

Fructooligosaccharides exert intestinal anti-inflammatory activity in the CD4+ CD62L+ T cell transfer model of colitis in C57BL/6J mice

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Received: 3 February 2015 / Accepted: 10 June 2015 / Published online: 8 July 2015
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Abstract

Purpose Fructooligosaccharides (FOS) are used as functional foods due to their prebiotic effects. Intestinal anti-inflammatory activity has been established in most, but not all, studies in animal models of colitis, using mainly chemically induced inflammation. Our goal was to test the effect of FOS (degree of polymerization 2–8) in the chronic, lymphocyte-driven CD4+ CD62L+ T cell transfer model of colitis.

Methods Colitis was induced by transfer of CD4+ CD62L+ T cells to C57BL/6J Rag1^{-/-} mice. FOS (75 mg day⁻¹) was administered by gavage as a post-treatment. Three groups were established: non-colitic (NC), colitic control (C, CD4+ CD62L+ transferred mice treated with vehicle) and colitic+FOS (C+FOS, similar but treated with FOS). Mice were killed after 13 days.

Results Treatment of mice with FOS ameliorated colitis, as evidenced by an increase in body weight, a lesser myeloperoxidase and alkaline phosphatase activities, a lower secretion of proinflammatory cytokines by mesenteric lymph node cells ex vivo (IFN- γ , IL-17, and TNF- α), and a higher colonic expression of occludin (C+FOS vs. C, $p < 0.05$). Increased relative abundance of lactic acid bacteria was observed in FOS-treated mice ($p < 0.05$).

Conclusions FOS exert intestinal anti-inflammatory activity in T lymphocyte-dependent colitis, suggesting it

may be useful in the management of inflammatory bowel disease in appropriate conditions.

Keywords Fructooligosaccharides · Prebiotic · Colitis · CD4+ CD62L+ T cell · Alkaline phosphatase · Inflammatory bowel disease

Introduction

Inflammatory bowel disease (IBD) presents in two main clinical forms: Crohn's disease (CD) and ulcerative colitis (UC). Both disorders are characterized by chronic intestinal inflammation, typically intermittent, resulting in diarrhoea, abdominal pain, bloody faeces and, in children, growth arrest. CD can affect any part of the digestive tract, from mouth to anus, while UC is restricted to the colon and the rectum. Presently, there is a general consensus among IBD investigators that both CD and UC are the result of the combined effects of four basic components: global changes in the environment, the input of multiple genetic variations, alterations in the intestinal microbiota, and aberrations of innate and/or adaptive immune responses. There is also agreement on the conclusion that none of these four components can by itself trigger or maintain intestinal inflammation [1].

IBD is regularly managed pharmacologically with drugs that downregulate the inflammatory and/or immune response such as corticoids, infliximab, aminosalicylates, or azathioprine. These agents have a plethora of serious adverse effects which limit their application, and they are not effective in all patients. Hence, the search for new treatments with a low profile of adverse effects is much warranted [2]. Prebiotics are oligosaccharides that resist digestion and are thus capable of reaching the large intestine in

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substantial amounts, where they have the capacity to modulate the microbiota towards a host-friendly profile. Resistance to digestion is conferred by some types of sugar-to-sugar bond, such as $\beta(2 \rightarrow 1)$ in inulin-type fructans, which are not tackled by human enzymes, so that the molecule remains too big to be absorbed in the upper part of the intestine [3]. Inulin-type fructans are fructose oligomers or polymers which variable chain length [normally defined by the degree of polymerization (DP) parameter] gives rise to a range of different products. Unfortunately, their nomenclature is not standard. The term inulin as such is usually applied to higher DP polymers, i.e. >20 but sometimes encompassing polymers with a DP as low as 10. Fructooligosaccharides (FOS) are considered inulin-type fructans with DP <10 . Chain length is biologically relevant because it determines the preferential site of fermentation along the gastrointestinal tract, with lower DP fructans being fermented in the proximal colonic segment. DP may also be relevant to other effects such as TLR ligation [4, 5]. Fructans typically present a terminal glucose, but it may be also absent due to hydrolysis [3].

Since these compounds are amenable to hydrolysis by bacteria, which are present in large amounts in the colonic lumen, preferential fermentation at the distal segments of the gastrointestinal tract occurs. This process leads in turn to the production of short-chain fatty acids (SCFA) and a colonic pH decrease, as well as the acceleration of intestinal transit due to stimulation of colonic flora growth and, consequently, to increased gas production and water retention in faeces [6, 7]. Other properties of FOS include a favourable impact on lipidemia and metabolic syndrome, and a low caloric value [8, 9]. In addition, microbiota-independent, direct effects on the intestinal mucosa have been described [3, 4, 10].

Changes in the intestinal microbiota are the basis for the colonic anti-inflammatory activity of prebiotics, including FOS [11–15]. This has been evidenced by most [11, 16–18], but not all studies in animals [19, 20]. Clinical studies range from small evidence of benefit to no effect [21, 22], although in this scenario the experimental conditions are obviously less flexible, and therefore, it is possible to have an effective treatment fail, for instance, for an inadequate dose. Animal studies have employed chemically induced models of colitis, namely trinitrobenzenesulfonic acid (TNBS) and dextran sulphate sodium (DSS), as well as other gene-targeted rodent models of spontaneous colitis, such as HLA-B27 transgenic rats [16, 23]. There is no ideal animal model of IBD [24]. The TNBS and DSS models are widely used because of reproducibility, ease of use, and good characterization, especially for the testing of therapeutic interventions (pharmacological or nutritional), but also for research on pathophysiology such as ionic transport or motility [25]. The TNBS model is based on the

immune reaction against TNBS-haptenated mucosal proteins, while DSS is considered to work by disruption of the epithelial layer, enhancing mucosal uptake of luminal elements [26–28]. Other chemically induced models include iodoacetamide, carrageenan, acetic acid, and monochloramine colitis. These models present several disadvantages, starting from the need of a chemical insult, including the fact that they are not strictly chronic (i.e. they heal with time). In addition, they are not lymphocyte driven, as human disease is considered to be. In contrast, genetic models are based on the progressive development of colitis as a result of immunological bias, for instance, by deletion of the anti-inflammatory cytokine IL-10 in mice or by insertion of human HLA-B27 in rats, resulting in chronic colitis. Spontaneous colitis models also exist, including cotton-top tamarin (*Saguinus oedipus*) colitis and SAMP1/YitFc mouse ileitis [29]. The fact that successful therapeutic interventions in chemically induced models have resulted in poor bench to bedside translation has prompted some authors to advocate the use of the most immunologically relevant mouse models of IBD, including T cell transfer model of colitis, to achieve a better prediction of human bioactivity [30]. Hence, we set out to verify the anti-inflammatory effect of FOS in the CD4⁺ CD62L⁺ T cell transfer model of colitis.

Materials and methods

Reagents

Except where indicated, all reagents and primers were obtained from Sigma (Barcelona, Spain). Reverse transcription was achieved with the iScript™ cDNA Synthesis Kit, and iQ™ Sybr® Green Supermix was used for amplification (Biorad, Alcobendas, Madrid, Spain). All the primary antibodies used in the magnetic separation were purchased from BD Pharmingen™ (Madrid, Spain); MACS column, and anti-biotin and CD62L microbeads were provided by MACS Miltenyi Biotec (Cologne, Germany). Mouse ELISA kits (IL-6, TNF- α , IL-10, IFN- γ and IL-17) were obtained from eBioscience (San Diego, CA, USA). Reinforced clostridial agar, MRS agar, AnaeroGen™ and CO2Gen™ pouches, and plastic anaerobic jars were purchased from Oxoid (Hampshire, England); Wilkins-Chalgren agar from BD Pharmingen™ (Madrid, Spain); and blood agar from Panreac (Barcelona, Spain). FOS was kindly provided by BENEORafti® (Tienen, Belgium). Natural FOS generally consist of chains of fructose units linked together by $\beta(2 \rightarrow 1)$ linkages. Almost every molecule is terminated by a glucose unit. The total number of fructose or glucose units (degree of polymerization, DP) ranges mainly between 2 and 60. Orafti® P95 oligofructose

(FOS) is produced by the partial enzymatic hydrolysis of chicory-derived inulin, consisting mainly of molecules with DP between 2 and 8 (average: 4), with more than 25 % of molecules having DP > 5 and less than 75 % having DP < 4. The overall oligofructose content was 93.2 %, and the molecular weight ranged between 342 and 1638 Da. Due to enzymatic hydrolysis, the terminal glucose is present in less than half of the FOS molecules. FOS stock was prepared daily by dissolving in autoclaved tap water.

Animals and housing

All animal procedures in this study were approved by the Animal Welfare Committee of the University of Granada (registry number 710) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Seven female C57BL/6J wild-type (cell donors, 19.2 ± 0.7 g) and 22 female C57BL/6J Rag1^{-/-} mice (cell recipients, 21.5 ± 0.2 g) were obtained from Jackson Laboratory (CA, USA). Animals were maintained at the University of Granada Animal Facility (Biomedical Research Center, University of Granada, Granada, Spain) in air-conditioned animal quarters with a 12-h light–dark cycle. Animals were housed per groups in specific pathogen-free conditions, in individual ventilated cages with an air insufflation and exhalation system with dual filter (pre-filter and HEPA filter), and were given free access to autoclaved tap water and food (Harlan-Teklad 2014, Harlan Ibérica, Barcelona, Spain).

Induction of transfer colitis and experimental design

Female C57BL/6J mice were killed at 16 week of age by cervical dislocation, and the spleen was extracted aseptically. CD4⁺ CD62L⁺ T cells were purified from spleen mononuclear cells as previously described [31]. The isolation was performed using the CD4⁺ CD62L⁺ T Cell Isolation Kit II (Miltenyi Biotec, Cologne, Germany) following the protocol recommended by the manufacturer. The CD4⁺ CD62L⁺ T cells were eluted in 100 μ L of sterile PBS and administered intraperitoneally into recipients C57BL/6J Rag1^{-/-} mice (1×10^6 CD4⁺ CD62L⁺ T cells mouse⁻¹). The non-colitic control group (Rag1^{-/-} background) was administered sterile PBS (without CD4⁺ CD62L⁺ T cells).

The status of the animals was monitored by general examination and specifically controlling body weight (BW) evolution. Treatment was started when colitic animals underwent a 10 % of BW loss (about 8 week after the transfer), time when rectal prolapse was first observed. BW loss was calculated per individual animal. At this point, colitic mice were randomly assigned to each corresponding group.

Three groups were established: non-colitic (NC, $n = 6$), colitic control (C, $n = 8$, CD4⁺ CD62L⁺ transferred mice treated with vehicle), and colitic+FOS (C+FOS, $n = 8$, CD4⁺ CD62L⁺ transferred mice treated with 75 mg day⁻¹ of FOS). FOS were administered by gavage (Fig. 1). Treatment was maintained for 13 days after which animals were killed by cervical dislocation under isoflurane anaesthesia. The period of treatment was established on the basis of a stable therapeutic effect, as judged from BW evolution and overall animal status.

Assessment of colonic damage

The entire colon was removed, gently flushed with saline and placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed and its length measured under a constant load (2 g). The large intestine was longitudinally opened and scored for visible damage by a blinded observer on a 0–7 scale. The score was assigned as follows: adhesions (0–2), hyperaemia (0–2), fibrosis (0–2), and thickness (0–1). A small segment was dissected from the intestine and used for RNA isolation. The colon was subsequently divided longitudinally in several pieces for biochemical determinations. The fragments were immediately frozen in liquid nitrogen and kept at -80 °C until used.

Myeloperoxidase (MPO) and alkaline phosphatase (AP) activities

Colonic tissue homogenization was carried out with the protocol for intestinal tissue homogenization in the Bullet Blender[®] (Next Advance, Inc., NY, USA) in 50 mM

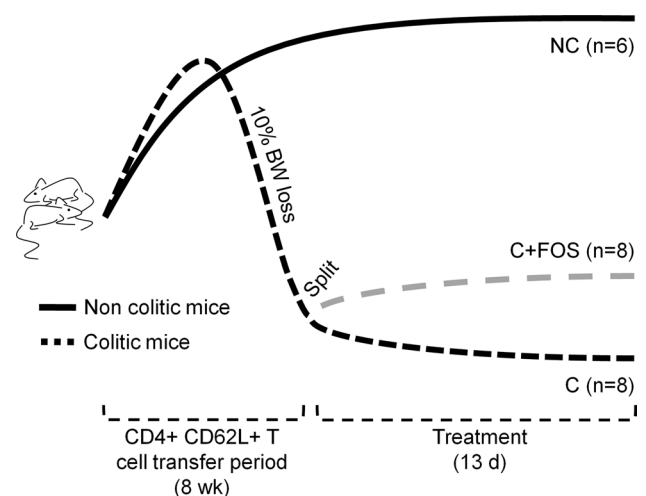


Fig. 1 CD4⁺ CD62L⁺ T cell transfer colitis experimental design. BW body weight, C colitic control, C+FOS colitic+ fructooligosaccharides, NC non-colitic

Tris base buffer with 0.5 % hexadecyl trimethyl ammonium bromide, pH 6.0. The activities of MPO and AP were measured spectrophotometrically as described previously [32, 33], and they are expressed as mU mg⁻¹ protein. In addition, the sensitivity to the AP inhibitor levamisole was assessed, and it is expressed as a % of inhibition.

RNA isolation and qRT-PCR analysis

Total RNA was isolated by the Trizol method (Invitrogen, Barcelona, Spain), checked for integrity by gel electrophoresis, and 1 µg was then subjected to reverse transcription, and specific RNA sequences were amplified with a Stratagene (La Jolla, CA, USA) MX3005P real-time PCR device. The following primers were used: *Gapdh* sense 5'-CAT TGA CCT CAA GTA CAT GG-3', antisense 3'-GTG AGC TTC CCG TTC AGC-5'; *Iilb* (IL-1β) sense 5'-AAG GGC TGC TTC CAA ACC TTT GAC-3', antisense 3'-TGC CTG AAG CTC TTG TTG ATG TGC-5'; *Iil10* (IL-10) sense 5'-CAG GAC TTT AAG GGT TAC TTG-3', antisense 3'-ATT TTC ACA GGG GAG AAA TC-5'; *S100* calcium-binding protein A8 (*S100a8*) sense 5'-GCC CTC TAC AAG AAT GAC TTC AAG-3', antisense 3'-ATC ACC ATC GCA AGG AAC TCC-5'; *Reg3g* (regenerating islet-derived protein 3 gamma, REG3-γ) sense 5'-CAG AGG TGG ATG GGA GTG GAG-3', antisense 3'-CAC AGT GAT TGC CTG AGG AAG AG-5'; *Ocln* (occludin) sense 5'-ACG GAC CCT GAC CAC TAT GA-3', antisense 3'-TCA GCA GCA GCC ATG TAC TC-5'; *Cldn4* (claudin 4) sense 5'-GAC TGT GCA AAG TTA CTA GC-3', antisense 3'-ACC AGC AAT TTG GAT GTA AG-5'; *Cldn5* (claudin 5) sense 5'-AAC AGT TCC TAC TGA GAT CC-3', antisense 3'-CTT TTT AAC ACG TCC CTC TG-5'. Results are expressed as 2^{-deltadeltaCt} using *Gapdh* as reference gene.

Mesenteric lymph node cells (MLNC) ex vivo culture

MLNC were extracted from the mice in the study as described elsewhere [31]. Cell viability was quantified with the trypan blue exclusion assay. Cell suspensions were diluted 1:10 in 0.4 % trypan blue in PBS and

incubated 2 min while shaking, and viable (unstained) and total cells were counted. 1 × 10⁶ cells mL⁻¹ were cultured and stimulated with concanavalin A (ConA) at a final concentration of 5 µg mL⁻¹ in a humidified 5 % CO₂ atmosphere at 37 °C. Cell culture medium was collected after 48 h, cleared by centrifugation (9300×g/10 min/4 °C), and frozen at -80 °C until assayed for cytokine content by commercial ELISA, following the protocols recommended by the manufacturer. The cytokines determined were IL-6, IL-10, IL-17, IFN-γ, and TNF-α. Plates (Nunc™ Immuno plate, Roskilde, Denmark) were read at 450 nm using a plate reader (Tecan, model Sunrise-basic, Austria).

Data and statistical analysis

In all the experiments, samples were run at least in triplicate and results are expressed as mean ± SEM. Differences among means were tested for statistical significance by one-way ANOVA and a posteriori Fisher's LSD test on all pairwise comparisons. Logarithmic transformation was applied to the PCR data before statistical analyses. Graphs were made with the OriginPro 8 program (OriginLab Corporation, Northampton, MA, USA). All analyses were carried out with the SigmaStat 3.5 program (Jandel Corporation, San Rafael, CA, USA). Differences were considered significant at $p < 0.05$.

Results

Colitis evolution and animal status

The animals selected for this study showed a 10.4 ± 0.9 % average BW loss around week 8 and were then randomized for treatment with FOS or vehicle (day 0) (Fig. 1). C+FOS maintained a relatively stable BW, while group C continued to lose weight (6.1 ± 3.0 % from day 0, $p < 0.05$ vs. NC). As expected, NC mice gained weight steadily throughout the experimental period (Table 1; Fig. 2). Food intake was comparable in the three groups (Table 1),

Table 1 BW evolution, food and water intake in C57BL/6J Rag1^{-/-} mice during CD4+ CD62L+ T cell transfer and treatment period

	Transfer period		Treatment		Food intake	Water intake
	% BW		% BW			
	BW week 0	BW week 4–6	BW day 0	BW day 12	g mouse ⁻¹ day ⁻¹	mL mouse ⁻¹ day ⁻¹
NC	91.62 ± 1.34	95.14 ± 0.63	100 ± 3.05	106.50 ± 0.95	2.97 ± 0.13	2.90 ± 0.20
C	103.67 ± 3.40*	111.72 ± 1.04*	100 ± 3.81	93.94 ± 2.96*	3.04 ± 0.17	4.05 ± 0.19*
C+FOS	101.04 ± 1.20*	109.72 ± 1.28*	100 ± 2.41	103.42 ± 1.94 [#]	2.83 ± 0.07	3.24 ± 0.08 [#]

* $p < 0.05$ versus NC. [#] $p < 0.05$ versus C. C ($n = 8$), C+FOS ($n = 8$), NC ($n = 6$). 100 % BW has been normalized to the day 0

Fig. 2 BW evolution during CD4+ CD62L+ transfer period (8 week) and treatment (13 days). BW is expressed as means (g) ± SEM, C (n = 8), C+FOS (n = 8), NC (n = 6). Within each day, *p < 0.05 versus NC; #p < 0.05 versus C. BW body weight, C colitic control, C+FOS colitic+ fructooligosaccharides, NC non-colitic

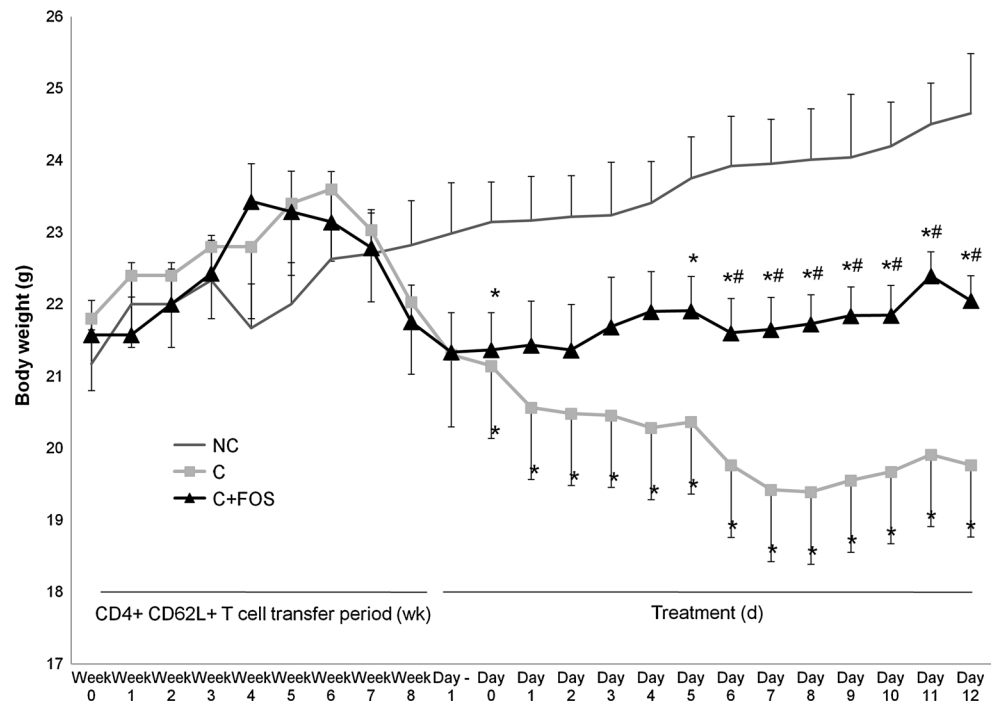


Table 2 Morphological indicators of inflammation in C57BL/6J Rag1^{-/-} mice after the kill

	Colonic damage score Arbitrary units	Colon weight: length ratio (mg cm ⁻¹) × 1000	Spleen weight (g spleen BW mouse ⁻¹) × 1000
NC	0.00 ± 0.00	21.60 ± 1.36	1.34 ± 0.06
C	2.00 ± 0.84*	39.39 ± 7.87*	3.82 ± 1.48
C+FOS	1.37 ± 0.44*	43.41 ± 4.13*	4.84 ± 2.30

* p < 0.05 versus NC. C (n = 8), C+FOS (n = 8), NC (n = 6)

while C drank more water than NC (C vs. NC, p < 0.05, Table 1). This was effectively counteracted by FOS treatment (p < 0.05).

Colonic inflammatory status

Group C exhibited a hyperaemic mucosa with bowel wall thickening and increased adhesions and rigidity but no necrosis, resulting in a significantly augmented damage score (C vs. NC, p < 0.05, Table 2). Treatment with FOS did not ameliorate these visible signs of colitis significantly (C+FOS vs. C, Table 2). Enhanced neutrophil recruitment to the mucosa was evidenced by a twofold increase in colonic MPO activity, a widely used inflammatory marker, compared with NC (C vs. NC, p < 0.05, Fig. 3a). This was fully prevented by FOS treatment (C+FOS vs. C, p < 0.05, Fig. 3a). Colonic AP activity, a marker of intestinal inflammation and epithelial stress [33–35], was also augmented threefold in group C, associated with a dramatic (over fivefold) increase in the sensitivity to the specific inhibitor

levamisole in vitro (C vs. NC, p < 0.05, Fig. 3b, c). FOS treatment resulted in a 35 % significant reduction in AP activity and a parallel effect on the sensitivity to levamisole (C+FOS vs. C, p < 0.05, Fig. 3b, c).

Colonic expression of inflammatory and barrier function markers assessed by qRT-PCR

Group C showed a diminished (≥tenfold) expression of the tight junction components *Cldn4* and *Cldn5* (claudins 4 and 5, p < 0.05), whereas *S100a8*, the cytokines *Il1b* (IL-1β) and *Il10* (IL-10), the antimicrobial peptide *Reg3g* (Reg3γ), and the tight junction protein *Ocln* (occludin) were not significantly affected (C vs. NC, Table 3). A nonsignificant trend to increase was noted in the case of *S100a8* and *Reg3g*. Occludin was upregulated in C+FOS compared with the C group (p < 0.05, Table 3). FOS had no significant effect on *Cldn4* or *Cldn5* compared with group C. A trend towards a lower *S100a8* expression level was observed in FOS-treated mice.

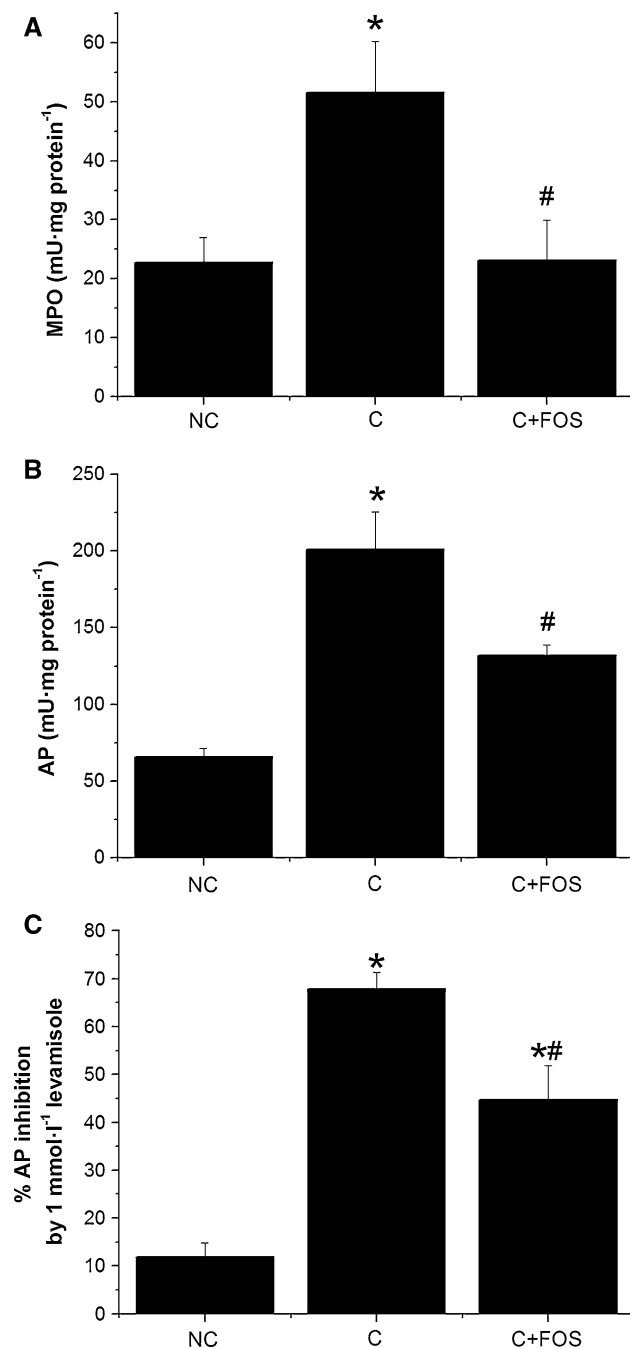


Fig. 3 Inflammatory markers in C57BL/6J Rag1^{-/-} mice. Colon MPO (a) and AP (b, c) activities. Enzymatic activity (mU mg protein⁻¹) and the sensitivity of AP to the specific inhibitor (% AP inhibition), levamisole, are shown, C ($n = 8$), C+FOS ($n = 8$), NC ($n = 6$). * $p < 0.05$ versus NC; # $p < 0.05$ versus C. AP alkaline phosphatase, C colitic control, C+FOS colitic+ fructooligosaccharides, MPO myeloperoxidase, NC non-colitic

Cytokine secretion by MLNC ex vivo

This model of colitis is characterized by a progressive expansion of the transferred T lymphocyte population, with

a predominance of Th1/Th17 cells and a paucity of Treg cells, and accordingly, basal and ConA-stimulated MLNC of C exhibited a heightened secretion of IFN- γ and IL-17 compared to NC samples (C vs. NC, $p < 0.05$, Fig. 4). The basal levels of IL-6, TNF- α , and IL-10 were not significantly affected (C vs. NC, Fig. 5). ConA elicited nonetheless a robust (5- to 42-fold) response in group C in all cases (C vs. NC, $p < 0.05$, Fig. 5). FOS treatment had no effect on spontaneous cytokine secretion by MLNC, but it diminished IFN- γ , IL-17, and TNF- α ConA-evoked secretion by ~50 % (C+FOS vs. C, $p < 0.05$, Figs. 4; 5). There was no effect, however, on IL-6 or IL-10.

Discussion

IBD is an obvious possible target of nutraceuticals because of the important adverse effects associated with drug therapy. Although it seems unrealistic to manage IBD on the basis of nutraceuticals or functional foods alone, these products may be useful adjuvants due to their extremely low toxicity, as an add-on to regular nutrition. We set out to test the intestinal anti-inflammatory activity of FOS using the lymphocyte transfer model [30, 36]. As mentioned above, there is no ideal single model of IBD. Instead, a number of various models with different advantages and drawbacks are available. FOS have been shown to exert colitis protective actions in chemical models [11, 16–18], with some exceptions [19, 20], and in HLA-B27 transgenic rat colitis [23]. The advantages of lymphocyte transfer colitis are its chronic course and the lymphocyte-driven pathology. Hence, establishing therapeutic efficacy of FOS in this model strengthens its translational potential.

Lymphocyte transfer colitis develops slowly, and once established, it may remain relatively stable for weeks or may deteriorate slowly until animal death (spontaneous or by euthanasia due to ethical reasons). We chose to apply a post-treatment protocol, i.e. FOS was administered after the colitis was well established, namely 8 weeks after the transference of lymphocytes, when mice had lost 10 % of their body weight. A crucial point in our experiment was the selection of the intervention starting point. Therefore, we examined mice that were transferred in parallel to the ones in this experiment and killed them at an earlier time point (five weeks after lymphocyte transfer, i.e. 3 weeks before the intervention started in our experiment). These animals showed an augmented colon weight: length ratio, colonic damage score and splenomegalia, greater ConA-stimulated MLNC cytokine secretion, and so forth (independent experiments, data not shown). This indicates that although no weight loss had been observed at this time point, intestinal inflammation had already started. Nevertheless, we waited for three more weeks until the animals had lost a

Table 3 Colonic expression of inflammatory and barrier function markers assessed by qRT-PCR in C57BL/6J Rag1^{-/-} mice

	NC	C	C+FOS
	Fold change		
<i>Il1b</i>	1.0 ± 0.2	1.5 ± 0.5	0.3 ± 0.1
<i>Il10</i>	1.0 ± 0.5	1.0 ± 0.1	2.3 ± 0.9
<i>Sl100a8</i>	1.0 ± 0.5	7.7 ± 3.0	0.2 ± 0.1
<i>Reg3 g</i>	1.0 ± 0.4	19.8 ± 12.8	12.0 ± 7.6
<i>Ocln</i>	1.0 ± 0.4	1.0 ± 0.6	3.7 ± 1.1*
<i>Cldn4</i>	1.0 ± 0.5	0.1 ± 0.0 ⁺	0.0 ± 0.0 ⁺
<i>Cldn5</i>	1.0 ± 0.5	0.0 ± 0.0 ⁺	0.1 ± 0.1 ⁺

⁺ $p < 0.05$ versus NC; * $p < 0.05$ versus C. C ($n = 8$), C+FOS ($n = 8$), NC ($n = 6$). Results are expressed as fold change of gene expression calculated from $2^{-\Delta\Delta Ct}$ using *Gapdh* as reference gene. Logarithmic transformation was applied to the PCR data before statistical analyses

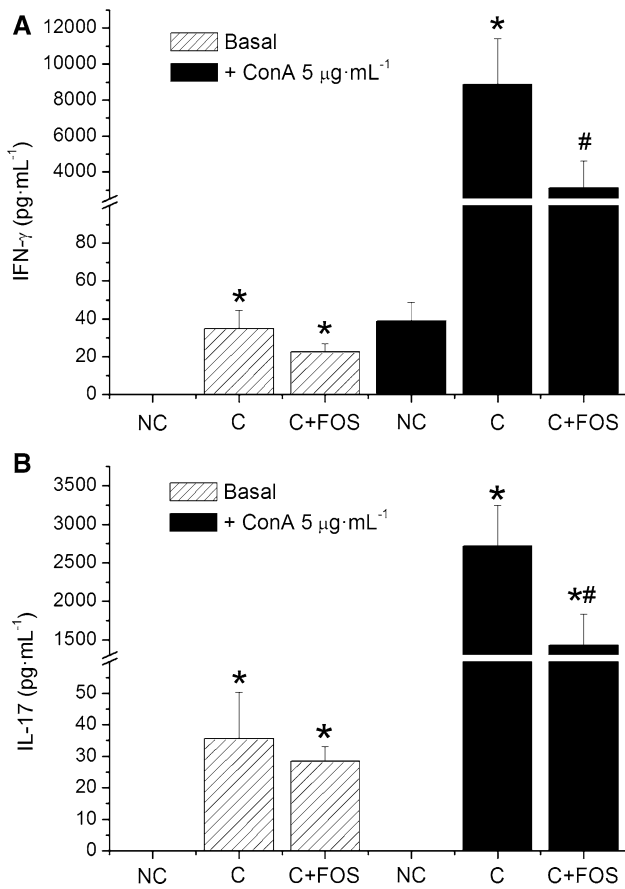


Fig. 4 Cytokine secretion by MLNC. Cytokines measured are IFN- γ (a) and IL-17 (b). Values are means (pg mL^{-1}) \pm SEM, C ($n = 8$), C+FOS ($n = 8$), NC ($n = 6$). Within basal or stimulated values, * $p < 0.05$ versus NC; # $p < 0.05$ versus C. C colitic control, ConA concanavalin A, C+FOS colitic+ fructooligosaccharides, MLNC mesenteric lymph node cells, NC non-colitic

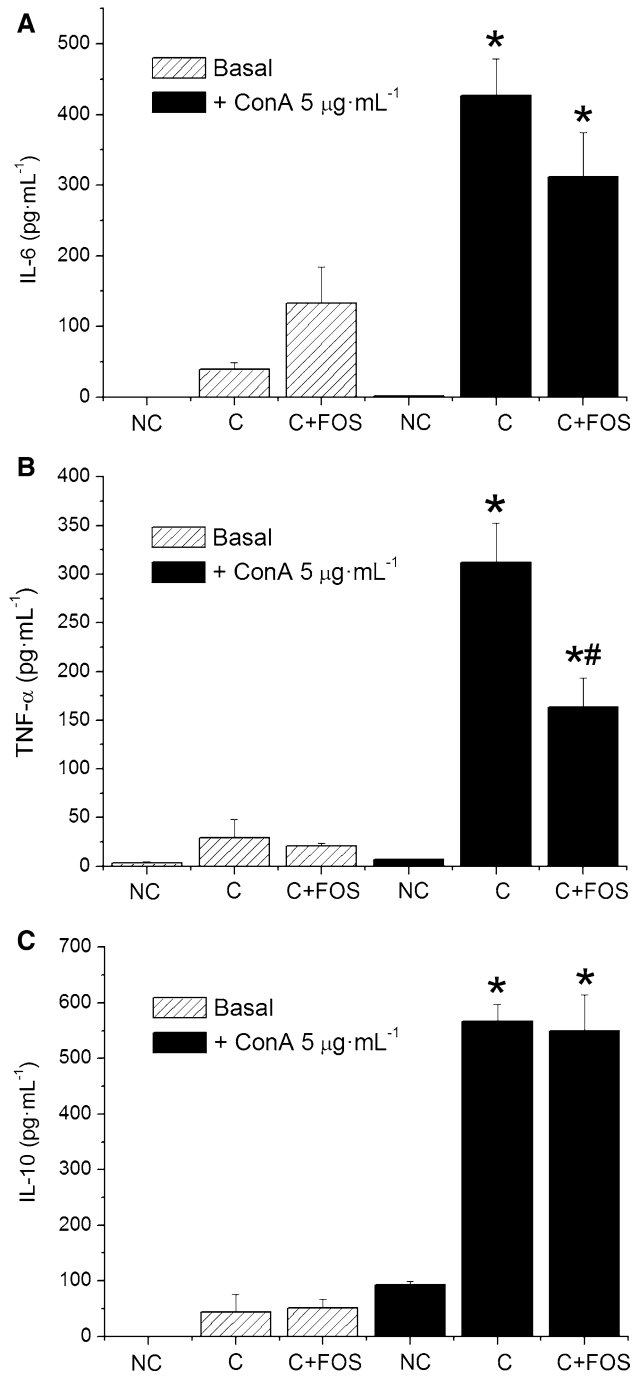


Fig. 5 Cytokine secretion by MLNC. Cytokines measured are IL-6 (a), TNF- α (b) and IL-10 (c). Values are means (pg mL^{-1}) \pm SEM, C ($n = 8$), C+FOS ($n = 8$), NC ($n = 6$). Within basal or stimulated values, * $p < 0.05$ versus NC; # $p < 0.05$ versus C. C colitic control, ConA concanavalin A, C+FOS colitic+ fructooligosaccharides, MLNC mesenteric lymph node cells, NC non-colitic

10 % of their body weight and interpreted this as a sign of a systemic response. The 10 % weight loss threshold was chosen following the ethical criterium that indicates that

maintaining a weight loss over 20 % in animal models of colitis implies unnecessary suffering. Following the same ethical criteria, the experiment was stopped before colitic control animals reached this limit.

Continued weight loss is consistent with a systemic response and the establishment of chronic colitis, a trend that was promptly counteracted by FOS treatment, resulting in a 9 % difference in body weight between the C+FOS and C groups. Although not tested in the transfer model, experimental colitis has been shown to be associated with augmented systemic levels of IL-1 β and leptin, resulting in anorexia and weight loss [37, 38]. Since there was no anorexia in the colitic group in the present study, inflammation and acute stress-associated cachexia is a logical explanation for weight loss and for FOS protection. It is possible also that FOS supplementation provides a relevant caloric input (in the form of SCFA), thus contributing to protection against body wasting. It is, however, uncertain to what extent this factor is considerable given that FOS supplementation represented only 2.5 % of dietary intake. Of note, dietary supplementation with similar or higher amounts of fructans has been shown to be weight neutral [39–41] or to lead to attenuated weight gain in obesity models [42, 43].

This initial benefit was confirmed by significantly reduced colonic MPO and AP activities, lower *S100a8* expression (albeit nonsignificantly), and ConA-elicited IFN- γ , IL-17, and TNF- α secretion by MLNC ex vivo. It should be noted in this regard that lymphocyte transfer colitis is relatively mild compared with chemically induced models. To our knowledge, there is hardly any precedent of the use of the model for the testing of drugs or functional foods. So we needed a score criterium, and we adapted the one we used for TNBS and DSS in rats and mice [31]. At any rate, FOS treatment clearly failed to diminish colonic thickening. In this model, this is accounted for in part by crypt enlargement, a typical sign of colitis, but also by mucosal immune maturation as a consequence of lymphocyte expansion, and therefore, it may not be a sensitive sign of inflammation. In fact, the small intestine is also thickened in this model without apparent inflammation (not shown).

FOS are thought to dampen intestinal inflammation by modulation of the enteric microbiota. Therefore, we looked at changes in the microbiota to confirm that FOS behave as a prebiotic in our experimental conditions. Indeed, we found a significant increase in lactic acid bacteria in FOS-treated mice (not shown). It should be noted, however, that the changes observed in the colitic group are relatively modest [12]. Although not explored so far as we can tell, it is possible that the contribution of dysbiosis is reduced in this model. In addition, we have established that FOS

have direct immunomodulatory actions on intestinal epithelial cells and monocytes in the absence of bacteria [4, 10], consistent with stimulation of innate immune defence. Although apparently incongruent, such an effect may be associated with a reduced inflammatory response by a prompt and efficient control of mucosa invading microorganisms and antigens [44]. Therefore, FOS may act by both microbiota-dependent and microbiota-independent effects.

Our data additionally suggest that FOS treatment has a protective effect on the epithelium. This is based on the one hand on the observation that both AP enzyme activity and sensitivity to levamisole are decreased by FOS, consistent with reduced AP isoform shift in enterocytes, a known feature of epithelial cells under inflammatory conditions [33, 35]. On the other hand, FOS augmented the expression of occludin, which is an integral plasma membrane protein that is important part of the tight junction complex in intestinal epithelial cells, among other tissues. This is a specific effect, since the expression of other epithelial proteins such as *Cldn4/5* or *Reg3 g* was not changed significantly by the treatment. An increase in occludin expression enhances mucosal barrier function [45]. Although not formally tested in our study, it is interesting to note in this regard that FOS reduces the invasion of intestinal epithelial cells in vitro by *E. coli* LF82 enteroinvasive bacteria, due at least in part to an effect on enterocytes (unpublished results). We are currently evaluating FOS effects on epithelial permeability.

The dose used in this study, 75 mg day⁻¹, corresponds to 0.5 g day⁻¹ in the rat or 29 g day⁻¹ in humans on a body surface basis. In rats, FOS have been shown to exert colonic anti-inflammatory effects at doses ranging between 1 and 2 g day⁻¹, i.e. up to fourfold higher than the dose used in this study [11, 16–18], while at least some of the studies which failed to find therapeutic benefit employed lower doses [19]. In particular, a recent study showing beneficial effects of FOS in the HLA-B27 transgenic model of rat colitis used a dose of 8 g kg⁻¹, equivalent to roughly three times the dose used by us after correction for body surface [16]. This may be relevant specially when considering the evidence available in clinical studies. Recently, Benjamin et al. found no improvement in CD patients with FOS despite evidence of mucosal immunomodulation [22]. However, the dose used was 15 g day⁻¹, i.e. about half of the equivalent dose in our study and a sixth of that applied in HLA-B27 rats [16]. Therefore, it is entirely possible that clinical studies may miss efficacy because of insufficient dosage. Of course we cannot rule out that human IBD responds differently to FOS than animal models. Nevertheless, the increase in doses administered to IBD patients should be carefully examined since a plethora of secondary effects including flatulence, borborygmi, and abdominal pain, were described in that study [22].

In conclusion, our study demonstrates that FOS are effective in lymphocyte transfer colitis at the dose of 75 mg day⁻¹ when given as a post-treatment.

Acknowledgments We are grateful to Beneo-Orafti (Tienen, Belgium). This work was funded by Fundación Ramón Areces, by the Ministerio de Economía y Competitividad (SAF2008-01432, AGL2008-04332, SAF2011-22922 and SAF2011-22812), and by Junta de Andalucía (CTS164 and CTS6736). BO and CJA are funded by Ministry of Education. AA was funded by Junta de Andalucía. CIBERehd (Centro de Investigación Biomédica en Red, Enfermedades Hepáticas y Digestivas) is funded by the Instituto de Salud Carlos III.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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