 Afterward, samples were immediately immersed in boiling water for 10 min to
127 inactivate the enzyme. Then, the mixture of oligosaccharides was treated with yeasts to
128 eliminate monosaccharides following the method of Sanz et al.,[23] with some
129 modifications. Briefly, oligosaccharide reaction mixture 20% (w/v) was treated with
130 fresh Saccharomyces cerevisiae 1.5% (w/v) (Levital, Panibérica de Levadura,
131 Valladolid, Spain) at 30ºC for 48 h in an orbital shaker (300 rpm) and submitted to
132 vacuum filtration to remove the yeasts. Sample was concentrated at 38-40ºC in a rotary
133 evaporator (Büchi Labortechnik AG. Flawil, Switzerland). Mono- and disaccharides as
134 well as OsLu were analysed by GC-FID.[24] Degree of polymerization (DP) of the
135 oligosaccharides found in the purified fraction from the synthesis mixture was also
136 determined by matrix-assisted laser desorption ionization time-of-flight mass
137 spectrometry (MALDITOF-MS). Before yeast treatment, the transglycosylation
138 mixtures contained 76% of sugars which corresponded to 40% of monosaccharides, 6%
139 of lactulose and 30% of OsLu. After yeast treatment the composition of mixtures was
140 28% of monosaccharides, 12% of lactulose and 36% of OsLu (17% disaccharides, 13%

141 trisaccharides, 5% tetrasaccharides and 1% pentasaccharides. MALDI-TOF-MS
142 analysis allowed to detect oligosaccharides with a higher DP of 6, 7 and 8 which could
143 not be quantified by gas chromatography. Also, other analysis of OsLu showed the
144 presence 5% of salts and 1% of nitrogen with a dry matter of 82 %.

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

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

163 In another set of experiments, the efficacy of both lactulose and OsLu was
164 evaluated in the same experimental model for a longer period of time, three weeks. In

165 this assay, a second dose of 10 mg of TNBS dissolved in 50% ethanol was administered
166 two weeks after the initial TNBS colonic instillation, in an attempt to mimic the relapses
167 common in human IBD. Ten animals from each colitic group (control and prebiotic
168 treated) and five from the non-colitic group were sacrificed after 1, 2 and 3 weeks of
169 colitis induction. So the efficacy of Oslu after one week was performed twice.

170 **Assessment of colonic damage.** The body weight, water and food intake were
171 recorded daily throughout the experiment. Once the rats were sacrificed, the colon was
172 removed aseptically and placed on an ice-cold plate, longitudinally opened and luminal
173 contents were collected for the microbiological studies (see below). Afterwards, the
174 colonic segment was cleaned of fat and mesentery, blotted on filter paper; each
175 specimen was weighed and its length measured under a constant load (2 g). The colon
176 was scored for macroscopically visible damage on a 0-10 scale by two observers
177 unaware of the treatment, according to the criteria previously reported,[12] which take into
178 account the extent as well as the severity of colonic damage. Representative whole gut
179 specimens (0.5 cm^2) were taken from a region of the inflamed colon corresponding to
180 the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered
181 formaldehyde for the histological studies. Equivalent colonic segments were also
182 obtained from the non-colitic group. The colon was subsequently minced, aliquoted and
183 kept frozen at -80ºC until biochemical determinations and RNA extraction was
184 performed.

185 For the histological studies, cross-sections were selected and embedded in
186 paraffin. Full-thickness sections of 5 μm were obtained at different levels and stained
187 with hematoxylin and eosin. The histological damage was evaluated by a pathologist

188 observer, who was blinded to the experimental groups, according to the criteria
189 previously described by Arribas et al.[25]

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

195 The analysis of gene expression in the colonic samples was performed by real
196 time quantitative PCR (RT-qPCR). For this purpose total RNA from colonic samples
197 was isolated using Trizol® following the manufacturer's protocol. All RNA samples
198 were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer and 2
199 µg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton,
200 UK). RT-qPCR amplification and detection was performed on optical-grade 48well
201 plates in a EcoTM Real-Time PCR System (Illumina, San Diego, CA, USA) with 20 ng
202 of cDNA, the KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Inc.,
203 Wilmington, MA, USA) and specific primers at their annealing temperature (Ta) (**Table**
204 **1**). To normalize mRNA expression, the expression of the housekeeping gene,
205 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. The mRNA
206 relative quantitation was calculated using the ΔΔCt method.

207 **Microbial analysis of the colonic contents.** For DNA extraction, samples from
208 colonic contents were diluted 1:12 (w/v) in the lysis buffer ASL provided by the
209 QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), according to the
210 manufacturer's instructions. The suspensions were transferred to a Lysing Matrix E tube
211 that contained a mixture of ceramic and silica particles designed to efficiently lyse

212 microorganisms (Qiagen, Hilden, Germany). Tubes were shaken in a Precellys
213 apparatus (VWR, Villeurbanne, France) at 5.5 rpm/min for 30 s twice. DNA was then
214 extracted by using a QIAamp DNA stool minikit from Qiagen, as recommended by the
215 manufacturer (protocol for isolation of DNA for pathogen detection), except that a
216 supplemental mixture of enzymes (mutanolysin at 90 U and lysozyme at 9 mg/mL) was
217 added to the lysis buffer. RT-qPCR was used to characterize the bacterial DNA present
218 in the colonic content samples as reported previously.[28] For this purpose, a series of
219 genus-specific primer pairs were used (**Table 2**). PCR amplification and detection was
220 performed on optical-grade 48well plates in a Eco™ Real-Time PCR System (Illumina,
221 San Diego, CA, USA). In this case, each reaction mixture (10 µl) was composed of 5 µL
222 of KAPA SYBR ® FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA,
223 USA), 0.25 µl of each specific primer at a concentration of 10 µM and 2 µl of DNA
224 template. Standard curves were created using serial 10-fold dilutions of bacterial DNA
225 extracted from pure cultures with a bacterial population ranging from 2 to 9 log colony
226 forming units (CFUs), as determined by plate counts. One strain belonging to each of
227 the bacterial genera or groups targeted in this study was used to construct the standard
228 curve. More specifically, the strains from which the DNA was extracted were the
229 following: *Bifidobacterium longum* CECT 4551, *Clostridium coccoides* DSMZ 935,
230 *Bacteroides fragilis* DSMZ 2151, *Lactobacillus salivarius* CECT 2197. All of them
231 were obtained from the Spanish Collection of Type Cultures (CECT) or the German
232 Collection of Microorganisms and Cell Cultures (DSMZ).

233 **Short chain fatty acid (SCFA) determinations.** To quantify the SCFA
234 concentrations in the colonic luminal contents, the samples were homogenized with 150
235 mM $NaHCO_3$ (pH 7.8) (1:5 w/v) in an argon atmosphere. Samples were incubated for

236 24 h at 37 ºC and stored at −80 ºC until the extraction. To extract the SCFAs, 50 µL of
237 the internal standard 2-methylvaleric acid (100 mM), 10 µL of sulphuric acid and 0.3
238 mL of chloroform were added to 1 mL of the homogenate and, then, centrifuged at
239 10,000 × g for 5 min at 4 ºC. The organic layer was dehydrated with sodium anhydrous
240 sulfate and centrifuged at 10,000 × g for 5 min at 4 ºC. 1 µL of the supernatant was
241 injected into a gas chromatograph (Perkin-Elmer Autosystem GC-FID, Waltham, MA,
242 USA) equipped with a capillary column (CPWAX 52CB 60 m × 0.25 mm, 0,25 µm,
243 Varian, Middelburg, The Netherlands) and connected to a Star Chromatography
244 WorkStation program (version 6, Varian, Middelburg, The Netherlands) to quantify the
245 samples. Operating conditions were: injector 275ºC; detector 300 ºC; initial column
246 temperature 90ºC for 0.1 min then increasing 15ºC/min up to 245ºC and 4 min at 245ºC.
247 The flow of carrier gas (nitrogen) was 1 mL/min. The gas chromatography system had a
248 split ratio 1:33. Acetate, propionate and butyrate concentrations between 0.30-60 mM
249 were used to make the standard curve. A linear relationship was found between the peak
250 area ratio SCFA/IS using the same protocol than that for samples.

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

256

257 **RESULTS**

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

270 The histological evaluation of the colonic segments confirmed the intestinal anti-
271 inflammatory effect of both lactulose and the OsLu (**Figure 1**). The colonic samples
272 from the untreated colitic control group showed extensive ulceration of the mucosa that
273 typically affected over 75% of the surface. Also, severe inflammation that involved all
274 the intestinal layers was observed, and it was associated with diffuse leukocyte
275 infiltration, mainly composed of neutrophils in the mucosa layer and, to a lesser extent,
276 lymphocytes in the submucosa. In addition, the inflammatory process was associated
277 with severe goblet cell depletion (**Figure 1B**). In this group of rats, the grade of lesion
278 was considered as severe, with a score value (mean ± SEM) of 27.3 ± 3.3 (**Figure 1E**).
279 On the contrary, the histological assessment of the colonic specimens from rats treated
280 with either lactulose (**Figure 1C**) or OsLu (**Figure 1D**) showed an evident recovery of
281 the colonic tissue, displaying a significantly reduced score in comparison with untreated

282 colitic rats. Thus, most of the samples revealed a restoration of the epithelial cell layer,
283 being only affected a maximum of 25% of the epithelium in contrast to the extensive
284 ulceration observed in TNBS control group. In addition, goblet cells appeared
285 replenished with their mucin content, and it was also observed a reduction in the
286 inflammatory infiltrate, which was slight to moderate with a patchy distribution.

287 The intestine anti-inflammatory effect was confirmed biochemically, since both
288 treated groups showed significantly reduced colonic MPO activity (**Table 3**), which
289 agrees with the reduction of neutrophil infiltration observed in the histological study and
290 thus demonstrates the intestine anti-inflammatory effect of the treatment.[29] In addition,
291 the administration of lactulose or OsLu partially restored colonic glutathione content, a
292 tripeptide with antioxidant properties, which was depleted in colitic rats as a
293 consequence of the colonic oxidative stress induced by the inflammatory process,
294 similarly to that previously reported in this model of experimental colitis[12] (**Table 3**).

295 The colonic inflammatory status in control colitic rats was also characterized by
296 an increased expression of the different pro-inflammatory markers evaluated, including
297 the cytokines IL-1β, IL-6, IL-12, IL-17 and IL-23, the chemokines CINC-1 and MCP-1,
298 and the inducible enzyme iNOS, in comparison with the non-colitic group. The
299 evaluation of the cytokines revealed that the pretreatment of colitic rats with OsLu was
300 able to significantly reduce the expression of all of them, whereas lactulose
301 administration only decreased the expression of IL-6 in the colonic tissue, without
302 showing statistical differences with the other cytokines assayed in comparison with
303 untreated colitic rats (**Figure 2**). These results suggest that the amelioration of the
304 altered immune response that characterizes the colonic inflammatory process is
305 involved in the beneficial effect observed in both treated groups, but OsLu showed a

306 higher efficacy than lactulose. Similarly, only the treatment of colitic rats with OsLu
307 resulted in a significant reduction of the expression of colonic iNOS (**Figure 2**), and the
308 chemokines CINC-1 and MCP-1 (**Figure 3**), that could support the lower infiltration of
309 either neutrophils and macrophages in the inflamed tissue, that was evidenced both
310 biochemically and histologically. Of note, both lactulose and OsLu managed to
311 completely restore the expression of proteins involved in colonic epithelial integrity
312 (MUC-2, MUC-3 and TFF-3), which were significantly reduced in colitic rats when
313 compared to the healthy ones (**Figure 3**) and could promote the recovery of the
314 epithelial layer observed in the histological studies.

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

330 week after the first administration of TNBS, whereas only the lactulose derivative OsLu
331 was able to significantly restore its colonic content after the reactivation of the colonic
332 damage (**Figure 4**).

333 The evaluation of the different biochemical markers by qPCR, confirmed the
334 results described above, for both lactulose and OsLu, when the inflammatory process
335 was evaluated one week after the first administration of TNBS. The evaluation after two
336 weeks revealed that the expression of the proinflammatory cytokines and chemokines
337 were reduced in the control colitic group in comparison with the data observed in the
338 same group at the first week, although most of them were still significantly higher than
339 in healthy rats. However, the evaluation of the proteins related with the epithelial
340 integrity (MUC-2, MUC-3 and TFF-3) revealed that only MUC-2 showed a significant
341 reduced expression in comparison with non-colitic rats (**Figures 5** and **6**). Since the
342 amelioration of the colonic inflammatory process took place at this time due to its
343 normal resolution, the beneficial effects exerted by the treatments were less evident than
344 in the first week of treatment. In fact these were noticeable for both compounds when
345 IL-6 or IL-17 were considered, and only for OsLu when IL-12 or the chemokines MCP-
346 1 and CINC-1 or the mucin MUC-2 were evaluated (**Figures 5** and **6**).

347 The reactivation of the inflammatory process typically resulted in a new
348 increment of the expression of most of the pro-inflammatory cytokines, including IL-1β,
349 IL-6, IL-12 and IL-23, as well as for iNOS, together with a reduction of the expression
350 of the mucins and TFF-3. At this time, OsLu also showed a higher beneficial effect than
351 lactulose, since the derivative reduced the expression of all the cytokines whose
352 expression was upregulated one week after the second administration of TNBS, whereas
353 lactulose only reduced IL-6 (**Figure 5**). At this time, only MUC-2 expression was

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354 restored after the treatment with both lactulose and OsLu, without showing a significant
355 effect in comparison with colitic control group, when MUC-3 or TFF-3 were considered
356 (**Figure 6**).

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

372 The reactivation of the inflammatory process after the second dose of TNBS
373 resulted in a new reduction of lactobacilli and bifidobacteria after one week, while the
374 number of clostridia and bacteroides were increased. OsLu was again able to
375 significantly increase the counts of lactobacilli and bifidobacteria in comparison with
376 the control colitic rats, whereas lactulose was devoid of any significant effect. Also, no

377 significant modifications were observed among colitic groups when clostridia or
378 bacteroides were considered (**Figure 7**).

379 As a consequence, the colonic inflammatory process was associated, at week 1
380 and 3, with a significant reduction in the production of the different SCFA evaluated
381 (acetate, propionate and butyrate) (**Figure 8**), as it has been previously reported in the
382 same model of experimental colitis.[25] The beneficial effects exerted by the treatments of
383 lactulose and OsLu in colitic rats were associated with an increased production of all the
384 SCFA one week after the first administration of TNBS. Moreover, OsLu was able to
385 restore these levels without showing statistical differences with non-colitic rats (**Figure
386 8**). However, one week after colitis reactivation, only OsLu was able to raise the
387 production of the SCFA in comparison with non-treated colitic rats (**Figure 8**).

388

389 **DISCUSSION**

390 A decrease in bacterial diversity has been reported to occur in IBD patients,
391 characterized by reduced levels of protective species with relative predominance of
392 pathobionts, thus promoting a deregulation of the immune response. In consequence, an
393 interesting approach to the treatment of intestine inflammation would be the restoration
394 of microbiota balance.[2]

395 In the present study we described the beneficial effects exerted by
396 oligosaccharides derived from lactulose (OsLu) in the TNBS model of rat colitis,
397 comparing them with those achieved by its precursor, lactulose. OsLu has been
398 characterized by a slower fermentation by the colonic microbiota than lactulose, so the
399 oligosacharides are able to reach distal colon without great alterations.[19,20]

400 Theoretically, these properties could be of great interest for the treatment of human
401 IBD, since distal areas of the large intestine are typically affected in UC and very
402 frequently in Crohn's disease. Furthermore, the delayed fermentation could account for
403 a better patient tolerability than lactulose, since the effects derived from its fermentation
404 in proximal colon would be avoided. Actually, both lactulose and OsLu were able to
405 reduce the colonic damage observed one week after colonic induction with TNBS.
406 Previous studies carried out with different oligosaccharides also displayed an
407 improvement in the macroscopic scores, indicating changes in the colitic rats receiving
408 the pretreatment with oligosaccharides. Thus, for example, colitic rats pretreated with
409 goat milk oligosaccharides[30] or FOS[31] showed significant amelioration of the intestine
410 inflammatory process in comparison with non-treated groups. It is interesting to note
411 that this beneficial effect was also evidenced when the two prebiotics were administered
412 for a longer period of time to colitic rats with a reactivation of the intestinal
413 inflammation with a second instillation of TNBS, showing both prebiotics a similar
414 efficacy. Unfortunately, a curative protocol with this type of products is not convenient
415 since previous observations have shown a deleterious effect of lactulose or OsLu when
416 the administration starts once the colitis is established. Other authors have reported
417 similar observations with different treatments in experimental colitis in rodents.[32]

418 As expected, the treatments were able to modify intestine bacteria composition
419 that was altered due to the colitic process, mainly derived from their ability to increase
420 the counts of either lactobacilli or bifidobacteria. In addition, the beneficial effect on
421 microbiota composition exerted by both treatments was associated with an improvement
422 of the defensive mechanisms of the intestine epithelial barrier, whose architecture
423 appeared restored in the histological studies. The administration of lactulose or OsLu

424 normalized the expression of the mucins MUC-2 and MUC-3, primary constituents of
425 the mucus layer in the colon,[33] as well as of TFF-3, a bioactive peptide involved in
426 epithelial protection and repair.[34] This could be one of the mechanisms for preserving
427 the mucus-secreting layer that covers the epithelium and acts as a physical barrier
428 protecting its integrity. This effect may be key for the intestine anti-inflammatory
429 capacity exerted by these prebiotics. Epithelial barrier function impairment is
430 considered as one of the initial steps in intestinal inflammation that facilitates the access
431 of antigens from the intestinal lumen, and hence generates an exacerbated immune
432 response.[35,36] Moreover, human IBD has been associated with a defective colonic mucus
433 layer and a reduced number of goblet cells.[37]

434 Furthermore, both lactulose and OsLu were able to increase the reduced
435 production of SCFA observed in colitis rats, which may help to the regeneration of the
436 inflamed mucosa, given the key role attributed to these acids in the intestine
437 homeostasis and immune response.[38] This may be responsible for the modifications in
438 the biochemical markers of the immune response observed in the colonic tissue. It is
439 interesting to note the differences observed between OsLu and lactulose on the Th17
440 pathway that could account for the higher anti-inflammatory efficacy of OsLu. IL-17
441 pathway is crucial in the pathogenesis of IBD.[39] Thus, IL-17 contributes to neutrophil
442 migration, expansion and function, and enhances dendritic cell maturation, T cell
443 priming, and the production of inflammatory mediators from different cell types.
444 Furthermore, IL-17 can synergize with other cytokines to promote the release of
445 additional proinflammatory cytokines, thus being essential for maintaining the
446 inflammatory response in the intestine. As confirmed in the present study, experimental
447 colitis has also been reported to be associated with an increased IL-17 production;[40] in

448 fact, in the TNBS colitis, the infiltration of CD4 lymphocytes and neutrophils, mostly
449 driven by a Th1/Th17 cytokine response, is one of the predominant features in the
450 initiation and perpetuation of the inflammatory process.[41] Furthermore, the inhibition in
451 the expression of chemotactic mediators CINC-1 and MCP-1 in the inflamed tissue,
452 probably contributes to lower the leukocyte infiltration and activation that could also
453 account for the higher efficacy showed by OsLu since it is well described that, in the
454 first steps of the gut inflammation, margination and extravasation of circulating
455 leukocytes probably result in the perpetuation of the inflammatory process.[42] Other
456 mechanisms may also explain the differences displayed by both prebiotics, and one of
457 these could be related to the ability of OsLu, but not lactulose, to significantly inhibit
458 colonic iNOS expression in the inflamed intestine, thus avoiding the deleterious effect
459 that NO overproduction may exert on the colonic tissue in these intestinal conditions.[43]

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

467

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477

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481 **Notes**

482 The authors declare no competing financial interest

483

484 **ABBREVIATIONS USED**

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

490

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Table 1
Primer sequences used in real-time PCR (RT-qPCR) assays in colonic tissue.

Gene	Sequence 3'-5'	Annealing temperature (°C)
GAPDH	FW:CCATCACCATCTTCCAGGAG RV:CCTGCTTCACCACCTTCTTG	60
IL-1β	FW: GATCTTTGAAGAAGAGCCCG RV: AACTATGTCCCGACCATTGC	59
IL-6	FW: CTTCCAGCCAGTTGCCTTCTTG RV: TGGTCTGTTGTGGGTGGTATCC	60
IL-12	FW: ATCCAGTGTGGTGATGGTTGTG RV: TGTCCGAGTCCAGCAGGTG	60
IL-17	FW:TGGACTCTGAGCCGCAATGAGG RV: GACGCATGGCGGACAATAGAGG	60
IL-23	FW: ATCCAGTGTGGTGATGGTTGTG RV: TGTCCGAGTCCAGCAGGTG	60
iNOS	FW: AAGAGACGCACAGGCAGAGG RV: AGCAGGCACACGCAATGATG	60
CINC-1	FW: CCGAAGTCATAGCCACACTCAAG RV: TCACCAGACAGACGCCATCG	60
MCP-1	FW: TCTTCCTCCACCACTATGC RV: TCTCCAGCCGACTCFATTG	60
TFF-3	FW: ATGGAGACCAGAGCCTTCTG RV: ACAGCCTTGTGCTGACTGTA	59
MUC-2	FW: ACCACCATTACCACCACCTCAG RV: CGATCACCACCATTGCCACTG	60
MUC-3	FW: CACAAAGGCAAGAGTCCAGA RV: ACTGTCCTTGGTGCTGCTGAATG	60

Table 2. Primer sequences used for microbiological analysis in real-time PCR (RT-qPCR) assays in the colonic contents.

Target bacterial Group	Sequence (5'–3')	Annealing temperature (ºC)
Bacteroides group	g-Bfra-F: ATAGCCTTTCGAAAGRAAGAT g-Bfra-R: CCAGTATCAACTGCAATTTTA	50
Clostridium cluster XIVa–XIVb	g-Ccoc-F: AAATGACGGTACCTGACTAA g-Ccoc-R: CTTTGAGTTTCATTCTTGCGAA	50
Bifidobacterium group	g-Bifid-F: CTCCTGGAAACGGGTGG g-Bifid-R: GGTGTTCTTCCCGATATCTACA	50
Lactobacillus group	Lab 159: GGAAACAG(A/G)TGCTAATACCG Lab 677: CACCGCTACACATGGAG	61

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.

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

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.

	1 week		2 weeks		3 weeks	
Group	Score (0-10)	W/L (mg/cm)	Score (0-10)	W/L (mg/cm)	Score (0-10)	W/L (mg/cm)
Non-colitic (n=5)	0	68.7±3.1	0	72.1±2.9	0	75.7±4.9
TNBS control (n=10)	7.6±0.5	148.4±3.0	5.3±0.3	155.5±17.2	7.7±0.2	161.3±9.8
Lactulose (n=10)	5.7±0.4*	124.1±13.9	3.6±0.6*	111.9±23.1	5.5±0.2*	96.7±5.8*
OsLu (n=10)	5.1±0.8*	104.8±9.1*	4.0±0.4*	116.1±13.8	5.4±0.4*	105.7±9.9*

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.[31] 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).

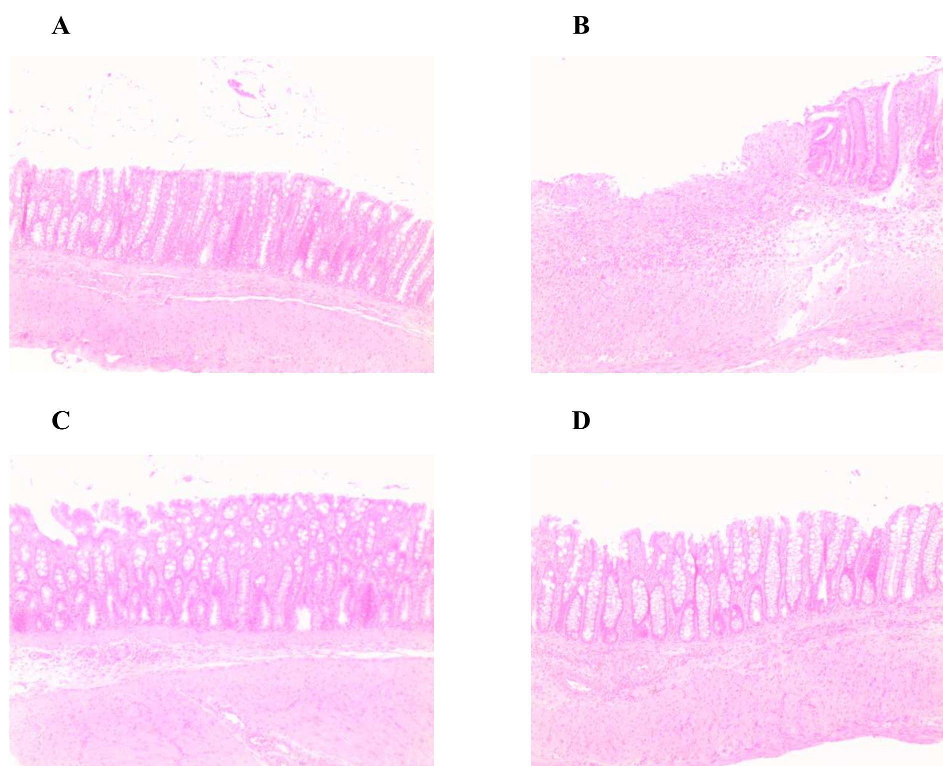
Figure 5. Effects of lactulose and oligosaccharides derived from lactulose (OsLu) on the colonic expression of the cytokines IL-1β, IL-6, IL-12, IL-17 and IL-23, and inducible nitric oxide synthase (iNOS) analyzed by RT-PCR in reactivated TNBS rat colitis. Data (n=10 per group) are expressed as means ± 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



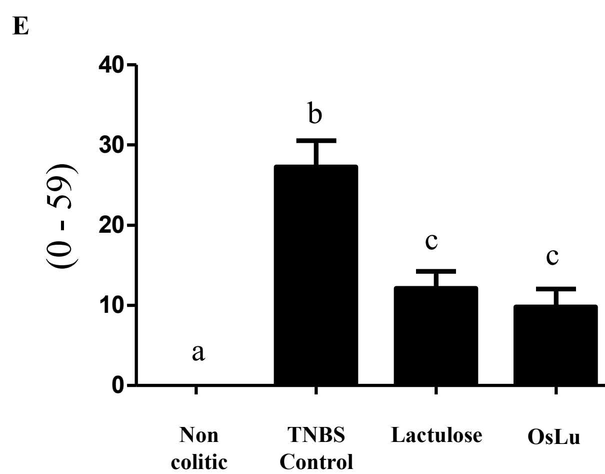


Figure 2

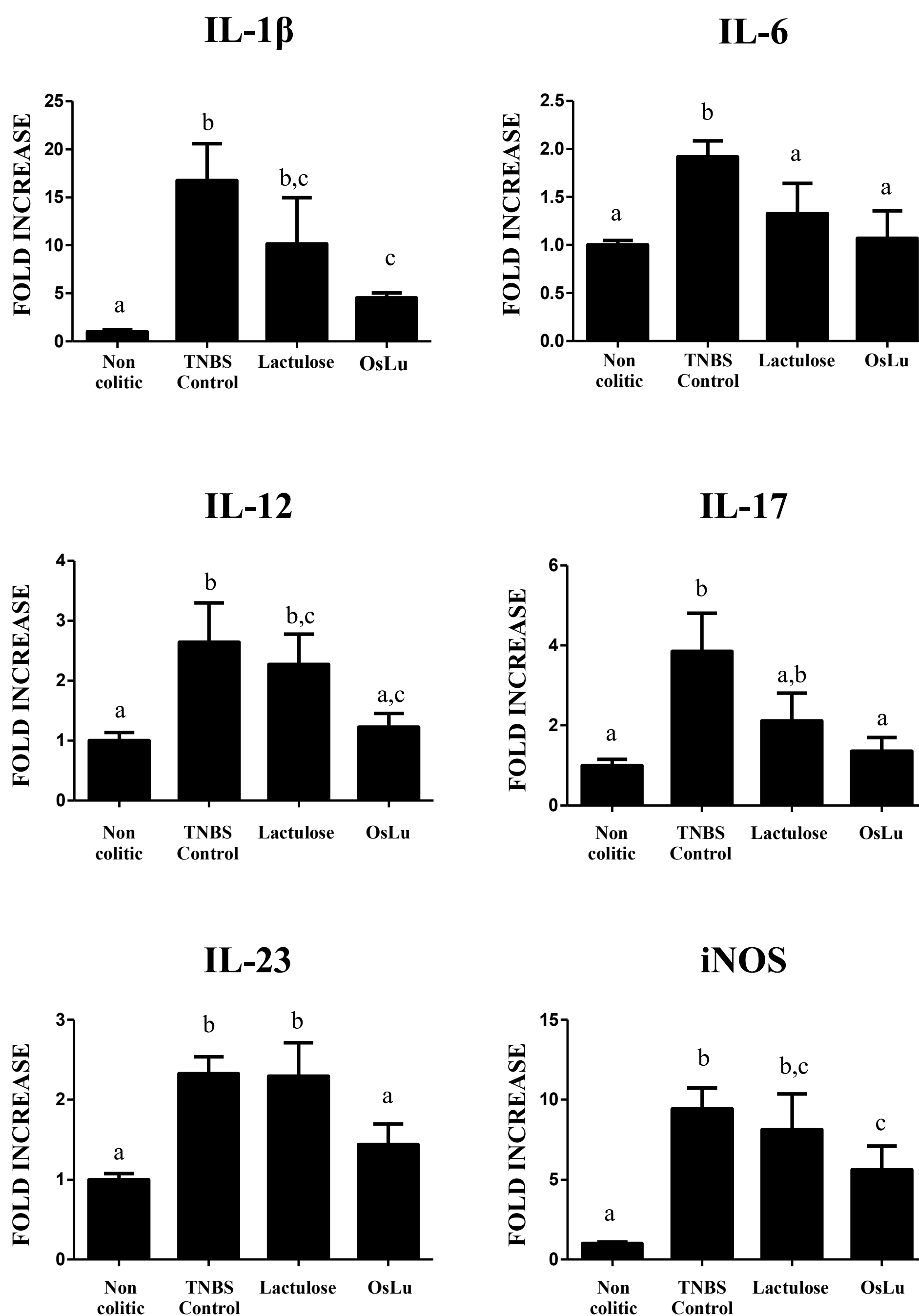


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Figure 3

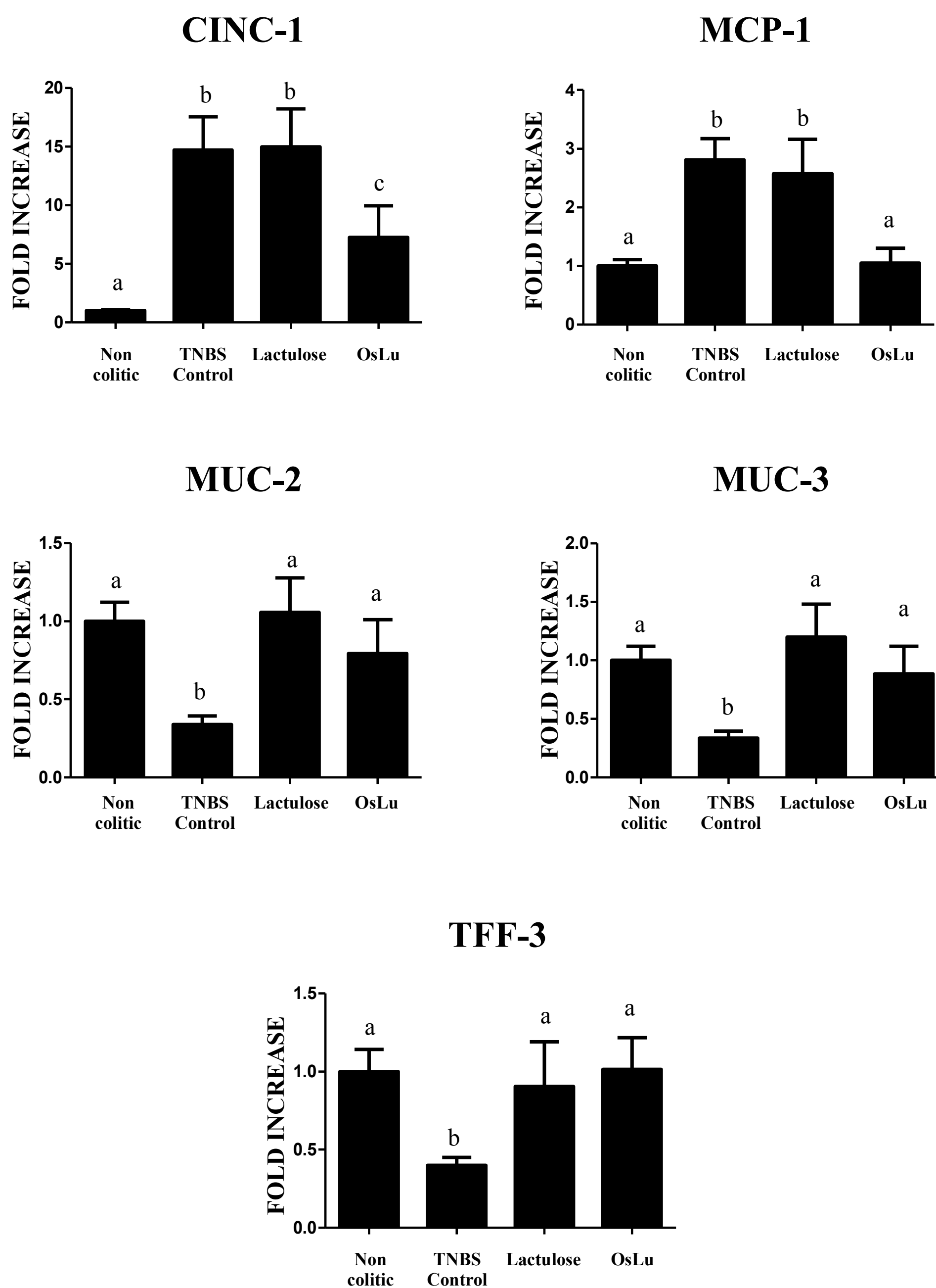


Figure 4

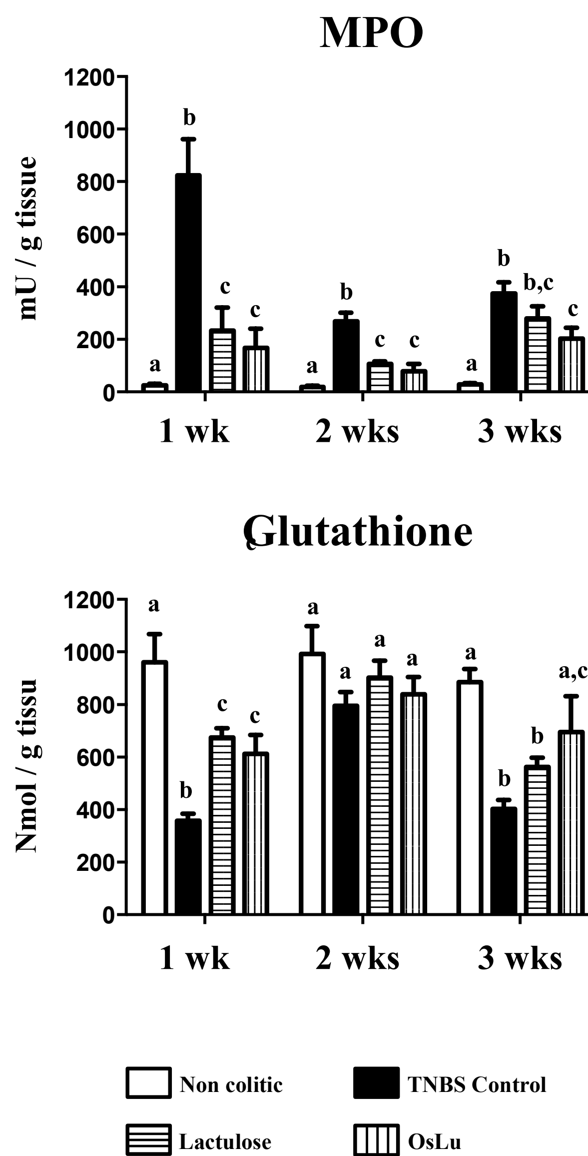
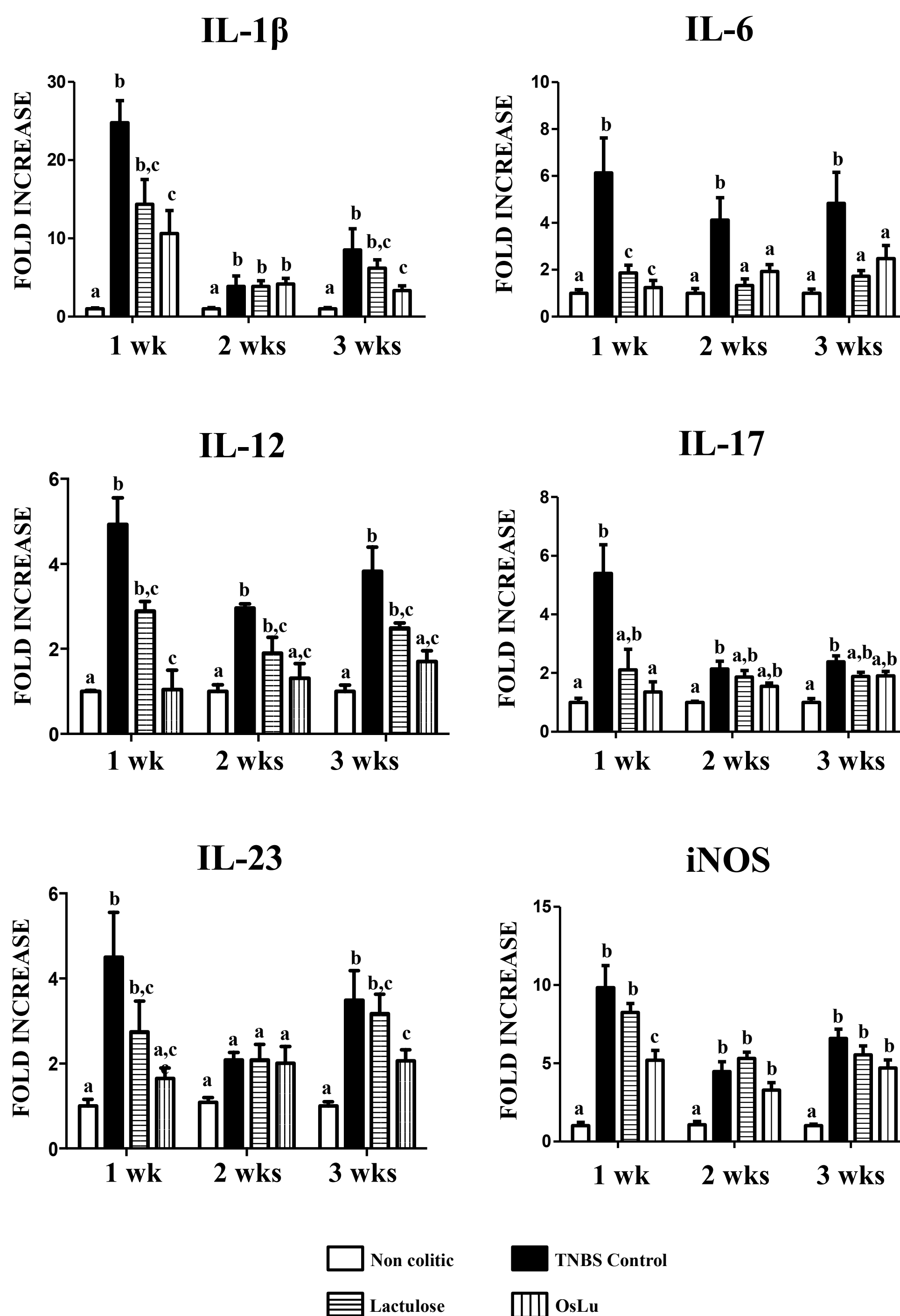


Figure 5



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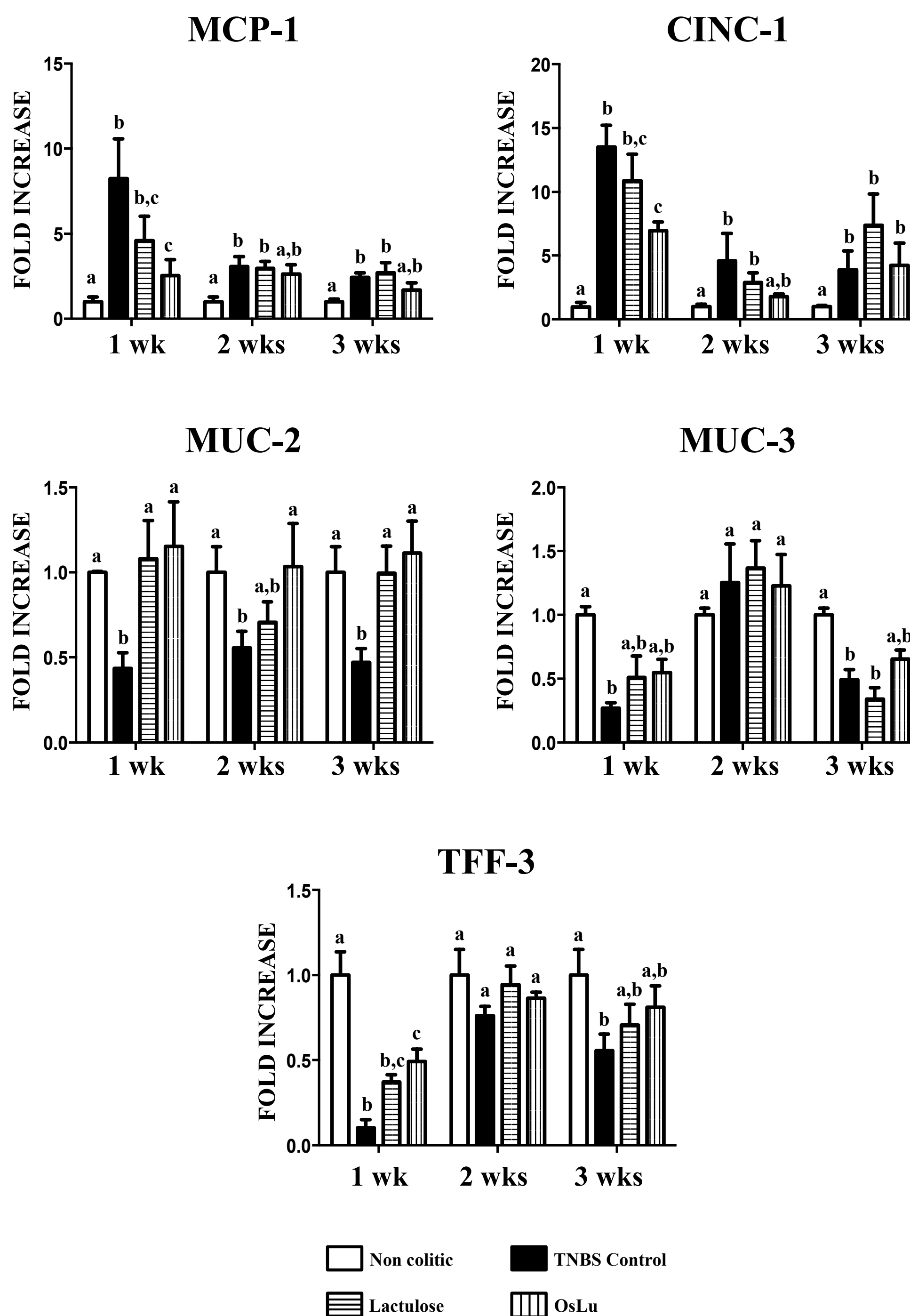


Figure 7

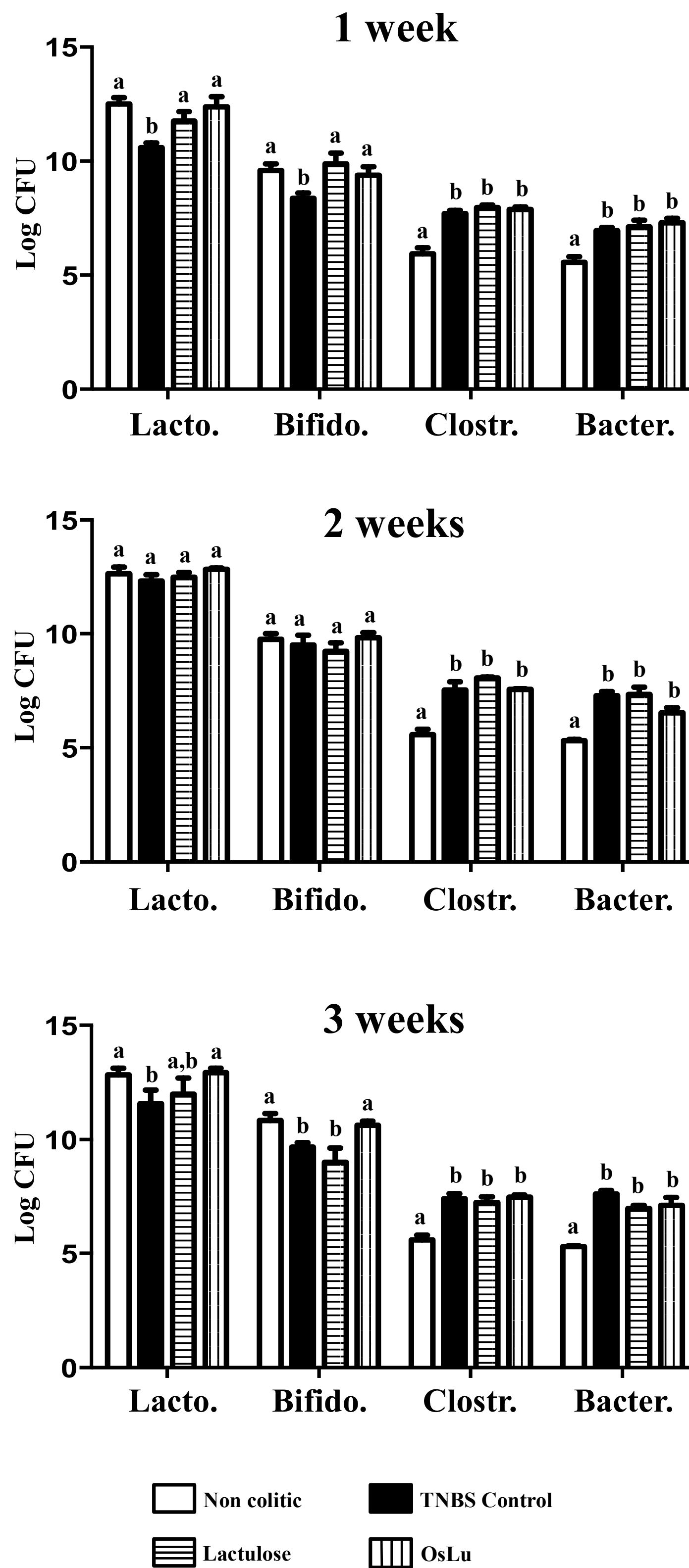
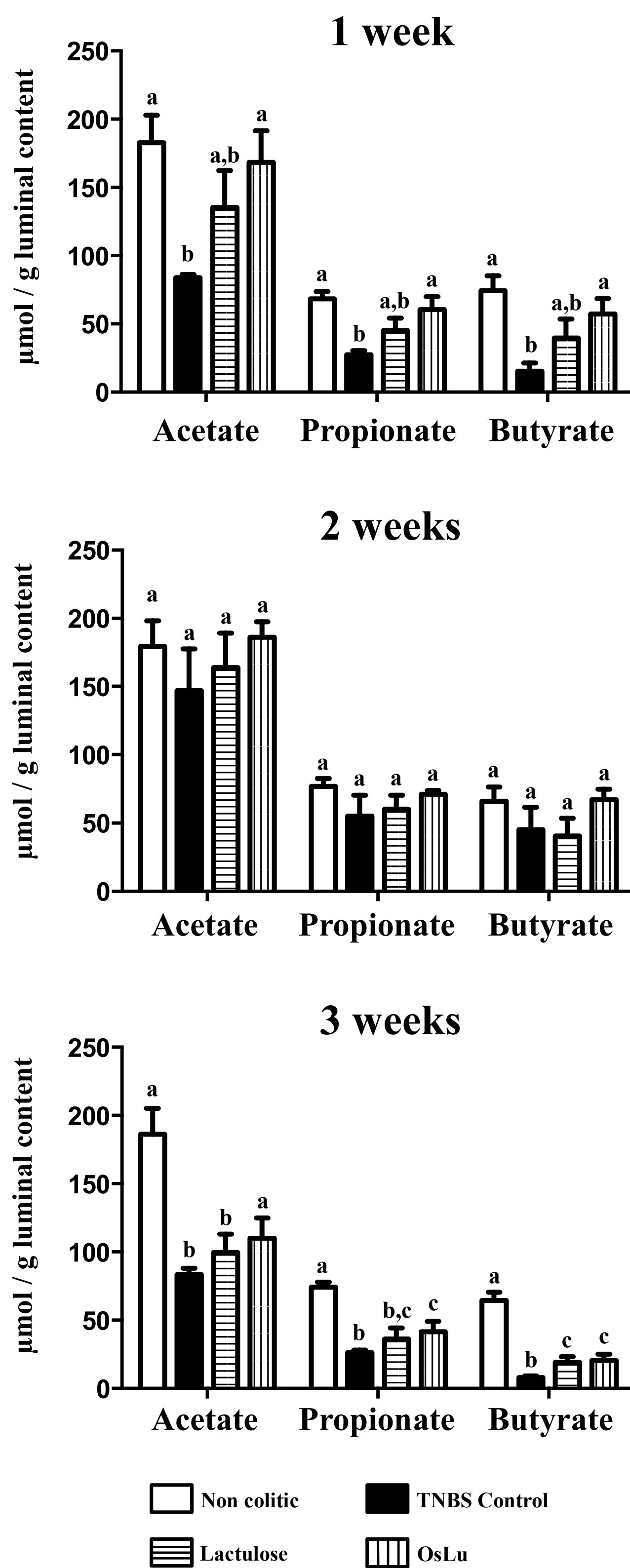
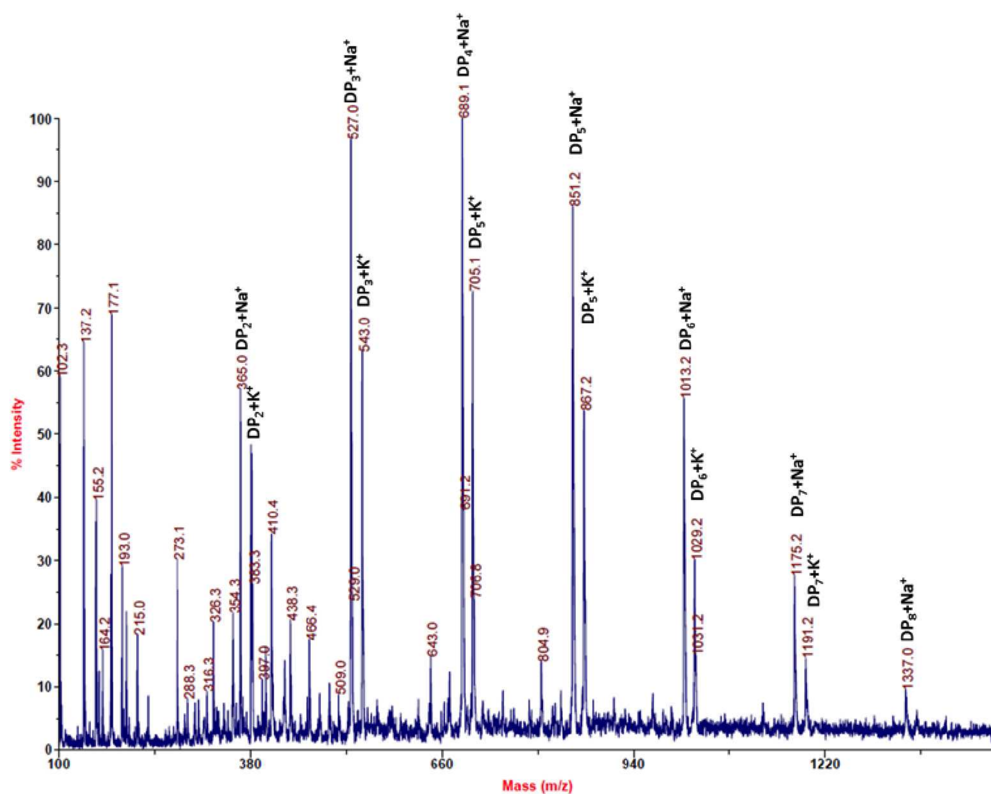


Figure 8



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