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Specific inulin-type fructan fibers protect against autoimmune diabetes by modulating gut immunity, barrier function and microbiota homeostasis

Kang Chen¹, Hao Chen¹, Marijke M. Faas², Bart J. de Haan², Jiahong Li¹, Ping Xiao³, Hao Zhang¹, Julien Diana⁴,⁵, Paul de Vos²,**, Jia Sun¹,⁶,**

¹State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, P. R. China

²Immunoenocrinology, Division of Medical Biology, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

³Institute of Clinical Medical Research, Affiliated Foshan Hospital of Sun Yat-sen University, Foshan 528000, P.R. China

⁴Université Paris Descartes, Sorbonne Paris Cité, Paris, France

⁵Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 1151, Institut Necker-Enfants Malades (INEM), Centre National de la Recherche ITF(1)enctifique, Unité 8253, Paris, France

⁶School of Medicine, Jiangnan University, Wuxi 214122, P.R. China

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Abstract

**Scope:** Dietary fibers capable of modifying gut barrier and microbiota homeostasis, affect the progression of type 1 diabetes (T1D). Here we aim to compare modulatory effects of inulin-type fructans (ITFs), natural soluble dietary fibers with different degrees of fermentability from chicory root, on T1D development in non-obese diabetic (NOD) mice.

**Methods and results:** Female NOD mice were weaned to long and short chain ITFs [ITF(l) and ITF(s), 5%] supplemented diet up to 24 weeks. T1D incidence, pancreatic-gut immune responses, gut barrier function and microbiota composition were analyzed. ITF(l) but not ITF(s) supplementation dampened the incidence of T1D. ITF(l) promoted modulatory T cell responses, as evidenced by increased CD25+ Foxp3+ CD4+ Treg cells, decreased IL17A+CD4+ Th17 cells and modulated cytokine production profile in the pancreas, spleen and colon. Furthermore, ITF(l) suppressed NLRP3-caspase-1-p20-IL-1β inflammasome in the colon. Expression of barrier reinforcing tight junction proteins occludin and claudin-2, antimicrobial peptides β-defensin-1 and CRAMP as well as SCFA production were enhanced by ITF(l). Next-generation sequencing analysis revealed that ITF(l) enhanced Firmicutes/Bacteroidetes ratio to an anti-diabetogenic balance and enriched modulatory Ruminococcaceae and Lactobacilli.

**Conclusion:** Our data demonstrate that ITF(l) but not ITF(s) delays the development of T1D via modulation of gut-pancreatic immunity, barrier function and microbiota homeostasis.
Modulatory effects of long and short chain ITFs [ITF(l) and ITF(s), 5%] were compared on type 1 diabetes development in non-obese diabetic (NOD) mice. Both types of ITFs were supplemented in diet to female NOD mice. It is found that ITF(l) but not ITF(s) supplementation suppresses type 1 diabetes by modulating gut-pancreatic immunity, barrier function and microbiota homeostasis.

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Keywords:

Barrier integrity; Dietary fibers; Gastro-entero pancreatic regulation, Gut microbiota homeostasis; Gut microbiota; Immunomodulation, Nutritional intervention, Pancreatic-gut immunity; Type 1 Diabetes; Type 1 diabetes pathophysiology

Abbreviations

CRAMP  Cathelicidin-related antimicrobial peptide
DP     Degree of polymerization
Fn     Fructose chain, n = number of fructose subunits
GFn    fructose chain terminated with a Glucose molecule, n = number of fructose subunits
HPAEC  High performance anion exchange chromatography
ITF    Inulin-type fructans
ITF(l)  Long chain inulin-type fructans
ITF(s)  Short chain inulin-type fructans
MAPK   Mitogen-activated protein kinase
NLRP3  NOD like receptor protein 3
PMA    Phorbol myristate acetate
T1D    type 1 diabetes
TGF-β  Transforming growth factor-β
Introduction

Autoimmune diabetes or type 1 diabetes (T1D) is characterized by autoreactive T cell-mediated selective destruction of pancreatic β-cells. Diet affecting intestinal immunity, barrier function and gut microbiota is a critical modulator in the pathogenesis of β-cell autoimmunity and T1D [1]. Dietary fibers may be fermented by the gut microbiota to produce health-promoting short chain fatty acids (SCFAs) and modify intestinal barrier function and microbiota homeostasis. Therefore, consumption of dietary fibers represents an effective strategy to modulate the development of T1D.

In general, increased dietary fiber intake is associated with reduced incidence of immunological diseases [2] but in NOD mice, it has been shown that some complex dietary fibers enhance autoimmunity against β-cells by conditioning microbiota and by disruption of gut barrier and immune function [3, 4]. We have hypothesized that this might be related to the chemistry of the consumed fibers as we and others have shown that not all but only specific types of dietary fibers are immunomodulating or improving the intestinal barrier function [5, 6]. However, whether this holds consequences for possible prevention of diabetes and effects on gut homeostasis has to the best of our knowledge not been studied yet.
A family of dietary fibers for which clear chemistry-dependent effects are demonstrated are inulin-type fructans (ITFs) [7]. Previous studies have demonstrated that the degree of polymerization (DP, i.e. chain length) of ITFs determines the immunological effects on human immune cells [5, 8] and its impact on gut epithelial cell barrier function [9]. The chemistry-dependent effects were determined by the magnitude by which ITFs can activate toll-like receptor (TLR)2 which was stronger with long chain (high-DP) inulins than with short chain inulins. In addition, ITFs have different effects on the gut microbiota as long-chains are predominantly fermented in the colon while short chains are already fermented in the small intestine [6, 10].

Dietary fibers may influence the diabetogenic process via effects on gut immunity, barrier and microbiota that modulate T cells involved in autoimmunity [11, 12]. T helper (Th)1 cells that secrete interferon-γ (INF-γ) can activate autoreactive CD8⁺ T cells. Additionally, IL-17-producing CD4⁺ T helper (Th17) cells are pathogenic during autoimmune diabetes [13]. Some gut microbiota can support generation of Foxp3⁺ CD4⁺ regulatory T (Treg) cells and correct the imbalance between Treg and Th17 cells and closely related autoreactive T cells [11, 12]. Based on these findings, we hypothesized that dietary fibers that beneficially influence the gut microbiota involved in maintaining T cell balances might be instrumental in slowing down or preventing progression of autoimmune diabetes.

Here we compared the effects of long chain ITF(l) and the more readily fermentable short chain ITF(s) on the frequency of autoimmune diabetes in NOD mice. In the mice we studied insulitis and the frequency of Treg and Th17 cells in the pancreas, spleen and colon as well as their functions by quantifying production of IL-10, IL-1β and IL-17. Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein (NLRP) 3 recently implicated in the pathogenesis of T1D [14] were also examined. Additionally, we studied colon integrity as a possible mechanism by which ITF(l) influence immunity by
determining expression of gut-epithelial tight junctions (TJ) and barrier reinforcing, immunomodulatory antimicrobial peptides [15, 16]. Finally, in ITF(l)-treated animals, we studied changes to the microbiota derived metabolites short chain fatty acids (SCFAs) and modification of gut microbiota composition with relation to T1D [17, 18].

Materials and methods

Fibers and structural characterization The applied short and long chain ITFs: ITF(s) (Frutalose OFP, 2 < DP < 25) and ITF(l) (FrutafitTEX, 10 < DP < 60) extracted from chicory roots were provided by Sensus B.V. (Roosendaal, The Netherlands). Their specific chain length profiles (range and distribution) were characterized by high performance anion exchange chromatography (HPAEC) as previously described [5].

Animals Female NOD/LtJ mice were purchased from Su Pu Si Biotechnology Co., LTD (Suzhou, Jiangsu, China) and maintained in specific pathogen-free environment at the Animal Housing Unit of Jiangnan University (Jiangsu, China) under a controlled temperature (23-25°C) and a 12-h light/12-h dark cycle. All experimental protocols were approved by the Institutional Animal Ethics Committee of Jiangnan University (JN.No 20150331-0410) and carried out in compliance with national and international guidelines for the Care and Use of Laboratory Animals. Three-week-old female NOD mice were fed with AIN-93G control diet [19] or diet supplemented with 5% (wt/wt) ITF(l) or ITF(s) until 24 weeks. Before the blood glucose test, mice were fasted for 6 h and blood glucose levels were determined with an Accu-check glucosemeter (Roche Diagnostics, Almere, The Netherlands). Overt diabetes was confirmed by a glycemia > 13.3 mmol/L in two consecutive days.

ELISA assays Serum was collected by allowing the blood to coagulate at ambient temperate for 25 min, and subsequently centrifuging the samples at 3,000 g for 10 min at 4°C. Tissue samples were homogenized in 0.9% saline by a homogenizer (Scientz-48, Scientz
Biotechnology, Ningbo, Zhejiang, China) and centrifuged at 900 g for 20 min at 4°C. Supernatant collected and serum were assayed for inflammatory mediators using a sandwich ELISA kits for IL-17, IL-1β and IL-10 (DiAo, Biotechnology, Shanghai, China). Absorbance was measured at 450 nm within 30 min, using an automated microplate reader (Multiskan™ GO; Thermo Fisher Scientific Oy, Vantaa, Finland). Data are expressed as pg/ml.

**Western blot analysis** Tissue lysates were prepared by homogenization in chilled radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail (BioVision Inc., Milpitas, CA, USA) and phosphatase inhibitor cocktails (Sigma Aldrich, Shanghai, China), followed by centrifugation at 9600 g for 10 min at 4°C. Tissue lysates separated on 10 % SDS polyacrylamide gel and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. Nonspecific binding was blocked by 1 h incubation of the membranes, at room temperature, in 5% Non-fat dry Milk in Tris Buffered Saline with Tween 20 (NMTBST; 0.1 % Tween 20 in Tris Buffered Saline). The blots were then incubated overnight at 4 °C with the primary antibodies (at a 1:1000 dilutions in NMTBST). Antibodies against caspase-1-p20, occludin, ZO-2, β-actin were purchased from Santa Cruz Biotechnology (CA, USA), antibodies against NLRP3, phosphor-p38, p38, NFkB p65 were obtained from Cell Signaling Technology (Beverly, USA), and the antibody against claudin-2 was purchased from ThermoFisher Scientific (Shanghai, China). The membranes were then washed three times with TBST and finally incubated for 2 h at room temperature with goat anti-rabbit HRP-conjugated secondary antibody at 1:2000 dilutions in NMTBST. The blots were developed for visualization using enhanced chemiluminescence detection kit.

**Histology** Freshly harvested pancreatic and colon tissues were fixed with 4 % polyformaldehyde overnight. The tissues were then washed with ddH₂O, dehydrated with gradient ethanol solutions and embedded in paraffin and cut into 5 μm sections. The sections were subsequently stained with hematoxylin/eosin (H&E) and examined under a DM2000.
light microscope (Leica Microsystems GmbH, Wentzler, Germany) at 200× magnification. Insulitis was quantified by evaluating the degree of infiltration and categorized as follows: 0-no insulitis, 1-peri-insulitis with or without minimal infiltration in islets, 2-insulitis with < 50% infiltration of islets, 3-invasive insulitis with > 50% in filtration of islets [19].

**RNA isolation and qPCR** Total RNA was isolated from the colon samples using Trizol (Invitrogen, Thermo Scientific, Massachusetts, USA). Complementary DNA was prepared by reverse transcription of 2 μg total RNA using a Reverse Transcription reagent kit (RT reagent Kit with gDNA Eraser RR047A, TaKaRa, Dalian, Liaoning, China). SYBR Green PCR reagents (BIO-RAD) were used to determine the mRNA levels. Calculations were made based on the comparative cycle threshold method (2^−ΔΔCt). Relative expression ratios were calculated as normalized ratios to β-actin internal control gene. Detailed primers sequences are shown in Table 1.

Table 1. Primers used for qPCR

<table>
<thead>
<tr>
<th>gene</th>
<th>forward</th>
<th>reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-defensin-1</td>
<td>5’-GCACAAGAAGGTCACACGGA-3’</td>
<td>5’-CTAAGGTTGCAGATGGGGTGT-3’</td>
</tr>
<tr>
<td>CRAMP</td>
<td>5’-CTTCAAGGAACAGGGGTTGG-3’</td>
<td>5’-CTTGAACGAAAGGGCTGTG-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-CCCAGGCATTGCTGACAGG-3’</td>
<td>5’-TGAAGGTGGACAGATGGGC-3’</td>
</tr>
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**Analysis of SCFAs and next-generation sequencing of gut microbiota** Colon and its content were pooled from NOD mice. Concentrations of SCFAs were measured by gas chromatography coupled mass spectrometer (GC-MS). Briefly, colon content samples (50 mg) were first homogenized in 500 μl of saturated NaCl solution. Thereafter, samples were
acidified with 40 μl 10% sulfuric acid. Diethyl ether (1ml) was added to the samples to extract SCFAs. Samples were then centrifuged at 14,000 g for 15 min at 4°C and supernatants were used for analysis. We used parameters of GC-MS as previously described [20]. Next-generation sequencing was performed on an Illumina MiSeq system. For each genomic DNA sample, the V4 region of 16S rRNA gene (250 bp) was amplified by using Kapa HiFi PCR kit (Kapa Biosystems, Wilmington, MA, USA) and Applied Biosystems® 2720 Thermal Cycler (Thermo Fisher Scientific, Massachusetts, USA). The quality of libraries was confirmed by using Bioanalyzer 2100 (Agilent, Santa Clara, MA, USA) and the dataset was analyzed by Trimmomatic V0.30.

Flow cytometry After 24-week treatment, mice were sacrificed and immune cell populations were studied in pancreas, spleen and colon. Antibodies were purchased from BD Pharmingen (California, USA) and detailed information is listed in Table S1. Pancreas, colon and spleen samples were harvested and cut into small pieces then digested in 1.4 mg/mL collagenase-P (Boehringer Mannheim, Germany). Pancreas and colon samples were digested at 37 °C for 30 min. Digested organ tissues were filtered through 75 μm polypropylene mesh with syringe plunger. Then red blood cells in single cell suspensions were lysed by Red Blood Cell Lysis Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Single cell suspensions were stained for 30 min at 4°C in PBS with the following mAbs: anti-CD4 and -CD25. For intracellular cytokine analysis of IL-17A and Foxp3, the cells from pancreas, spleen and colon (1 × 10^6 cells/ml) were stimulated with PMA (30 ng/ml; Sigma Aldrich) and ionomycin (1 mg/ml; Sigma Aldrich). One hour later, monensin (5 mg/ml; Sigma Aldrich) was added for 4 h for blocking transport processes at the Golgi Complex/Endoplasmic Reticulum facilitating accumulation of the intracellular proteins. Isotype-matched controls were included in all experiments. Flow cytometry was performed on an Attune NxT (Thermo Fisher Scientific, Massachusetts, USA), and data were analyzed using Attune NxT software.
Statistical analysis Normal distribution was confirmed using the Kolmogorov-Smirnov test. Diabetes incidence was plotted according to the Kaplan-Meier method. Incidences between each group were compared with the log-rank test. Statistical analysis between two groups was performed by independent t-test, or when multiple comparisons were made, by one-way analysis of variance followed by Tukey’s post-hoc test using GraphPad Prism (v5; GraphPad Software Inc., San Diego, CA, USA). P <0.05 were considered as a statistically significant difference.

Results

Long chain but not short chain inulin is protective against the development of autoimmune diabetes We first characterized the chemical composition of two ITF formulations using HPAEC. Figure 1A describes the oligosaccharide range and relative response per oligomer of the ITFs. ITF(s) and ITF(l) have very different profiles with only a partial overlap (Figure 1A). ITF(s) consist of fructooligosaccharide (FOS) with mostly chain lengths of 2 < DP < 10, while ITF(l) may be described as inulin due to their broad range of chain lengths up to DP60. ITF(s) contain mostly chain types of GF3, GF4, and GF5 (i.e. starting with a glucose molecule followed by 3, 4, or 5 fructose subunits) and F3, F4, or F5 (chains consisting only of 3, 4, or 5 fructose moieties). ITF(s) consists of both GFn and Fn fructans while ITF(l) consists solely of the GFn type fructans. Glucose and fructose monomers, GF and GF2 (i.e. dimers of glucose and fructose subunits and trimers made up of one glucose subunit and two fructose subunits) are present in both ITFs.

The effects of long and short chain ITF on diabetogenesis were then examined over a period of 24 weeks. ITF(l) prevented the development of autoimmune diabetes as diabetes incidences were only 10% in ITF(l) treated animals (P < 0.01) and 40% in ITF(s) treated animals (Figure 1B). Accordingly, lower frequencies and reduced severity of insulitis were
observed in ITF(l) but not in ITF(s) fed animals compared to the control animals (Figure 1C and D).

**Long chain ITF modulates pancreatic and systemic Treg and Th17 responses** Next we examined and compared ITF(1) and ITF(s) supplementation on cellular immune responses in the pancreas and quantified T regulatory cells and Th17 cells (Figure 2A). ITF(l) fed mice had increased Treg cells and decreased Th17 cells in the pancreas. Also, we quantified levels of IL-10, IL-1β and IL-17 in the pancreas. ITF(l) significantly enhanced the production of the regulatory cytokine IL-10 (P < 0.0219) and decreased the production of proinflammatory IL-17 (P < 0.0129) and IL-1β (P < 0.0085) when compared to the control animals (Figure 2B).

Similar modulatory effects of ITF(l) were found in the spleen and serum. Treg in the spleen was enhanced (P < 0.0184), while Th17 was reduced (P < 0.0211) in ITF(l) fed NOD mice compared to the control animals. The pro-inflammatory mediators IL-17 (P < 0.0005) and IL-1β (P < 0.0064) were decreased in the serum by ITF(l) (Figure 3A), although a significant change in systemic IL-10 was not observed (Figure 3B).

**Long chain ITF(l) modulates colonic T cell responses and modulatory cytokine release** As ITF(l) are fermented in the colon [21] and influence immunity by either direct interaction with intestinal cells [22] or by modulating the immune active fermentation products of microbiota [21], we next studied the colonic immunity, colonic integrity, microbiota and their short-chain fatty acids fermentation products in ITF(l)-treated animals with reduced diabetes frequency.

In the colon, we observed that ITF(l) supplementation enhanced Treg cell and downregulated Th17 cells compared with control NOD mice (Figure 4A). In addition, ITF(l)
fed NOD mice had higher colonic levels of IL-10 ($P < 0.0130$), but lower levels of IL-1β ($P < 0.0060$) and IL17 ($P < 0.0115$) (Figure 4B).

Reduction in IL-1β production in the colon was at least in part caused by suppression of the NLRP3 inflammasome and caspase-1 pathway. ITF(l) supplementation downregulated the NLRP3 and caspase-1 expression (Figure 4C). We also examined mitogen-activated protein kinase (MAPK) p38 and nuclear factor (NF)κB p65 phosphorylation and activation in the colon to determine whether the reduction in inflammation was through suppression of these pathways. However, there were no distinction in p-p38 and p-p65 expression between the NOD control and ITF(l) fed groups (Figure S1).

**ITF(l) improves gut integrity and barrier function** A healthier colon with a longer length, thicker colon mucosa and deeper crypts were observed in ITF(l) fed NOD mice compared to controls (Figure 5A and B). The enhanced colon integrity in ITF(l) fed mice was further substantiated by higher levels of the TJ proteins occludin and claudin-2 (Figure 5C) but not of ZO-2 (Figure S2). In addition, ITF(l) enhanced the expression of the antimicrobial peptides β-defensin-1 and CRAMP, important in the defense against pathogens and for maintaining the gut barrier function (Figure 5D).

**ITF(l) promotes SCFA production and modifies gut microbiota composition** Shifts in gut microbiota and SCFA production have been associated with development of autoimmune diabetes [17]. Consequently, we studied the effect of ITFs on SCFA production and gut microbiota composition. The data showed that ITF(l) increased acetate, propionate and butyrate production compared to the control group (Figure 6A). Notably by 16S rRNA gene sequencing analysis, ITF(l) rich diets indeed modified gut microbiota and increased the ratio of *Firmicutes* to *Bacteroidetes* at the phylum level in NOD mice (Figure 6B). Further analysis demonstrated that ITF(l) modified lineages of *Firmicutes* and *Bacteroidetes* microbiota at the family and genus levels (Table 2). The mucin-degrading *Ruminococcaceae*
and Lactobaccilli with profound anti-inflammatory properties [23] were significantly more abundant in ITF(l) fed mice. ITF(l) supplementation altered gut microbiota composition with a much greater α and β diversity in the bacterial community of ITF(l) fed group compared with normal chow fed mice (Figure 6C and D).

Discussion

Our study demonstrates that dietary supplementation with ITF(l) but not ITF(s) dampens the incidence of autoimmune diabetes in NOD mice. In ITF(l)-fed NOD mice, we observed protection from insulitis and absence of infiltration of immune cells. ITF(l) promotes modulatory pancreatic and systemic T cell responses as illustrated by increased Treg/Th17 ratio and modulatory cytokine production. Furthermore, ITF(l) improves intestinal immune homeostasis and barrier integrity by selective regulation of TJ proteins and the antimicrobial peptide β-defensin-1. Lastly, ITF(l)-fed mice show enhanced SCFA production as well as a more diverse and balanced gut microbiome.

The finding that ITF(l) but not ITF(s) dampens the incidence of autoimmune diabetes in NOD mice leads to the postulation that only specific dietary fibers promote immunity and intestinal homeostasis in autoimmune prone NOD mice. This finding does not seem to corroborate earlier findings of Toivonen et al [3] that fermentable dietary fibers promote the development of autoimmune diabetes in NOD mice. Notably, however, fibers with totally different structures than that of ITFs were used. In addition, it is suggested that T1D promoting or modulating effects of dietary fibers are related to their influence on Firmicutes which are associated with Treg generation. Here we support this by showing that long-chain ITF(1) enhances the abundance of Firmicutes which may be one of the mechanisms by which this dietary fiber ameliorates diabetes in NOD mice [24].
Previously, differential prebiotic and immunomodulatory effects have been demonstrated by ITFs of varying chain length. Prolonged fermentation of long chain inulin (DP > 10) compared with short chain inulin (DP < 10) ensures more endurable and profound prebiotic and immunomodulatory effects, locally on the colon and systemically, which are largely determined by the fermenting microbiota [25]. Here in NOD mice, we first report that dietary supplementation with ITF dampens the incidence of autoimmune diabetes in a fiber-chemistry dependent fashion. The protective effects by long chain ITF is found to be associated with modification of gut microbiota composition, strengthening of intestinal mucosal barrier integrity and resultant modulatory pancreatic and systemic immune responses. As such enrichment of the diet with these types of dietary fibers may reduce or slow down the progress of autoimmune destruction of β-cells.

CD4+ Foxp3+ Treg cells are responsible for maintaining self-tolerance and immune homeostasis [26]. Foxp3+ Treg cells, which suppress autoreactive T cells [27] and Th17 cells/IL-17 indispensable in the pathogenesis of autoimmune diabetes [13] are dysregulated. With the observations that ITF(l) increases Treg and decreases Th17 cell frequencies in pancreas, spleen, and colon, accompanied by a regulatory cytokine milieu, we confirm that ITF(l) promotes a modulatory pancreatic and systemic regulatory T cell responses. Reinforced immunomodulatory effects of ITF(l) in the colon may be transmittable to the pancreas [28].

The development of T1D is thought to have an intestinal origin [1, 29]. Alterations in intestinal permeability and structure have been reported in human subjects either preceding T1D onset or concomitant with the disease [30]. Dietary intervention by hydrolyzed casein to restore impaired intestinal barrier function modulates the development of β-cell autoimmunity and decreases the risk of T1D in humans and in Biobreeding diabetes-prone rat model [1, 31]. TJ proteins maintain intestinal epithelial integrity, inhibit microbial antigens
passing and hence are closely related to diabetes development. NOD mice show disturbances in colon integrity compared to nondiabetic C57BL/6 [32]. In our study, ITF(l) increases the expression of the TJs claudin-1 and occludin. Differential regulation of TJ proteins has been observed after dietary interventions [33]. Fermentable fibers such as ITF(l) may be consumed by colonic microbiota and transformed into SCFAs including butyrate that influence colonic barrier function [34] and maintain gut immunological homeostasis [35]. Reduced butyrate production has been associated with the development of autoimmune diabetes [36]. Butyrate produced in the intestine induces differentiation of colonic Treg cells [24] and also enhances systemic Treg cell generation. Butyrate also exerts anti-inflammatory effects and suppresses NFκB activation in colonocytes [37]. ITF(l)-induced SCFA production and especially that of butyrate as we observed may have been a causative factor in the enhanced TJ expression and beneficial immunomodulatory effects in the NOD mice.

Composition of the gut microbiome and the abundance of certain bacteria are important factors to influence the progress of T1D in NOD mice [38]. Our microbiome analysis demonstrated that ITF(l) modulate the composition and increased diversity of microbiota. In particular, ITF(l) increased Firmicutes and decreased Bacteroidetes at the phylum level and enriched modulatory Ruminococcaceae and Lactobacilli. Earlier studies have shown that Bacteroidetes promote the development of T1D [17, 39]. Dietary interventions that accelerated T1D have caused a decreased Firmicutes to Bacteroidetes ratio [40, 41]. Prediabetic children harbor more bacteriodetes compared to the healthy children [42]. ITF(l)-induced decrease in Bacteroidetes can therefore exert additional anti-diabetogenic effect of ITF(l). Lactobacilli is one of potential probiotic members that alleviate inflammation as demonstrated after co-culturing dendritic cells with species of lactobacilli with formation of Treg cells as a consequence [23]. Many bacteria including Lactobacilli have been identified to have effects on gut barrier function [43-45]. Modulated microbiome
could reverse impaired integrity of the intestinal barrier and hence protect gut immunity from microbial antigens escaping the gut barrier and provoking intestinal immune responses during T1D. An unbalanced mucosal immunity is a major contributor to the failure to form tolerance, resulting in the autoimmunity in T1D [29].

In conclusion, we show that modulation of the autoimmune process with dietary fibers in a feasible option but at the same time our data warrant selection of specific immune modulatory and colon acting fibers. Our findings emphasize the role of intestinal homeostasis in the modulation of T1D, supporting the hypothesis that strengthening gut barrier may be a promising target to hinder the development of T1D. As for cellular molecular changes in NOD mice that connect diet to gut and immune homeostasis, this work may herald promising dietary approaches with the ITF(l) prebiotic to prevent or slow down progression of autoimmune diabetes.

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Author Contributions
KC, HC, MMF and BJH performed experiments and analyzed data, with general assistance from XP for FACS experiments and analysis. JL, HZ and JD provided intellectual inputs, contributed to the data acquisition and critically reviewed the manuscript. JS and PDV designed and interpreted experiments. KC, JS and PDV wrote the paper.

Conflict of interest

The authors have declared no conflict of interest.

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Figure legends

Figure 1. ITF(l) but not ITF(s) supplementation is protective against autoimmune diabetes in NOD mice. A: The HPAEC profiles depict the fructose (F) and glucose (G) monomers, dimers, and fructan oligomer present in ITF(l) and ITF(s) formulations. The n represents the number of fructose moieties in the chain. B: Female NOD mice were fed on 5% ITF(l) or ITF(s) supplemented diets until 24 weeks. Shown are Kaplan-Meier survival curves and the percentage of diabetes-free mice, n=12-18 mice per group. C: Histological examination of pancreatic islet infiltration by immune cells in female NOD mice at 24 weeks. Representative islets from five independent mice in each group were shown. D: Insulitis score was quantified as described. Data are means ± SEM. *: P < 0.05, **: P < 0.01.
Figure 2. ITF(l) but not ITF(s) supplementation modulates pancreatic immune responses. A: Percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> Treg and CD4<sup>+</sup>IL-17A<sup>+</sup> Th17 cells in the pancreas. Symbols represent individual mice and data are the median ± interquartile range from five independent mice per group. B: Pancreatic production of anti-inflammatory and inflammatory cytokines. Data are means ± SEM. *: P < 0.05, **: P < 0.01.
**Figure 3.** ITF(l) but not ITF(s) supplementation modulates systemic immune responses. A: Percentage of CD25+ Foxp3+ Treg and CD4+ IL-17A+ Th17 cells in the spleen. Symbols represent individual mice and data are the median ± interquartiles from five independent mice per group. B: Serum levels of anti-inflammatory and inflammatory cytokines. Data are means ± SEM. *: P < 0.05 and ***: P < 0.001.
Figure 4. ITF(I) supplementation modulates colonic immune responses in NOD mice. A: Percentage of CD25⁺ Foxp3⁺ Treg and CD4⁺ IL-17A⁺ Th17 in the colon. Symbols represent individual mice and data are the median ± interquartile range from five independent mice per group. B: Production of anti-inflammatory and inflammatory cytokines in the colon. Data are means ± SEM. *: P < 0.05 and **: P < 0.01. C: Western blot and densitometry analysis of caspase-1-NLRP3 inflammasome activation. β-actin: housekeeping protein. Data are representative from three independent experiments with three animals in each group. *: P < 0.05 and **: P < 0.01.
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**Figure 5.** ITF(l) supplementation strengthens gut integrity and barrier function in NOD mice.  

A: Representative sections of colon tissues from six independent mice in NOD and ITF(l) fed groups were shown. Arrows indicate crypt lengths. Scale bar: 50 μm. B: Colonic length, mucosa thickness and depth of crypt were quantified. C: Western blot and densitometry analysis of occludin and claudin-2 protein expression in NOD and ITF(l) fed NOD mice in the colon. β-actin: housekeeping protein. Representative blots were shown from three independent experiments with three animals in each group. *: $P < 0.05$ and **: $P < 0.01$. D: Expression of the antimicrobial peptides β-defensin1 and CRAMP were assessed by quantitative PCR analysis in the colon. Data are means ± SEM. *: $P < 0.05$.  

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**Figure 6.** Effects of ITF(l) supplementation on gut microbiota composition and its metabolites. A: Concentration of total SCFAs, acetic acid, propionic acid and butyric acid. Data are means ± SEM from five independent mice in each group. B-D: Colon content samples from NOD mice fed with normal AIN-93G diet and ITF(l)-supplemented diet were used for taxonomic analysis by 16S rRNA sequencing. B: The taxonomic compositions of gut bacteria were shown in phylum (n=6). C: α-Diversity of gut microbiota was shown by Shannon evenness. Two-tailed independent t-test was used. Data are means ± SEM. D: β-Diversity, shown by principal component analysis (PCoA; unweighted) of taxonomic families of gut microbiota in normal chow fed (red circles, n=6) and ITF(l) supplemented NOD mice (green circles, n=6). Principal components PC1 and PC2 explained 9.91% of the total variance. *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. 
**Figure S1.** Effects of ITF(l) or ITF(s) supplementation on MAPK p38 and NFκB p65 pathways. Western blot analysis of p38, p-p38 and p-p65 expression in the colon. β-actin: housekeeping protein. Representative blots were shown from three independent experiments with three animals in each group.

**Figure S2.** ITF(l) did not affect ZO-2 expression in NOD mice. Expression of ZO-2 protein in NOD and ITF(l) fed NOD mice by Western blot analysis in the colon. β-actin: housekeeping protein. Representative blots were shown from three independent experiments with three animals in each group.
Table 2 Significant differences in taxa (genus and species level) among NOD and NOD+ITF(l) mice

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
<td>Lachnospiraceae</td>
<td>Other</td>
<td>-</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
<td>Lachnospiraceae</td>
<td>Acetatifactor</td>
<td>-</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
<td>Ruminococcaceae</td>
<td>uncultured</td>
<td>-</td>
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<td>Lactobacillaceae</td>
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<td>Bacteroidetes</td>
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<td>Rikenellaceae</td>
<td>Alistipes</td>
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<td>Clostridiales</td>
<td>Lachnospiraceae</td>
<td>Other</td>
<td>Other</td>
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<td>Lachnospiraceae</td>
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<td>Clostridia</td>
<td>Clostridiales</td>
<td>Lachnospiraceae</td>
<td>Blautia</td>
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<td>Clostridiales</td>
<td>Ruminococcaceae</td>
<td>uncultured</td>
<td>Other</td>
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<td>Clostridia</td>
<td>Clostridiales</td>
<td>Ruminococcaceae</td>
<td>Intestinimonas</td>
<td>uncultured</td>
</tr>
</tbody>
</table>

Uncultured, inability to assign given operational taxonomic unit (OTU) into single taxonomic group; Other, absence of information about given OTU in the database.

*Values were by two-tailed independent t-test.
Table 2: Significant differences in taxa (genus and species level) among NOD and NOD+ITF(l) mice

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>NOD mean abundance</th>
<th>NOD+ITF(l) mean abundance</th>
<th>P value</th>
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<td>Lachnospiraceae</td>
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<td>6.28%</td>
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<td>Lachnospiraceae</td>
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<td>-</td>
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<td>0.0318</td>
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<td>-</td>
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<td>0.50%</td>
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<td>Lachnospiraceae</td>
<td>Other</td>
<td>Other</td>
<td>4.17%</td>
<td>6.28%</td>
<td>0.0083</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
<td>Lachnospiraceae</td>
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<td>Other</td>
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<td>0.87%</td>
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</tbody>
</table>

Uncultured, inability to assign given operational taxonomic unit (OTU) into single taxonomic group; Other, absence of information about given OTU in the database. *Values were by two-tailed Student t test.