RESEARCH ARTICLE

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

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Received: November 17, 2016
Revised: January 7, 2017
Accepted: February 2, 2017

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 nonobese diabetic mice.
Methods and results: Female nonobese diabetic 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 $CD25^+Foxp3^+CD4^+$ regulatory T cells, decreased $IL17A^+CD4^+$ Th17 cells, and modulated cytokine production profile in the pancreas, spleen, and colon. Furthermore, ITF(l) suppressed NOD like receptor protein 3 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 cathelicidin-related antimicrobial peptide as well as short-chain fatty acid production were enhanced by ITF(l). Next-generation sequencing analysis revealed that ITF(l) enhanced *Firmicutes/Bacteroidetes* ratio to an antidiabetogenic 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.

Keywords:
Barrier integrity / Dietary fibers / Gastroentero pancreatic regulation / Gut microbiota homeostasis / Immunomodulation / Nutritional intervention / Pancreatic-gut immunity / Type 1 diabetes pathophysiology

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Abbreviations: **DP**, degree of polymerization; **ITF**, inulin-type fructans; **ITF(l)**, long-chain ITFs; **ITF(s)**, short-chain ITFs; **NOD**, nonobese diabetic; **NLRP3**, NOD like receptor protein 3; **SCFA**, short-chain fatty acid; **T1D**, type 1 diabetes; **Th**, T helper; **TJ**, tight junction; **Treg**, regulatory T

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1 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 nonobese diabetic (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 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 can activate autoreactive $CD8^+$ T cells. Additionally, IL-17-producing $CD4^+$ 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 (ITF(l)) and the more readily fermentable short-chain ITF (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 (NOD like receptor 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 (TJs) and barrier reinforcing immunomodulatory antimicrobial peptides [15, 16]. Finally, in ITF(l)-treated animals, we studied changes to the microbiota derived metabolites SCFAs and modification of gut microbiota composition with relation to T1D [17, 18].

2 Materials and methods

2.1 Fibers and structural characterization

The applied 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 as previously described [5].

2.2 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-chek glucosemeter (Roche Diagnostics, Almere, The Netherlands). Overt diabetes was confirmed by a glycemia > 13.3 mmol/L in 2 consecutive days.

2.3 ELISA assays

Serum was collected by allowing the blood to coagulate at ambient temperate for 25 min, and subsequently centrifuging the samples at 3000 *g* for 10 min at 4°C. Tissue samples were homogenized in 0.9% saline by a homogenizer (Scientz-48; Scientz Biotechnology, Ningbo, Zhejjiang, China) and centrifuged at 900 *g* for 20 min at 4°C. Supernatant collected and serum were assayed for inflammatory mediators using

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Table 1. Primers used for qPCR

Gene	forward	reverse
β-Defensin-1	5′-GCACAAGAAGGTCACACGGA-3′	5′-CTAAGGTTGCAGATGGGGTGT-3′
CRAMP	5′-CTTCAAGGAACAGGGGGTGG-3′	5′-CTTGAACCGAAAGGGCTGTG-3′
β-Actin	5′-CCCAGGCATTGCTGACAGG-3′	5′-TGGAAGGTGGACAGTGAGGC-3′

CRAMP, cathelicidin-related antimicrobial peptide.

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^{TM} GO; Thermo Fisher Scientific Oy, Vantaa, Finland). Data are expressed as pg/mL.

2.4 Western blot analysis

Tissue lysates were prepared by homogenization in chilled radioimmunoprecipitation assay 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 were separated on 10% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride 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, zonula occludens-2, and β-actin were purchased from Santa Cruz Biotechnology (CA, USA), and antibodies against NLRP3, phosphor-p38, p38, and NFκB 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 an enhanced chemiluminescence detection kit.

2.5 Histology

Freshly harvested pancreatic and colon tissues were fixed with 4% polyformaldehyde overnight. The tissues were then washed with ddH_2O, dehydrated with gradient ethanol solutions, and embedded in paraffin and cut into 5 μm sections. The sections were subsequently stained with hematoxylin/eosin 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].

2.6 RNA isolation and qPCR

Total RNA was isolated from the colon samples using Trizol (Invitrogen, Thermo Scientific, MA, 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 ($2^{-\Delta\Delta Ct}$). Relative expression ratios were calculated as normalized ratios to β-actin internal control gene. Detailed primers sequences are shown in Table 1.

2.7 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 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 (1 mL) 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, MA, 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.

2.8 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 (CA, USA) and detailed information is listed in Table 1 in the

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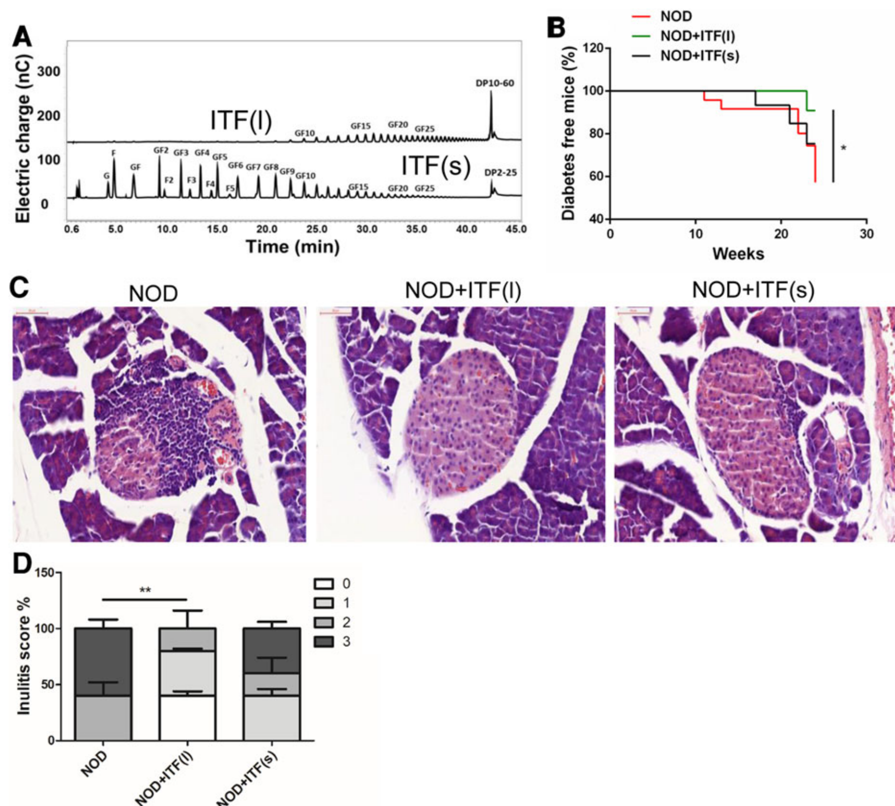

Figure 1. ITF(l) but not ITF(s) supplementation is protective against autoimmune diabetes in NOD mice. (A) High-performance anion exchange chromatography (HPAEC) profiles depict the fructose (F) and glucose (G) monomers, dimers, and fructan oligomer present in ITF(l) and ITF(s) formulations. The n represents 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 mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

Supporting Information. Pancreas, colon, and spleen samples were harvested and cut into small pieces, and 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 phorbol myristate acetate (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/ER 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), and data were analyzed using Attune NxT software.

2.9 Statistical analysis

Normal distribution was confirmed using the Kolmogorov–Smirnov test. Diabetes incidence was plotted according to the Kaplan–Meler 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). $P < 0.05$ was considered as a statistically significant difference.

3 Results

3.1 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 high-performance anion exchange chromatography. 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 (Fig. 1A). ITF(s) consist of fructooligosaccharide 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

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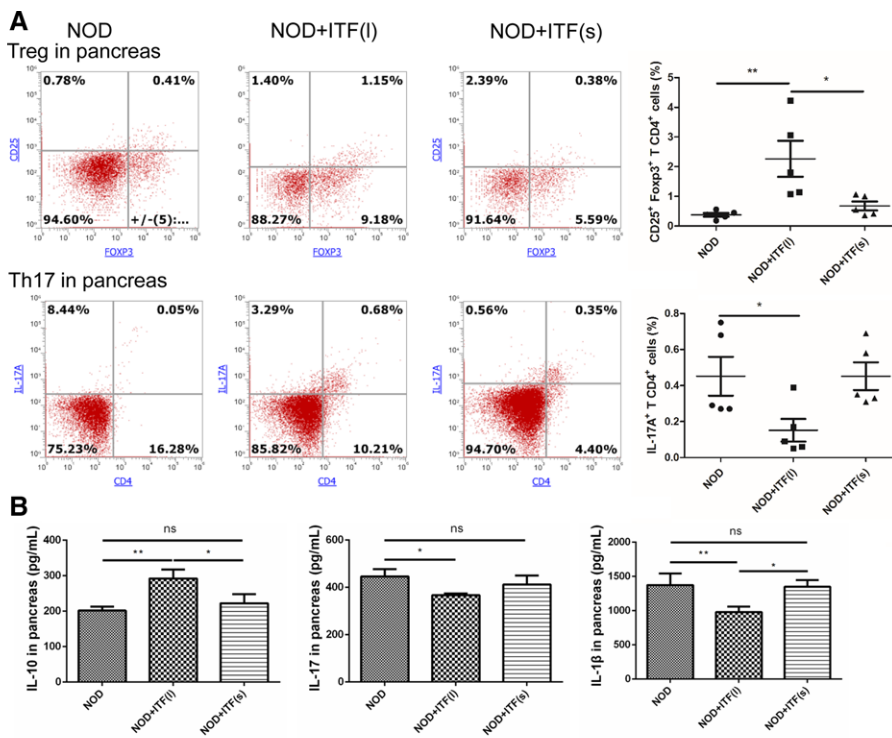

Figure 2. ITF(l) but not ITF(s) supplementation modulates pancreatic immune responses. (A) Percentage of $CD25^+$ $Foxp3^+$ Treg and $CD4^+$ IL-$17A^+$ Th17 cells in the pancreas. Symbols represent individual mice and data are the median ± interquartile range from five independent mice per group. (B) Pancreatic production of anti-inflammatory and inflammatory cytokines. Data are mean ± SEM. $*p < 0.05$, $**p < 0.01$.

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 ITF(l) and ITF(s) on diabetogenesis were then examined over a period of 24 weeks. ITF(l) prevented

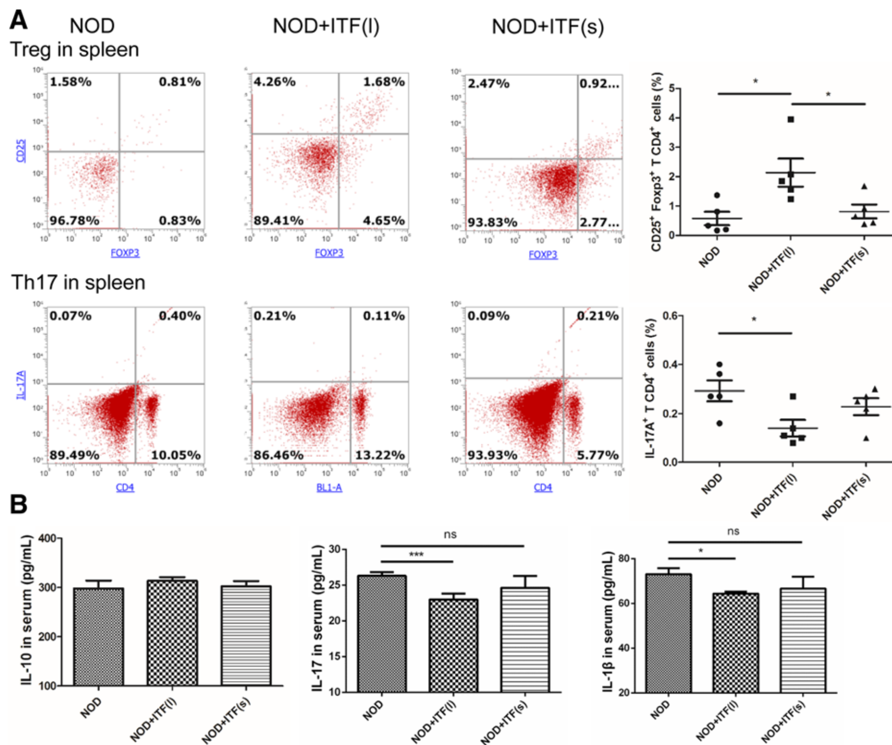

Figure 3. ITF(l) but not ITF(s) supplementation modulates systemic immune responses. (A) Percentage of $CD25^+$ $Foxp3^+$ Treg and $CD4^+$ IL-$17A^+$ Th17 cells in the spleen. Symbols represent individual mice and data are the median ± interquartiles from five independent mice per group. (B) Serum levels of anti-inflammatory and inflammatory cytokines. Data are mean ± SEM. $*p < 0.05$ and $***p < 0.001$.

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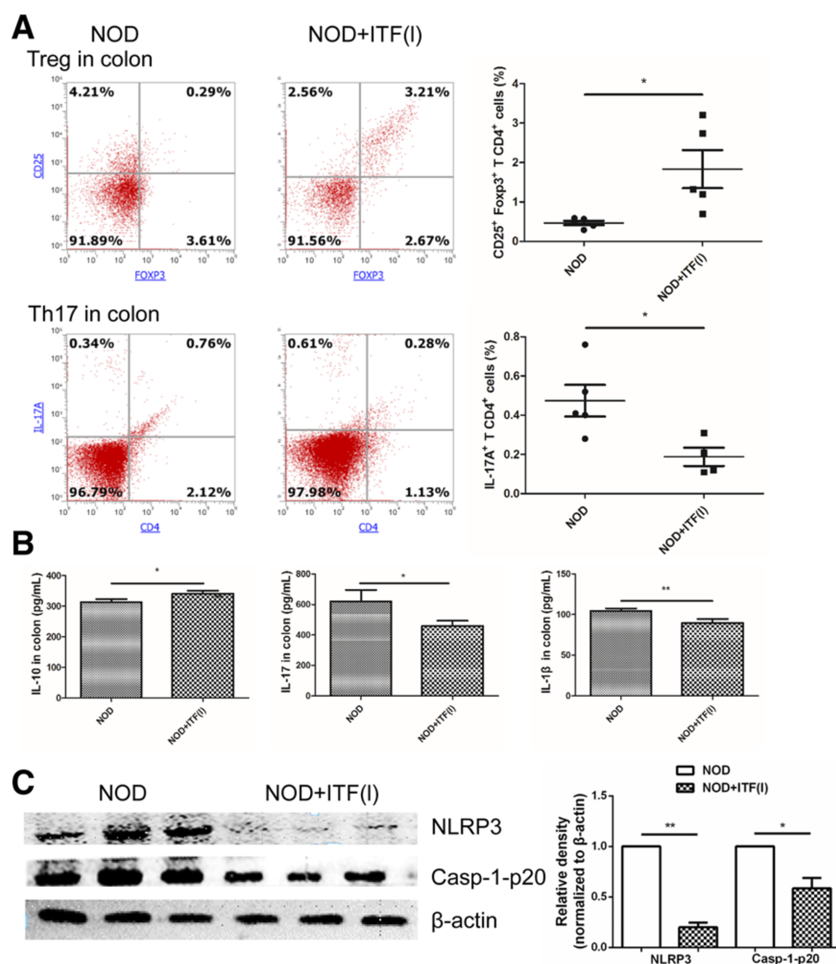

Figure 4. ITF(l) supplementation modulates colonic immune responses in NOD mice. (A) Percentage of $CD25^+$ $Foxp3^+$ Treg and $CD4^+$ $IL\text{-}17A^+$ Th17 in the colon. Symbols represent individual mice and data are the median ± interquartile range from five independent mice per group. (B) Production of anti-inflammatory and inflammatory cytokines in the colon. Data are mean ± 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 $^{**}p < 0.01$.

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 (Fig. 1B). Accordingly, lower frequencies and reduced severity of insulitis were observed in ITF(l)-fed but not in ITF(s)-fed animals compared to the control animals (Fig. 1C and D).

3.2 ITF(l) modulates pancreatic and systemic Treg and Th17 responses

Next, we examined and compared ITF(l) and ITF(s) supplementation on cellular immune responses in the pancreas and quantified T regulatory cells and Th17 cells (Fig. 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 (Fig. 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) (Fig. 3A), although a significant change in systemic IL-10 was not observed (Fig. 3B).

3.3 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 SCFAs 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 (Fig. 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 ($P < 0.0115$) (Fig. 4B).

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Table 2. Significant differences in taxa (genus and species lever) among NOD and NOD + ITF(l) mice

Phylum	Class	Order	Family	Genus	Species	NOD mean abundance (%)	NOD + ITF(l) mean abundance (%)	p value
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Other	–	4.17	6.28	0.0083
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Acetatifactor	–	0.27	0.02	0.0318
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Uncultured	–	0.57	1.67	0.0191
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	–	0.08	0.50	0.0463
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes	Uncultured	4.70	2.43	0.0556
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Other	Other	4.17	6.28	0.0083
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Uncultured	Other	0.58	1.90	0.0219
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	Other	1.58	4.52	0.0121
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Uncultured	Other	0.38	1.33	0.0300
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Intestinimonas	Uncultured	1.37	0.87	0.0130

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

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 (Fig. 4C). We also examined mitogen-activated protein kinase 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,

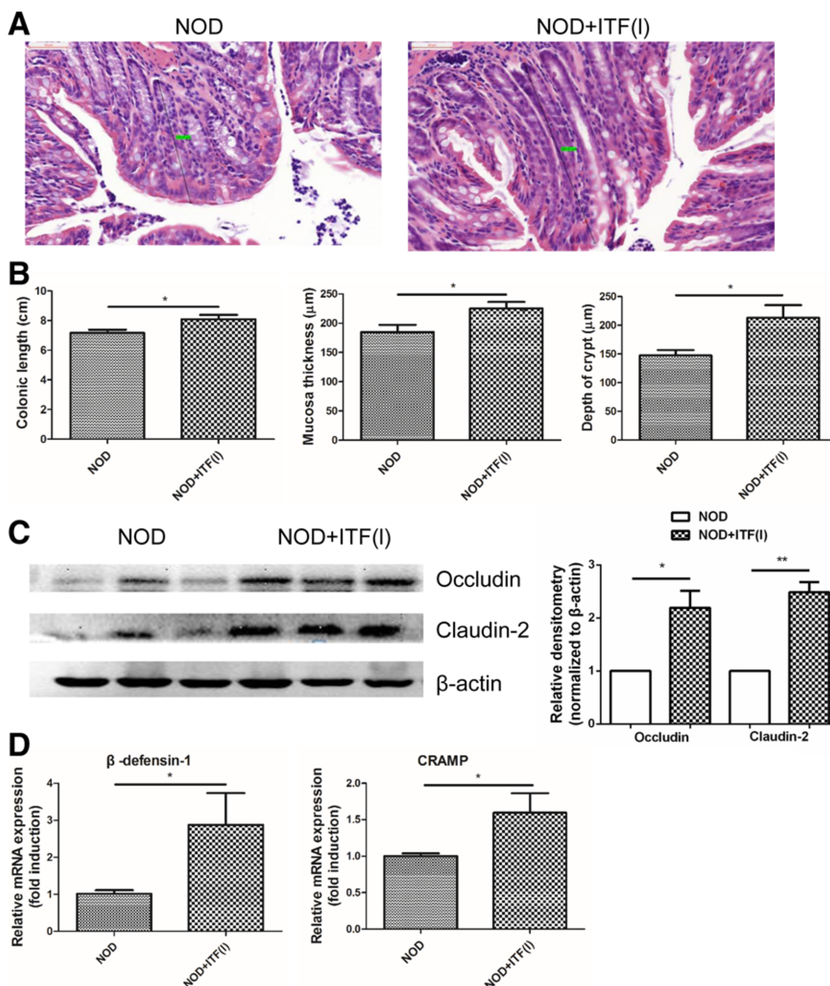

Figure 5. ITF(l) supplementation strengthens gut integrity and barrier function in NOD mice. (A) Representative sections of colon tissues from six independent mice in NOD and ITF(l)-fed groups were shown. Arrows indicate crypt lengths. Scale bar: 50 μm. (B) Colonic length, mucosa thickness, and depth 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-defensin-1 and cathelicidin-related antimicrobial peptide (CRAMP) were assessed by quantitative PCR analysis in the colon. Data are mean ± SEM. $*p < 0.05$.

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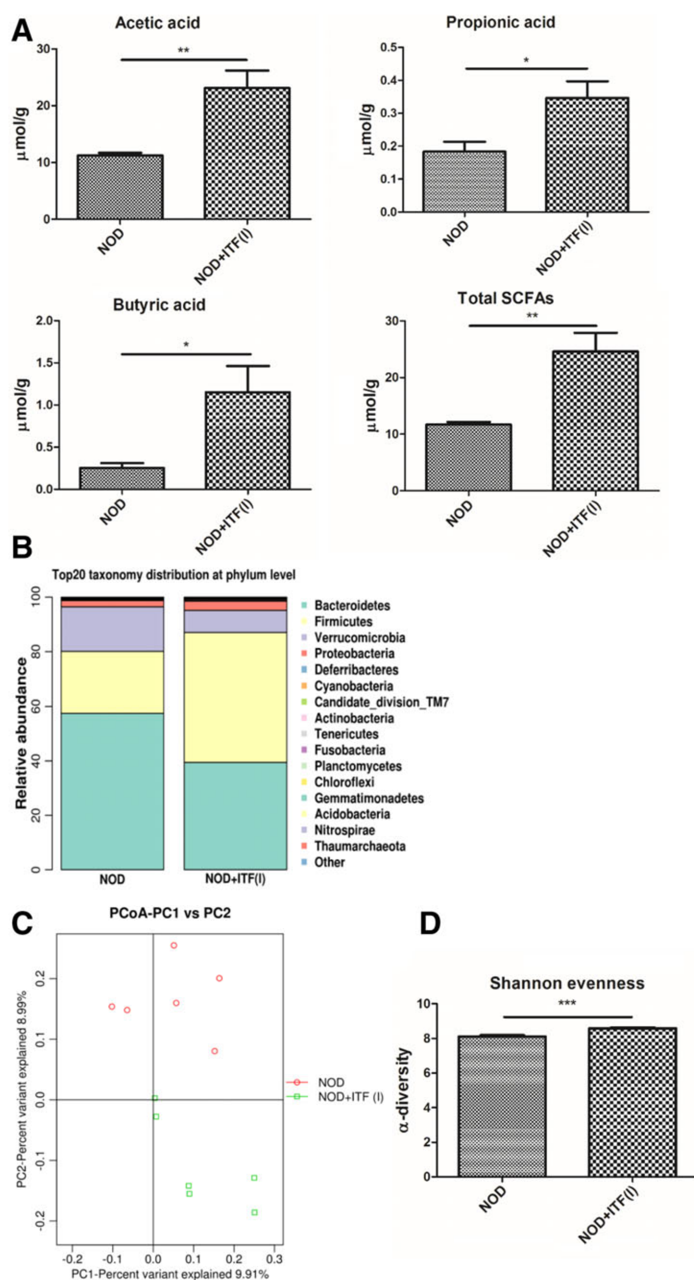

Figure 6. Effects of ITF(l) supplementation on gut microbiota composition and its metabolites. (A) Concentration of total SCFAs, acetic acid, propionic acid, and butyric acid. Data are mean ± SEM from five independent mice in each group. (B–D) Colon content samples from NOD mice fed with normal AIN-93G 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 mean ± 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 $^{***}p < 0.001$.

there were no distinction in p-p38 and p-p65 expression between the NOD control and ITF(l)-fed groups (Fig. 1 in the Supporting Information).

3.4 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 (Fig. 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 (Fig. 5C) but not of zonula occludens-2 (Fig. 2 in the Supporting Information). In addition, ITF(l) enhanced the expression of the antimicrobial peptides β-defensin-1 and cathelicidin-related antimicrobial peptide, important in the defense against pathogens and for maintaining the gut barrier function (Fig. 5D).

3.5 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].

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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 (Fig. 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 (Fig. 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* [3] 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 (Fig. 6C and D).

4 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 ITF(l) 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 ITF(l) 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 Treg 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*

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compared to the healthy children [42]. ITF(l)-induced decrease in *Bacteroidetes* can therefore exert additional antidiabetogenic 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.

K.C., H.C., M.M.F., and B.J.H. performed experiments and analyzed data, with general assistance from XP for FACS experiments and analysis. J.L., H.Z., and J.D. provided intellectual inputs, contributed to the data acquisition, and critically reviewed the manuscript. J.S. and P.D.V. designed and interpreted experiments. K.C., J.S., and P.D.V. wrote the paper.

The present work was supported by the Dutch Diabetes Foundation and by funds from the National Natural Science Foundation of China (grant nos. 91642114, 31570915, and 31400779), Jiangsu Province Recruitment Plan for High-level, Innovative and Entrepreneurial Talents, and Key Program of Fundamental Research Funds for the Central Universities (grant no. JUSRP51613A) to J.S. Henk A. Schols of the Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands is acknowledged for analysis of the inulins.

The authors have declared no conflict of interest.

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