Microbiota-independent immunological effects of non-digestible oligosaccharides in the context of inflammatory bowel diseases

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List of abbreviations:

NDO, non-digestible oligosaccharides; IBD, inflammatory bowel diseases; CD, Crohn's disease; UC, ulcerative colitis; IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; FOS, fructooligosaccharides; GOS, galactooligosaccharides; HMO, human milk oligosaccharides; AOS, arabinooligosaccharides; MOS, mannanoligosaccharides; XOS, xylooligosaccharides; POS, pectic oligosaccharides; DP, degree of polymerisation; SCFA, short chain fatty acids; STAT1, signal transducer and activator of transcription 1; PPARγ, peroxisome proliferator-activated receptor gamma; DC, dendritic cells; MLN, mesenteric lymph node; scGOS/lcFOS, short chain GOS/long chain FOS; Caco-2 cells, human epithelial colorectal adenocarcinoma cells; Gal, galactose; HUVEC, human umbilical vein endothelial cells; MCP-1, monocyte chemoattractant protein-1; MIP-3α, macrophage inflammatory protein-3 alpha; NF-κB, nuclear factor kappa B; GMO, goat milk oligosaccharides; LPS, lipopolysaccharide; MoDC, monocyte-derived dendritic cells; TLR4, toll-like receptor 4; PBMC, peripheral blood mononuclear cells; GRO-α, growth-regulated oncogene alpha; MIP-2, macrophage inflammatory protein 2; TGF-β, transforming growth factor beta;

Abstract

The aim of this paper is to review the effects of non-digestible oligosaccharides (NDO) on immunity, focusing on their microbiota-independent mechanisms of action, as well as to explore their potential beneficial role in inflammatory bowel diseases (IBD). IBD are chronic, inflammatory conditions of the gastrointestinal tract. Individuals with IBD have an aberrant immune response to commensal microbiota, resulting in extensive mucosal inflammation and increased intestinal permeability. NDO are prebiotic fibres well known for their role in supporting intestinal health through modulation of the gut microbiota. NDO reach the colon intact and are fermented by commensal bacteria, resulting in the production of short chain fatty acids with immunomodulatory properties. In disease states characterised by increased gut permeability, prebiotics may also bypass the gut barrier and directly interact with intestinal and systemic immune cells, as demonstrated in patients with IBD and in infants with an immature gut. *In vitro* models show that fructooligosaccharides, inulin and galactooligosaccharides exert microbiota-independent effects on immunity by binding to toll-like receptors on monocytes, macrophages and intestinal epithelial cells and by modulating cytokine production and immune cell maturation. Moreover, animal models and human supplementation studies demonstrate that some prebiotics, including inulin and lactulose, might reduce intestinal inflammation and IBD symptoms. Although there is convincing preliminary data to support NDO as immunomodulators in the management of IBD, their mechanisms of action are still unclear and larger standardised studies need to be performed using a wider range of prebiotics.

Inflammatory bowel diseases (IBD)

Inflammatory bowel diseases (IBD) are chronic and relapsing conditions affecting the gastrointestinal tract. Based on the location of inflammation, IBD are classified as Crohn's disease (CD) or ulcerative colitis (UC). While CD is associated with deep inflammation in any part of the gastrointestinal tract, UC is linked to severe tissue damage of the colon and rectum[1]. In the 21st century, IBD has become a major burden in Western countries, with over 1.5 million and 2 million people affected in North America and Europe, respectively[2]. Although the exact pathogenesis is unclear, there is evidence that IBD develop in genetically susceptible individuals who have been exposed to environmental insults that lead to an aberrant immune response to commensal gut microbiota and chronic inflammation[3]. Despite both CD and UC having a genetic basis, UC is more strongly affected by environmental factors than genetic factors, compared to CD[4]. Relevant environmental factors include lifestyle (e.g. smoking, diet and stress), host microbiota (dysbiosis), pharmacologic agents (e.g. antibiotics), ecological factors (e.g. pollution) and surgery (e.g. appendectomy), with smoking having the greatest level of evidence for association with IBD than other factors according to the 2009 Oxford Centre for Evidence-based Medicine levels of Evidence[3]. Genetic and environmental factors drive alterations to the gut immune response, leading to reduced ability to clear pathogenic bacteria[5], lower levels of goblet cells, Paneth cell dysfunction and increased secretion of inflammatory mediators, such as interleukins (IL-1β, IL-6, IL-17, IL-18, IL-22), tumour necrosis factor (TNF-α) and interferon (IFN-γ)[6]. These processes can cause damage to the intestinal epithelium, which becomes permeable and susceptible to the commensal flora and their metabolites. Whereas in healthy conditions the gut microbes live in mutualistic relationship with the host (homeostasis), in IBD this balance is altered (dysbiosis) and the host responds to the commensal bacteria with an aberrant immune response. In recent years, prebiotic oligosaccharide fibres have been studied for their role in maintaining gut health, supporting the growth of health-promoting bacteria and positively modulating gut immunity[7]. There is an increasing interest in using prebiotics as a preventive or supportive therapy in IBD, as this is a condition for which there is currently no cure but only maintenance treatments to mitigate the symptoms.

Non-digestible oligosaccharides (NDO)

Non-digestible oligosaccharides (NDO) are fermentable dietary fibres with a role as prebiotics. According to the International Scientific Association for Probiotics and Prebiotics consensus statement[8], prebiotics are "substrates that are selectively utilised by host microorganisms

conferring a health benefit". Fructooligosaccharides (FOS), inulin and galactooligosaccharides (GOS) are the most researched prebiotics. Additionally, lactulose-derived oligosaccharides, human milk oligosaccharides (HMO), arabinooligosaccharides (AOS), mannanoligosaccharides (MOS), xylooligosaccharides (XOS), pectic oligosaccharides (POS) and glucose-derived oligosaccharides are emerging prebiotics, as the level of evidence of their health benefits is lower than for FOS, inulin and GOS[8-10]. NDO are carbohydrates made up by 3 to 10 monosaccharide units, usually glucose, galactose, fructose, and xylose. The number of monomeric units constituting NDO is also referred to as the degree of polymerisation (DP). The carbons of the monosaccharide units are linked by covalent β-glycosidic bonds rather than by α-glycosidic bonds found in digestible oligosaccharides. This β configuration of the bonds makes NDO indigestible by human salivary and digestive enzymes[11]. FOS and inulin are made by fructosyl monomers linked by β-(2,1) bonds attached to a terminal glucosyl residue by an α-(1,2) bond. They differ in terms of the length of sugar chains, which are shorter for FOS (2-8 units) than for inulin (up to 60 units)[12]. GOS are made up by galactopyranosyl units linked by β-(1,4) or β-(1,6) bonds and terminating with a glucosyl residue linked by β-(1,4) bonds (Figure 1)[13]. Dietary sources of NDO are plants and include chicory, garlic, Jerusalem artichoke, leeks, onions, bananas, barley and wheat[14]. FOS, inulin and GOS are also produced industrially either by trans-glycosylation of monosaccharides or by enzymatic hydrolysis of polysaccharides[11].

Prebiotic mechanism of action

Due to their chemical structure, prebiotic NDO resist low gastric pH and hydrolytic enzymes and reach the colon virtually intact, where they are degraded by specific gut bacteria collectively referred to as the 'gut microbiota'[15].

Lactobacilli and bifidobacteria break down NDO via saccharolytic reactions and use them as energy sources to support their growth. In doing so, short chain fatty acids (SCFA) are generated as volatile end-products of the fermentation process. These SCFA, which include acetate, propionate and butyrate[16], exert beneficial health effects for the host such as inhibition of pathogens, maintenance of gut barrier integrity, regulation of glucose and lipid metabolism and modulation of immunity[17-21].

Traditionally, immunomodulatory effects of prebiotics were thought to result exclusively from the actions of SCFA and other metabolites (e.g. bactericidal molecules) produced by the microbiota. For example, SCFA are known to modulate cytokine production and immune cell functions (dendritic cells, T cells) as well as to inhibit several pro-inflammatory pathways, as extensively

reviewed elsewhere[7, 22-24], There is growing interest in understanding whether NDO can also modulate immunity in a non-prebiotic manner, especially in those individuals with increased gut permeability, by directly interacting with systemic and gut immune cells. Addressing the aim of this review, microbiota-independent effects of NDO on immunity will be discussed in more detail.

Microbiota-independent effects of NDO on immunity

Evidence for intestinal transportation of NDO

Prebiotics may pass through the gut barrier and enter in direct contact with gut and systemic immune cells when the gut is immature, such as in infants[25, 26], or in other situations characterised by increased gut permeability, such as IBD[27], obesity[28, 29], type 1 diabetes[30] and non-alcoholic fatty liver disease[31]. It is conceivable that NDO may be transported across the intestinal barrier also in individuals exposed to lifestyle-associated stressors that have been linked to alterations in gut permeability, such as high fat Western diet, alcohol consumption and use of medications[32].

Studies *in vitro* using cell lines demonstrated that neutral and acidic HMO are transported across the Caco-2 monolayer via transcellular and/or paracellular pathways[33] and that short chain GOS/long chain FOS (scGOS/lcFOS) are transferred with rates of 4-14% depending on their molecular size and structure[34]. In human supplementation studies, HMO, FOS and GOS with a DP between 3 and 9 were found in plasma, urine and stool of infants fed with supplemented human milk or formula containing FOS or GOS[35, 36], confirming transport of intact oligosaccharides across the intestinal epithelium, as summarised in Table 1 and Table 2. Although it is plausible that prebiotics are transported across the intestinal epithelium not only in infants but also in adults with increased gut permeability, there is an important gap in the research field that needs addressing to support this hypothesis.

Evidence for direct effects of NDO on immunity

A systematic literature search was conducted in Ovid MEDLINE(R) from 1946 through December 2019 and EMBASE from 1947 through December 2019. The search terms used included "Prebiotic* or Fibre* or Fiber* or Oligo?saccharide* or Oligo?saccharide* fraction* or Human?milk oligosaccharide* or HMO* or Human milk-derived oligo?saccharide* or non?digestible oligosaccharide* or NDO" and "Monocyte* or Lymphocyte* or Dendritic cell* or

monocyte* derived dendritic cell* or Intestinal epithel*" and "Gastro?intestinal epithelial transfer or Gastro?intestinal adj2 epithelial adj2 transfer or epithelial transport* or in?vitro transfer or CaCo-2 cell monolayer* or intestinal epithelial cell* or IEC*". After deduplication, relevant papers were selected and bibliographies of the retrieved articles were searched to identify additional articles of interest.

Twelve *in vitro* studies involving human or animal cell lines and/or primary cultures and one *in vivo* study using germ-free mice were reviewed to assess whether NDO could directly affect immunity (Table 3 – Table 5). Three out of thirteen studies focused on HMO[37-39], whereas the other studies focused on other NDO including FOS, inulin, GOS and/or a combination of these[40-46]. Of those studies that looked at effects of NDO on human cell lines, HMO acidic fractions (12.5-125 µg/mL) showed anti-inflammatory effects by dose-dependently inhibiting leukocyte rolling and adhesion, which are two inflammatory processes involved in tissue damage, to human umbilical vein endothelial cells (HUVEC)[37]. Treatment with HMO and GOS (both 5 g/L) attenuated TNF-α-, IL-1β- and pathogen-induced inflammatory cytokines (IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-3 alpha, MIP-3α) in H4 cells, a cell model for human immature intestine[39]. When the same experiment was conducted on T84 and NCM-460 cell lines to mimic mature intestinal cells, TNF-α-induced MIP-3α was reduced by HMO and GOS treatment. However, TNF-α-induced IL-8 was inhibited in NCM-460, but not T84, cells only by HMO[39]. Inflammatory nuclear factor kappa B (NF-κB) signalling was attenuated in H4 and NCM-460 cell lines[39]. A reduction in the expression of pro-inflammatory IL-8, as well as IL-12 and TNF-α, was seen after treatment of human epithelial colorectal adenocarcinoma cells (Caco-2 cells) with α3-sialyllactose HMO (50 mg/L) and FOS (50 g/L), whose anti-inflammatory effects are likely linked to the induction of PPARγ[40]. When the same cell line was incubated with lower concentrations (5 g/L) of FOS, inulin, GOS and goat milk oligosaccharides (GMO), reduced production of MCP-1, but not IL-8, was observed. However, in the same study, stimulation with FOS, inulin and GMO but not GOS resulted in a higher IL-8 production by human colon cancer cell lines (HT-29), in contrast to the studies on H4, NCM-460 and Caco-2 cells[43]. Overall, *in vitro* work conducted on cell lines consistently supports the hypothesis that NDO are able to modulate cytokine production (e.g. IL-8, MCP-1) in a microbiota-independent manner. However, their anti-inflammatory effects appear to be cell-line specific and the various NDO structures, doses and stimulation times used in the studies may contribute to the different outcomes observed.

Among the *in vitro* studies that used animal cell cultures, when GOS and GOS/FOS were tested at higher concentrations (5 - 20 g/L) on LPS-challenged equine peripheral blood mononuclear cells (PBMC), an increase in TNF-α was found. However, a mixture of GOS/FOS/AOS showed a

biphasic effect when used at different doses, with a reduction in TNF-α and IL-10 at higher concentrations and an increase in the same mediators at lower concentrations[45]. Stimulation of rat splenic macrophages and T cells with FOS, inulin and GOS (all 5 g/L) increased the levels of TNF-α, IL-6, and IL-10 and reduced the levels of LPS-induced IFN-γ and IL-17, with effects that may be related to the modulation of TLR4[42]. Research conducted on rat small intestinal cell lines (IEC18 cells) confirmed the hypothesis that FOS, GOS and goat milk oligosaccharides may act as TLR4 ligands. Indeed, there was a reduction in cytokine secretion (growth-regulated oncogene alpha (GRO-α), MCP-1 and macrophage inflammatory protein 2 (MIP-2)) seen after treatment with FOS, GOS and goat milk oligosaccharides (5 g/L) when the TLR4 gene was knocked down in IEC18 cells[43]. Interaction between NDO and TLR4 was demonstrated not only in rat intestinal cells but also in mouse-derived bone marrow DC, whose maturation and cytokine secretion (TNF-α, IL-6, IL-10 and IL-12) was enhanced by treatment with feruloylated oligosaccharides from rice bran tested at different concentrations (6.25-100 μg/mL)[46].

One *in vivo* study on germ-free mice receiving short chain or long chain β2→1 fructans for 5 days showed that the immunomodulatory effects seen were partially mediated by the microbiota and partially microbiota-independent. Both short chain and long chain fructans increased the numbers of T helper cells in the Peyer's patches. Short chain fructans increased regulatory T cells and $CD11b^-$ $CD103^-$ dendritic cells in the mesenteric lymph node and reduced their CD80 expression in the Peyer's patches, whereas LC fructans modulated the B cell responses. These effects were independent from the gut microbiota and/or their SCFA production[47].

Research performed on human primary cells revealed that treatment of cord blood mononuclear cells with acidic HMO (1 μg/mL) lead to an increase in IFN-γ- and IL-13-producing T helper and/or cytotoxic T cells and stimulated T helper cell maturation, as shown by higher expression of the activation marker CD25[38]. Culture with FOS and inulin (5 g/L) increased IL-10 and TNF-α, but not IL-1β and IL-8, production by adult peripheral blood monocytes compared to control, and an upregulation in the response to lipopolysaccharide (LPS) was seen when those cells were co-stimulated with NDO and LPS[42]. Higher levels of IL-10 were found after stimulation of human immature monocyte-derived DC (MoDC) from peripheral blood with 5 g/L of scGOS/lcFOS, through a mechanism that involved NDO binding to toll-like receptor 4 (TLR4)[44].

Overall, there is evidence to conclude that NDO can exert direct, microbiota-independent effects on immune cells. HMO, FOS, inulin and GOS were consistently shown to directly modulate cytokine production (IL-6, IL-8, IL-10, IL-12, MCP-1, MIP-3α and TNF-α) and immune cell maturation (lymphocytes, DC) in *in vitro* models, with mechanisms that seem to involve TLR ligation. One *in vivo* study on germ-free mice reinforced the *in vitro* evidence for microbiota-independent effects of

NDO on immunity. However, not all NDO appear to have the same effect (anti-inflammatory *vs* pro-inflammatory) and within a class of NDO inconsistent effects have been reported. Whereas HMO displayed clear anti-inflammatory properties *in vitro*, which might at least partially explain their protective effects against allergy and infection *in vivo*, FOS, inulin and GOS showed various outcomes on immunity, including anti-inflammatory as well as pro-inflammatory effects. The use of different doses and types of prebiotics as well as various cell culture models may explain the differences in cytokine production observed *in vitro*. Additionally, the chain length of oligosaccharides appears to have an important role in inducing either anti-inflammatory or pro-inflammatory responses. Vogt *et al.*[41] demonstrated that short chain FOS (2-5 units) elicited the production of anti-inflammatory cytokines to a greater extent than long chain FOS (>8 units). Additionally, only half of the studies assessed the LPS content of oligosaccharide fractions used in culture[34, 37, 38, 44-46]. Because the binding of LPS to TLR4 on monocytes, macrophages and B cells leads to strong, pro-inflammatory responses including the secretion of TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-15 and transforming growth factor (TGF-β)[48], it is important to determine whether the direct effects of oligosaccharides on cytokines are due to the action of the oligosaccharide fractions or to the presence of LPS within prepared fractions. Although dietary oligosaccharides bind and signal via TLR4 in monocytes, macrophages and intestinal epithelial cells[41, 43, 44, 46] often eliciting a pro-inflammatory cytokine production *in vitro*, they are not necessarily pro-inflammatory *in vivo*. This is most likely due to differences between *in vitro* and *in vivo* conditions. Therefore, more research needs to be carried out to understand the mechanisms through which oligosaccharides affect immunity.

Effects of prebiotics on IBD: animal models and human clinical trials

IBD patients have lower numbers of bifidobacteria and lactobacilli in the gut, lower concentrations of faecal SCFA and high mucosal inflammation primarily induced by pro-inflammatory cytokines[49]. Prebiotics are promising in the management of IBD for their role in restoring gut microbiota homeostasis and affecting cytokine production and immune cell maturation. In recent years, animal and human studies on the role of prebiotics in IBD have been extensively reviewed[50-53].

Murine experimental models of colitis associated either with epithelial barrier disruption or with immune cell defects have been developed to mimic IBD in humans [54]. Rodent experimental colitis models showed that both short term (<1 week) and long term (>1 month) treatment with prebiotics including lactulose, inulin and goat milk oligosaccharides reduced colonic damage and

inflammation, whereas no convincing reduction of inflammation was seen in animal studies that used GOS or FOS[52]. When GOS and FOS were used in association with other soluble and insoluble polysaccharides, a decrease in inflammatory cytokines as well as an increase in IL-10 and regulatory T cells in the mesenteric lymph nodes were observed[55]. Together, these results indicate that various prebiotics may have different potential in attenuating inflammation, and that more studies using the experimental colitis model are needed[52].

Human clinical trials on prebiotic use in IBD are available for FOS and inulin (Table 5), but not for GOS and other NDO. In a double-blind placebo controlled trial on individuals with chronic pouchitis (an inflammation in the lining of an artificial rectum created after UC surgery), treatment with 24 g/d inulin for 3 weeks resulted in lower endoscopic and histological inflammation, higher faecal butyrate concentration and a tendency for lower concentrations of secondary bile acids in faeces[56]. Similarly, patients with active UC receiving 12 g/d oligofructose-enriched inulin for 2 weeks displayed a reduction in disease activity and in faecal calprotectin (a marker of intestinal inflammation) but no changes in circulating inflammatory mediators (e.g. IL-8), compared to control[57]. In another double-blind randomised control trial, supplementation with 10 g/d inulin for 4 weeks increased the numbers of bifidobacteria, lead to higher concentrations of faecal acetaldehyde and butyrate and decreased disease activity in CD patients, although there was a higher dropout rate for those undergoing supplementation compared to placebo[58]. In an open-label trial, treatment with 15 g/d FOS for 3 weeks reduced disease activity in individuals with active ileo-colonic CD and increased the numbers of bifidobacteria as well as the numbers of DC from rectal biopsies expressing IL-10, TLR2 and TLR4[59]. These results were only partially confirmed by a larger double-blind randomised control trial, where patients with active CD supplemented 15 g/d FOS for 4 weeks showed higher numbers of IL-10^{+} DC from rectal biopsies but no differences in disease activity, numbers of bifidobacteria and levels of faecal calprotectin[60]. Several studies conducted on other non-prebiotic dietary fibres, such as ispaghula husk[61] and germinated barley foodstuff[62-64], showed that also these two polysaccharides may have a potential in attenuating UC and/or CD clinical symptoms. Overall, while inulin appears promising in reducing IBD symptoms and inflammation, there are currently few studies of FOS and no studies of GOS in IBD. More research using standardised methods needs to be conducted to explore the potential preventive and/or therapeutic use of prebiotics in the management of IBD.

Conclusions

Prebiotics have been extensively studied for their beneficial role in maintaining gut health and in supporting the growth of health-promoting bacteria. Additionally, prebiotics may positively modulate gut and systemic immunity via microbiota-dependent and microbiota-independent mechanisms, as reviewed in this paper. Whereas immunomodulatory effects of prebiotics via the production of SCFA have been extensively seen in the literature, fewer studies are available on direct prebiotic-immune cell interactions. *In vitro*, FOS, inulin and GOS were shown to exert microbiota-independent effects on immunity, including the binding to TLR on monocytes, macrophages and intestinal epithelial cells and consequent modulation of cytokines and immune cell maturation. In infants, there is good evidence to conclude that prebiotics can pass through the intestinal epithelium and directly modulate gut and systemic immune cells. Although it is logical to speculate that the same might happen in other conditions characterised by increased gut permeability, such as IBD, this hypothesis needs to be confirmed by further research. There is convincing preliminary data to suggest that inulin and lactulose can reduce IBD symptoms and inflammation in animal models and/or in human supplementation studies. However, the mechanisms of action of prebiotics in IBD are not fully understood, and there is a particular need for further research on a wider range of prebiotics, including FOS and GOS. The overall beneficial effects of prebiotics may be as a result of transfer across the permeable gut of IBD patients resulting in direct interaction with gut and systemic immune cells, or prebiotic actions may derive from microbiota-dependent mechanisms, or perhaps they might act through a combination of both these effects. An important first step will be to establish whether prebiotic oligosaccharides do in fact bypass the intestinal epithelium in IBD patients as has been established in infants and *in vitro* experiments.

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Conflict of Interest

None.

Authorship

S.D.F. performed a review of the literature and drafted the manuscript. P.C.C. and C.E.C. critically revised and approved the final version of the manuscript.

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Table 1. *In vitro* studies providing evidence for intestinal transportation of prebiotics.

Reference	Treatment	*In vitro* model	Study design	Findings
(33)	Neutral and acidic HMO fractions (5 mg/mL)	Caco-2 cells	Caco-2 cells grown on filter inserts in minimal essential medium. 200 µL of transport buffer with neutral and acidic HMO fractions applied. HPLC-MS analysis of HMO in basolateral compartment	Neutral HMO use transcellular and paracellular pathways to cross Caco-2 monolayer; acidic components use only paracellular pathways
(34)	scGOS/lcFOS	Caco-2 cells	Transfer of scGOS/lcFOS via Caco-2 monolayer measured by HPAEC-PAD. Sample preparation as in Ref. (33)	Transfer of scGOS/lcFOS detected with rate of transfer of 4-14%, depending on molecular size and structure

Abbreviations: HMO, human milk oligosaccharides; Caco-2 cells, human epithelial colorectal adenocarcinoma cells; HPLC-MS, high performance liquid chromatography mass spectrometry; scGOS/lcFOS, short chain galactooligosaccharides/long chain fructooligosaccharides; HPAEC-PAD, high-pH anion exchange chromatography with pulsed amperometric detection.

Table 2. Human studies supporting evidence for intestinal transportation of prebiotics.

Reference	Treatment	Population	Study design	Findings
(35)	Infant formula with FOS (3 g/L)	Term infants (*n* = 84) aged 1 to 8 (± 3) days	Controlled, randomised and blinded clinical study to determine safety of use of FOS and ability to detect oligosaccharides in urine and plasma of infants randomised to receive FOS-enriched formula, control formula or breast-feeding for 16 weeks. Anthropometric measures, urine, stool and plasma samples taken	No adverse effects with FOS supplementation. Prebiotic effect of FOS on lactobacilli. FOS with DP = 4 in plasma and urine of infants fed with FOS-enriched formula
(36)	HMO; fortified human milk; infant formula with FOS; infant formula with GOS or *B. animalis*	Mother-preterm infant dyads (*n* = 4)	Clinical study where preterm infants received human milk with Similac® Human Milk Fortifier or unsupplemented human milk followed by human milk with fortifier Prolact+4® or formula milk Similac® Special Care® 24 High Protein either with GOS or with *B. animalis*. Samples of milk, urine and stool collected for analysis by nanoflow LC-TOFMS	HMO and oligosaccharides with 3 < DP < 9 identified and quantified in urine and stool of infants

Abbreviations: FOS, fructooligosaccharides; DP, degree of polymerisation; HMO, human milk oligosaccharides; GOS, galactooligosaccharides; B. animalis, Bifidobacterium animalis; LC-TOFMS, liquid chromatography time-of-flight mass spectrometry.

1 Table 3. *In vitro* studies with human cell cultures showing direct effects of oligosaccharides on immunity.

Reference	Treatment	Human cells cultured	Study design	LPS testing	Findings
(37)	HMO fractions (12.5 - 125 μg/mL)	Monocytes, lymphocytes and neutrophils from PB + HUVEC	Monocytes, lymphocytes and neutrophils passed over TNF-α-activated HUVEC. Effects of HMO determined by video-microscopy	Yes (QC LAL assay)	Acidic fraction dose-dependently inhibited leukocyte rolling and adhesion to endothelial cells (125 μg/mL, $P < 0.001$; 87.5 μg/mL, $P < 0.001$; 50 μg/mL, $P < 0.001$; 25 μg/mL, $P < 0.001$)
(38)	Neutral HMO (10 μg/mL) and acidic HMO (1 μg/mL)	Term new born CBMC	CBMC cultured with HMO. Intracellular cytokines (IL-4, IL-6, IL-10, IL-13 and IFN-γ) and surface markers of T cells and maturation (CD3, CD4,CD8 and CD25) analysed by flow cytometry	Yes (LAL assay)	Acidic HMO ↑ % IFN-γ-producing T helper and cytotoxic T cells ($P < 0.05$), as well as IL-13 by cytotoxic T cells ($P < 0.05$). Acidic HMO ↑ CD25 expression on T helper cells ($P < 0.05$)
(40)	a3-sialyllactose (10, 50, or 100 mg/L) or FOS Raftilose p95 (0.05, 0.5, 1, 10, or 50	Caco-2 cells	Caco-2 cells cultured with a3-sialyllactose or Raftilose p95 or combination. Effects of treatments tested on expression of PGlyRP3, PPARγ and pro-inflammatory cytokines	None reported	a3-sialyllactose and Raftilose p95 ↓ IL-12, IL-8 and TNF-α ($P < 0.05$). Both induced ↑ PGlyRP3 expression ($P < 0.05$ for 50 mg/L and 50 g/L), linked to the induction of PPARγ

	g/L) or a combination (50 mg/L a3-sialyllactose + 50 g/L Raftilose p95)				
(41)	Fructans (1 µg/mL or 100 µg/mL) at different DP	Adult PBMC (*n*= 6) and TLR-engineered cell lines (*n*= 11)	PBMC stimulated with fructans. Effects on cytokines (IL-1Ra, IL-1β, IL-6, IL-10, IL-12p70, and TNF-α) evaluated. TLR-engineered cell lines stimulated with fructans. Cell activation measured	Yes (quantitative LAL assay)	Cytokine production dependent on dose and chain length of fructans. SC fructans (DP up to 10) induced regulatory cytokine balance *vs* to LC fructans (DP up to 60), as seen by IL-10/IL-12 ratio ($P < 0.05$ for PBMC stimulated with 1 µg/mL or 100 µg/mL fructans). Activation of TLR-engineered cell lines showed signalling was TLR-dependent
(42)	FOS, inulin, GOS and GMO (5 g/L)	Adult PB monocytes (*n*= 10)	PB monocytes pre-stimulated with LPS (1 µg/mL) or unstimulated, then cultured with NDO. Supernatants collected for cytokine analysis (IL-1β, IL-8, IL-10 and TNF-α) by ELISA	None reported	Treatment with FOS and inulin ↑ IL-10 and TNF-α ($P < 0.05$) but not IL-1β and IL-8. NDO ↑ LPS response when the cells where co-treated

(43)	FOS, inulin, GOS and GMO (5 g/L)	HT29 and Caco-2 cells	LPS (1-5 μg/mL) used for reference or as co-treatment. Cytokine secretion (IL-8 and MCP-1) measured by ELISA	None reported	FOS, inulin, and GMO, but not GOS, ↑ IL-8 production by HT29 cells ($P < 0.05$). No changes in IL-8 after treatment of Caco-2 lines. MCP-1 ↓after treatment of Caco-2 cells with NDO ($P < 0.05$)
(44)	scGOS/lcFOS (5 g/L)	Immature MoDC and T cells from PB	Immature MoDC stimulated with scGOS/lcFOS in presence or absence of LAB. IL-1α, IL-1β, IL-6, TNF-α, MIP-3α, IL-10 and IL-12p70 measured by ELISA and Luminex assay. MoDC co-cultured with naïve T cells, and Foxp3 expression evaluated by flow cytometry. Experiments with TLR4 antagonist included	Yes (quantitative LAL assay)	scGOS/lcFOS promoted IL-10 release by MoDC ($P < 0.01$). Blocking TLR4 abrogated IL-10 increase, suggesting that NDO act via TLR4. scGOS/lcFOS showed a tendency to ↑ regulatory T cells ($Foxp3^+$)
(39)	HMO (5 g/L) and GOS (5 g/L)	Immature H4 and mature T84, NCM-460	H4, T84 and NCM-460 cell lines treated with TNF-α or IL-1β or infected with *Salmonella*	None reported	HMO and GOS ↓ TNF-α-, IL-1β- and pathogen-induced IL-8, MCP-1 and MIP-3α in H4 cells ($P < 0.001$). In T84 and NCM-

	enterocyte cell lines	or *Listeria* and/or with HMO or GOS. Induction of IL-8, MCP-1 and MIP-3α by ELISA and mRNA by qPCR	460 cells, HMO and GOS ↓ TNF-α-induced MIP-3α ($P < 0.0005$). TNF-α–mediated IL-8 induction was ↓ by HMO in NCM-460 ($P < 0.0005$) but not in T84 cells. Galactosyllactosein HMO and GOS ↓ inflammatory NF-κB signalling in H4 and NCM-460 cell lines ($P < 0.0005$)

Abbreviations: LPS, lipopolysaccharide; HMO, human milk oligosaccharides; PB, peripheral blood; HUVEC, human umbilical vein endothelial cells; QC, quantitative chromogenic; TNF, tumour necrosis factor; LAL, Limulus Amoebocyte Lysate; CBMC, cord blood mononuclear cells; IL, interleukin; IFN, interferon; Caco-2 cells, human epithelial colorectal adenocarcinoma cells; PGlyRP3, peptidoglycan recognition protein 3; PPARγ, peroxisome proliferator-activated receptor γ; DP, degree of polymerisation; PBMC, peripheral blood mononuclear cells; TLR, toll-like receptor; SC, short chain; LC, long chain; FOS, fructooligosaccharides; GOS, galactooligosaccharides; GMO, goat milk oligosaccharides; ELISA, enzyme-linked immunosorbent assay; HT29 cells, human colorectal adenocarcinoma cells; MCP-1, monocyte chemoattractant protein-1; scGOS/lcFOS, short chain galactooligosaccharides/long chain fructooligosaccharides; MoDC, monocyte-derived dendritic cells; LAB, Lactic Acid Bacteria; MIP-3α, macrophage inflammatory protein-3 alpha; H4 cells, human normal foetal intestinal epithelial cells; T84 cells, human metastatic colonic epithelial cells; NCM-460 cells, human normal colon mucosal epithelial cells; mRNA, messenger ribonucleic acid; qPCR, quantitative polymerase chain reaction.

Table 4. *In vitro* studies with animal cell cultures showing direct effects of oligosaccharides on immunity.

Reference	Treatment	Cell culture	Study design	LPS testing	Findings
(45)	GOS, GOS+FOS and GOS+FOS+AOS (5 - 20 g/L)	Equine PBMC (*n*= 22)	Equine PBMC pre-incubated with oligosaccharides, then incubated with medium with 1 μg/mL LPS + oligosaccharides. IL-10 and TNF-α measured by ELISA	Yes (quantitative LAL assay)	Exposing PBMC to GOS or GOS+FOS caused a dose-dependent ↑ of TNF-α in LPS-challenged PBMC ($P < 0.05$ for 20 g/L dose of GOS or GOS+FOS vs LPS). Incubation with GOS/FOS/AOS dose-dependently ↓ TNF-α and IL-10 following LPS challenge ($P < 0.05$ for 20 g/L dose of GOS+FOS+AOS vs LPS). Mono- and disaccharide control fractions stimulated inflammatory response in LPS-challenged PBMC, though to a lesser extent than NDO
(42)	FOS, inulin, GOS and GMO (5 g/L)	Rat splenic T cells (*n*= 28) and WT/TLR4 KO mouse splenocytes (*n*= 10)	Rat splenic T cells and WT/TLR4 KO mouse splenocytes pre-stimulated with LPS 1 μg/mL, then cultured with oligosaccharides. Cell signalling inhibitors added prior to treatment with NDO. Supernatants	None reported	Prebiotics ↑ TNF-α, IL-6, and IL-10 secretion by mouse splenocytes ($P < 0.05$) but ↓LPS-induced IFN-γ and IL-17 ($P < 0.05$). Inulin ↑LPS-induced IL-10 ($P < 0.05$). TLR4 KO splenocytes had a depressed response.

			collected for cytokine analysis (rat cytokines: IL-1β, IL-2, IL-6, IL10, GRO-α, IFN-γ, TNF-α and mouse cytokines: IL-6, IL-10, IL-17, IFN-γ, and TNF-α) by ELISA		Prebiotics are TLR4 ligands/modulators in monocytes
(43)	FOS, inulin, GOS and GMO (5 g/L)	Rat IEC18 cells	LPS used for reference or as co-treatment with prebiotics. Cytokine secretion (IL-6, IL-8, MIP-2, MCP-1, GRO-α) measured by ELISA	None reported	IEC18 cells secreted GRO-α, MCP-1 and MIP-2 ($P < 0.05$) following treatment with prebiotics, with an efficacy similar to LPS-stimulated cells. Response was ↓ by TLR4 gene knockdown. Prebiotics are TLR4 ligands in IEC
(46)	Feruloylated oligosaccharides from rice bran (6.25-100 μg/mL)	Bone marrow DC from C3H/HeN or C57BL/6 mouse with normal or mutated TLR4 and TLR2	DC cultured with LPS or feruloylated oligosaccharides. Cytokines and chemokines (IL-6, IL-10, IL-12, MCP-1, TNF-α and RANTES) analysed by ELISA. Surface markers analysed by flow cytometry. Activity assaysfor NF-κB	Yes (QC LAL assay)	Feruloylated oligosaccharides induced maturation of DC, as shown by ↑ CD40, CD80/CD86 expression ($P < 0.01$ and $P < 0.001$, respectively) as well as ↑ TNF-α, IL-6, IL-10 and IL-12 (at the highest dose tested, $P < 0.001$ for all cytokines) via TLR4 and/or TLR2. This may be related to ↑ NF-κB activity

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Abbreviations: LPS, lipopolysaccharide; GOS, galactooligosaccharides; FOS, fructooligosaccharides; AOS, acidic oligosaccharides; PBMC, peripheral blood mononuclear cells;; IL, interleukin; TNF, tumour necrosis factor; ELISA, enzyme-linked immunosorbent assay; LAL, Limulus Amoebocyte Lysate; GMO, goat milk oligosaccharides; WT/TLR4 KO mice, wild type/toll like receptor 4 knockout mice; NDO, non-digestible oligosaccharides; GRO-α, growth-related oncogene-alpha; IFN, interferon; TLR, toll-like receptor; IEC18 cells, nontransformed rat small intestinal epithelial cells; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; IEC, intestinal epithelial cells; DC, dendritic cells; RANTES, regulated on activation, normal T cell expressed and secreted; NF-κB, nuclear factor κB; QC, quantitative chromogenic.

Table 5. *In vivo* study on germ-free mice showing direct effects of oligosaccharides on immunity.

Reference	Treatment	Study design	Duration	Findings
(47)	SC or LC β2→1-fructans	Conventional C57BL/6OlaHsd male mice or C57BL/6OlaHsd male GF mice (both 8 weeks old) received SC or LC β2→1-fructans. Immune cells in spleen, MLN and PP analysed by flow cytometry. Gene expression in ileum measured with microarray. Gut microbiota composition analysed with 16S rRNA sequencing of faecal samples	5 days	β2→1-fructans modulated immunity by microbiota and microbiota-independent effects. Effects dependent on chain length of fructans. Both SC and LC fructans ↑ Th1 cells in PP (both $P < 0.05$). SC fructans ↑ regulatory T cells and CD11b− CD103− DCs in MLN ($P < 0.01$). ↑ 2-α-l-fucosyltransferase 2 expression in ileum of conventional mice. Effects not associated with shifts in gut microbiota or SCFA. SC fructan induced ↓ CD80 expression by CD11b−

					CD103− DC in PP ($P < 0.05$), LC fructan modulated B cell responses in GF mice

Abbreviations: SC, short chain; LC, long chain; GF, germ free; MLN, mesenteric lymph nodes; PP, Peyer's patches; rRNA, ribosomial RNA; Th1, T helper 1; DC, dendritic cells; SCFA, short chain fatty acids;

Table 6. Human studies of prebiotic use for the management of IBD.

Reference	Treatment	Study design	Duration	Condition	Findings
(56)	Inulin 24 g/d	Double-blind placebo controlled trial (n= 20)	3 weeks	Chronic pouchitis	↓ endoscopic and histological inflammation, ↑ in faecal butyrate ($P < 0.01$), ↓ in faecal pH ($P = 0.02$), tendency in ↓ secondary bile acids in faeces
(59)	FOS 15 g/d	Open-label trial (n= 10)	3 weeks	Active ileocolonic CD	↓ disease activity ($P <$ 0.01), ↑ in faecal bifidobacteria ($P <$

					0.001),↑numbers of dendritic cells expressing IL-10, TLR2 ($P < 0.08$) and TLR4 ($P < 0.001$) from rectal biopsies
(60)	FOS 15 g/d	Double-blind randomised control trial (n= 103)	4 weeks	Active CD	No difference in disease activity, ↑ flatulence ($P = 0.004$) and abdominal pain ($P = 0.048$) than placebo. No difference in bifidobacteria, serum C-reactive protein or faecal calprotectin. ↑ in IL-10^{+} DC from rectal biopsies ($P < 0.05$)
(58)	Oligofructose-inulin (1:1) 10 g/d	Double-blind randomised control trial (n= 67)	4 weeks	Inactive or moderately active CD	↓ disease activity ($P <$ 0.048), but ↑ dropout rate than placebo. ↑ in bifidobacteria ($P =$ 0.03) and faecal acetaldehyde ($P =$

					0.0008) and butyrate (P = 0.0011) concentrations
(57)	Oligofructose-enriched inulin 12 g/d	Double-blind randomised controlled trial (n= 19)	2 weeks	Active UC	↓ disease activity (P < 0.05), ↓ in faecal calprotectin (P < 0.05) after just 1 week, no changes in inflammatory mediator release (IL-8, PGE-2)

Abbreviations: FOS, fructooligosaccharides; CD, Crohn's disease; IL, interleukin; TLR, toll-like receptor; DC, dendritic cells; UC, ulcerative colitis; PGE-2, prostaglandin-E2;