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

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Abstract

Purpose. Fructooligosaccharides (FOS) are used as functional foods due to their prebiotic effects. Intestinal antiinflammatory 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.

*Method*s. Colitis was induced by transfer of CD4+ CD62L+ T cells to C57BL/6J $Rag1^{-/-}$ mice. FOS (75 $mg \cdot d^{-1}$) was administered by gavage as a postreatment. 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 sacrificed after 13 d.

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 diarrhea, 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 substantial amounts, where they have the capacity to modulate the microbiota toward a host-friendly profile. Resistance to digestion is conferred by some types of sugar-to-sugar bond, such as β(2→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), giving 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 favorable 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 sulfate 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, and 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 antiinflammatory 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 has 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 BENEO Orafti® (Tienen, Belgium). Natural FOS generally consist of chains of fructose units linked together by β(2→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 raanged 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 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 sacrificed at 16 wk 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 μL of sterile PBS and administered intraperitoneally into recipients C57BL/6J Rag1$^{-/-}$ mice ($1 \cdot 10^6$ 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 wk 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·d^{-1} of FOS). FOS were administered by gavage (Fig. 1). Treatment was maintained for 13 d after which animals were sacrificed 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 to 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 ºC 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 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 $mU \cdot mg^{-1}$ 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, 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'; *Il1b* (IL-1β) sense 5'-AAG GGC TGC TTC CAA ACC TTT GAC-3', antisense 3'-TGC CTG AAG CTC TTG TTG ATG TGC-5'; *Il10* (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 $2^{-\text{deltadeltaCt}}$ 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, incubated 2 min while shaking, and viable (unstained) and total cells were counted. $1 \cdot 10^6$ cells·mL^{-1} were cultured and stimulated with concanavalin A (ConA) at a final concentration of 5 µg·mL^{-1} in a humidified 5% CO_2 atmosphere at 37 ºC. Cell culture medium was collected after 48 h, cleared by centrifugation (9,300 g/10 min/4 ºC) and frozen at -80 ºC 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™ Inmuno 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 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, Northhampton, 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 wk 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), 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 2-fold 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 3-fold in group C, associated with a dramatic (over 5-fold) increase in the sensitivity to the specific inhibitor levamisole *in vitro* (C *vs.* NC, $p < 0.05$, Fig. 3B and 3C). 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 and 3C).

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

Group C showed a diminished (≥ 10-fold) 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.

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, $p < 0.05$, Fig. 4 and 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 to drug therapy. Although it seems unrealistic to manage IBD on the basis of nutraceuticals or functional foods alone, these products may be useful coadjuvants 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 sacrificed 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 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 to augmented systemic levels of IL-1β 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 is not 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 behaves 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 has direct immunomodulatory actions on intestinal epithelial cells and monocytes in the absence of bacteria [10, 44], consistent with stimulation of innate immune defense. 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 [45]. Therefore FOS may act by both microbiota dependent and 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 *Reg3g* were not changed significantly by the treatment. An increase in occludin expression enhances mucosal barrier function [46]. 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 $mg \cdot d^{-1}$, corresponds to 0.5 $g \cdot d^{-1}$ in the rat or 29 $g \cdot d^{-1}$ in humans on a body surface basis. In rats, FOS has been shown to exert colonic anti-inflammatory effects at doses ranging between 1 and 2 $g \cdot d^{-1}$, *i.e.* up to 4-fold 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 3 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 $g \cdot d^{-1}$, *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 of 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 $mg \cdot d^{-1}$ when given as a post-treatment.

Conflict of interest

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

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Figure Legends

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

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

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

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

Figure 1
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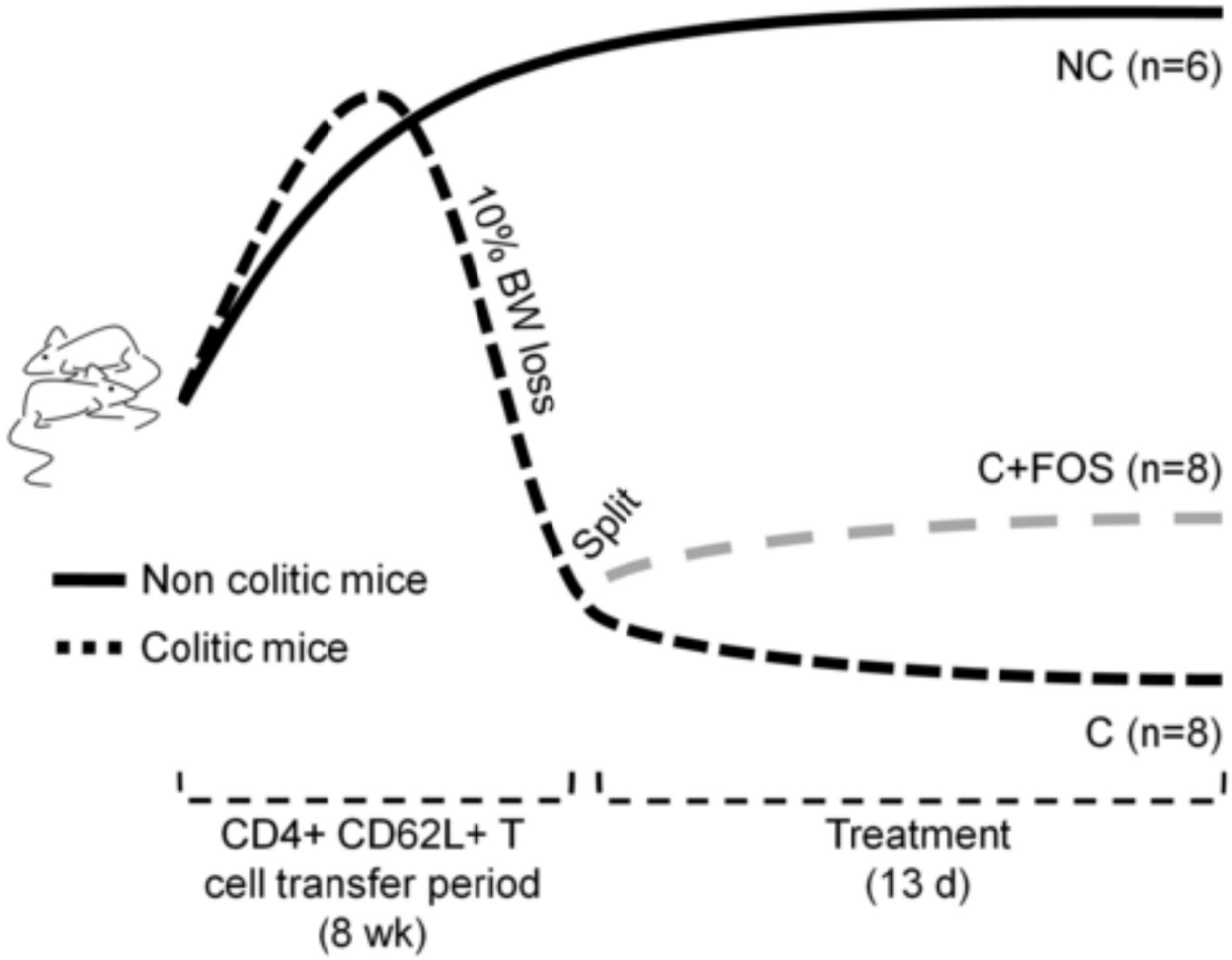

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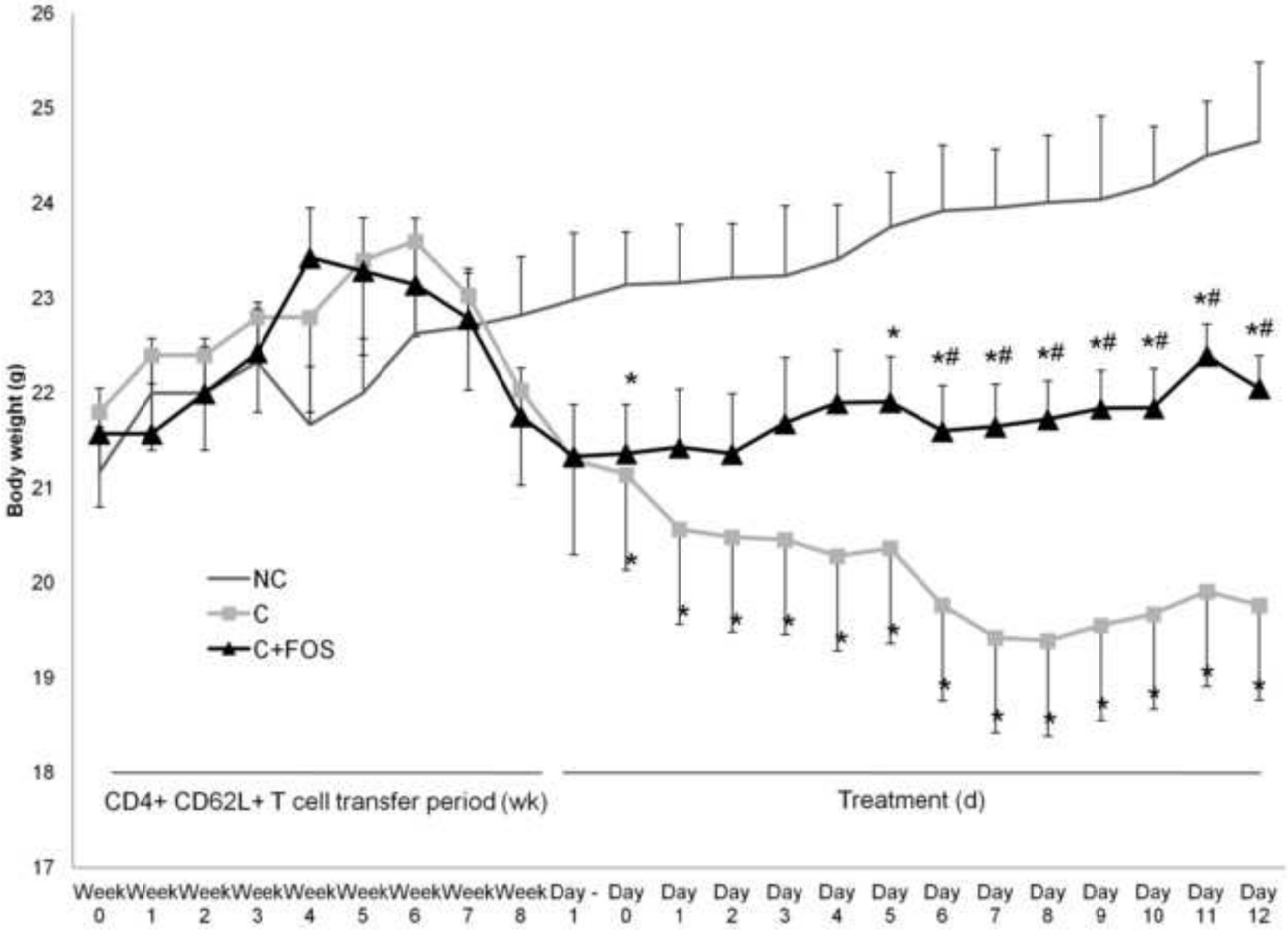

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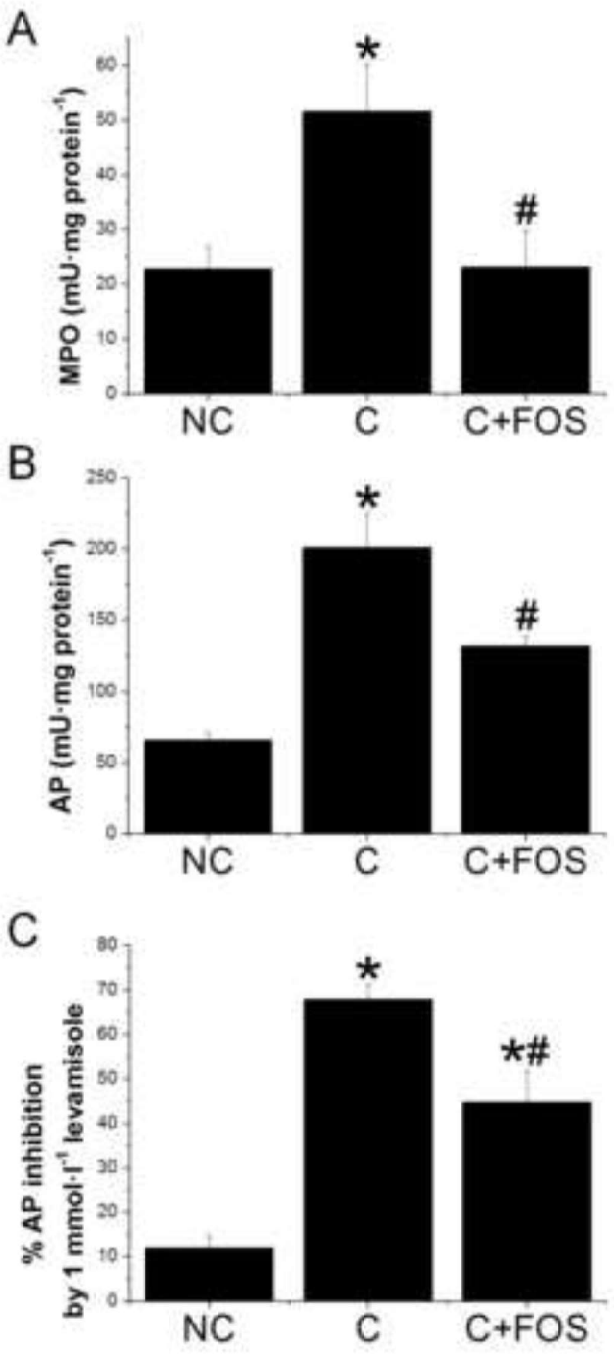

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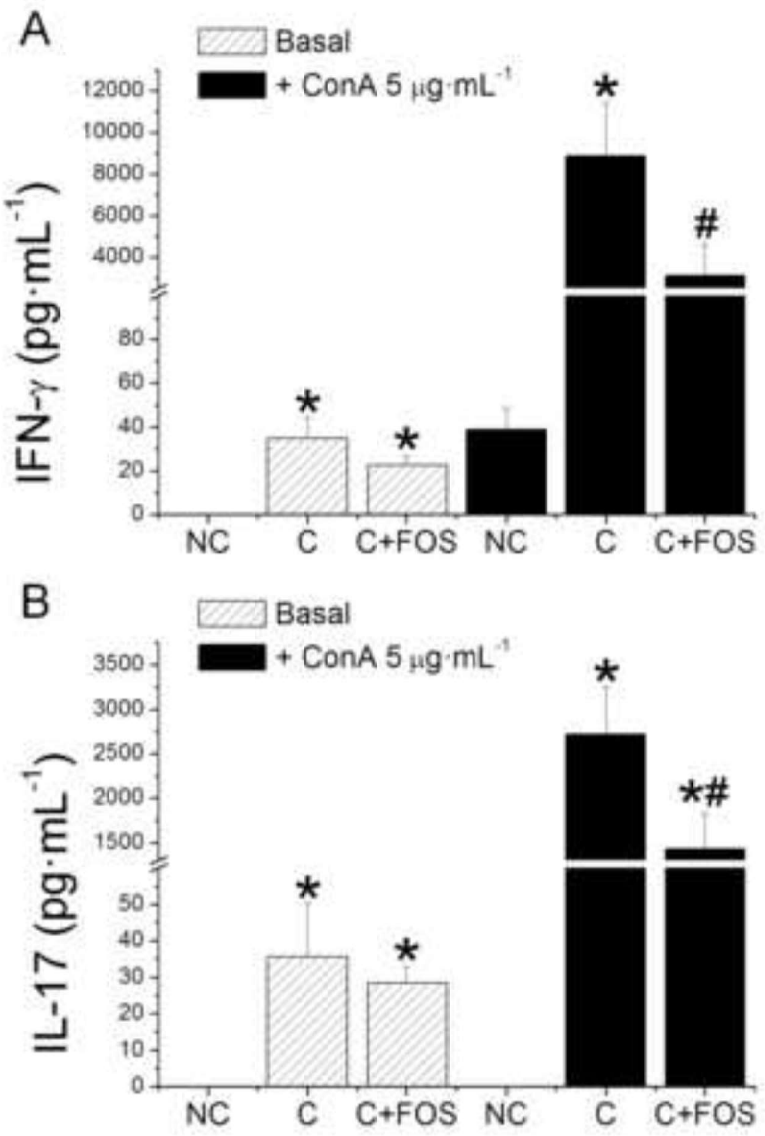

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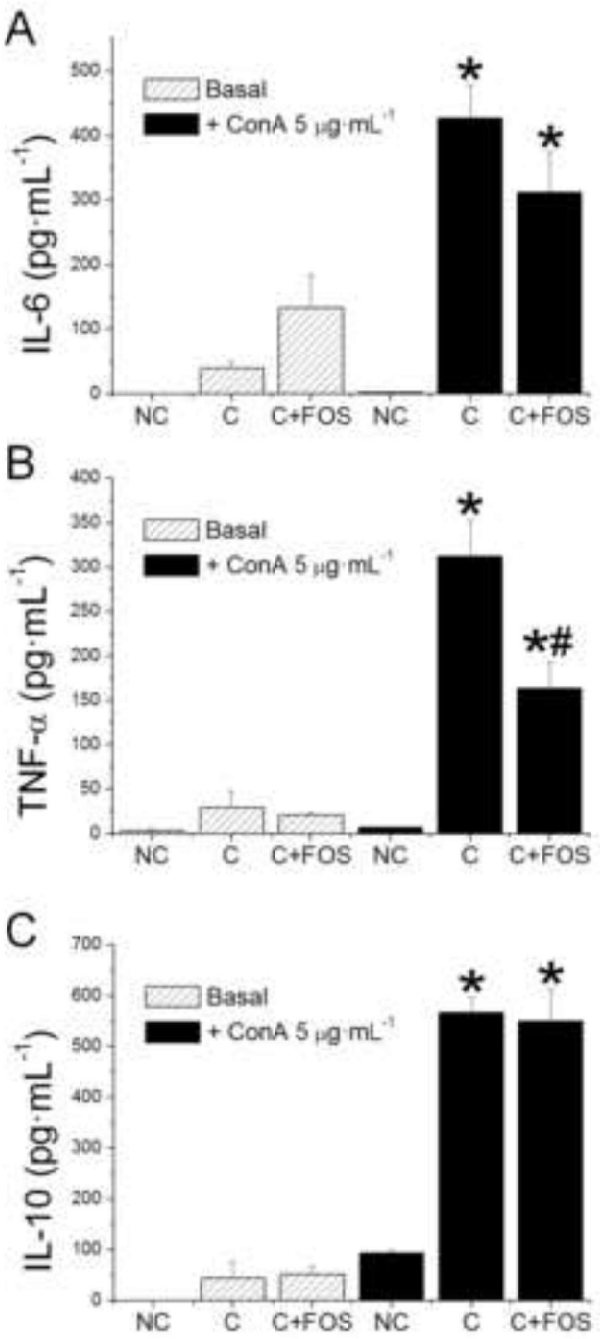

Table 1 BW evolution, food and water intake in C57BL/6J Rag1$^{-/-}$ mice during CD4+ CD62L+ T cell transfer and treatment period

	Transfer period		**Treatment**		**Food Intake**	**Water Intake**
	BW wk 0	**BW wk 4-6**	**BW d 0**	**BW d 12**		
	% BW		*% BW*		*g·mouse^{-1}·d^{-1}*	*mL·mouse^{-1}·d^{-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 #

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

Table 2

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

	Colonic damage score	**Colon weight:length ratio**	**Spleen weight**
	Arbitrary units	*(mg·cm^{-1})·1000*	*(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

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

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		
Il1b	1.0 ± 0.2	1.5 ± 0.5	0.3 ± 0.1
Il10	1.0 ± 0.5	1.0 ± 0.1	2.3 ± 0.9
S100a8	1.0 ± 0.5	7.7 ± 3.0	0.2 ± 0.1
Reg3g	1.0 ± 0.4	19.8 ± 12.8	12.0 ± 7.6
Ocln	1.0 ± 0.4	1.0 ± 0.6	3.7 ± 1.1*****
Cldn4	1.0 ± 0.5	0.1 ± 0.0^{+}	0.0 ± 0.0^{+}
Cldn5	1.0 ± 0.5	0.0 ± 0.0^{+}	0.1 ± 0.1^{+}

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