# The Fiberdia study: Effects of inulin-type fructans on gut microbiota and regulation of blood glucose and appetite in type 2 diabetes:

A randomised, placebo-controlled crossover trial

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# Table of Contents

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# Summary

Compared to a healthy population, the gut bacteria in type 2 diabetes is associated with a deviating composition, including lower concentrations of bifidobacteria and reduced diversity. Clinical trials report beneficial effects of prebiotic fibres on the composition of gut bacteria and on regulation of blood glucose and appetite in non-diabetic populations. Although such responses could benefit individuals with type 2 diabetes in particular, studies of the potential role of prebiotic fibres in this population are scarce.

This thesis includes data from a randomised and placebo controlled trial investigating prebiotic effects after six weeks treatment with 16 g inulin-type fructans per day in patients with type 2 diabetes.

The objective of paper I was to investigate changes in gut bacteria and short-chain fatty acids (SCFA). In paper II, we evaluated changes in glycaemic regulation and responses of glucagon-like peptide-1 and 2 (GLP-1 and 2) to a standardised mixed meal. The aim of paper III was to investigate changes in gut hormones regulating appetite during a standardised mixed meal, and subjective sensation of appetite and energy intake during an *ad libitum* lunch.

The prebiotics induced moderate changes in the faecal bacterial composition ($P$ = 0.045). A bifidogenic effect was most prominent, with highest positive effect on operational taxonomic units (OTUs) of *Bifidobacterium adolescentis*, followed by OTUs of *Bacteroides*. Significantly higher faecal concentrations of total SCFA, acetic acid, and propionic acid were also found after prebiotic consumption compared to placebo. The prebiotic fibre had no effects on the concentration of butyric acid or the overall bacterial diversity.

The prebiotic treatment did not positively affect GLP-1 concentrations. On the contrary, we found a reduction in GLP-1 response after consumption of inulin-type fructans, significantly different from an increased response after the control treatment. Fasting and postprandial glucose, insulin or GLP-2 concentrations remained unchanged.

Compared to control treatment, the prebiotic treatment had no effect on hunger hormone ghrelin or the ratings of hunger, satiety, fullness and prospective food consumption. The energy intake did not differ between the treatments either. Responses of the satiety hormone peptide YY however, increased unexpectedly after the control treatment, compared to the prebiotics that just barely affected the PYY concentrations, and in an unfavourable direction.

Our findings suggest a moderate potential of inulin-type fructans to improve the bacterial composition and to affect the bacterial fermentation in type 2 diabetes. However, the results do not support a role for inulin-type fructans in regulation of blood glucose or appetite in this population.

# Sammendrag

Effekt av inulinlignende fruktaner på tarmbakterier og regulering av blodglukose og appetitt ved diabetes type 2: En randomisert og placebokontrollert overkrysningsstudie

Denne avhandlingen presenterer resultater fra en randomisert og placebokontrollert studie som undersøker prebiotiske effekter hos pasienter med diabetes type 2 etter seks ukers behandling med et daglig tilskudd av 16 g inulinlignende fruktaner.

Tverrsnittsstudier viser at sammensetningen av bakterier i tarmen er avvikende hos pasienter med diabetes type 2 sammenlignet med hos friske. Bakteriefloraen har lavere konsentrasjoner av bakterier som har beskyttede effekt på tarmen og tarmbarrieren. I tillegg finner man økte konsentrasjoner av sykdomsfremkallende bakterier og økning i funksjoner relatert til oksidativ stressrespons.

Et tarmmiljø med slike trekk er assosiert med inflammatoriske tilstander og mistenkes å bidra i selve utviklingen av diabetes 2. I sirkulasjonen er det dessuten funnet økte nivåer av lipopolysakkarider, en komponent i celleveggen til gram-negative bakterier. Man tror at dette forårsakes av økt permeabilitet i tarmen. Enkelte studier rapporterer om lavere nivåer av det inkretine tarmhormonet glukagonlignende peptid-1 (GLP-1), imens andre finner normale nivåer.

Kostfiber som selektivt fermenteres av dokumentert helsefremmende bakterier i tarmen defineres som prebiotika. Under fermenteringen dannes kortkjedete fettsyrer som blant annet virker som signalmolekyler. De kan binde seg til reseptorer i enteroendokrine L-celler i tarmen og forårsake økt sekresjon av tarmhormonene GLP-1, peptid YY (PYY) og GLP-2 ved inntak av mat. GLP-1 kan forbedre reguleringen av blodglukose ved å fremme utskillelse av insulin og hemme utskillelse av glukagon fra pancreas. GLP-1 har også en beskyttende innvirkning på betacellene i pancreas, og både PYY og GLP-1 kan forbedre appetittreguleringen via innvirkning på det sentrale nervesystemet og mage-/tarmsystemet. GLP-2 har en vedlikeholdende funksjon på den intestinale barrieren og kan forhindre utvikling av systemisk inflammasjon.

Randomiserte, kliniske studier utført på populasjoner uten diabetes type 2 viser helsefremmende effekter av prebiotiske fibre på sammensetningen av tarmbakterier og på reguleringen av blodglukose og appetitt. Til tross for at slike effekter vil kunne gagne de med diabetes type 2 spesielt, er potensialet til prebiotiske fibre knapt studert i det hele tatt i denne populasjonen.

Målsetningen med artikkel I var å utforske endringer i bakteriefloraen og kortkjedete fettsyrer i tarmen. I artikkel II evaluerte vi endringer i reguleringen av blodglukose og responsen av GLP-1 og -2 i forbindelse med et standardisert måltid. Målsetningen med artikkel III var å undersøke endringer i responsen av tarmhormoner som regulerer appetitt i forbindelse med det standardiserte måltidet. I tillegg ble energiinntak og subjektiv opplevelse av appetitt undersøkt i forbindelse med en *ad libitum* lunsj.

Inklusjonskriteriene var; diagnosen diabetes type 2, BMI $\leq$ 40 kg/m$^2$, $HbA_{1c}$ < 86 mmol/mol (10.0%) og uten behandling med insulin eller GLP-1 analoger. Eksklusjonskriteriene var; inntak av kosttilskudd med prebiotika eller probiotika, behandling med antibiotika i løpet av de siste 3 månedene, vektendring > 3 kg i løpet av de siste 3 månedene, fiberinntak > 30 g per dag, svært høyt aktivitetsnivå, graviditet, alkohol- eller stoffmisbruk og lang reisevei. I tillegg var diagnoser som demens, kronisk tarmsykdom, irritabel tarmsyndrom, cøliaki og kreft i løpet av siste fem år også eksklusjonskriterier.

Trettifem deltagere ble inkludert i studien og randomisert til å starte med enten aktivt stoff eller et kontrollsupplement, hvorav 29 deltagere fullførte studien. De inntok 16 g inulinlignende fruktaner eller 16 g kontrollsupplement (maltodekstrin) i seks uker etterfulgt av en fireukers utvaskningsperiode. Deretter fulgte seks uker til med inntak av det motsatte supplementet av hva de startet med. Supplementene ble inntatt i tillegg til deltagernes vanlig kosthold.

Behandlingen med prebiotika ga moderate endringer i den fekale sammensetningen av bakterier. Mest fremtredende var økning i bifidobakterier med størst positiv effekt på operasjonelle taksonomiske enheter (OTU) av *Bifidobacterium adolescentis* etterfulgt av OTU av *Bacteroides*. Fekale konsentrasjoner av total mengde kortkjedete fettsyrer, samt acetat og propionat økte signifikant etter behandling med

prebiotika sammenlignet med kontrollsupplement. Prebiotika hadde derimot ingen effekt på konsentrasjonen av butyrat eller på bakteriell diversitet.

Vi fant heller ingen positiv effekt av prebiotika på GLP-1 respons. Tvert imot fikk deltagerne en reduksjon i GLP-1 respons etter inntak av prebiotika som skilte seg signifikant fra en påvist økning i GLP-1 respons etter inntak av kontrollsupplementet. Fastende og postprandiale konsentrajoner av glukose, insulin og GLP-2 forble uendret.

Sammenlignet med kontrollbehandlingen var det ingen effekt av prebiotika hverken på sulthormonet grelin eller subjektiv rangering av sult, metthet, oppfylthet og motivasjon for å spise. Det var heller ingen endring i energiinntaket I løpet av intervensjonen. I likhet med effekten på GLP-1, var det en uventet økning i responsen av metthetshormonet PYY etter inntak av kontrollsupplementet som skilte seg signifikant fra en liten nedgang i responsen av PYY etter inntak av prebiotika.

Resultatene fra denne studien tyder på at inulinlignende fruktaner kan ha en moderat kapasitet til å forbedre sammensetningen av tarmbakterier ved diabetes type 2. Denne studien bidrar derimot ikke med evidens for at inulinlignende fruktaner har noen positiv innvirkning på reguleringen av GLP-1, GLP-2, blodglukose eller appetitt i denne populasjonen.

# List of papers

**Paper I**: Birkeland E, Gharagozlian S, Birkeland KI, Valeur J, Måge I, Rud I, Aas AM. Prebiotic effect of inulin-type fructans on faecal microbiota and short-chain fatty acids in type 2 diabetes: a randomised controlled trial. Eur J Nutr. 2020

**Paper II:** Birkeland E, Gharagozlian S, Gulseth HL, Birkeland KI, Hartmann B, Holst JJ, Holst R, Aas AM. Effects of prebiotics on postprandial GLP-1, GLP-2 and glucose regulation in patients with type 2 diabetes. A randomised, double-blind, placebo-controlled crossover trial (submitted manuscript).

**Paper III:** Birkeland E, Gharagozlian S, Birkeland KI, Holm OKS, Thorsby PM, Aas AM. Prebiotic effect of inulin-type fructans on appetite in type 2 diabetes: A randomized controlled crossover trial (submitted manuscript).

# Abbreviations

| | |
|---|---|
| AgRP | Agouti-related peptide |
| ANOVA | Analysis of variance |
| ARC | Arcuate nucleus |
| ASCA | ANOVA simultaneous component analysis |
| CART | Cocaine-amphetamine-related transcript |
| DPP4 | Dipeptidyl peptidase |
| FFQ | Food frequency questionnaire |
| FOS | Fructo-oligosaccharides |
| GHS-R | Growth hormone secretagogue receptor |
| GIP | Glucose-dependent insulinotropic peptide |
| GLP-1 | Glucagon-like peptide-1 |
| GLP-2 | Glucagon-like peptide-2 |
| GOS | Galacto-oligosaccharides |
| GPR | G-protein-coupled receptor |
| $HbA_{1C}$ | Haemoglobin $A_{1C}$ |
| ITF | Inulin-type fructans |
| LADA | Latent autoimmune diabetes in adults |
| LMM | Linear mixed model |
| MCS | MiSeq control software |
| MODY | Maturity onset diabetes of the young |
| NPY | Neuropeptide Y |
| NST | Nucleus of the solitary tract |
| OTU | Operational taxonomic unit |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PLS-DA | Partial least squares discriminant analysis |
| PLSR | Partial least squares regression |
| POMC | Propiomelanocortin |
| PYY | Peptide YY |
| Q30 | Quality score of 30 |
| QIIME | Quantitative insight into microbial ecology |
| Q-Q | Quantile-quantile |
| SCFA | Short-chain fatty acids |

| | |
|---|---|
| VAS | Visual analogue scale |
| VIP | Variable importance in prediction |

# List of figures

# List of tables

# 1 Introduction and background

## 1.1 Type 2 diabetes

### 1.1.1 Classification

Diabetes is characterised by hyperglycaemia caused by impaired production of insulin in the pancreas and/or reduced sensitivity to insulin (1). Generally, diabetes is classified into type 1 and type 2 (2). Type 1 diabetes is caused by destruction of the beta cells in pancreas, which results in little to no secretion of insulin. Gestational diabetes, maturity onset diabetes of the young (MODY), latent autoimmune diabetes in adults (LADA), neonatal diabetes and secondary diabetes are other types of diabetes. Only type 2 diabetes will be regarded further in this thesis.

In type 2 diabetes, the insulin sensitivity is reduced and/or the pancreas fails to produce enough insulin (3). Type 2 diabetes may present with symptoms such as increased thirst, frequent urination, increased hunger, weight loss, fatigue, blurred vision, slow-healing sores and frequent infections.

### 1.1.2 Diagnosis and treatment

Type 2 diagnosis is made when glycated haemoglobin (HbA1c) > 48 mmol/mol (6.5%). Life-style interventions such as weight regulation, physical activity and dietary adjustments are considered cornerstones in the treatment of type 2 diabetes (4), and weight reduction may potentially lead to remission (5). The dietary recommendations include a diet rich in dietary fibres (6), which seems to have beneficial effects on glycaemic control, explained by slowed rate of nutrient absorption (7, 8). However, glucose-lowering medication is often required (9). In most patients, fasting blood glucose is sought to be kept between 5 and 6 mmol/L, with a blood glucose between 4.5 and 10.0 mmol/L during day and night, and HbA1c close to 53 mmol/mol (7%). Treatment goals are securing quality of life with a minimum of symptoms, and to avoid or delay development of long-term complications, involving eyes, kidneys, blood vessels, heart, and nerves.

### 1.1.3 Prevalence and risk factors

Until recent past, the disease was most common in the elderly, but increased prevalence of the disease in younger populations has followed the development of overweight and physical inactivity. Today, approximately 350 billion of the adult population worldwide is estimated to have type 2 diabetes (10). In Norway, approximately 216 000 have type 2 diabetes. Additionally, an unknown number of undiagnosed cases is suspected (11).

Heredity and unhealthy life-style are considered risk factors for development of type 2 diabetes (3).

## 1.2 Prebiotics

### 1.2.1 Definition and classification

In recent years, novel food ingredients have received increased attention as potential treatment strategy in obesity and metabolic disturbances, including type 2 diabetes (12-17). Among these, are the prebiotics defined as; substrates selectively utilised by host microorganisms conferring a health benefit (18). Prebiotics are in other words, an energy source for presumed health promoting bacteria, and thereby stimulate their growth and activity. The definition also opens for inclusion of non-carbohydrate substances, extraintestinal body sites, and categories other than food.

The inulin-type fructans (ITF) and galacto-oligosaccharides (GOS) are the most studied prebiotic fibres and ITF are extensively used as industrial food ingredients (18). Additional non-digestible and fermentable fibres have been investigated and suggested as canditate prebiotics, but are not yet formally classified.

The ITF are non-digestible, soluble and fermentable dietary fibres composed of 3 to 60 units of D-fructose linked with a β(2-1) bond, usually with a D-glucose unit at one end (Figure 1) (19).

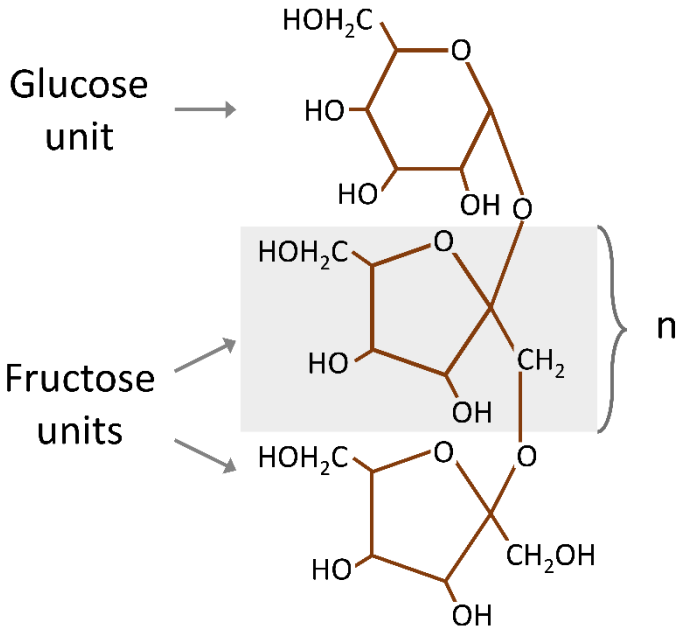


**Figure 1. Molecular structure of inulin-type fructans**

ITF occure naturally in food such as chickory root, artichokes, leeks, asparagus, garlic, onions, bananas and wheat. In food industry, the ITF are commonly extracted from chickory root or synthesised from sucrose. Daily intake of inulin has previously been estimated to be 3-11 g per day in Europe (20) and 1-4 g per day in the United States (21). Due to increased interrest in ITF as food ingredients, these estimates may be outdated.

The nomenclature of ITF varies in the litterature. The terms inulin, oligofructose and fructo-oligosaccharides (FOS) have been used interchangeably for all chain lenghts and origins of ITF. Often however, ITF-chains with fructose units < 10 are called oligofructose or FOS, with the latter sometimes reserved for ITF synthesised from sucrose.

### 1.2.2 Clinical effects

Human trials show several beneficial effects of prebiotic fibres on constipation, absorption of calcium, infections and allergic reactions in infancy, and travellers' diarrhoea (18, 22). Prebiotic fibres have also been investigated as a treatment strategy in obesity and metabolic disturbances. Systematic reviews and meta-analyses report favourable effects on regulation of glycaemic control and appetite, suppression of energy intake, and weight loss (12, 15-17, 23-25). The bulk part of

these studies was conducted in non-diabetic populations. However, one systematic review and one meta-analysis included populations with type 2 diabetes exclusively (16, 25). Due to multiple overlapping features (research groups, origin of study populations, design, and registration numbers), the results from these and most of their individual trials should be interpreted with caution.

### 1.2.3 Safety

During a risk assessment conducted in 2016, The Norwegian Scientific Committee for Food Safety found no serious adverse health effects at doses of 5-20 g per day in human studies. Negative effects reported were mild gastrointestinal symptomes, such as diarrhea, bloating, flatulence and cramping (26).

## 1.3 Human gut bacteria

### 1.3.1 Classification and prevalence

The term microbiota is often used in clinical science describing bacteria in humans or animals. Microbiota is more precisely defined as the microbial taxa associated with complex organisms (27). These taxa include bacteria, archaea, fungi, protists, and viruses (Figure 2), with bacteria comprising the bulk mass. Microbiome is the catalogue of these microbes and their genes, though the term is often used interchangeably with “microbiota”.

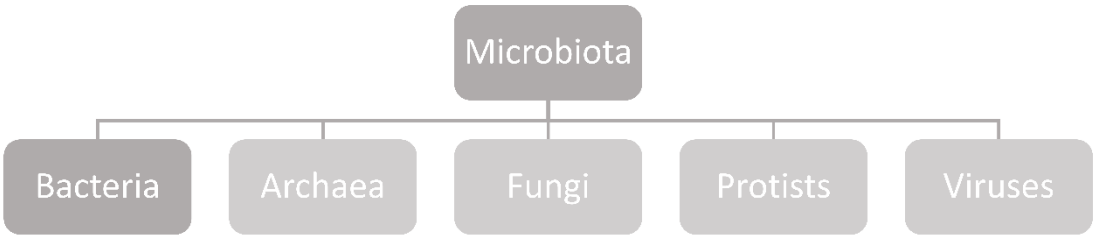


**Figure 2. Overview of microbiota**

The majority of bacteria throughout the human body reside in the colon. The prevalence of bacterial cells in the human gut has been estimated and repeatedly stated to outnumber human cells by tenfold. This estimate was recently questioned and suggested down-sized to $3.8 \times 10^{13,}$ and thus closer to the number of human cells in the body (28). The bacterial kingdom is further categorised into phylum,

order, class, family, genus and specie. The phylums firmicutes, bacteroidetes, actinobacteria and proteobacteria comprise the main bulk of the gut bacteria, of which firmicutes and bacteroidetes constitutes aproximately 90%. Other phylums includes cyanobateria, verrumicrobia, tennericutes and phylms not yet assigned (22).

### 1.3.2 Gut bacteria through life

The human gut bacteria changes through life and is affected by intrinsic and extrinsic factors such as age, mode of delivery, maternal microbiota, diet, environment, medication, and state of health (29). The diversity increases during childhood, remains relatively stable in adulthood and declines in old age. The diversity of microbes within a given body habitat can be defined as “the number and abundance distribution of distinct types of organisms” (30). In healthy adults, the gut bacteria appears relatively stable at phylum level, but considerably responsive at species level, although the bacterial composition generally returns to its original status after transient changes in diet or medication (31). The composition of the gut microbiota at species level varies widely between individuals, which complicates deciphering a common core microbiome in humans (30, 32). The composition appears on the other hand to be stable over longer periods (33) and bacterial functions seem to vary less between individuals than the actual species (34).

### 1.3.3 Gut bacteria in health and disease

In the last two decades, there has been an enormous developmental progress in methods for analysing bacteria. Genetic sequencing and taxonomic profiling of marker genes with faster and more cost efficient sequencing of DNA, have resulted in a rapidly evolving science field (22). This spiked an interest for the microbial community in the gut, additionally fuelled by observational studies revealing differences in gut bacteria between healthy people and people with sub-optimal health states (35, 36). Such differences are frequently referred to as dysbiosis.

The term dysbiosis however, does not seem to have a formal definition. It is variously explained as for instance; “imbalances in the composition and function of the intestinal microbes” (29), “a shift in the balance of microbiota composition such that it may become deleterious to host health” (37), and “any change to the composition of resident commensal communities relative to the community found in

healthy individuals" (38). Dysbiosis of the gut bacteria is also a controversial topic in itself, because it remains unclarified exactly what a healthy gut microbiota should constitute (39). Bacterial cross-feeding, high inter-individual variability of the microbial structure in the gut and confounding effects of medication complicate the process of establishing indicators of a healthy gut microbiota (30). So far, theories about healthy factors constituting a bacterial gut community appears mainly to be deduced from the corresponding opposite traits found to differentiate gut bacteria of overweight or diseased humans from healthy humans.

Metagenomic studies have found associations between low bacterial diversity and several states of health, such as obesity, irritable bowel syndrome, inflammatory bowel disease, and metabolic disorders (36, 40-46). Specific bacterial ratios at phylum-level have also been evaluated as possible unhealthy traits (35). Moreover, many of the studies reporting differences in the gut microbiota between healthy and diseased or overweight people are characterised by small sample sizes (35, 39).

Even though there is no clear consensus about what constitutes an optimal microbial community, some bacteria have been singled out as healthy over the years. These have gained formal status as probiotics and are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (47, 48). Species of the *Bifidobacterium* and *Lactobacillus* genera are examples of well-studied probiotics that have shown promising effects on glycaemic regulation in humans with type 2 diabetes (49). Furthermore, supplementation with bifidobacteria has also been shown to improve glucose tolerance in animal studies (35).

### 1.3.4 Gut bacteria in type 2 diabetes

Larger metagenomic, cross-sectional studies report that gut microbiota in type 2 diabetes differs from healthy individuals with lower diversity of the microbial community, less of the butyrate producing bacteria, and lower faecal concentrations of SCFA (41, 50, 51). Elevated levels of pathogenic bacteria, and functions related to oxidative stress response, such as enrichment of catalase and increased production of the antioxidant glutathione have also been found (50). Alongside these discoveries, the perception of the gut bacteria's role have changed from relatively passive inhabitants to active conduits with the ability to affect the hosts' metabolism

and defence against diseases. Belated identification of confounding medication commonly used in metabolic disturbances, may have weakened some of these associations, but not necessarily dismissed them (52). The question regarding causality also remains unanswered, although several inventive trials have been conducted in search for clarification (29, 51). All undetermined assumptions aside, alterations in gut homeostasis are currently under suspicion of contributing to the pathophysiology of type 2 diabetes (53).

The development in analysis of gut bacteria and discovery of changed bacterial composition in disease have motivated the research of various strategies of manipulating the gut bacteria to promote health. In addition to treatment with prebiotics and probiotics, effects of faecal transplantation have been investigated in human and animal studies (51, 54-57).

## 1.4 Short-chain fatty acids

The prebiotic fibres are fermented by gut bacteria into short-chain fatty acids (SCFA) in the colon. The SCFA mainly comprise acetic, propionic and butyric acid, but include formic and lactic acid as well (58). The acetic, propionic and butyric acids are commonly present in faeces in the molar ratio 60:20:20 (59). Lactate can also be metabolised to acetate, propionate and butyrate by cross-feeding. SCFA are used as energy source by the colonocytes, as substrates in the hepato-metabolic pathways, and are involved in regulation of transcription factors and the immune system (53, 60, 61). As depicted in figure 3, the SCFA may also act as signaling molecules (60). Studies conducted mainly in cells and animals have provided valuable insight into explanatory mechanisms and mapping of potential molecular pathways. Although questions remain unanswered and theories need confirmation, this scientific terrain is gradually expanding. By binding to the G-protein-coupled receptors (GPR), GPR41 and GPR43, they may cause various effects depending on the tissues affected (62, 63). In enteroendocrine L-cells, the SCFAs have been reported to increase release of the gut hormones glucagon-like peptide-1 and 2 (GLP-1 and 2) and peptide YY (PYY) in response to feeding (53), with the potential to improve regulation of blood glucose and appetite, as well as preserving intestinal integrity (53, 64).

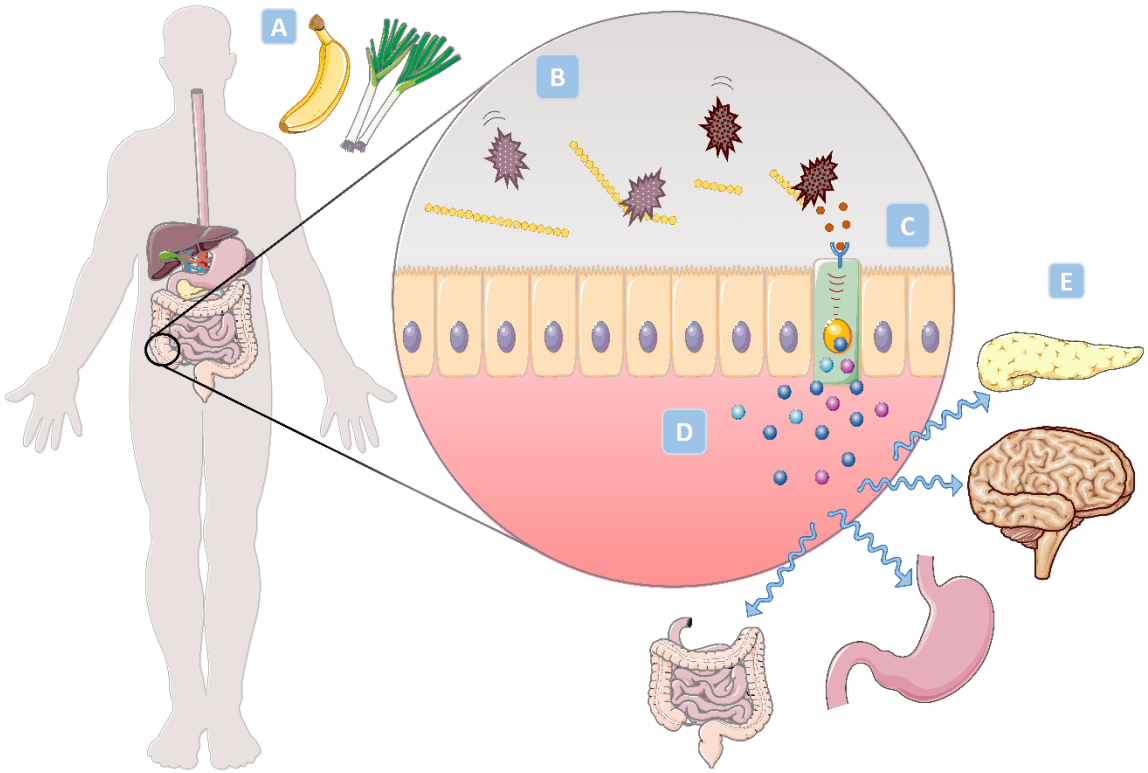


**Figure 3. Effect of prebiotics on glycaemic control and appetite, and possible pathways.** A) Prebiotic fibres escape digestion in the small intestine and B) are fermented into SCFA acids by gut bacteria in the colon. C) The SCFA bind to G-protein coupled receptors in enteroendocrine L-cells. D) This causes increased secretion of GLP-1, GLP-2, and PYY in response to a meal. E) GLP-1 improves regulation of blood glucose by enhancing release of insulin and suppressing release of glucagon from pancreas. GLP-1 also protects the beta-cells. GLP-1 and PYY enhance satiety by affecting the brain and the gastrointestinal system. GLP-2 maintains the intestinal barrier and may thus prevent systemic inflammation. GLP-1 and 2, glucagon-like peptide 1 and 2; PYY, peptide YY; SCFA, short-chain fatty acids. Figure was produced using Servier Medical Art

Butyric acid is of particular interest in type 2 diabetes as animal studies report it improves glucose homeostasis by inducing gut production of GLP-1 and PYY as well as protecting the barrier function in the gut (59, 65-67). The ability of butyric acid to induce proliferation of healthy cells as well as apoptosis of cancer cells in the colon, is referred to as the “butyrate paradox” (68).

The propionic and acetic acids appear less studied than butyric acid, but have been linked to mechanisms maintaining glycaemic control and may have anti-carcinogenic properties (68). Propionic acid may also reduce visceral and liver fat (68).

## 1.5 Gut hormones

The gastrointestinal tract is an endocrine organ that produces and secretes a wide array of hormones, including ghrelin, leptin, cholecystokinin, glucose-dependent insulinotropic peptide (GIP), GLP-1, GLP-2, and PYY. Studies conducted in rodents in particular, but also humans, have contributed with information about their possible involvement in regulation of appetite and blood glucose (69). Ghrelin, GLP-1, GLP-2 and PYY are the only gut hormones that will be further covered in this thesis.

### Ghrelin

Ghrelin is a 28-amino acid acylated peptide cleaved from preproghrelin, and the only gut hormone known to stimulate hunger (70). It is primarily produced in the stomach, and binds to the growth hormone secretagogue receptor (GHS-R), that is expressed in the brain and peripheral tissues. Ghrelin stimulates orexigenic neurons within the hypothalamus (71). During fasting, ghrelin concentration in plasma increases, while it declines postprandial. Although luminal nutrients are not the only suppressants of ghrelin, proteins and carbohydrates appear more efficient than fat. Studies conducted mainly in rodents have provided evidence for a strong role for ghrelin in meal-time hunger and meal initiation as well as long-term regulation of body weight (70). Ghrelin is mostly known as a hunger hormone, but is also involved in the regulation of blood glucose with inhibition of insulin secretion from the beta-cells (72).

### Glucagon-like peptide-1, GLP-1

GLP-1 is a 30 and 31-amino acid peptide with two biologically active forms (7-36) and (7-37), of which GLP-1 (7-36) is the predominant (73). It is cleaved from proglucagon and secreted from the enteroendocrine L-cells in response to feeding, and more efficiently from large than small meals (73). The GLP-1 receptor is expressed in pancreatic islets, brain, heart, kidney, and the gastrointestinal tract. The hormone has an important incretin role in the regulation of postprandial glucose concentrations in the circulation (74). In the pancreas, GLP-1 induces increased secretion of insulin from the beta-cells in a glucose-dependent manner and inhibits release of glucagon. GLP-1 also has a protective effect on the beta-cells. It inhibits apoptosis and promotes proliferation and differentiation of the cells (73).

GLP-1 promotes satiety by stimulating anorexigenic neurons in the hypothalamus, delaying gastric emptying. It also appear to be involved in the “ileal brake” effect, an endocrine inhibition of functions in the upper gut offset by nutrients in the lower gut (73, 74). GLP-1 is rapidly deactivated by the enzyme dipeptidyl peptidase IV (DPP4) (73). GLP-1 -analogues and DPP4-inhibitors are used in medical treatment of type 2 diabetes (75).

#### Glucagon-like peptide-2, GLP-2

GLP-2 is a 33-amino acid peptide cleaved from proglucaon (76). Similarly, to GLP-1, it is secreted from the enteroendocrine L-cells in response to feeding and is rapidly deactivated by DPP4. GLP-2 is on the other hand, mainly known as an intestinal growth factor (77). It binds to the receptor GLP-2R expressed by enteric neurons. The hormone maintains the intestinal integrity through several mechanisms. It has a trophic and protective effect on the intestine, and appears to enhance the barrier function, as well as to preserve the enteric nervous system during intestinal inflammation (76). GLP-2 has been approved for treatment of short bowel syndrome (77). GLP-2 may play a role in regulation of appetite and glycaemic control, but to what extent remains unclear (76).

#### Peptide YY, PYY

PYY is co-secreted with GLP-1 and GLP-2 from enteroendocrine L-cells in response to feeding (73). PYY is secreted as PYY (1-36) and is cleaved to the active form PYY (3-36) by DPP4. It binds to the Y2-receptor expressed in the hypothalamus and stimulates anorexigenic neurons. PYY also appear to be involved in the “ileal brake” effect (78). Concentrations are low during fasting, increase after feeding and remain elevated for two hours until slowly declining.

### 1.5.1 Appetite regulation

Readily available and palatable food challenges self-control, cognitive reasoning and impulsivity on a regular basis. Additional factors influence appetite in a complex orchestration, which is still poorly understood (79). Yet, involvement of the hypothalamus was studied as early as the mid-1900s (80). Studies of rodents in

particular, have contributed with further explanatory mechanisms of appetite regulation (69).

### Gut-brain axis

The communication between the gut and the brain is often referred to as the gut-brain axis. It is facilitated by efferent and afferent nerves and regulates the motility, in addition to secretory and sensory functions in the gastrointestinal tract (22, 69). The rate of gastric emptying is believed to constitute a major impact on appetite. The enteric nervous system innervating the entire gastrointestinal tract is connected to the brain, but may also control the gastrointestinal tract independently. The arcuate nucleus (ARC) within the hypothalamus has been acknowledged as central in the regulation of appetite and energy homeostasis. It contains two major clusters of neurons, the orexigenic agouty-related peptide (AgRP)/neuropeptide Y (NPY)-expressing neurons, that stimulates food intake and suppresses energy expenditure and the anorexigenic propiomelanocortin (POMC)/cocaine-amphetamine-related transcript (CART)-expressing neurons, that supresses food intake and increases energy expenditure.

Incoming information from the gut to the ARC in form of nutrients and gut hormones are processed and translated into state of appetite and metabolic status of the organism. The gut hormones may influence the ARC directly through the blood-brain barrier, or indirectly via afferent nerves (mainly vagal) that project the nucleus of the solitary tract (NTS) in the brainstem (Figure 4). The NTS is a sensory complex that registers smell, taste and vision. The NTS forwards the signals to the ARC. The ARC may also signal information to the NTS. Exchange of information between AgRP/NPY and POMC/CART and between ARC and NST involve positive and negative feed-back with complex fine-tuning and coordinating of signals before the summarised information is passed on from both the ARC and NTS to higher centres in the brain. The transferred information is sequentially integrated with hedonic information (reward-based sensations), which ultimately results in regulation of responses, such as feeding behaviour, energy expenditure and gastric emptying.

In addition to fermenting ITF into SCFA, the gut bacteria are capable of deconjugating primary bile acids into secondary bile acids. This prevents

recirculation of the bile acids. The secondary acids may act as signalling molecules by binding to the G-protein coupled receptor TGR5 in L-cells and muscles (81, 82). The resulting increased secretion of GLP-1 and enhanced energy expenditure improve the insulin sensitivity. Furthermore, SCFA and deconjugated bile acids may also modulate the neuronal activity in the gut-brain axis (22).

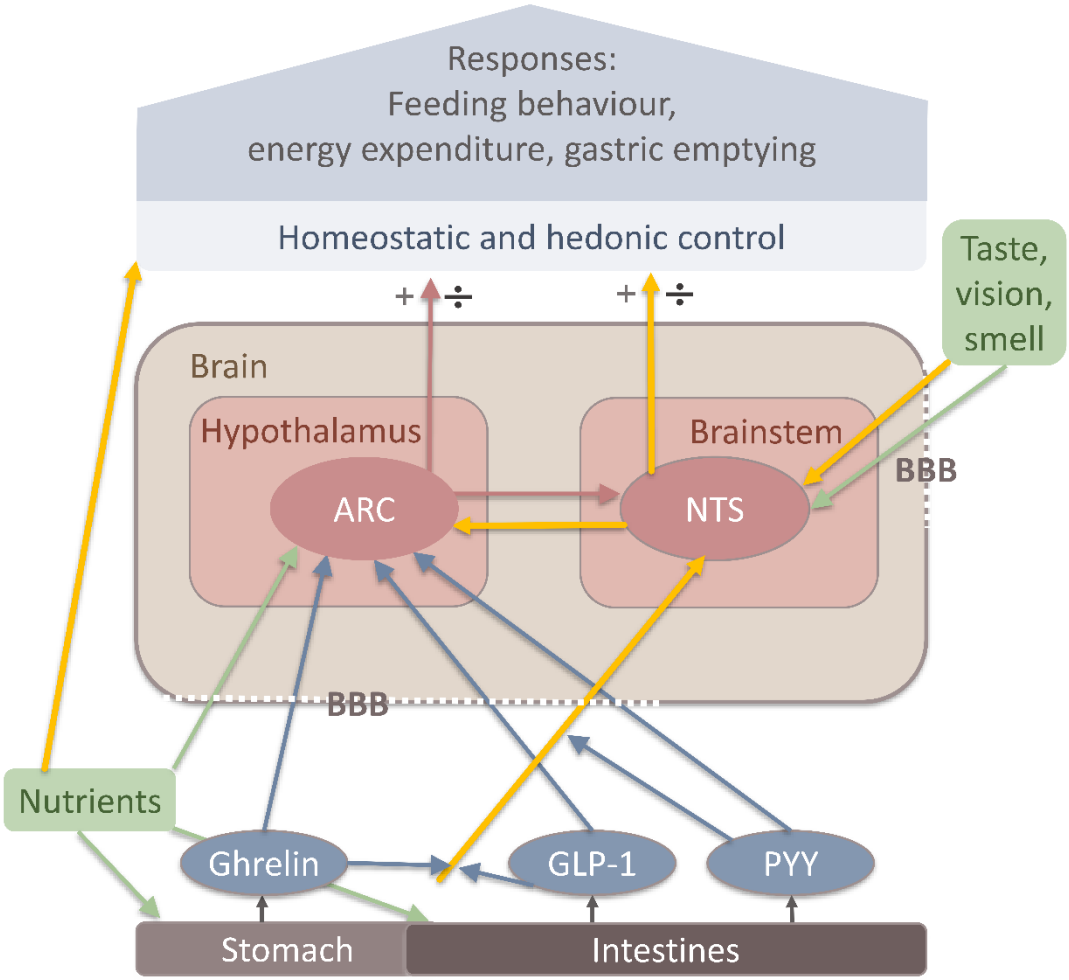


**Figure 4. Diagram of ghrelin, GLP-1 and PYY regulating appetite via the gut-brain axis.** Gut hormones may influence appetite in the hypothalamus directly via the blood-brain barrier or indirectly via afferent nerves. Yellow arrows indicate signalling via vagal nerves. Other arrows indicate transfer of signalling molecules. ARC, arcuate nucleus; NTS, Nucleus of the solitary tract; BBB, blood-brain barrier; GLP-1, glucagon-like peptide 1; PYY, peptide YY. Figure was produced using Servier Medical Art

## 1.6 Rationale of this thesis

Numerous trials report that inulin-type fructans supplemented in daily doses between 5 and 30 g may increase the abundance of bifidobacteria and SCFA in faeces, and enhance microbial diversity in healthy people and in non-diabetic patients (83-93).

Consumption of ITF have also been shown to enhance GLP-1 response, suppress excursions of blood glucose and increase fasting GLP-2 in healthy adults (94, 95). Additionally, increased GLP-2 response and reduced intestinal permeability have been demonstrated in obese mice after consumption of oligofructose (96). Although enhanced GLP-1 response, improved regulation of glucose and appetite, suppression of energy intake and weight loss could benefit patients with type 2 diabetes in particular, studies of the potential role of prebiotic fibres in this population are scarce. Moreover, we were not able to identify any clinical trials investigating the effect of inulin-type fructans on gut microbiota and fermentation in this population.

# 2 Aims

## 2.1 General aims and hypotheses

The overall aim of this thesis was to evaluate the prebiotic effect of inulin-type fructans on regulation of glucose and appetite in patients with type-2 diabetes.

We hypothesised that treatment with inulin-type fructans for six weeks would induce positive changes in the composition of gut microbiota, such as enriched concentrations of bifidobateria and butyrate producers, increased bacterial diversity, and increased concentrations of faecal SCFA. Furthermore, we hypothesised that increased production of SCFA would enhance release of the gut hormones GLP-1, PYY, and GLP-2, and suppress concentrations of ghrelin. This was in turn hypothesised to induce improved glycaemic control and improved regulation of appetite.

## 2.2 Spesific aims

### 2.2.1 Paper I

The aim was to investigate the effect on faecal microbiota and SCFA.

### 2.2.2 Paper II

The aim was to investigate the effect on responses of glucose, insulin, GLP-1, and GLP-2 to a meal tolerance test.

### 2.2.3 Paper III

The aim was to investigate the effect on appetite measured by responses of ghrelin and PYY to a standardised mixed meal, energy intake at an *ad libitum* lunch, and subjective ratings of appetite before and after the lunch.

# 3 Methods

## 3.1 Study design

This thesis is based on the Fiberdia study (paper I - III), a single-centre clinical trial investigating the effect of inulin-type fructans in patients with type 2 diabetes. The trial has a randomised, placebo controlled, and double-blind crossover design, and was conducted between February 2016 and December 2017 at the Diabetes Research Laboratory, Oslo University Hospital, Aker.

Two master students in clinical nutrition contributed in data collection, supervised by the PhD student and PhD supervisor. Prior to the intervention, the students received practical training at the Diabetes Research laboratory, Oslo University Hospital, Aker, supervised by clinical dietitians and research nurses.

## 3.2 Sample size

The sample size was calculated based on expected effects on the primary outcome measurement: GLP-1-response to a standardised meal. However, in the planning of this study, few data were available in the literature for a power calculation. Hence, the sample size was calculated based on changes in area under the curve (AUC) for GLP-1 response after a pharmaceutical intervention in patients with type 2 diabetes where mean (95% CI) difference between treatment and placebo was 2.34 (1.32, 3.35) pmol/L*min (97). This provided a tentative sample size of 23 individuals to achieve 80% power at alpha = 0.05. To account for dropouts and a possibly lower treatment effect because of differences in study design and intervention, we added 12 individuals, giving a total of 35 participants required for randomisation.

## 3.3 Participants

Adult men and women with type 2 diabetes were invited consecutively as they attended the Diabetes Outpatient Clinic at the Department of Endocrinology, Morbid Obesity, and Preventive Medicine at Oslo University Hospital. Participants were also recruited by advertisement in social media, posters in the hospital lobby and pharmacies, and from general practices.

Eligibility for participation was determined at a screening visit at a minimum of four weeks prior to enrolment. Eligible patients had a BMI $\leq$ 40 kg/m$^2$, $HbA_{1c}$ < 86 mmol/mol (10.0%), and were not treated with insulin or glucagon-like peptide-1 (GLP-1) analogues. Exclusion criteria were consumption of dietary supplements containing prebiotics or probiotics, treatment with antibiotics within the last 3 months, weight changes of > 3 kg within the last month, fibre intake > 30 g per day, performance of high intensity exercise, planned or present pregnancy, drug or alcohol dependence, and long distance from home to the study centre. Patients diagnosed with either dementia, chronic bowel diseases, irritable bowel syndrome, celiac disease, or cancer within the last five years, were not included in the study.

In total, 131 patients were assessed for eligibility and 35 were randomly allocated to start with either ITF or control supplement (Figure 5). Long distance from home was the main reason for exclusion.

Of those who were allocated, four participants were excluded or withdrew before initiating the first intervention period. Two additional participants were diagnosed with illness during the intervention, and were excluded from all analysis.

A total of 29 participants were included in the analyses for glucose, insulin, visual analogue scale (VAS) scores, and energy intake. Analyses for all gut hormones and SCFA were performed only for the 25 participants that attended all four visits. Due to one one sample with low amounts of extracted DNA, data from 24 of these participants were included in the analyses of gut microbiota.

Among the four participants who did not attend all four visits; two withdrew for personal reasons, one was diagnosed with dementia and one started treatment with antibiotics for an infection during the washout period.

None of the participants was excluded or withdrew for reasons related to the intervention.

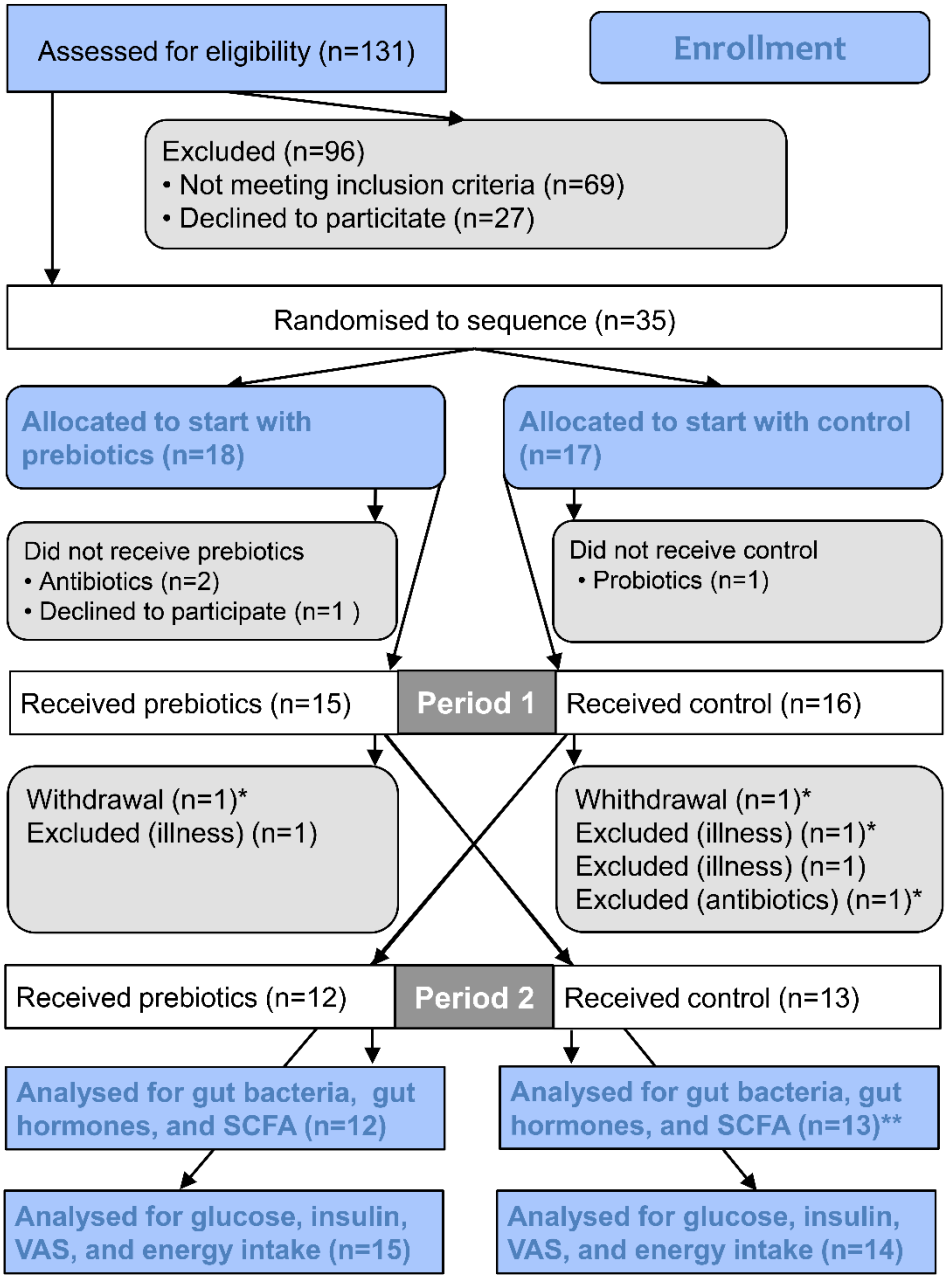


**Figure 5. Flow chart showing all subjects approached for the study**. *Included in analyses for glucose, insulin, appetite scores and portions; **For microbiota analysis n=12 due to one low-quality sample; SCFA, short-chain fatty acids; VAS, visual analogue scale

## 3.4 Randomisation and blinding

Randomisation lists were generated by a statistitian (not involved in the conduct of the study) using a randomisation command for 2 x 2 crossover studies in Stata version 14 software. The treatment allocation was concealed for both participants and clinical investigators.

## 3.5 Intervention

For two periods of six weeks, separated by a four-week washout, the participants consumed 16 g per day of inulin-type fructans (a 50/50 mixture oligofructose and inulin; Orafti® Synergy1, Beneo GmbH, Germany) and a control supplement (maltodextrin) in randomised order (Figure 6). The supplements were consumed in addition to their ordinary diet.

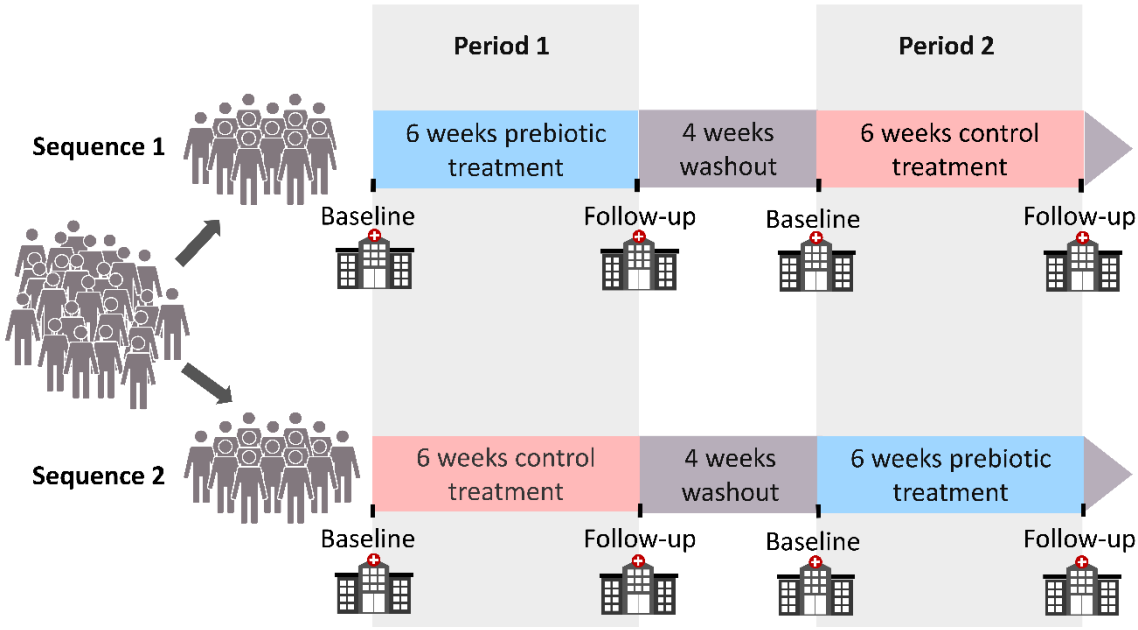


**Figure 6. Overview of the study design**

The supplements were powdered, similar in appearance and taste, and wrapped in unlabelled and identical opaque sachets of 8 g. To allow for adaptation, the participants were instructed to consume one sachet per day the first week, and increase to two daily sachets for the remaining 5 weeks. They mixed the supplements into food or drinks, and ingested whenever convenient. For estimation of compliance, the participants were asked to return all unused sachets.

Furthermore, the participants were repeatedly reminded to maintain their habitual life-style during the trial and to avoid making changes regarding medication. Two days prior to the visits, diabetes medication was discontinued, and the participants were instructed to avoid strenuous exercise one day in advance.

## 3.6 Outcome measures

### 3.6.1 Data collection

#### Faecal collection

The participants were provided with sterile plastic containers for collection of faecal samples at home one day prior to each of the four visits. They were instructed to instantly store the samples in a freezer. The samples were transported to the clinic in cooler bags containing freezer blocks.

Before and after both intervention periods, the participants attended the hospital for examinations after an overnight fast (Figure 7). On arrival, they delivered the faecal samples that were immediately stored at -80℃ for later analyses of gut bacteria and SCFA. They also delivered food frequency questionnaire (FFQ) for assessment of diet at the first baseline and both follow-up visits. Changes in GI-symptoms were reported after both intervention periods.

During the morning, a meal tolerance test was performed, proceeded by an appetite test (Figure 7) initiated at lunch time.

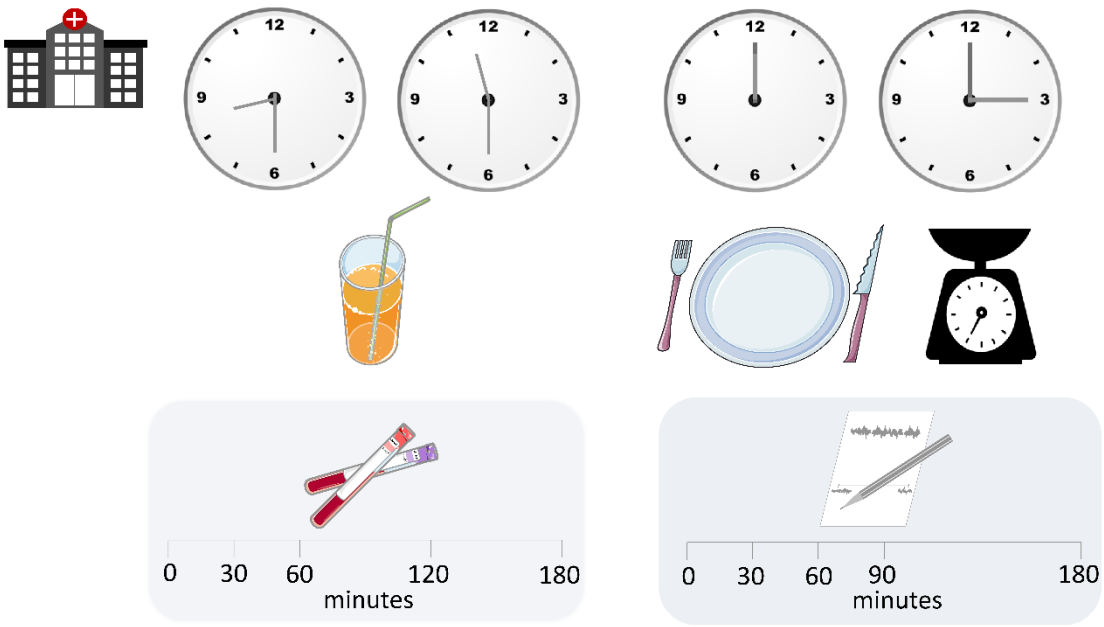


**Figure 7. Overview of time line for tests during visits.** Figure was produced using Servier Medical Art

#### Meal tolerance test

An intravenous catheter was inserted in an antecubital vein. Blood samples for glucose, insulin, GLP-1, and GLP-2 measurements were collected in fasting state (time 0) and 15, 30, 45, 60, 90, 120, 150, and 180 min after initiation of a standardised mixed meal. Blood samples for measurement of ghrelin and PYY were collected and at time 0, 30, 60, 120, and 180 minutes. The meal consisted of two nutritional drinks (200 ml Fresubin 2 kcal Drink vanilla and 100 ml Fresubin Jucy Drink apple), constituting: 550 kcal, 78.5 g carbohydrate, 24 g protein and 15.6 g fat. The drinks were consumed within 12 minutes.

#### Appetite test

After the three hour meal tolerance test, the participants were served an *ad libitum* lunch. The meal was a mixed casserole dish of pasta with meatballs (Fjordland, ready meals) constituting: 114 kcal, 17.3 g carbohydrate, 4.9 g protein, and 2.5 g fat per 100 grams. The participants were instructed to eat as much as desired, and the lunch was consumed within 30 minutes. Water was served unrestricted with the meal. All food ingested was weighed and registered by the participants on a kitchen scale with accuracy of 1.0 g. Subjective feeling of appetite was measured before (time 0) and 30, 60, 90 and 180 min after meal initiation using VAS (98-100).

### 3.6.2 Clinical data

#### Blood pressure

Blood pressure was measured in sitting position using an automatic blood pressure monitor (Omron HEM-711 DLX) with the cuff positioned at the upper arm. Three measurements were performed, with the second and third measurements averaged and used.

#### Medication

Use of medication was assessed at the screening and at the visits along with a reminder to keep the medication unchanged during the trial.

#### Anthropometric measurements

Height was measured with standard altimeter at the screening visit. A body composition analyser (Tanita BC-418 MA Segmental Body Composition Analyzer) was used for assessment of weight and bioimpedance at every visit. The participants were examined with bare feet and light clothing.

### 3.6.3 Laboratory analyses

#### Microbiota

Analysis of gut bacteria was performed at Nofima-Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås. Bacterial DNA was extracted from faecal content (approximately 100 mg) by mechanical and chemical lysis using the DNeasy PowerSoil HTP 96 Kit (Qiagen), following the manufacturer's protocol. The mechanical lysis step with bead beating was done twice using the FastPrep®-96 homogenizer (MP Biomedicals) for 60 seconds at 1600 rpm. Then samples were centrifuged for 6 minutes at 4500 x g as described in the protocol. The microbiota was analysed by 16S rRNA amplicon sequencing (2 x 150 bp) of the variable region 4 following an in-house protocol (101), which is presented in detail in supplementary methods of Caporaso *et al.* (102). The current primers (103-105) have been modified from the original 515F–806R primer pair, with barcodes now on the forward primer and degeneracy added to both the forward and reverse primers to remove known biases. The sequencing was done on a MiSeq (Illumina) at Nofima using pooled polymerase chain reaction (PCR) samples, which were based on triplicate PCRs per DNA sample using sample-specific barcoded forward primers. PhiX Control v3 was included and accounted for 10% of the reads. The MiSeq Control Software (MCS) version used was RTA 1.18.54.

Data processing of the sequencing reads was performed using the pipelines in Quantitative Insight Into Microbial Ecology (QIIME) v.1.9 (106). Briefly, the total number of reads was 15 217 265 followed by 9 007 278 reads after joining forward and reverse reads and removal of barcodes that failed to assemble. The sequences were demultiplexed into representative sample taqs and quality filtered, allowing zero barcode errors and a quality score of 30 (Q30), resulting in 7 550 212 sequences. Reads were assigned to their respective bacterial taxonomy (operational taxonomic unit: OTU) by clustering them against the Greengenes reference sequence collection

(gg_13_8) using a 97% similarity threshold. Reads that did not hit a sequence in the reference sequence collection were clustered *de novo*. Chimeric sequences were removed using ChimeraSlayer, and all OTUs that were observed fewer than 2 times were discarded. This resulted in an OTU table containing 15 168 different OTUs, which was based on a total of 6 642 085 read counts. The OTU table was used for microbial (alpha) diversity analysis using equal number of sequences across samples, i.e. alpha rarefaction, where the OTU table was resampled to an even depth of 13 000 sequences per sample. Summary tables at phylum, order, family and genus levels were constructed from the OTU table (i.e. OTU level/species level). The data was transformed by centered log2 ratios, in order to stabilise the variation and remove dependencies between abundance variables. At any taxonomic level, bacteria groups that were present in less than 50% of the subjects were combined into one group (called “rare”), as it is not possible to make statistical inference on individual rare bacteria groups. Square brackets around taxonomic names (e.g. [Ruminococcus]) are taxa proposed by Greengenes based on genomic trees, but are not verified taxonomies.

### Short-chain fatty acids (SCFA)

Analyses of SCFA were performed at Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital in Oslo. Upon analysis, 0.5 g of the faecal material was homogenised after addition of distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of $H_2SO_4$; 2.5 mL of the homogenate was vacuum distilled, according to the method of Zijlstra *et al.* (107), as modified by Høverstad *et al.* (108). The distillate was analysed with gas chromatography (Agilent 7890 A, CA, USA), using a capillary column (serial no. USE400345H, Agilent J&W GC columns, CA, USA), and quantified using internal standardisation. Flame ionization detection was employed. The following SCFA were analysed: acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic and isocaproic acids. The results were expressed in mmol/kg wet weight. In addition, we calculated the proportional distribution of individual SCFA to total SCFA.

### Glucose

Whole-blood glucose was measured concurrently with sampling by a glucose oxidase method (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH), and plasma glucose concentrations calculated (whole-blood glucose x 1.119) (109).

### Insulin

Blood for insulin analysis was sampled in tubes without anticoagulant. Serum was separated by centrifugation at 3500 × g at room temperature for 12 min. Insulin was measured at the Hormone Laboratory, Oslo University Hospital, Aker using Modular Analytics E170 (Roche, Switzerland) (110). The minimum detectable concentration of the assay was 1.39 pmol/L, and inter-assay CV was ≤ 4% for insulin analysis.

### GLP-1 and GLP-2

Blood for GLP-1 and GLP-2 analyses was collected in EDTA tubes added 40 μl DPP-IV inhibitors (Merck Millipore, Germany) and 40 μl Pefabloc® SC (Merck Millipore, Germany). Plasma was separated by centrifugation at 3500 × g at 4°C for 10 minutes and aliquots stored at -80°C in biobank for later analysis.

GLP-1 and GLP-2 were measured at Department of Biomedical Sciences, University of Copenhagen, Denmark. All samples were extracted in a final concentration of 70% (GLP-1) or 75% (GLP-2) ethanol before measurements. Total GLP-1 was measured as described by Orskov *et. al* (111) using a radioimmunoassay (antibody code no 89390) specific for the C-terminal of the GLP-1 molecule and reacting equally with intact GLP-1 and the primary (N-terminally truncated) metabolite. Intact GLP-2 was measured using a radioimmunoassay originally described by Hartmann *et. al* (112). The antiserum (code no. 92160) is directed against the N-terminus of GLP-2 and therefore measures only fully processed, active GLP-2 of intestinal origin. Sensitivity for both assays was below 1 pmol/l, and intra assay coefficient of variation below 10%.

### Ghrelin and PYY

Forty μl DPP-IV inhibitors (Merck Millipore, Germany) and 40 μl Pefabloc® SC (Merck Millipore, Germany) were added to the EDTA tubes in advance. Plasma was separated by centrifugation at 3500 rpm at 4°C for 10 minutes and aliquots stored at

-80°C in biobank for later analysis of acylated ghrelin and total PYY at the Hormone Laboratory, Oslo University Hospital.

Acylated ghrelin and total PYY were analysed in duplicates using Human Metabolic Hormone Magnetic Bead Panel (Metabolism Multiplex Assay, Merck Millipore, Germany) and Luminex 200 Technology (Invitrogen, Thermo Fisher, USA). The minimum detectable concentration of the assay was 13.7 pg/mL for acylated ghrelin and 41.2 pg/mL for total PYY. For both hormones, the intra- and inter-assay coefficient of variation was < 10% and < 15%, respectively.

### 3.6.4 Patient-reported outcome measures

#### Food frequncy questionnaire (FFQ)

The FFQ used was a validated, self-administered questionnaire assessing the total diet (113, 114). Participants were instructed to fill in questionnaires based on their eating habits during the last six weeks. The FFQs were scanned by a master student in Clinical Nutrition at Department of Nutrition, Faculty of Medicine, University of Oslo.

#### Subjective sensation of appetite

To assess the subjective sensation of appetite a questionnaire with VAS was used. The VAS-questionnaire used in this trial was a validated, self-administered questionnaire (98-100) and consisted of four questions (Table 1). The participants answered by drawing a vertical mark on a 100 mm line with opposing terms at the ends.

**Table 1. Questions in the visual analogue scale**

| Questions | | Answer at 0 mm | Answer at 100 mm |
|---|---|---|---|
| Hunger: | How hungry do you feel? | I am not hungry at all | I have never been more hungry |
| Satiety: | How satisfied do you feel? | I am completely empty | I cannot eat another bite |
| Fullness: | How full do you feel? | Not full at all | Totally full |
| Prospective food consumption: | How much do you think you can eat? | Nothing at all | A lot |

### Gastrointestinal symptoms

After finishing eacht treatment, the participants answered a non-validated questionnaire with simple basic questions about changes in gastrointestinal symptoms concerning the last six weeks (abdominal discomfort, diarrhoea, constipation, bloating, and flatulence) with a word rating scale: much worse, worse, unchanged, better, and much better.

## 3.7 Statistical analyses

Data analyses were perfomed using IBM SPSS Statistics version 25.0 and 26.0 software (IBM SPSS Inc,. Armonk, NY: IBM Corp.), MATLAB (R2018b, The MathWorks Inc.), Minitab Statistical Software version 18.1 and R Statistical Software version 3.6.1.

In all papers, baseline characteristics were reported as mean (range), (SD) or n (%). Reported *P*-values are two-sided and $P < 0.05$ was considered significant for all tests.

Analysis of gastointestinal symptoms was performed with Wilcoxon Signed Rank test. Spearman's rank correlation test was used to explore relations between

measures assessed at baseline in the first period, including concentrations of fecal SCFA, gut hormones, glycaemic regulation and ratings of appetite.

### 3.7.1 Paper I

#### Gut bacteria

The statistical analysis of gut bacteria was performed by statistician at Nofima - Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås. Since the gut microbiota consist of many interrelated bacteria groups, multivariate methods were used for these analyses. The intervention effect was analysed by a multivariate version of ANOVA, called analysis of variance simultaneous component analysis (ASCA) (115). This method is a combination of fixed-effects ANOVA and principal component analysis (PCA), and shows how the experimental factors affect the microbial community as a whole. Significance of the multivariate effects is estimated by permutation testing, and interpretation of the effects can be done by inspecting scores and loadings from PCA for each experimental factor. Identification of individual bacteria groups that differed in abundance between the placebo and treatment group was done by further analysis of the ASCA treatment effect, using partial least squares discriminant analysis (PLS-DA) (116) combined with variable importance in prediction (VIP). The VIP value represents the contribution of each bacteria group on the group discrimination, and it is usal practise to set a cutoff-value close to one.

The microbial diversity can be estimated by various metrics such as observed OTUs, phylogenetic distance (PD) whole tree and chao1. These are usually highly correlated, and because of their univariate nature they were analysed with linear mixed model (LMM). ‘Treatment‘ (control/prebiotics), ‘Day‘ (baseline/6 weeks follow-up), and ‘Period‘ were defined as fixed effects and ‘Subject‘ as random.

The multivariate regression method Partial least squares regression (PLSR) was used to analyse the assosiation between the bacteria and other endpoints (e.g. SCFA) and baseline characteristics (e.g. medication and initial fiber intake). Variable importance was also here estimated by the VIP method.

#### Short-chain fatty acids (SCFA)

The statistical analysis of SCFA was performed by the PHD student. Data from 25 participants were included. The variables total SCFA and the individual SCFA were skewed, and log transformation did not improve their distribution. Concequently, the effects of inulin-type fructans on SCFA were analysed using Wilcoxon Signed Rank test and $P < 0.05$ (two-tailed) was considered statistically significant. The results from SCFA analyses were reported as medians ($25^{th}$-$75^{th}$ percentiles).

### 3.7.2 Paper II

The statistical analyses were performed by a statistician at Oslo Centre for Biostatistics and Epidemiology, Faculty of Medicine, University of Oslo. Data from 25 participants were incuded in the analyses of gut hormones and data from 29 particiants were included in the analyses of glucose and insulin.

For each of the four parameters of interest, trajectories across the 9 measurement points were averaged over individuals at baseline and at each of the four visits for both the active and the control treatment.

The uni-modal and potentially asymmetrical shapes were accommodated by letting the response vary with combinations of Time, $Time^2$ and log(Time). Various normalising transformations of the responses were considered. Analyses were performed using LMM and repeated measures were accounted for. Besides the time variables the models included effects of ‘Day’ (baseline/6 weeks follow-up), ‘Treatment’ (control/prebiotics). The curves were allowed to attain different shapes for the two treatments by including terms for interactions with the time variables. GLP-1, GLP-2 and insulin were log-transformed whereas glucose was fitted untransformed. GLP-1 and GLP-2 used Time and log(Time) as dependent time variables. Glucose and insulin used Time and $Time^2$ as independent time-variables. The models were controlled for effects of period, and order of treatment, and were adjusted for age and sex. Finally, we checked the models for goodness of fit by residual plots and quantile-quantile (QQ) plots. Not all curves were tractable for integration, and hence we used predicted mean curves for calculation of empirical AUC values and bootstrapping (117) for assessing the uncertainties of the AUCs.

A potential correlation between GLP-1 and the associated microbial data was assessed by considering the residuals from the mixed model, constrained to the data

from the control arm of the trial. The model was adjusted for age and gender and time variables, and used subject as a random effect with period and day as random coefficients.

The results were reported as model based means ± SE or with 95% confidence intervals.

### 3.7.3 Paper III

The statistical analyses were performed by the PhD student. Data from 25 participants were included in the analyses of appetite hormones and data from 29 participants were included in the analyses of VAS-scores and portion size. The results were reported as model based means ± SE or with 95 % confidence intervals.

The VAS-scores, ghrelin, PYY, and energy intake were analysed using LMM. For all outcomes, mean differences between prebiotics and the control supplement (between treatments) and between baseline and 6 weeks (within treatments) were analysed. Repeated measures were accounted for, according to best model fit. Fixed effects in the models were ‘Treatment‘ (prebiotics/control), ‘Day‘ (baseline/6 weeks), with their interactions, and ‘Minutes‘. The effect of period was tested in all models, and removed if not significant. As potential confounding factors, we evaluated the effects of gender, age, baseline BMI, and metformin. The impact of portion size on VAS scores was investigated as well. *Post hoc* comparisons of treatments at individual time points were tested with LMM and Bonferroni correction.

Moreover, area under the curves (AUC) for hormones and VAS-scores were calculated by the trapezoidal rule. AUCs and energy intake at the lunch were analysed with the same approach as described above, but without minutes as fixed effect. Normality of residuals was investigated with QQ plots and Shapiro-Wilk test, and the outcome measures transformed if appropriate.

## 3.8 Ethics and funding

This trial was approved by the Regional Ethics Committee for Medical and Health Research, and registered at clinicaltrials.gov (NCT02569684). Prior to study

enrolment, all patients were thoroughly informed about the study procedure, expected benefits and potential adverse effects. Written informed consent was obtained from all included subjects. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and later amendments.

The research was supported by the DAM Foundation (20132--267) and the Norwegian Diabetes Association (36660), the Norwegian Levy on Agricultural Products (FFL; Project NFR 262300, 262306 and 262308) and Mills AS (36660), Oslo, Norway. Synergy1 and placebo were provided free of charge by Beneo, Mannheim, Germany.

The funders of the trial had no influence on study design, interpretation of results, or writing or publication of the papers.

# 4 Main results – summary of papers

The mean (± SD) age of the 29 participants that completed the trial, was 61.5 ± 11.7 years, and BMI 28.9 ± 4.5 kg/m$^2$. Their mean HbA1C was 52 ± 10.9 mmol/mol (6.9 ± 1.0%), diabetes duration 5.1 ± 4.4 years and eight subjects (27.6%) did not use glucose lowering medication. Apart from a reported higher intake of dietary fibre (mean 31.5 ± 10.2 g/day), the participants characteristics seemed to be representative of Norwegian patients with type 2 diabetes. The baseline characteristics did not significantly differ between the 25 subjects that attended all visits and the total study population.

The compliance was high, and only mean (range) 3.3% (0-20.8%) of the prebiotic sachets and 4.3% (0-22.1%) of the control sachets were returned. After treatment with prebiotics, 16 participants (64%) reported passage of gas and flatulence to be worse or much worse than before, while only 2 of the participants (4%) expressed the same complaints after the control treatment ($P < 0.001$). There were no significant changes in other gastrointestinal symptoms or any adverse effects during the trial.

The test of correlations at baseline in the first period showed that the GLP-1 response (GLP-1 AUC) to the standardised mixed meal was inversely related to the sensations of hunger (Hunger AUC) assessed at the *ad libitum* lunch (Figure 8). Similarly, the response of ghrelin (Ghrelin AUC) was inversely related to the sensations of fullness (Fullness AUC).

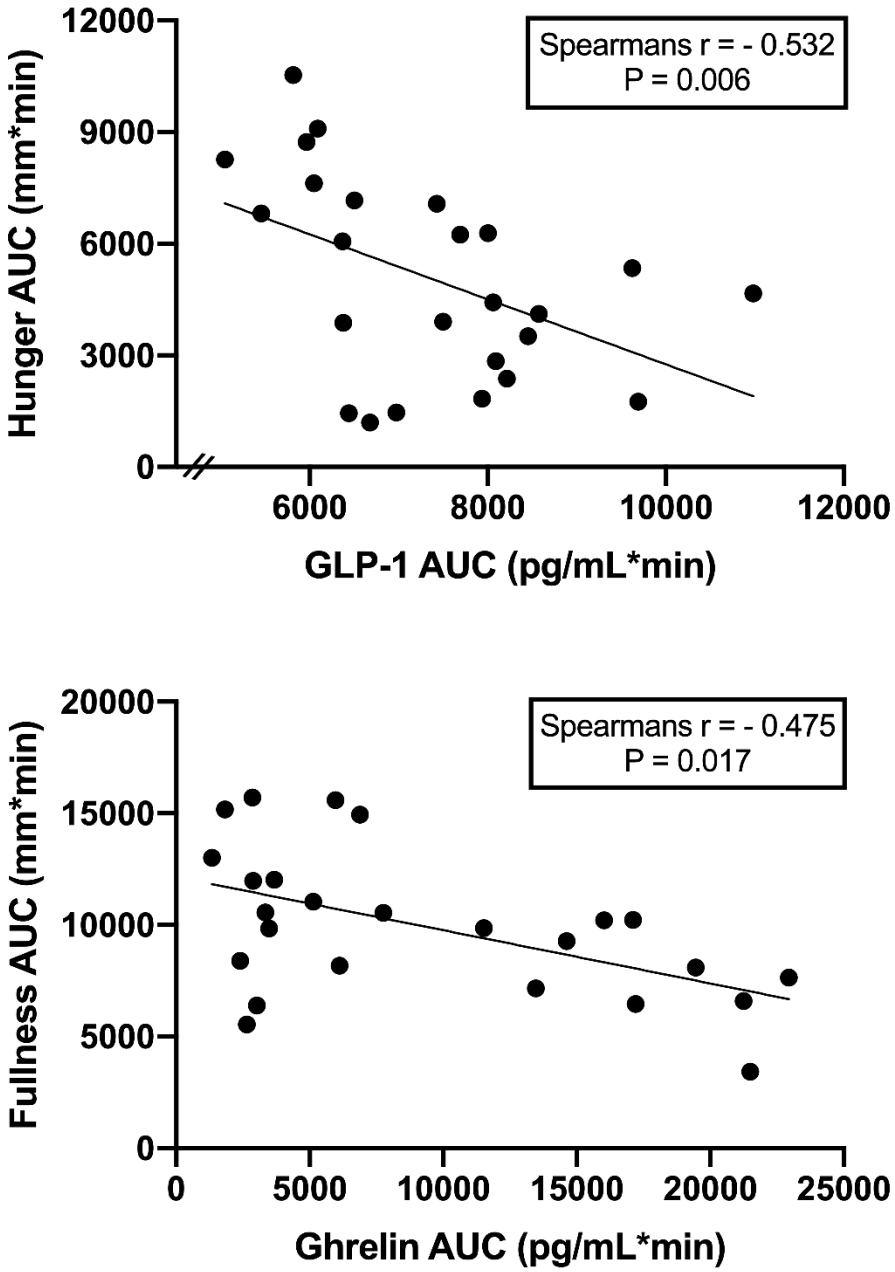


**Figure 8. Correlations at the baseline in the first period.** Upper: Inverse relation between responses of GLP-1 and sensation of hunger. Lower: Inverse relation between responses of ghrelin and sensation of fullness. GLP-1, glucagone-like peptide-1. AUC, area under the curve

## 4.1 Paper I

Prebiotic effect of inulin-type fructans on faecal microbiota and short-chain fatty acids in type 2 diabetes: a randomised controlled trial

At phylum level, the overall 2.2% change after prebiotic treatment, did not reach significance when compared to the control treatment (*P* = 0.091), although, the phylum Actinobacteria (VIP 1.32) was significantly affected by prebiotics compared to control. At species level, we found an overall change of 1.5% after prebiotics, significantly different from the control (*P* = 0.045). Thirty-two OTUs were significantly affected by the prebiotics. Of these, *Bifidobacterium* was the most altered genus, with highest positive effect on OTUs of *Bifidobacterium adolescentis*.

The participants had significantly higher faecal concentrations of total SCFA (*P* = 0.04), acetic acid (*P* = 0.02), and propionic acid (*P* = 0.04) after prebiotic consumption compared to control. The prebiotic fibre did not appear to have any effect on the concentration of butyric acid or the overall microbial diversity.

When investigating relations between OTUs and SCFA, some patterns emerged. In general, acetic acid related positively with OTUs that increased after the prebiotic consumption and negatively with OTUs that decreased. Interestingly, the OTUs of *Bifidobacterium adolescentis* that increased after prebiotic treatment related negatively with butyric acid. Among the prebiotic affected OTUs, only Lachnospiraceae OTU514272 was positively related to butyric acid. Valeric acid related positively to OTUs that declined after prebiotic consumption.

## 4.2 Paper II

### Effects of prebiotics on postprandial GLP-1, GLP-2 and glucose regulation in patients with type 2 diabetes. A randomised, double-blind, placebo-controlled crossover trial

Consumption of 16 g inulin-type fructans per day for six weeks did not affect fasting or postprandial glucose, insulin or GLP-2 concentrations. There was however a 4.8% reduction in plasma GLP-1 concentrations after the prebiotic treatment significantly different from the 8.6% increase after the control treatment ($P < 0.001$). These differences did not remain significant in the corresponding AUCs.

We found no effect of metformin on the GLP-1 response, and the *post hoc* analysis displayed no correlations between changes in GLP-1 responses and the gut bacteria at any taxonomical level.

## 4.3 Paper III

Prebiotic effect of inulin-type fructans on appetite in type 2 diabetes: A randomised controlled crossover trial

We found no effect of ITF over the control supplement on ghrelin or the ratings of hunger, satiety, fullness or prospective food consumption. Energy intake at the *ad libitum* lunch and the daily energy intake during the study did not differ between the treatments either. However, the response of PYY increased significantly after the control treatment with (mean ± SEM) 11.1 ± 4.3 pg/mL when compared to the prebiotic treatment -0.3 ± 4.3 pg/mL ($P = 0.013$). The habitual energy intake assessed with FFQ appeared unchanged during the trial.

Throughout the trial, male participants had 58.7 ± 25.5 pg/mL higher concentrations of PYY (P = 0.03), rated satiety 21.4 ± 7.4 mm higher ($P = 0.001$), and sensation of fullness 33.2 ± 8.5 mm higher ($P < 0.001$) than the females. More expectedly, they also consumed more than females from the *ad libitum* lunch throughout the trial, with a mean difference of 200.6 ± *85.2* kcal ($P = 0.03$). Analysing the genders separately did not change any conclusions. The corresponding AUCs mainly displayed similar traits as the marginal means. The covariates age, baseline BMI and metformin did not affect any of the outcomes.

Regardless of treatment order, PYY increased by 5.5 ± 2.3 pg/mL ($P = 0.019$), and hunger ratings decreased by 5.5 ± 1.2 mm ($P < 0.01$) between the first and second period.

# 5 Discussion of methods

Randomised controlled trials are considered the gold standard, but are not excluded from methodological considerations (118). Potential sources of errors, such as selection bias, confounding and information bias are discussed in the following sections along with the countermeasures taken.

## 5.1 Study design

The crossover approach was chosen due to previously reported large inter-individual variability in microbial response to dietary interventions (22), allowing each participant to serve as their own control. Furthermore, the crossover design is suitable in trials investigating chronic diseases such as type 2 diabetes, where patients' underlying condition is relatively stable and the anticipated treatment effect is relief of symptoms rather than a cure (119, 120).

Drawbacks concerning crossover trials are the prolonged duration for participation and doubling of the workload burden for the participants (121). This increases the risk of missing data due to participants dropping out for various reasons, such as illness, need for antibiotics, or weariness. This was a constant consideration in the planning of the study and multiple steps were taken to enhance compliance and prevent dropouts. The participants were provided with a phone number to call or text message, and an e-mail address for whenever they had any questions or concerns. In addition to admission letters with the appointments sent in the mail, the participants also received notifications by text messages on their phones (Table 2).

**Table 2. Notifications sent to participants by text messages to their phones**

| Time sent | Message |
|---|---|
| Two days in advance of a hospital visit | It is time to produce a faecal sample and avoid strenuous exercise |
| One day in advance of a hospital visit | It is time to (fill out the FFQ,)[a] stop taking diabetes medication, and initiate fasting at midnight. Remember to bring the faecal sample(, FFQ)[a] (, unused sachets of supplement)[b] (, and the 180 min-VAS from the previous hospital visit)[c] |
| 180 minutes after initiation of the *ad libitum* lunch | It is time to fill out the 180 min VAS (, and remember to start taking the supplement today, one sachet per day)[d] (, and remember to send it in the mail)[e] |
| One week after initiation of an intervention period | It is time to increase to two sachets of supplement per day |

[a]Applicable to visit nr. 1, 2, and 4. [b]Applicable to visit nr. 2 and 4. [c]Applicable to visit 1, 2 and 3. [d]Applicable to visit nr. 1 and 3. [e]Applicable to visit nr. 4

Filling out the FFQs was expected to constitute a major workload for the participants and the questionnaire was mainly ment for mapping the background diet in this trial. Concidering this, we decided against administering the questionnaire prior to the third visit, resulting in one baseline for the FFQ.

Another concern in crossover studies is the possibility of a carryover effect. This can be countermeasured by implementing a washout period sufficiently long to avoid remnant effects from the first intervention period lingering into the second period. In crossover studies with only two periods, it is not possible to statistically distinguish between carryover effects and the period*treatment effects. The current recommendation is concequently to include a sufficient washout periode and commit to the assumption of no carryover effect (121). The present trial included a washout period of four weeks. The bacterial response in the gut to dietary changes occurs within a few days and returns to the original state at the same rate when the intervention is discontinued (122). We thus regarded a washout period of four weeks ample time to minimise the risk of carryover effects. Additionally, no differences between baseline concentrations before and after the washout was found.

## 5.2 Sample size

Calculation of sample size was only performed for the primary outcome measurement (GLP-1 response) and not on expected effects on secondary outcomes. However, bifidogenic effect on gut bacteria have been found with similar and lower sample sizes in comparable studies that evaluated effects of inulin-type fructans (83, 84, 86, 87). Moreover, the fact that moderate changes in total microbiota (1.5%) were observed with relatively low p-values (< 0.05) implies that the sample size was sufficient. However, a *post hoc* evaluation of the sample size capacity to detect changes in ghrelin, PYY, and subjective ratings of appetite (accounted for correlating observations) with 80 % power was performed. The result indicated that the sample size was sufficiently high for detecting potential differences slightly above moderate effect size (Cohen's convention for a moderate effect (d = 0.3 – 0.5)).

## 5.3 Participants

Only 35 of 131 patients (26,7%) were eligible for inclusion. This is clearly a potential source of selection bias and a limitation of the external validity. More than 50% of the patients were not included because they lived too far away from the hospitable. Whereas a little more than 40% of those not included, declined to participate. The main bulk of these expressed concerns about the workload related to participation, others were unable to get the four days for the visits off from work, and some declined because they considered 16 weeks without need for antibiotics unlikely. We cannot rule out the possibility that the study population may be healthier and more conscious about their health than the general Norwegian population with type 2 diabetes. Indeed, results from the FFQ assessment showed that our participants slightly exceeded the criteria for allowed fibre intake (mean of 32.2 g per day). This implies that the study population had higher habitual fibre intake than the general population with type 2 diabetes in Norway, and were to the contrary adherent to the Norwegian dietary recommendations of 25-35 g fibre per day (123). Nevertheless, apart from a high fiber intake, the participants' baseline characteristics seemed representative of patients with type 2 diabetes in Norway. Yet, the results may not extrapolate to patients with lower intake of fiber. We also acknowledge that the

participants living in a high-income country with advanced health care system and primarily being Caucasians, may limit the generalisability of this trial.

## 5.4 Randomisation and blinding

Computer randomisation facilitates allocation devoid of unintentional influence of researchers. Blinding of participants and research personnel, as well as the close similarity between active treatment and control supplement was a strength to this trial. The statistician that performed the computer randomisation and the research nurse administering the supplements, were in no other way involved in the study. The randomisation key was not broken, until all data was collected, the database washed, and the laboratory analyses performed.

## 5.5 Intervention

### 5.5.1 Active treatment

We decided to use ITF as active treatment because they are the most studied prebiotics, and have shown promising effects on the outcomes of interest for the present trial, such as blood glucose and appetite (18). A combination of inulin and oligofructose was chosen because a mixture of long and short-chain inulin-type fructans have been proposed to minimise gastrointestinal symptoms (124, 125). The 16 g dose was decided after considering the amounts of ITF sufficient to induce positive and clinically significant changes in gut microbiota and GLP-1 response against doses low enough to minimise gatrointestinal discomfort. Studies with healthy adults have shown significant increases in bifidobacteria with doses of ITF from 5 g per day (83, 126), and that 10 g per day is preferred over 20 g when also concidering side-effects (126). Moreover, Cani *et al*. reported that 16 g inulin-type fructans per day increased the response of GLP-1 with only minor gatrointestinal symptoms in healthy adults (94). The effect on bifidobacteria in the present trial is in line with other trials that used doses of ITF between 5 and 30 g per day, in healthy people and in non-diabetic patients (83-93). Hence, we belive a dose of 16 g per day to be sufficient.

### 5.5.2 Choice of control treatment

Maltodextrin is the most common choice of placebo in trials investigating effects of prebiotics (7, 12-14, 16, 17, 23-25). It is a highly absorbable carbohydrate and in that sense, a suitable placebo.

### 5.5.3 Length of intervention

We limited the intervention to six weeks to avoid a prebiotic induced weight loss that potentially could have confounded the other outcome measures (127, 128). Six weeks may on the other hand have been too short to enhance microbial diversity.

## 5.6 Outcome measures

### 5.6.1 Data collection

#### The meal tolerance test

The oral glucose tolerance test with administration of 75 g glucose solution is commonly used in clinical trials for studying postprandial glucose regulation, and to diagnose type 2 diabetes (129). As our study population already was diagnosed with type 2 diabetes, a mixed meal tolerance test constituting proteins and fats along with carbohydrates was chosen instead, in attempt to limit the excursion of blood glucose. A mixed meal tolerance test is not a standardised tool however, but may be a more physiologically relevant test than an oral glucose tolerance test (130, 131). We decided on a liquid meal over a typical breakfast meal, as it is easier to standardise and administer while simultaneously preparing for blood samples to be drawn and handled appropriately on a tight time schedule.

#### The ad *libitum* lunch

For practical reasons, a relative homogenous dish of pasta with meatballs was chosen over a free-choice buffet for the *ad libitum* lunch. The simultaneously weighing and registration of food servings could potentially have induced more measurement errors during a free-choice buffet compared to a meal from a set casserole. The ready-made meal also ensured equal propotions of nutrients at every visit compared to meals cooked at the facility. In addition, the dish of pasta with meatballs was without pork, and a type of dish we considered palatable to most people. Unfortunately, the chosen ready meal unexpectedly went out of production

midways during the trial and had to be changed. However, we managed to find another ready meal of pasta and meatballs with nutrient content, taste and appearance very similar to the first meal (Table 3). Only four participants were served a dish in the second intervention period that was different from the dish in the first period, and no one was served different dishes within one intervention period. Hence, we do not believe the swap in dishes made an impact on the results with regard to ratings of appetite and energy intake in, but we cannot exclude the possibility.

**Table 3. Difference in nutrient content between the two ready meals used at the test lunch**

| Nutrients | Difference per 100 g |
|---|---|
| Energy (kcal) | - 6.1 |
| Protein (g) | + 0.8 |
| Carbohydrate (g) | - 3.4 |
| of which sugars (g) | - 0.4 |
| Fat (g) | + 0.3 |
| of which saturates (g) | - 0.4 |
| Fibre (g) | - 0.9 |

We acknowledge that the fasting since midnight in addition to only nutrient drinks for breakfast may have affected the appetite in terms of VAS scores and energy intake at the test lunch. One could thus argue that the two tests should have been conducted on separate days. On the other hand, the workload burden for the participants was a constant concern, and conducting the test lunch directly after the mixed meal tolerance test ensured prolonged standardised pre-conditions at the test lunch.

### 5.6.2 Clinical data

Trained research personnel performed assessments of clinical data. Only blood pressure assessed at baseline was used and reported in patient characteristics in the results chapter. However, the measurements were performed at each visit as a

routine control, and a medical doctor was consulted if the blood pressure was > 160/90 mmHg.

### 5.6.3 Laboratory analyses

Trained research personnel performed all laboratory analyses, and each outcome measure was analysed in one single laboratory and by the same people. This minimises risk of measurement errors.

#### Microbiota

The best way of assessing gut bacteria is not necessarily the most practical, let alone achievable, when taking costs and patient acceptance into account (132). Faecal samples are however, regarded acceptable as proxy for the colonial content in clinical trials (132). A certain time duration from sampling until analysis is clearly also inevitable. Several steps were taken to minimise deterioration of sample quality. The participants received thorough instruction for stool sampling, storage and transport, and were provided with appropriate equipment. Upon arrival at the hospital, the samples were stored immediately at -80℃.

Moreover, several precautions were made to circumvent possible confounders known to affect microbiota. These include the criteria for participation concerning antibiotics, probiotics, and prebiotics, and instructing the participants to avoid changes in habitual life-style and medication during the trial. Medication previously identified as possible confounders, such as metformin, proton pump inhibitors and laxatives (32, 52, 133) were also accounted for.

Marker gene analysis, such as 16S rRNA amplicon method, is based on targeting an amplicon of only one gene instead of attempting to sequence most genes in a sample (132). This method is relatively cheap and fast, and the gene is suitable for taxonomic classification, though prone to lower accuracy at lower taxonomic levels, especially species level. The well-established protocol from the Earth microbiome project (https://earthmicrobiome.org/protocols-and-standards/16s/), was chosen to ensure high quality data. The protocol is based on sequencing the variable region 4 (V4) of the 16S rRNA gene, which is one of the most common regions used to differentiate between bacteria. At the time of sequencing, a longer sequencing

protocol of V4-V5 was launched, and also tested in the project since it might improve the taxonomic resolution to some degree. However, the sequencing run revealed high amounts of chimeric sequences (artefacts), and the data was discarded.

### Short-chain fatty acids

Changes in faecal SCFA may indicate changed bacterial activity in the gut, and is thus a valuable measurement when exploring the effect of prebiotic supplements. It is however considered a poor proxy for the colonic production, as most of the SCFA are absorbed during transit, and only few percents remain in faeces (68). SCFA concentrations in faecal samples are determined by multiple factors, including substrate availability, absorption rate into the systemic circulation and portal vein, transit time through colon, and cross-feeding establishments among the gut bacteria (68). Results from these analysis must concequently be enterpreted with caution.

### GLP-1 and GLP-2

Due to rapid degradation by DPP4, circulating concentrations of GLP-1 and GLP-2 are very low (77). Measurement of these hormones are consequently technically challenging. The analysis methods used in this trial were especially tailored to circumvent these obstacles (111, 112), as shown by sensitivity below 1 pmol/l and intra assay coefficient of variation below 10% for both assays.

### Glucose and insulin

Analyses of glucose and insulin were performed instantly after blood sampling, and the protocol for analysis at our laboratories have been chosen due to low inter and intra-assay variation (109, 110).

### Ghrelin and PYY

Ghrelin and PYY were analysed with multiplex assay. The advantage of this method is the capacity of simultaneous quantification of several targets. The minimum detectable concentration of the assay was 13.7 pg/mL for ghrelin and 41.2 pg/mL for PYY, resulting in 3.6% of PYY and 8.1% of ghrelin below the detection limit. However, the intra- and inter-assay coefficient of variation for both hormones was < 10% and < 15%, respectively, which is considered satisfactory.

### 5.6.4 Patient-reported outcome measures

#### Evaluation of fibre intake at the screening

At the screening, the patient's fibre intake, level of physical activity and alcohol consumption were evaluated with simple, basic questions. The purpose was to detect extreme behaviour, rather than to determine exact levels. For assessment of fibre inntake, the participants were asked how often they consumed certain food items, known to be important sources of fibre in the Norwegian diet, and their portion sizes. In hindsight, this unrefined assessment method turned out to be too crude for detemining a cut-off level of 30 g dietary fiber. Indeed, the results from the FFQ at the first visit (baseline) showed that our participants slightly exceeded the study criteria for allowed fibre intake by mean 2.2 g per day. Presuming the fiber intake measured with FFQ is reliable, this indicates that the study populations′ habitual fibre intake was higher than in the general population with type 2 diabetes (123).

#### Food frequency questionnaire

The FFQ used in the present study, is a retrospective method for dietary assessment. It is validated for measuring habitual diet in an adult Norwegian population and designed to capture the intake during the preceding year (113, 114). When filling out the questionnaires, our participants were on the other hand instructed to recall their diet for the last 6 weeks. It is unlikely that this may have impaired the validity of the FFQ in the present trial, and may even have limited the level of recall bias, which is a known weakness to retrospective metods for dietary assessments. Nevertheless, because of the participants' knowledge of the nature of the study, we cannot exclude a reporter bias. All dietary assesment methods are known to be biased by both over- and underreporting. This is illustrated by the reported intake of dietary fibre ranging between 9.6–54.7 g per day at baseline. Hence, the data on dietary fibre intake should only be interperted on group level and not individually. FFQs' predictability as sole evaluation method for changes over time may be limited (134) and the results on changes in energy intake assessed with FFQ during the trial must be interpreted with caution.

#### Visual analogue scale

VAS scores for appetite have been found to show an acceptable degree of within-subject reliability and validity. The method is sensitive to experimental manipulations and reliable for research of appetite (98-100). Subjective sensation of appetite may however be affected by environmental factors and day-to-day variations, of which only the former can be controlled to some extent. In the present trial, several steps were taken to standardise the pre-test conditions as well as the environmental factors during the test lunch: 1) The participants had all been fasting since midnight and were given the same amount of nutrient drink during the standardised meal proceeding the test lunch. 2) On the rare occasions when a participant did not have company of other participants during a visit, one of the researchers participated in the test lunch. 3) The group of participants attending the visits were, with few exceptions, kept the same. 4) Other practical conditions during the test lunches, such as meal environment, were replicated as good as possible. 5) All participants had been instructed to avoid strenuous exercise two days in advance.

The ability to understand the method of expressing hunger and satiety with VAS scores conceptually may be considered as limitation. As a countermeasure, the participants received thorough instructions before each test lunch about how to score VAS, and a researcher was present for supervision, reminding the participants to weigh and register their servings, and when and how to score the VAS.

Another limitation to the test lunch, was allowing the participants to leave after scoring the 90-minutes VAS, consequently scoring the 180-minutes VAS unsupervised. At departure however, they were reminded not to eat anything until after finishing the last VAS scores. They also received a notification by text message when it was time to fill in scores. All the 180-minutes VAS except one was filled out and returned as instructed. The inverse association between appetite regulating hormones measured before noon and ratings of appetite measured in the afternoon imply that the VAS captured the participants' sensation of appetite

## 5.7 Statistical analyses

Intention to treat is considered the gold standard (135) in clinical trials. Excluding participant from the analyses increases the risk of over-estimating the effect of the intervention.

A limitation to this trial was failure to analyse bacteria and SCFA in faeces and hormones in blood sampled from the participants that did not attend all four visits. Traditionally, the exclusion of data sets from participants with missed visits have been a common approach in crossover trials. In this regard, enhanced risk of missed observations and visits due to the prolonged period of participation and extra workload on the participants constitutes an unfortunate paradox. The LMM however, ensure realistic estimates of the uncertainties and can thus utilise data from all included participants, despite missing observations (136).

# 6 Discussion of results

Measured by number of unused sacets, the level of compliance was high, and there were no dropouts related to the intervention. This indicates that the effort invested in enhancing compliance during the trial was sufficient, and that the dose of ITF was tolerable despite reports of some level of gastrointestinal side-effects.

## 6.1 Paper I: Effect of inulin-type fructans on gut bacteria

### 6.1.1 Faecal gut bacteria

Despite the increased interest in manipulating gut microbiota with prebiotic supplements in clinical trials the resent years, the present trial appears to be the first studying the effect of inulin-type fructans on faecal microbiota and SCFA in patients with type 2 diabetes. Although we expected some beneficial effects on microbiota composition, diversity and SCFA production, the microbiota analysis should be considered explorative.

The bifidogenic effect found in the present study is in line with other trials supplementing doses of ITF varying between 5 and 30 g per day to healthy people and to non-diabetic patients (83-93, 137, 138). The observed change in bacterial composition accounted for only a few percentage of variation. Large inter-individual variation in microbiota is a possible explenation (> 60% of total variation). However, our result concure with the scale of bacterial change and inter-individual variation found in a trial supplementing ITF to healthy adults (91). We also note that some of the top covariates previously identified in a large population-level study of microbial variation displayed comparable effect sizes (32).

Contrary to our hypothesis, the prebiotic treatment did not affect the bacterial diversity in this trial. Three other clinical trials supplementing ITF to healthy adults also failed to increase the diversity of gut bacteria (88, 90, 91), whereas one study reported increased diversity after a lower dose than the other studies and a notably long treatment duration of three months (138). This may imply that it takes longer to alter microbial diversity than to increase the abundance of bifidobacteria in the gut when consuming inulin-type fructans, and that six weeks may be inadequate in this regard.

### 6.1.2 Short-chain fatty acids

Total SCFA, acetic acid and propionic acid were significantly enriched after consumpiton with ITF compared to control, indicating changed bacterial activity in the gut. Butyric acid did not appear positively affected by the intervention. Of note, a conciderable varibility in the SCFA changes was found, which may result from differences in baseline microbiota, diet and absorption. Unlike the present trial, other clinical trials measuring faecal SCFA in normal or overweight healthy adults after 2-12 weeks consumption of 5-16 g ITF per day were unable to detect increased concetrations (87, 88, 90, 92, 93). Only one study reported increased concentrations of total SCFA in faecal samples from healty adults treated with 20 g inulin per day for two weeks, albeit with no changes in acetic or propionic acid, separately (93). The failure to increase concentrations of butyric acid in the present trial is in agreement with the previously mentioned trials that reported no change or even decline in butyric acid in fecal samples from healthy individuals (88-90).

### 6.1.3 Associations between changes in gut microbiota and SCFA

The bifidobacteria are uncapable of producing butyric acid themselves, but play an important role in cross-feeding establihments where various species metabolise non-digestible carbohydrates through several steps. The bifidobacteria contribute by degrading the fructan chains in preparation for other species to complete the fermentation (88). The extensive healt benefits of bifidobacteria are well documented (139) and studies also confirm effects of particular interest in type 2 diabetes (48, 140). In addition to positive effects on blood lipids and anti-carcinogenic properties, trials in mice and humans have shown that the bifidobacteria may improve glycaemic control, prevent endotoxemia, and defend the barrier function in the gut (48, 140-142).

The increase in OTUs assigned to *Bifidobacterium adolescentis* was inversely related to butyric acid. Stimulation of *Bifidobacterium adolescentis* is in line with other trials investigating the effect of ITF as substrates (93, 143-146). The genomic capacity to ferment both long and short-chain fructans may have been an advantage of *Bifidobacterium adolecentis*, an ability that is species and strain-dependent among the bifidobacteria (146). Nonetheless, bacterial metabolic activity induced in strictly

controlled *in vitro* studies may not occur in less predictable environment such as *in vivo* studies. The prebiotic treatment also stimulated species of *Bacteroides*, including *Bacteroides ovatus*. The genus *Bacteriodes* is known for its capacity to ferment several polysaccharides into acetic and propionic acid. Ability to ferment short and long-chain ITF was previously shown for *Bacteroides ovatus* (147). One could also speculate that the observed enrichment of *Bacteroides* species was due to *Bacteroides* being the dominating genus among participants. The butyrate-producing *Faecalibacterium prausnitzii* was slightly enriched in the present trial. In some human trials, this specie has also been stimulated by consumption of inulin-type fructans (88, 93, 138). Furthermore, an increased OTU of *Lachnospiraceae* was positively related to butyric acid. Yet, the increase did not significantly affect concentrations of faecal butyric acid. Low levels of taxas capable of producing butyric acid are a known feature of the type 2 diabetes gut, and this may also explain why we were unable to find significant increase in faecal concentration of butyric acid (50, 52).

### 6.1.4 Factors known to confound gut bacteria

The majority of participants (68%) used metformin during the trial, all with a dose kept unchanged, and we found no difference in the overall faecal microbiota between participants using metformin or not. None of the participants used proton pump inhibitors or laxatives. A relatively high fiber intake may have affected the baseline microbiota composition and diversity, and consequently its responsivness to the prebiotic supplement. However, we found no significant correlation between baseline data such as fibre intake, microbial diversity or concentrations of bifidobacteria on the bifidogenic response. This contradicts results from other studies that reported more pronounced bifidogenic response with higher habitual fibre intake (88) and lower baseline levels of bifidobacteria (83, 84, 148, 149).

## 6.2 Paper II: Effect of inulin-type fructans on response of GLP-1 and GLP-2, and glycaemic regulation

The six weeks consumption of 16 g ITF per day did not positively affect GLP-1, glucose, insulin or GLP-2 responses to a mixed meal in this trial. Instead, the GLP-1

response declined after the prebiotic treatment and increased after the control treatment.

Few trials have focused on effects of prebiotics on glycaemic regulation in type 2 diabetes, and we were only able to identify one trial investigating the response of GLP-1 in this population, and none that measured GLP-2 excursions. In line with our results, Roshanravan *et al*. found no significant difference in glycaemic control or GLP-1 response in patients with type 2 diabetes after 6 weeks treatment with 10 g inulin per day (150). Pedersen *et al.* and Luo *et al.* also reported no changes in glycaemic regulation after supplementing GOS (151) and FOS (152) to patients with type 2 diabetes. Others however, found reduced fasting glucose in type 2 diabetes after treatment with 10 g ITF (153, 154). The treatment duration in the latter trials lasted two weeks longer than in our trial. In healthy adults on the other hand, Cani *et al.* found increased GLP-1 response and reduced excursions of blood glucose after consumption of 16 g ITF per day for only two weeks (94). One could thus speculate that it may take longer to induce positive changes in GLP-1 response and glucose regulation in patients with type 2 diabetes compared to healthy people. Alternatively, meal-induced GLP-1 secretion may be deficient in type 2 diabetes and prebiotic supplement may not have the same effect as in healthy people. This theory has previously been postulated by several research groups and sequentially questioned and refuted by Nauck *et al.* (75). Anyhow, effects of dietary fibres with prebiotic quality, not yet formally classified as prebiotics, have also been investigated in type 2 diabetes. Human trials report promising effects of resistant starch (155, 156), resistant dextrin (157), and arabinoxylan (158) on glucose regulation and of resistant starch on GLP-1 responses (155) in this population.

We were not able to identify any trials evaluating the effect of prebiotics on GLP-2 concentrations in type 2 diabetes. In healthy adults however, a slight increase in fasting GLP-2, but unchanged GLP-2 response to a standardised meal was found after consumption of 11 g ITF for five weeks (95). Moreover, Nilsson *et al.* reported improved insulin sensitivity and GLP-1 and -2 responses in healthy subjects after consumption of resistant starch for three days (159). Increased GLP-2 response and reduced intestinal permeability have also been demonstrated in obese mice after consumption of oligofructose (96).

## 6.3 Paper III: Effect of inulin-type fructans on appetite

Six weeks consumption of 16 g of ITF per day did not induce changes in ghrelin or PYY in our participants. Nor did it improve subjective ratings of appetite or suppress energy intake. Interestingly however, we observed an increase in PPY response after the control treatment. This was significantly different from the prebiotic treatment.

We could not find any trials investigating effects of ITF on ghrelin, PYY, energy intake, or subjective ratings of appetite in populations with type 2 diabetes. Studies with non-diabetic populations are on the other hand abundant. The inconsistent results they report, may partly be explained by extended heterogeneity regarding design and methods and do not allow firm and general conclusions to be made at present.

### 6.3.1 Ghrelin

Our results on ghrelin concur with the findings of Rebello *et al.* who reported unchanged concentrations in overweight adults after four weeks treatment with 4 g inulin per day (160). Parnell *et al.* on the other hand, found declined AUC for ghrelin in an overweight population after daily consumption of 21 g oligofructose for 12 weeks (127). Another trial reported a significant dose dependent relationship between ITF and ghrelin after one week of daily treatment with 0, 15, 35 and 55 g oligofructose in healthy, normal weight adults. The effect on ghrelin appeared close to significant, although not with 15 g per day (161). This suggests that the ITF dose of 16 g per day administered in the present trial may be too low to suppress ghrelin.

### 6.3.2 PYY

Results from trials evaluating the effect on PYY also seem to support a dose dependent effect of ITF. Rebello *et al.* found no effect on PYY after consumption of 4 g ITF per day for four weeks (160), while Parnell et al. showed increased response of PYY after treatment with 21 g ITF per day for 12 weeks (127), with overweight adults in both trials. Pedersen *et al*. reported increased responses of PYY after daily treatment with 35-55 g ITF for one week, but not with 15 g, and Verhoef *et al.* found increased PYY response after 16 g, but not 10 g ITF per day for 13 days, with

healthy adults attending both trials (162). One trial interrupts this pattern by failing to detect an impact of daily treatment with 20 g ITF for one week in patients with gastroesophageal reflux disease (163). However, this may be explained by the limited sample size of nine participants. Contrary to the present study, Verhoef *et al.* found that a daily dose of 16 g ITF was sufficed to induce increased PYY response in normal weight adults, even when administered over a comparably short time span of 13 days (162). This may imply that treatment with 16 g ITF per day for as long as six weeks should have sufficed to enhance the response of PYY in the present trial, and suggests that ITF may have a different impact in type 2 diabetes compared to non-diabetic populations.

### 6.3.3 Subjective rating of appetite and energy intake

Similar to the present study, several studies have found no effect of ITF on subjective rating of appetite (127, 162, 164, 165), whereas other studies report that ITF suppress appetite (94, 137, 160, 161, 166-168).

Energy intake assessed at the ad libitum lunch and in the habitual diet measured with FFQ remained unchanged during the present trial. This is in agreement with other studies measuring changes in energy intake in type 2 diabetes after consumption of ITF (150, 152) and galacto-oligosaccharides (GOS) (151) that found no effect of the supplement on energy intake. In non-diabetic populations, two studies reported suppressed energy intake in normal weight adults treated with 16 g ITF per day for two weeks and overweight adults treated with 21 g ITF per day for twelve weeks (127, 166). Other studies in non-diabetic populations, found unchanged energy intake after treatment with ITF when compared to placebo, seemingly regardless of dose or length of intervention (94, 137, 161, 162, 167-169).

## 6.4 Linking changes in microbiota to clinical outcomes

In this trial, we tried to link the effect of ITF, not only to regulation of blood glucose and appetite, but to changes in the gut bacteria as well. However, the bifidogenic effect induced by the prebiotics did not have further beneficial impact on regulation of glucose, gut hormones, appetite sensations or energy intake in our participants. The *post hoc* analysis also dismissed any correlations between changes in GLP-1

responses and gut microbiota. A possible explanation is that the effects we found on the gut bacteria were too weak to induce further beneficial changes. There are only few human trials linking the effects of prebiotic fibres to metabolic outcomes concurrently with changes in the gut microbiota. Similar to our study, others have demonstrated positive effects on gut microbiota after supplementing prebiotics to overweight and prediabetic adults, with no further implications on fasting GLP-1 or glucose regulation (170, 171). Pedersen *et al*. investigated gut microbiota in addition to glycaemic regulation and energy intake after supplementing prebiotics to patients with type 2 diabetes, but found no changes in neither outcomes. (151). The authors suggested the low dose of 5.5 g per day and failure to account for use of metformin, as possible explanations. Reimer *et al.* on the other hand, reported bifidogenic effect in addition to beneficial effects on appetite ratings in overweight adults after daily consumption of 16 g ITF for 12 weeks (137).

The daily intake of maltodextrin constituted 268 kJ (64 kcal) as opposed to 142 kJ (34 kcal) from the prebiotics. The possibility that maltodextrin, in an amount of carbohydrates comparable to less than a tablespoon of sucrose per day for six weeks, could induce long-term effects on responses of GLP-1 and PYY in our participants, is questionable. The possibility of 16 g maltodextrin acutely affecting GLP-1 response nine hours after ingestion seems unlikely as well. The two daily sachets of supplements were consumed separately or simultaneously, and at any time during the day, but not after initiation of the fast at midnight proceeding a visit. The last intake of either supplements may thus have been maximum 16 g and a minimum of nine hours prior to a standardised mixed meal. Undeniably, however, two outcomes appeared affected by maltodextrin during the present trial. Among comparable trials using maltodextrin as placebo and study populations with type 2 diabetes, we were unable to find reports of any effects of the placebo, although none had measured GLP-1 or PYY responses. Yet, we cannot firmly rule out the possibility that maltodextrin in fact made an impact on outcomes in the present trial.

# 7 Main conclusions

In the present study a daily supplement of inulin-type fructans induced a moderate, but significant increase in faecal levels of bifidobacteria, total SCFA, acetic acid and propionic acid in patients with type 2 diabetes. We were not able to detect any effects on the overall microbial diversity or faecal butyric acid. Our findings imply a moderate potential for these prebiotic fibres to improve the intestinal microenvironment in type 2 diabetes.

However, we did not find evidence to support a potential of inulin-type fructans in regulation of GLP-1, GLP-2, glucose, or insulin, or in suppression of appetite in this population.

Perhaps ITF affect people with type 2 diabetes differently than other populations. There may also have been effects of the ITF too small for our study to detect, but the implications of smaller effects may on the other hand be of little practical interest.

# 8 Clinical implications and future perspective

Results from merely one trial do not suffice as evidence to state that treatment with ITF fail to induce improved regulation of blood glucose and appetite in type 2 diabetes. Compared to the vast clinical trials conducted in non-diabetic populations, the effects of prebiotics on gut bacteria and regulation of glucose and appetite are clearly understudied in patients with type 2 diabetes. Hence, further studies are warranted to validate or revise our findings, and preferably with larger sample sizes. Intervention of longer duration may also be necessary for changes in the gut microbiota to take hold, and for the bacteria to establish cross-feeding arrangements beneficial to the host. In the years to come, the increasing interest for studying the functional capacity of the gut microbiome, may also expand our knowledge of which bacterial compositions in the gut that are to the host‘s advance. Moreover, many gaps in our knowledge about this field need to be filled. For instance, we need a better understanding of the relationship between gut bacteria and human health. There are numerous metabolic pathways and mechanism explaining how gut bacteria affect body and health that remains to be untangled. Of note, it is also not to be ignored that the bacteria are accompanied by several other microbes in the gut. Future explorations of the remaining intermicrobial gut community may add valuable insight into human pathology and means to improve health.

A diet rich in fibres in general has been shown as beneficial for glycaemic regulation in type 2 diabetes (7, 8). Which components of such diets that actually causes these effects and to what extent related food components are involved, are yet to be uncovered. The potential of dietary adjustments as treatment in type 2 diabetes shows too many promises to be overlooked and further explorations of these mysteries should be encouraged. In terms of economics, the cost of dietary adjustments is far lower than the cost of diabetes medication, and prevention or delay of the long-term complications menacing type 2 diabetes is by any means a worthwhile investment.

Current results from clinical trials conducted in patients with type 2 diabetes are too scarce to either suggest or dismiss recommending prebiotic supplements to this population.

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# Errata list

Name of candidate: Eline Birkeland

Title of thesis: The Fiberdia study: Effects of inulin-type fructans on gut microbiota and regulation of blood glucose and appetite in type 2 diabetes: A randomised, placebo-controlled crossover trial

Abbreviation for type of corrections: Cor – correction of language

| Page | Line | Original text | Type of correction | Correctede text |
|---|---|---|---|---|
| 2 | 4 | …billion… | Cor | …million… |
| 46 | 14 | …two… | Cor | …one… |
| 49 | 2 | …sacets… | Cor | …sachets… |

# Appendix: Paper I, II, and III

# I

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ORIGINAL CONTRIBUTION

# Prebiotic effect of inulin-type fructans on faecal microbiota and short-chain fatty acids in type 2 diabetes: a randomised controlled trial

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## Abstract

**Purpose** Compared to a healthy population, the gut microbiota in type 2 diabetes presents with several unfavourable features that may impair glucose regulation. The aim of this study was to evaluate the prebiotic effect of inulin-type fructans on the faecal microbiota and short-chain fatty acids (SCFA) in patients with type 2 diabetes.

**Methods** The study was a placebo controlled crossover study, where 25 patients (15 men) aged 41–71 years consumed 16 g of inulin-type fructans (a mixture of oligofructose and inulin) and 16-g placebo (maltodextrin) for 6 weeks in randomised order. A 4-week washout separated the 6 weeks treatments. The faecal microbiota was analysed by high-throughput 16S rRNA amplicon sequencing and SCFA in faeces were analysed using vacuum distillation followed by gas chromatography.

**Results** Treatment with inulin-type fructans induced moderate changes in the faecal microbiota composition (1.5%, $p=0.045$). A bifidogenic effect was most prominent, with highest positive effect on operational taxonomic units (OTUs) of *Bifidobacterium adolescentis*, followed by OTUs of *Bacteroides*. Significantly higher faecal concentrations of total SCFA, acetic acid and propionic acid were detected after prebiotic consumption compared to placebo. The prebiotic fibre had no effects on the concentration of butyric acid or on the overall microbial diversity.

**Conclusion** Six weeks supplementation with inulin-type fructans had a significant bifidogenic effect and induced increased concentrations of faecal SCFA, without changing faecal microbial diversity. Our findings suggest a moderate potential of inulin-type fructans to improve gut microbiota composition and to increase microbial fermentation in type 2 diabetes.

**Trial registration** The trial is registered at clinicaltrials.gov (NCT02569684).

**Keywords** Prebiotics · Type 2 diabetes · SCFA · Faecal bacteria · 16S rRNA sequencing

## Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| ASCA | ANOVA simultaneous component analysis |
| FFQ | Food frequency questionnaire |
| FOS | Fructooligosaccharides |
| GLP-1 | Glucagon-like peptide-1 |

Ida Rud and Anne-Marie Aas share last authorship

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s00394-020-02282-5) contains supplementary material, which is available to authorized users.

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| | |
|---|---|
| MCS | MiSeq Control Software |
| OUT | Operational taxonomic unit |
| PCR | Polymerase chain reaction |
| PLS-DS | Partial least squares discriminant analysis |
| PLSR | Partial least squares regression |
| PYY | Peptide YY |
| Q30 | Quality score of 30 |
| QIIME | Quantitative Insight into Microbial Ecology |
| SCFA | Short-chain fatty acids |
| VIP | Variable importance in prediction |

## Introduction

Advice on diet and physical activity are the cornerstones of treatment of type 2 diabetes for regulation of blood glucose and prevention of long-term complications. Dietary recommendations include a diet rich in dietary fibres [1]. Dietary fibres may have several beneficial effects on glycaemic control, including slowing the rate of nutrient absorption [2, 3], and modifying the gut microbiota. Prebiotic fibres evade degradation in the small intestine and are fermented into short-chain fatty acids (SCFA) in the colon by presumed health promoting gut bacteria, stimulating their growth and activity [4]. The wide-ranging health benefits of bifidobacteria in particular, are well documented [5].

The SCFA produced by gut bacteria, mainly the acetic, propionic and butyric acids, are used as an energy source for the colonocytes and substrates for the hepato-metabolic pathways [6, 7]. The SCFA may also act as signalling molecules by binding to receptors on the enteroendocrine cells, with the potential to increase postprandial secretion of gut hormones and improve regulation of blood glucose [7]. Thus, increased production of SCFA, especially butyric acid, is considered favourable [8–10].

Observational studies have shown that gut microbiota in type 2 diabetes differs from healthy individuals with lower diversity of the microbial community, less of the butyrate-producing bacteria, and lower faecal concentrations of SCFA [11–13]. Elevated levels of pathogenic bacteria, and functions related to oxidative stress response, such as enrichment of catalase and increased production of the antioxidant glutathione were also found [12]. Alterations in gut homeostasis such as these are suspected to contribute to the pathophysiology of type 2 diabetes [7].

Improvement of the microbial profile in the gut could benefit individuals with type 2 diabetes in particular, by enhancing the production of SCFA. The inulin-type fructans and galactooligosaccharides are the most studied prebiotic fibres and inulin-type fructans are also extensively used as industrial food ingredients. Numerous trials show that inulin-type fructans supplemented in doses varying between 5 and 30 g per day may increase the abundance of bifidobacteria and SCFA in faeces, and enrich microbial diversity in healthy people and in non-diabetic patients [14–24]. Interestingly, lower levels of bifidobacteria have been reported in individuals with type 2 diabetes compared to healthy individuals, and probiotic supplementation with this genus has been reported to improve glucose tolerance in animal studies [25]. Furthermore, studies conducted in type 2 diabetes patients have also shown that dietary fibres with and without prebiotic abilities could improve glucose metabolism [2, 26]. Yet, to the best of our knowledge, it has not been investigated whether inulin-type fructans have different impact on gut microbiota and fermentation in people with diabetes than in healthy individuals.

The aim of this study was, thus, to evaluate the prebiotic effect of inulin-type fructans on faecal microbiota and SCFA in patients with type 2 diabetes. We hypothesised that treatment with inulin-type fructans for 6 weeks would induce positive changes in the composition of gut microbiota, such as enriching concentrations of bifidobacteria and butyrate producers, increasing the microbial diversity, and increasing concentrations of faecal SCFA.

## Methods

### Trial design

We conducted a randomised, placebo controlled and double-blind crossover trial between February 2016 and December 2017 at the Diabetes Research laboratory, Oslo University Hospital, Aker. Due to high inter-individual variability in the microbial response to dietary interventions, the crossover approach was selected over a parallel design, allowing the participants to serve as their own controls. This study is part of a trial where the primary aim was to investigate the effect of prebiotics on GLP-1 response. These results are not yet published. The trial was approved by the Regional Ethics Committee for Medical and Health Research and registered at clinicaltrials.gov (NCT02569684). Written informed consent was obtained from all patients prior to their inclusion in the study. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

### Participants

Adult men and women with type 2 diabetes were invited consecutively as they attended the Diabetes Outpatient Clinic. Participants were also recruited from advertisement in social media, the hospital lobby and pharmacies, and from general practices.

Eligibility for participation was determined at a screening visit at least 4 weeks prior to enrollment. Eligible

patients had a BMI ≤ 40 kg/m$^2$, $HbA_{1c}$ < 10.0% (86 mmol/mol), and were not treated with insulin or glucagon-like peptide-1 (GLP-1) analogues. Exclusion criteria were fibre intake > 30 g per day, performance of high-intensity exercise, weight changes of > 3 kg within the last month, planned or present pregnancy, drug or alcohol dependence, treatment with antibiotics within the last 3 months, long distance from home to the study centre, and consumption of dietary supplements containing prebiotics or probiotics. At screening, the fibre intake was assessed based on a simplified approach where we asked the potential participants how often they consumed food items known to be important sources of fibre in the Norwegian diet, and their portion sizes. Patients diagnosed with either dementia, organic or functional gastrointestinal diseases, or had cancer within the last 5 years were not included.

In total, 131 patients were assessed for eligibility and 35 were randomised to start with either inulin-type fructans or placebo, of whom 25 completed the intervention (Online Resource 1). Of the ten patients who were randomised, but did not start or complete the intervention, no individuals were excluded or withdrew because of side effects from the supplements or other study-related procedures. One participant was excluded in the faecal microbiota analysis due to one sample with low amounts of extracted DNA.

## Dietary intervention

For two periods of 6 weeks separated by a 4-week washout, the participants consumed 16 g per day of inulin-type fructans (a 50/50 mixture of oligofructose and inulin; Orafti® Synergy1, Beneo GmbH, Germany) and placebo (maltodextrin 16 g per day) in addition to their ordinary diet and in a randomised order. The dose of 16 g was decided after considering the amounts of prebiotics sufficient to induce positive and significant changes in gut microbiota and GLP-1 response against doses low enough to avoid adverse side effects and minimise gastrointestinal discomfort. Trials with healthy adults have demonstrated significant increases in bifidobacteria with doses of inulin-type fructans from 5 g per day [14, 27] and that 10 g per day is preferred rather than 20 g when also taking side effects into consideration [27]. Furthermore, Cani et al. demonstrated that 16-g inulin-type fructans per day induced increased response of GLP-1, and only minor gastrointestinal symptoms in healthy adults [28]. The supplements were powdered, similar in colour and taste, and were wrapped in identical and non-transparent portion packages of 8 g. For adaptation, the participants consumed only 8 g per day during the first week and progressed to 16 g per day for the remaining 5 weeks. The participants added the supplements to food or drinks and consumed it whenever they preferred. They returned unused supplement packages, and the number of unused sachets was used as an estimate of compliance.

## Outcomes and data collection

Before and after the 6-week intervention periods, the participants attended the hospital for visits, where they delivered faecal samples for analysis of microbiota and SCFA. For a comprehensive assessment of diet, the participants filled out food frequency questionnaires (FFQ) before the first intervention period. The participants were instructed to avoid making changes in habitual lifestyle during the trial and to avoid strenuous exercise one day in advance of the visits. They were also told not to make any changes regarding medication during the study and to discontinue diabetes medication two days prior to the visits.

### Anthropometric measurements

Weight and bioimpedance were measured using a body composition analyser (Tanita BC-418 MA Segmental Body Composition Analyzer) at the four visits, before and after the intervention periods. Height was measured with a standard altimeter. Participants were examined with bare feet wearing light clothing.

### Assessment of diet

The FFQ is a validated, self-administered, paper-based optical mark readable questionnaire assessing the total diet [29, 30]. Participants were instructed to fill in questionnaires based on eating habits during the last 6 weeks.

### Faecal collection

The participants were provided with sterile plastic containers to collect faecal samples at home, and instructed to store these instantly in a freezer one day prior to each of the four visits. The samples were brought to the clinic in cooler bags containing freezer blocks and immediately stored at – 80 ℃ for later analysis.

## Microbiota analysis

### DNA extraction and microbiota analysis

Bacterial DNA was extracted from faecal content (approximately 100 mg) by mechanical and chemical lysis using the DNaeasy PowerSoil HTP 96 Kit (Qiagen), following the manufacture's protocol. The mechanical lysis step with bead beating was done twice using the FastPrep®-96 homogenizer (MP Biomedicals) for 60 s at 1600 rpm. Then, samples were centrifuged for 6 min at 4500 × *g* as described in

the protocol. The microbiota was analysed by 16S rRNA amplicon sequencing (2 × 150 bp) of the variable region 4 following an in-house protocol [31], which is presented in detail in supplementary methods of Caporaso et al. [32]. The current primers [33–35] have been modified from the original 515F–806R primer pair, with barcodes now on the forward primer and degeneracy added to both the forward and reverse primers to remove known biases. The sequencing was done on a MiSeq (Illumina) at Nofima using pooled polymerase chain reaction (PCR) samples, which were based on triplicate PCRs per DNA sample using sample-specific barcoded forward primers. PhiX Control v3 was included and accounted for 10% of the reads. The MiSeq Control Software (MCS) version used was RTA 1.18.54.

#### Data processing of sequencing data

Data processing of the sequencing reads was performed using the pipelines in Quantitative Insight Into Microbial Ecology (QIIME) v.1.9 [36]. Briefly, the total number of reads was 15,217,265 followed by 9,007,278 reads after joining forward and reverse reads and removal of barcodes that failed to assemble. The sequences were demultiplexed into representative sample taqs and quality filtered, allowing zero barcode errors and a quality score of 30 (Q30), resulting in 7,550,212 sequences. Reads were assigned to their respective bacterial taxonomy (operational taxonomic unit: OTU) by clustering them against the Greengenes reference sequence collection (gg_13_8) using a 97% similarity threshold. Reads that did not hit a sequence in the reference sequence collection were clustered de novo. Chimeric sequences were removed using ChimeraSlayer, and all OTUs that were observed fewer than 2 times were discarded. This resulted in an OTU table containing 15,168 different OTUs, which was based on a total of 6,642,085 read counts. The OTU table was used for microbial (alpha) diversity analysis using equal number of sequences across samples, i.e. alpha rarefaction, where the OTU table was resampled to an even depth of 13,000 sequences per sample. Summary tables at phylum, order, family and genus levels were constructed from the OTU table (i.e. OTU level/species level). The data were transformed by centred log2 ratios, to stabilize the variation and remove dependencies between abundance variables. At any taxonomic level, bacteria groups that were present in less than 50% of the subjects were combined into one group (called "rare"), as it is not possible to make statistical inference on individual rare bacteria groups. Square brackets around taxonomic names (e.g. [Ruminococcus]) are taxa proposed by Greengenes based on genomic trees, but are not verified taxonomies.

### SCFA analysis

Upon analysis, 0.5 g of the faecal material was homogenised after addition of distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of $H_2SO_4$; 2.5 mL of the homogenate was vacuum distilled, according to the method of Zijlstra et al. [37], as modified by Høverstad et al. [38]. The distillate was analysed with gas chromatography (Agilent 7890 A, CA, USA), using a capillary column (serial no. USE400345H, Agilent J&W GC columns, CA, USA), and quantified using internal standardisation. Flame ionisation detection was employed. The following SCFA were analysed: acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic and isocaproic acids. The results were expressed in mmol/kg wet weight. In addition, we calculated the proportional distribution of individual SCFA to total SCFA.

### Gastrointestinal symptoms

After both interventions the participants completed a questionnaire about changes in gastrointestinal symptoms concerning the last 6 weeks (abdominal discomfort, diarrhoea, constipation, bloating, and flatulence) with a word rating scale: much worse, worse, unchanged, better, and much better.

### Sample size

The sample size was calculated based on the expected effects on the primary outcome measurement from the main study, which was change in GLP-1-response to a standardised meal. This estimation was based on results from a drug trial in patients with type 2 diabetes, where changes in GLP-1 response were the primary endpoint [39]. This provided a tentative sample size of 23 patients to achieve 80% power at alpha = 0.05. To account for drop-outs and a possible lower treatment effect due to differences in intervention and design, we added 12 patients, giving a total of 36 patients required for randomisation.

### Randomisation and blinding

Staff not involved in the study performed subject randomisation and product distribution. Randomisation lists were generated using a randomisation command for two by two cross-over studies in Stata 14. All participants and clinical researchers were blinded to treatment allocation and the randomisation key was not broken before all data were collected, the database was washed and the laboratory analyses were performed.

### Statistical analyses

SPSS version 25.0 software was used for descriptive statistics and analyses of biochemical responses. Baseline characteristics are reported as mean (range), (SD) or *n* (%). The variables, total SCFA as well as the individual SCFA, were skewed and their distribution did not improve with log transformation. The effects of inulin-type fructans on SCFA were, thus, analysed using Wilcoxon Signed Rank test and $P < 0.05$ (two tailed) was considered as statistically significant. The results from SCFA analyses are reported as medians (25th–75th percentiles).

The observed variation in microbiota at different taxonomic levels were decomposed by analysis of variance (ANOVA) simultaneous component analysis (ASCA) [40]. The carry-over effect was originally included in the model by the effects *Period* + *Treatment* × *Week* × *Period*, but were removed as they were non-significant. The final ASCA model contained a *Subject* effect, accounting for the between subjects variation, and a intervention-specific *Treatment* × *Week* effect. Post hoc comparisons between factor levels of the intervention design were performed using partial least squares discriminant analysis (PLS-DA) after removing the between-subjects variation [41]. Bacteria that discriminate the prebiotic fibres from placebo and baseline levels were identified by variable importance in prediction (VIP) combined with Pearson correlations between individual bacteria's group means and class labels [41]. A cutoff of 1.2 was used for VIP and 0.9 for correlation. Effect sizes were calculated as difference between means after prebiotic treatment compared to placebo treatment and baseline values combined.

The microbial diversity, represented by the metrics Observed OTUs, Phylogenetic Distance (PD) whole tree and Chao1, was analysed using a Mixed-Effects Model in Minitab®18.1. Treatment, Week and Period were defined as fixed effects and Subject as random.

Partial least squares regression (PLSR) was used to analyse the relationship between microbiota (OTU level) and the different SCFA/metformin users (yes or no) without taking the intervention into account and validated by cross-validation [41]. Variable importance was estimated by the VIP method. Individual variation in effect size of the intervention (subject-specific effect sizes) on the *Bifidobacterium* genus and its OTUs were used to relate against baseline data, i.e. initial level of *Bifidobacterium*, microbial diversity and fibre intake (g/day) characteristics. Data of none identified relationships (i.e. metformin, *Bifidobacterium*, microbial diversity and fibre intake) are not presented. The multivariate statistical analyses were performed using MATLAB (R2018b, The MathWorks Inc.).

**Table 1** Baseline characteristics of study participants

| | (*n* = 25) |
|---|---|
| Women | 10 (40.0) |
| Age (years) | 63.1 (41–73) |
| Fasting glucose (mmol/L) | 8.7 (4.0–12.8) |
| BMI (kg/m$^2$) | 29.1 (19–39) |
| HbA1C (%) | 6.9 (5.1–9.6) |
| (mmol/mol) | 51.9 (32.2–81.4) |
| Energy (kcal/day) | 2338 (1315–4658) |
| Proteins (E%) | 18.1 (9.6–22.9) |
| Fat (E%) | 36.9 (21.7–44.7) |
| Carbohydrates (E%) | 38.9 (27.4–60.3) |
| Dietary fibre (g/day) | 32.2 (9.6–54.7) |
| Diabetes duration (years) | 4.7 (0.2–20.0) |
| Diabetes treatment | |
| Diet | 8 (32.0) |
| Metformin | 17 (68.0) |
| SLGT2 inhibitors[a] | 2 (8.0) |
| DPP-4 inhibitors[a] | 5 (20.0) |
| Sulfonylureas[a] | 1 (4.0) |
| Proton pump inhibitors | 0 (0) |

Values are mean (range) or *n* (%)

[a]Medication used in addition to Metformin

## Results

### Patient characteristics

Baseline characteristics of the 25 participants who completed the intervention are presented in Table 1. Forty percent were women, the overall mean age was 63.1 years, BMI 29.1 kg/m$^2$, $HbA_{1C}$ 6.9% [52 mmol/mol], and diabetes duration was 4.7 years. Two thirds of participants received glucose lowering medications. The intake of dietary fibre assessed with FFQ at the first visit (baseline) turned out to be higher than expected, as the evaluation of fibre intake at the screening was based on a simpler approach with questioning about how often a few certain food items were consumed and their portion sizes. Apart from a reported higher intake of dietary fibre (mean 32.2 ± 10.3 g/day), the participants characteristics seemed to be representative of patients with type 2 diabetes in Norway.

The compliance was excellent with mean (range) 96.7 (79.2–100.0)% of the prebiotic supplement and 95.7 (77.9–100.0)% of the placebo consumed.

Individual faecal microbiota and effects of inulin-type fructans.

The faecal microbiota was analysed from 24 participants who completed the two crossover periods with four sampling times per individual. Statistical overview of the microbiota data is presented in the online supporting material (Online

Resources 2, 4 and 5), also confirming no differences in microbiota composition nor microbial diversity between crossover periods.

The microbiota data show abundant inter-individual variability of microbiota composition, (explaining > 60% of total variation) and minor effect of the prebiotic fibre (explaining < 2.5%) (Online Resource 2). Overview of the inter-individual variation of phyla at baseline is presented in Fig. 1, showing the gradient distribution of the dominating Bacteroidetes (mean abundance of 69%), with a trade-off with Firmicutes (26%) as the second dominating phylum. Indeed, except for two participants, Bacteriodetes accounted for more than 50% of the microbiota present in the individuals. Tenericutes (1.5%), Proteobacteria (1.2%), Actinobacteria (0.9%), Verrucomicrobiota (0.7%) and Cyanobacteria (0.3%) were also present to a variable degree between individuals.

The moderate changes in microbiota composition after intervention with the prebiotic fibre were explained by only 2.2% and 1.5% of the total variation at phylum and OTU (species) levels, respectively (Online Resource 2). The overall microbiota effect did not reach significance at the phylum level ($p = 0.091$), although Actinobacteria (VIP 1.32) was significantly positively affected by prebiotic fibre compared to placebo and baselines after the 6 weeks of intervention (Online Resource 3). However, the prebiotic fibre had significant effect at the OTU level ($p = 0.045$), with significant impact on 32 OTUs (Online Resource 4). These are presented in Fig. 2, with their representative effect sizes.

Indeed, the three OTUs with highest positive effect sizes were of Actinobacteria and assigned to *Bifidobacterium adolescentis*. *Bifidobacterium adolescentis* OTU559527 was the most abundant of these OTUs (0.6%). The remaining OTUs positively related to prebiotic fibre intake were not that highly ranked and with less effect sizes, and were mostly of Bacteroidetes origin or Firmicutes. Especially, OTUs within *Bacteroides* were among these, including one dominating OTU assigned to *Bacteroides ovatus*, and three OTUs within Clostridiales, including Lachnospiraceae and *Faecalibacterium prausnitzii*. The OTUs that decreased with the prebiotic fibre were of Firmicutes, including dominating OTUs assigned to the families Ruminococcaceae (*Ruminococcus*) and Lachnospiraceae ([*Ruminococcus*]), all with high effect size. In addition, an OTU of Erysipelotrichaceae declined with the prebiotic fibre.

Microbial diversity was not affected by the prebiotic fibre after the 6-week intervention (Online Resource 5), as exemplified with the metrics observed OTUs (Fig. 3).

## Effects of inulin-type fructans on faecal SCFA

The intervention resulted in a significant increase in faecal concentrations of total SCFA ($p = 0.04$), acetic acid ($p = 0.02$), and propionic acid ($p = 0.04$) as compared to placebo (Table 2). There was no difference in effect on butyric acid between the treatments ($p = 0.19$).

## Relationship between microbiota and SCFA

The relationship between microbiota and the SCFA (acetic, propionic, butyric and valeric acid) is presented in a heatmap, only including the OTUs significantly affected by the prebiotic intervention (Fig. 4). A general trend was that acetic acid was positively related to OTUs that increased with the prebiotic fibre. The opposite trend was observed for the OTUs that declined with the prebiotic treatment. Interestingly, the prebiotic affected OTUs of *Bifidobacterium adolescentis* were negatively related towards butyric acid. Only Lachnospiraceae OTU514272 was positively related to butyric acid among the prebiotic affected OTUs. Another trend was that valeric acid was positively related to the OTUs that declined with the prebiotic fibre.

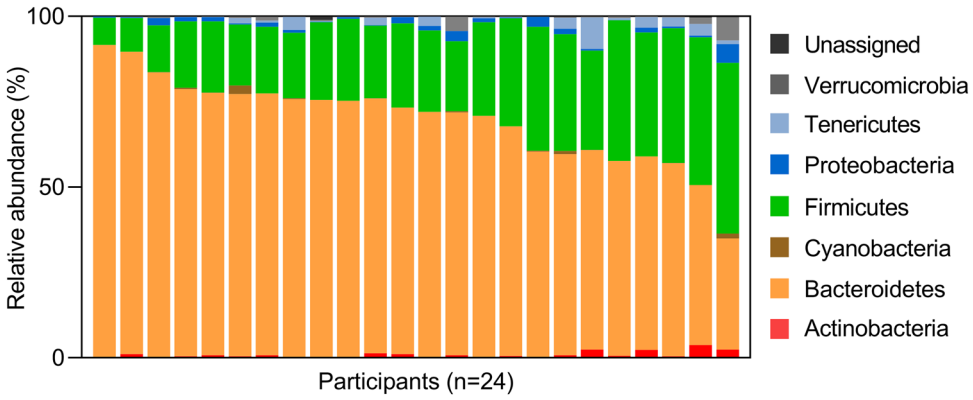


**Fig. 1** Relative abundance (%) of the dominating phyla in faeces of the participants at baseline

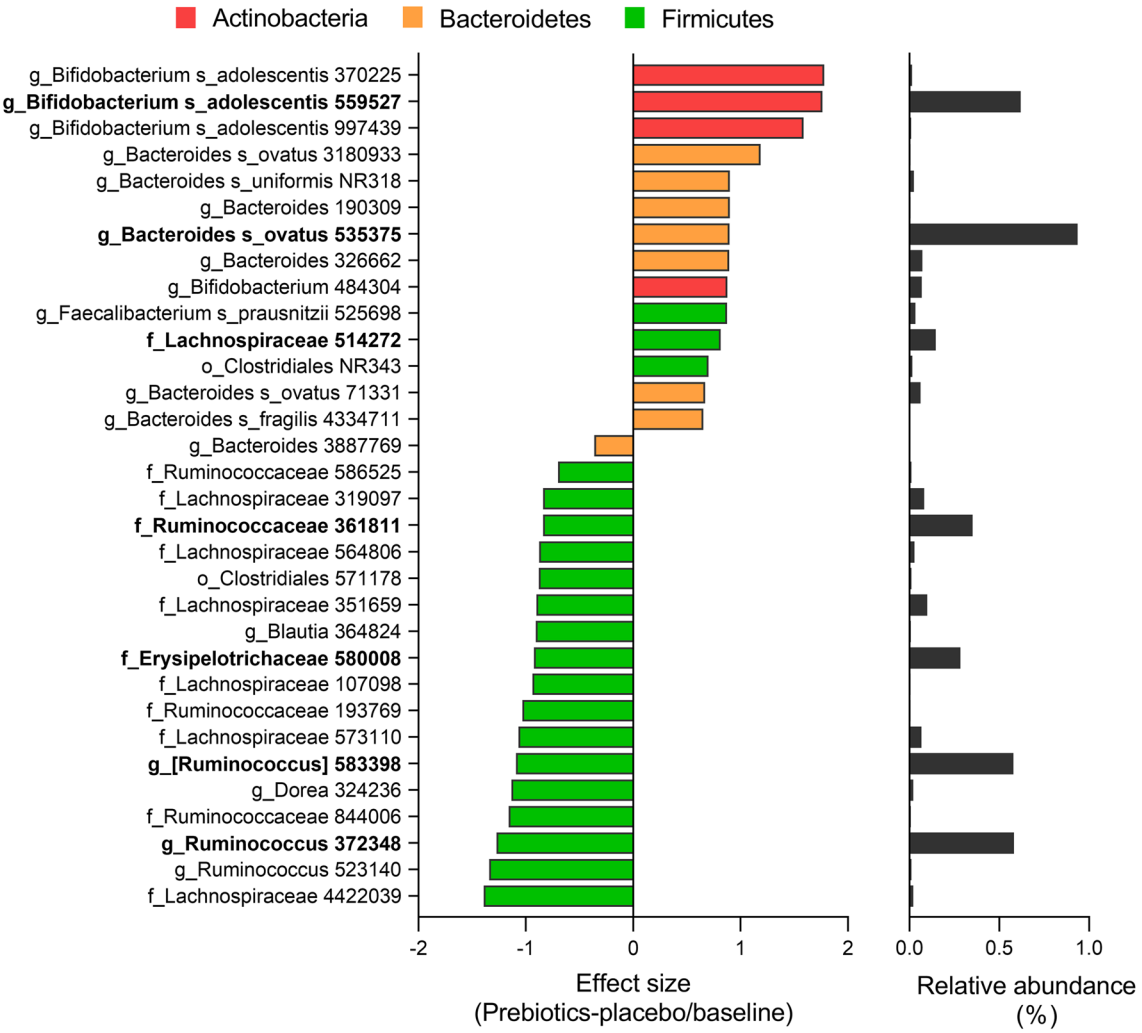


**Fig. 2** OTUs affected by the prebiotic intervention for 6 weeks sorted by effect size. Effect size is the differences between prebiotic intervention period compared to placebo period /baseline (log2). Dominating OTUs (>0.1%) are indicated in bold, and the relative average abundance of the OTUs is included at the right. Brackets indicate candidate taxonomy. Bars are coloured according to representative phylum

## Discussion

In this randomised controlled trial in patients with type 2 diabetes, we found that 16 g per day of a 50/50 mixture of inulin and oligofructose supplemented for 6 weeks caused an increase in bifidobacteria and SCFA in faeces, compared to maltodextrin. However, the prebiotic fibre had no effect on butyric acid or the overall microbial diversity. To the best of our knowledge, this is the first trial studying the effect of inulin-type fructans on faecal microbiota and SCFA in people with type 2 diabetes.

In planning the present trial, we decided on inulin-type fructans as choice of prebiotic fibres. These are the most studied among prebiotics and a mixture of both long- and short-chain inulin have been proposed to minimise the expected gastrointestinal symptoms [42, 43]. The bifidogenic effect found in the present trial is in accordance with other human studies with doses of inulin-type fructans varying between 5 and 30 g per day, in healthy people and in non-diabetic patients [14–24]. We thus belive a dose of 16 g per day to be sufficient. However, the prebiotic effect on microbiota composition in the present study was moderate, accounting for only a few percentage of variation in the microbiota. This has also been demonstrated in healthy humans given inulin as prebiotic [22], and may be explained by the large individual variation in microbiota between the participants.

Although bifidobacteria are unable to produce butyric acid themselves, they are valuable in cross-feeding where various species metabolise non-digestible carbohydrates through several steps. The bifidobacteria contribute with their ability to degrade fructan chains and, thus, prepare

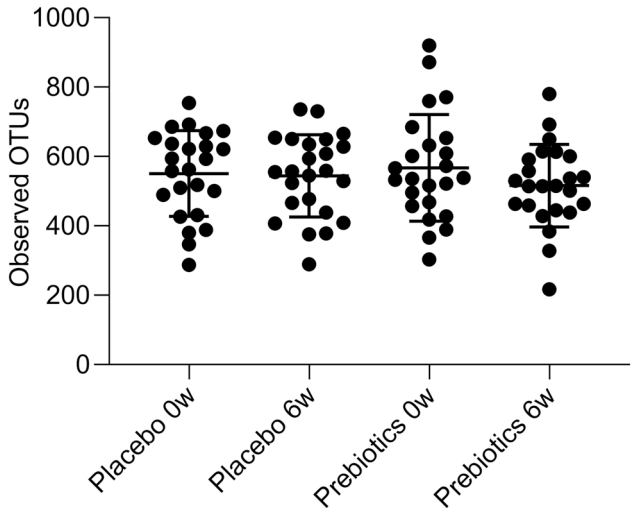


**Fig. 3** Microbial diversity shown as number of observed OTUs between prebiotics and placebo at baseline (0w) and after treatment period of 6 weeks (6w)

for other species to complete the fermentation [19]. The extensive health benefits of bifidobacteria are well documented [5]. Studies also confirm bifidogenic health benefits of particular interest in type 2 diabetes [44, 45]. Apart from anti-carcinogenic properties and positive effects on blood lipids, trials in humans and mice report that bifidobacteria also may prevent endotoxemia and improve regulation of blood glucose [44–46].

The prebiotic treatment did not have the desired effect of increased microbial diversity in our participants. Tandon et al. found increased diversity of faecal bacteria in a healthy population after supplementing fructooligosaccharides (FOS) [47], but others found no or even decreased effect of inulin-type fructans [19, 21, 22]. These studies were all conducted in healthy adults, but with varying treatment doses and degrees of polymerization. The trial performed by Tandon et al. however, stands out with a particular long treatment duration (3 months) and lower treatment dose. This may indicate that it takes longer to affect the microbial diversity than to enhance the abundance of bifidobacteria in the gut when supplementing inulin-type fructans. We chose to limit the duration of the intervention period to 6 weeks to avoid a prebiotic effect of weight loss previously reported [48, 49], as weight loss could potentially have confounded other outcome measures.

Even though the effect of prebiotic fibre on the microbiota composition was moderate, enhanced faecal concentrations of SCFA was detected, indicating changed microbial metabolic activity in the gut. Total SCFA, acetic acid and propionic acid increased significantly. This contrasts the findings in the majority of other clinical trials that measured faecal SCFA after supplementing inulin-type fructans. Only Baxter et al. found increased concentrations of total SCFA in faecal samples from healthy individuals supplemented with 20-g inulin per day for 2 weeks, despite no changes in acetic or propionic acid, separately [24]. Others found no or even decreased concentrations of faecal SCFA in healthy adults with normal or overweight after treatment with 5–16-g inulin-type fructans per day for durations between 2 and 12 weeks [18, 19, 21, 23, 24]. Acetic and propionic acid have been linked to mechanisms preserving or improving glucose homeostasis and appear to be anti-carcinogenic, and propionic acid is able to reduce visceral and liver fat [50]. Butyric acid is of particular interest in type 2 diabetes as animal studies report it improves glucose homeostasis by inducing gut production of GLP-1 and peptide YY (PYY) [9] as well as protecting the gut barrier function [51]. However, no significant increase in faecal concentration of butyric acid was detected in the present study. This is in line with the previously mentioned human trials with inulin-type fructans showing no change or even decrease in faecal butyric acid in healthy individuals [19–21]. It is worth noticing that there was a large variability in the measured change in all SCFA, which may be due to individual differences in baseline microbiota, diet and absorption. This can also explain some of the inconsistent findings between studies.

The bifidogenic effect in the present study was related to increase in OTUs assigned to *B. adolescentis*, which were negatively related to butyric acid. Stimulation of *B. adolescentis* is in agreement with other studies using oligofructose and inulin as substrates [24, 52–55]. Fermentability of both the short- and long-chain fructans may have been an advantage of *B. adolescentis*, a capacity shown to be species- and strain dependent among the bifidobacteria [55]. However, bacterial metabolic activity reported in strictly controlled in vitro studies may not occur in the less predictable environment associated with in vivo studies.

Species of *Bacteroides*, e.g. *B. ovatus,* were also enriched by the prebiotic fibre. This genus is known for its genomic capacity to ferment a wide range of polysaccharides into acetic and propionic acid. Capability to ferment both FOS and inulin has previously been shown for *B. ovatus* both genetically and physiologically [56]. It could be speculated that the observed increase in *Bacteroides* species was enhanced by *Bacteroides* being the dominating genus among the type 2 diabetes patients. The butyrate-producing *F. prausnitzii* has in some human studies also been shown to be stimulated by intake of inulin-type fructans [19, 24, 47]. Indeed, *F. prausnitzii* was slightly enriched in this study, as well as an OTU of *Lachnospiraceae* that was also positively related to butyric acid. Still, the increase did not significantly affect the levels of faecal butyric acid. Low levels of butyrate-producing taxas are a well known feature of the type 2 diabetes gut and this may also explain why we did not see significant increase in faecal concentration of butyric acid

**Table 2** Effects of prebiotics on faecal SCFA in type 2 diabetes

| | Prebiotics | | | Placebo | | | Between-*p* value |
|---|---|---|---|---|---|---|---|
| | Baseline | Δ 6w | Within-*p* value | Baseline | Δ 6w | Within-*p* value | |
| Total SCFA (mmol/kg) | 53.95 (43.89–75.23) | 8.80 (− 4.7 to 27.44) | 0.07 | 66.53 (58.40–83.50) | − 4.98 (− 24.94 to 12.70) | 0.35 | **0.04** |
| Acetic acid (mmol/kg) | 31.57 (25.38–40.14) | 6.11 (− 3.14 to 13.34) | **0.02** | 39.95 (33.95–42.87) | − 4.23 (− 11.94 to 7.45) | 0.34 | **0.02** |
| % Acetic acid | 56.19 (47.71–60.33) | 2.24 (− 6.16 to 9.80) | 0.22 | 53.58 (46.81–62.46) | 1.07 (− 3.72 to 6.78) | 0.46 | 0.58 |
| Propionic acid (mmol/kg) | 10.02 (6.46–13.33) | 1.72 (− 2.49 to 8.57) | 0.17 | 11.75 (8.96–16.25) | − 1.52 (− 3.78 to 1.19) | 0.12 | **0.04** |
| % Propionic acid | 16.54 (14.81–19.84) | 0.06 (− 3.40 to 1.92) | 0.96 | 17.97 (15.26–20.64) | 0.26 (− 2.31 to 1.99) | 0.90 | 0.90 |
| Isobutyric acid (mmol/kg) | 1.33 (0.87–1.67) | 0.05 (− 0.81 to 0.85) | 0.99 | 1.42 (0.91–2.92) | − 0.33 (− 0.61 to 0.21) | 0.09 | 0.70 |
| % Isobutyric acid | 2.55 (1.76–3.77) | − 0.05 (− 1.39 to 0.41) | 0.19 | 2.34 (1.38–3.43) | 0.03 (− 0.60 to 0.33) | 0.49 | 0.14 |
| Butyric acid (mmol/kg) | 9.06 (7.36–15.08) | 2.35 (− 1.81 to 4.81) | 0.17 | 10.63 (7.99–16.68) | − 1.18 (− 6.58 to 6.04) | 0.53 | 0.19 |
| % Butyric acid | 17.70 (14.20–22.53) | − 0.90 (− 4.33 to 3.86) | 0.53 | 16.30 (14.21–22.31) | − 1.00 (− 5.13 to 4.72) | 0.68 | 0.95 |
| Isovaleric acid (mmol/kg) | 2.05 (1.32–2.58) | − 0.01 (− 1.51 to 1.16) | 0.76 | 2.01 (1.37–4.38) | − 0.40 (0.94–0.38) | 0.15 | 0.82 |
| % Isovaleric acid | 3.91 (2.61–5.92) | − 0.15 (− 2.29 to 0.86) | 0.15 | 3.52 (1.99–5.32) | 0.04 (− 1.01 to 0.77) | 0.62 | 0.09 |
| Valeric acid (mmol/kg) | 1.61 (1.06–2.26) | 0.06 (− 0.78 to 0.98) | 0.80 | 1.57 (1.19–2.99) | − 0.13 (− 0.50 to 0.28) | 0.33 | 0.44 |
| % Valeric acid | 2.93 (2.34–3.42) | − 0.43 (− 1.20 to 0.29) | 0.12 | 2.81 (1.96–3.30) | − 0.02 (− 0.34 to 0.41) | 0.80 | 0.08 |
| Isocaproic acid (mmol/kg) | 0.00 (0.00–0.00) | 0.00 (0.00–0.00) | 0.32 | 0.00 (0.00–0.00) | 0.00 (0.00–0.00) | 1.00 | 0.32 |
| % Isocaproic acid | 0.00 (0.00–0.00) | 0.00 (0.00–0.00) | 0.32 | 0.00 (0.00–0.00) | 0.00 (0.00–0.00) | 1.00 | 0.32 |
| Caproic acid (mmol/kg) | 0.09 (0.00–0.62) | 0.00 (− 0.14 to 0.05) | 0.98 | 0.15 (0.00–0.15) | 0.00 (− 0.17–0.17) | 0.98 | 0.90 |
| % Caproic acid | 0.20 (0.00–0.50) | 0.00 (− 0.45 to 0.08) | 0.33 | 0.23 (0.00–1.59) | 0.00 (− 0.33–0.10) | 0.98 | 0.38 |

Data are median (25th–75th percentiles). Wilcoxon signed rank test

Significant differences in bold

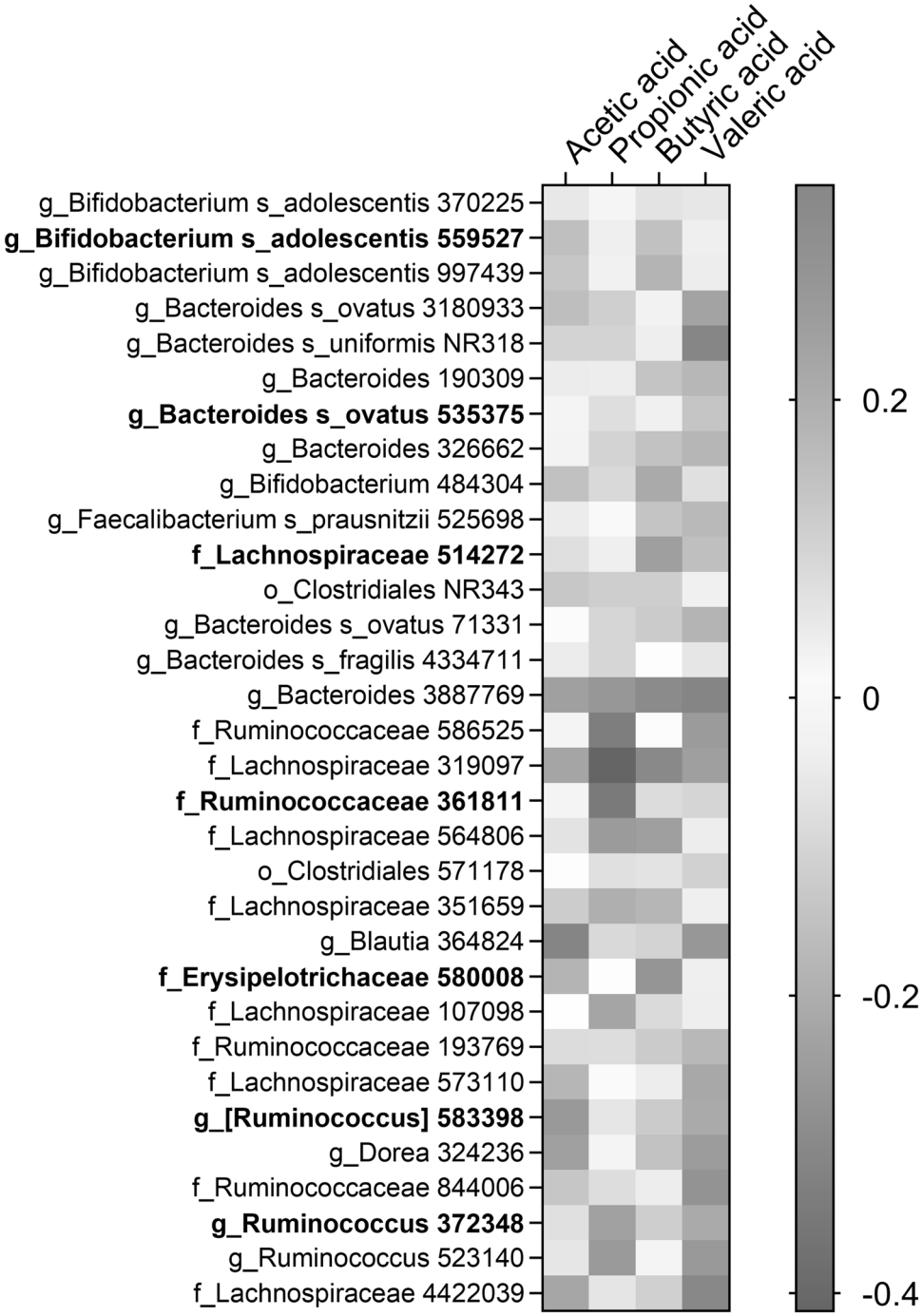


**Fig. 4** Heatmap of OTUs related to SCFA by PLS regression. Only OTUs affected by the prebiotic intervention are presented and sorted by their effect sizes (as in Fig. 2). Correlation is estimated with Spearman's rho coefficient, where red is a positive and blue is a negative relation. Asterisk indicates significant relationship (VIP > 1.2). Dominating OTUs (> 0.1%) are indicated in bold

[11, 12]. Importantly, the faecal concentrations of SCFA is only an estimate of colonic SCFA production. Inulin is rapidly fermented in the proximal colon and most of the SCFA produced are absorbed during transit through the colon, and only few percents remain in the faeces [50]. Apart from the substrate availability, SCFA concentrations in faecal samples are also determined by the absorption rate into the systemic circulation and portal vein, transit time through colon and cross-feeding establishments in the microbiota. Changed faecal SCFA is rather an indication of changed bacterial activity in the gut and thus a valuable measurement when exploring the effect of prebiotic supplements.

Lately, metformin has been shown to affect the gut microbiota, and may, thus, confound the results in clinical trials investigating the composition of gut bacteria in populations with type 2 diabetes [11]. The majority of the participants in our study (68%) used metformin during the intervention, all with a dose that was kept unchanged, and we found no difference in the overall faecal microbiota between participants using metformin or not.

The strengths of this study include the randomised double-blind crossover design, high level of compliance, no dropouts related to the intervention, and assessment of habitual diet and medication known as possible confounders. To minimise the risk of carry-over effects, we included a washout period of 4 weeks. The bacterial response in the gut to dietary intervention occurs within few days and returns to its original state at the same rate when the intervention is discontinued [57]. A remaining effect of prebiotics on faecal SCFA after a 4-week-long washout is, thus, unlikely and no differences between baseline concentrations before and after the washout were found (Online Resource 6).

One clear limitation of this study is measuring of faecal SCFA as a proxy for the colonic production of SCFA. The treatment duration of 6 weeks may also have been too short to enhance the microbial diversity. Another limitation is that the sample size was calculated based on expected effects on the primary outcome measurement from the main study (GLP-1 response) and not on expected effects on composition of the microbiota. However, bifidogenic effect on gut bacteria has been found in comparable studies that have evaluated the effects of inulin-type fructans, both with similar and lower sample sizes [14, 15, 17, 18]. Although we expected some beneficial effects on microbiota composition, diversity and SCFA production, the microbiota analysis should be considered as explorative. Hence, it does not make sense to perform power analysis on selected bacteria groups post hoc. There is also no established method for calculating the power of a multivariate analysis, although some simulation-based approaches have been suggested. However, the fact that moderate changes in total microbiota (1.5%) were observed with relatively low $p$ values ($<0.05$) indicate that the sample size is sufficiently high.

Results from the FFQ assessment at baseline also showed that our participants slightly exceeded the criteria for allowed fibre intake (mean of 32.2 g per day). This indicates that the study population had higher habitual fibre intake than the general population with type 2 diabetes in Norway, and were on the other hand adherent to the Norwegian dietary recommendations of 25–35-g fibre per day [58]. This may have affected the baseline microbiota composition and diversity and thus its responsiveness to the prebiotic fibre. However, no significant correlation was found between baseline data such as fibre intake, microbial diversity or bifidobacteria levels on the bifidogenic response in the study. This is in contrast to other studies that reported more pronounced bifidogenic response with higher habitual fibre intake [19] and lower baseline levels of bifidobacteria [14, 15, 59, 60]. Nevertheless, regarding the results from the FFQ, we cannot exclude a reporter bias due to the participants' knowledge of the nature of the study. All dietary assessment methods are known to be biased by both over- and underreporting. This is clearly illustrated by some of the extreme reported intakes of fibre in this study (Table 1). Hence, the data on dietary fibre intake should only be interpreted on group level and not individually.

## Conclusions

In the present study, a daily supplement of inulin-type fructans induced a moderate, but significant increase in faecal levels of bifidobacteria, total SCFA, acetic acid and propionic acid in patients with type 2 diabetes. We were not able to detect any effects on the overall microbial diversity or faecal butyric acid. Our findings imply a moderate potential for these prebiotic fibres to improve the intestinal microenvironment in type 2 diabetes.

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**Author contributions** EB: researched data, performed clinical assessments, and drafted the manuscript; SG and AA: initiated and designed the study and researched data; KIB: researched data; IR: performed microbiota analysis and data processing; JV: was responsible for laboratory analysis of faecal SCFA; IM: performed analysis of microbial data. All authors participated in manuscript writing, review, editing and discussion and have read and approved the final version.

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**Availability of data and material** Data described in the manuscript and analytic code will be made available upon request pending application and approval.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** The trial was approved by the Regional Ethics Committee for Medical and Health Research and registered at clinicaltrials.gov (NCT02569684). The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients prior to their inclusion in the study.

**Consent to participate** Written informed consent was obtained from all patients prior to their inclusion in the study.

**Consent for publication** This manuscript is not being simultaneously submitted elsewhere and no portion of the data has been published elsewhere.



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# Electronic Supplementary Material

Prebiotic effect of inulin-type fructans on fecal microbiota and short-chain fatty acids in type 2 diabetes: A randomized controlled trial

European Journal of Nutrition

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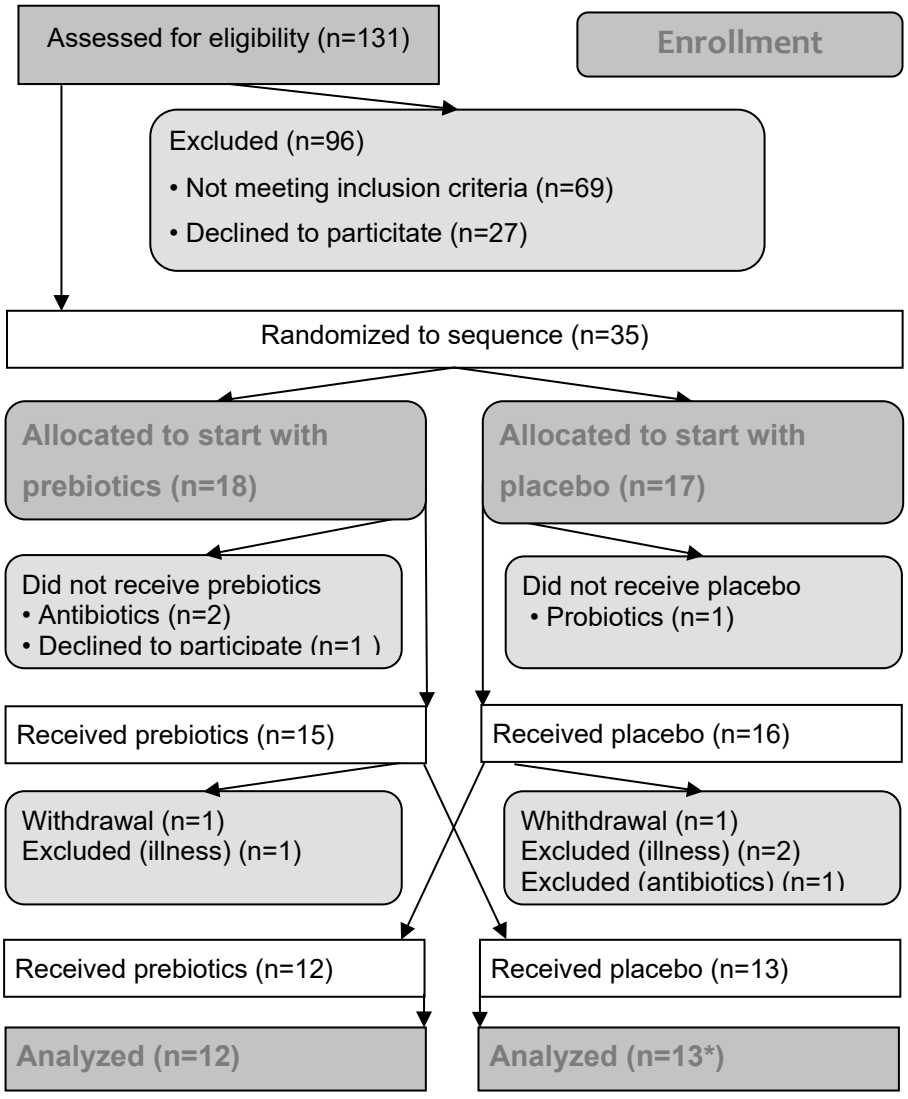


**Online Resource 1.** Flow chart showing all patients approached for the study. *n=12 for microbiota analysis

# Electronic Supplementary Material

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**Online Resource 2**. ANOVA table and post-hoc level comparisons of the intervention design at different taxonomic levels.

| | Multivariate Analysis of Variance (ASCA) | | | Post-hoc comparisons between Treatment x Week levels (PLS-DA) | |
|---|---|---|---|---|---|
| | Explained variance (%) | | | Explained variance (%) (cross-validated) | |
| Taxonomic level | Between subjects effect | Treatment x Week effect | Error | Treatment vs baseline/placebo | Baseline1 vs baseline2 |
| L2 | 64.9 ($p < 0.001$) | 2.2 ($p = 0.091$) | 32.9 | 8.0 | 0.0 |
| L4 | 65.7 ($p < 0.001$) | 1.7 ($p = 0.195$) | 32.5 | 0.0 | 0.0 |
| L5 | 73.9 ($p < 0.001$) | 1.1 ($p = 0.457$) | 25.0 | 0.0 | 0.0 |
| L6 | 73.2 ($p < 0.001$) | 1.3 ($p = 0.123$) | 25.5 | 6.0 | 0.0 |
| L7 | 69.9 ($p < 0.001$) | 1.5 ($p = 0.049$) | 28.6 | 43.0 | 0.0 |

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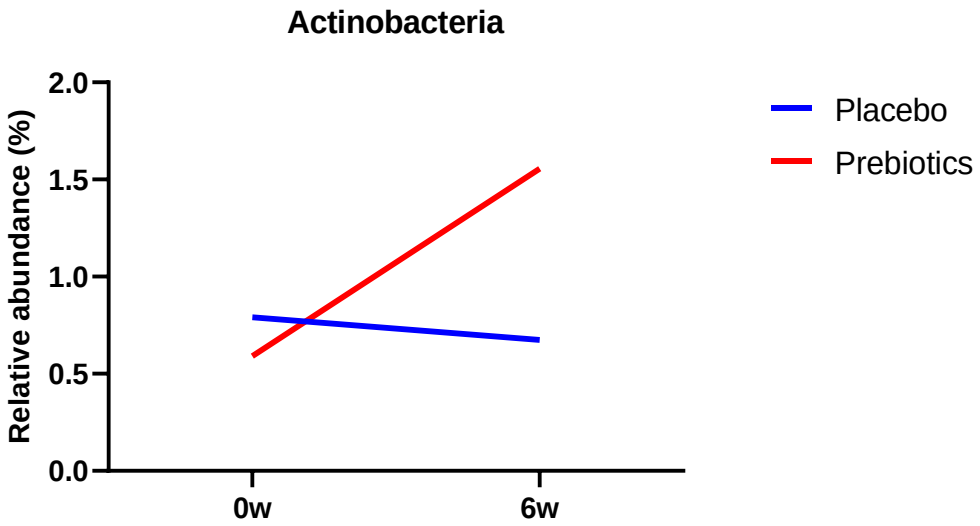


**Online Resource 3.** Interaction plot of the prebiotic effect on Actinobacteria (VIP 1.32)

# Electronic Supplementary Material

Prebiotic effect of inulin-type fructans on fecal microbiota and short-chain fatty acids in type 2 diabetes: A randomized controlled trial

European Journal of Nutrition

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Ida Rud and Anne-Marie Aas share last authorship

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**Online Resource 4**. Overview of prebiotic affected OTUs determined by PLS-DA and their correalations to SCFAs (%)

| **Bacterial OTUs** | | | **Prebiotic affected OTUs determined by PLS-DA** | | | **PLS regression of prebiotic affected OTUs and SCFA (%)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Acetic acid | | Propionic acid | | Butyric acid | | Valeric acid | |
| | | | | | | Explained variance: 11% | | Explained variance: 27% | | Explained variance: 18% | | Explained variance: 31% | |
| OTU | Taxonomy | Average abundance (%) | VIP | Pearson correlation with interaction | Effect size (Prebiotics - placebo/baseline) | Spearmans rho | VIP | Spearmans rho | VIP | Spearmans rho | VIP | Spearmans rho | VIP |
| 370225 | p_Actinobacteria; g_Bifidobacterium; s_adolescentis | 0.0140 | 3.05 | 0.99 | 1.78 | 0.05 | 0.43 | -0.02 | 0.42 | -0.07 | 0.60 | 0.05 | 0.56 |
| 559527 | p_Actinobacteria; g_Bifidobacterium; s_adolescentis | 0.6200 | 3.19 | 0.99 | 1.76 | 0.15 | 0.92 | -0.04 | 0.23 | -0.15 | 1.28 | -0.04 | 0.61 |
| 997439 | p_Actinobacteria; g_Bifidobacterium; s_adolescentis | 0.0089 | 2.64 | 0.99 | 1.59 | 0.13 | 0.98 | -0.03 | 0.27 | -0.18 | 1.33 | 0.04 | 0.30 |
| 3180933 | p_Bacteroidetes; g_Bacteroides; s_ovatus | 0.0059 | 1.79 | 0.99 | 1.19 | 0.15 | 1.31 | -0.12 | 0.52 | -0.03 | 0.29 | -0.23 | 0.95 |
| NR318* | p_Bacteroidetes; g_Bacteroides; s_uniformis | 0.0239 | 1.57 | 0.90 | 0.90 | 0.10 | 1.06 | 0.10 | 1.05 | -0.04 | 0.65 | -0.31 | 1.50 |
| 190309 | p_Bacteroidetes; g_Bacteroides; s_ | 0.0062 | 1.36 | 0.99 | 0.90 | -0.05 | 0.31 | 0.04 | 0.45 | 0.14 | 0.86 | -0.18 | 0.50 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 535375 | p_Bacteroidetes; g_Bacteroides; s_ovatus | 0.9360 | 1.37 | 0.92 | 0.90 | 0.02 | 0.04 | -0.08 | 0.60 | 0.03 | 0.48 | -0.14 | 0.79 |
| 326662 | p_Bacteroidetes; g_Bacteroides; s_ | 0.0728 | 1.36 | 0.92 | 0.90 | 0.03 | 0.06 | -0.11 | 1.12 | 0.14 | 1.07 | -0.18 | 0.95 |
| 484304 | p_Actinobacteria; g_Bifidobacterium; s_ | 0.0695 | 1.57 | 0.93 | 0.88 | 0.15 | 1.24 | 0.08 | 0.46 | -0.21 | 1.61 | -0.07 | 1.30 |
| 525698 | p_Firmicutes; g_Faecalibacterium; s_prausnitzii | 0.0338 | 1.35 | 0.92 | 0.88 | 0.04 | 0.23 | -0.02 | 0.18 | 0.14 | 0.96 | -0.17 | 0.50 |
| 514272 | p_Firmicutes; f_Lachnospiraceae; g_; s_ | 0.1467 | 1.48 | 0.94 | 0.82 | -0.08 | 0.45 | -0.04 | 0.47 | 0.24 | 1.43 | -0.16 | 1.03 |
| NR343* | p_Firmicutes; o_Clostridiales; f_; g_; s_ | 0.0154 | 1.26 | 0.98 | 0.70 | 0.13 | 1.25 | -0.12 | 0.75 | -0.12 | 0.89 | -0.04 | 1.06 |
| 71331 | p_Bacteroidetes; g_Bacteroides; s_ovatus | 0.0624 | 1.32 | 0.96 | 0.67 | -0.01 | 0.05 | 0.09 | 0.79 | 0.12 | 0.54 | -0.18 | 1.19 |
| 4334711 | p_Bacteroidetes; g_Bacteroides; s_fragilis | 0.0039 | 1.26 | 0.94 | 0.65 | -0.05 | 0.14 | 0.09 | 0.81 | -0.01 | 0.18 | -0.06 | 0.41 |
| 3887769 | p_Bacteroidetes; g_Bacteroides; s_ | 0.0037 | 1.26 | -0.97 | -0.36 | 0.24 | 1.78 | 0.28 | 1.36 | -0.30 | 2.11 | -0.31 | 1.60 |
| 586525 | p_Firmicutes; f_Ruminococcaceae; g_; s_ | 0.0114 | 1.29 | -0.90 | -0.70 | -0.03 | 0.30 | -0.33 | 1.81 | 0.01 | 0.29 | 0.26 | 0.97 |
| 319097 | p_Firmicutes; f_Lachnospiraceae; g_; s_ | 0.0818 | 1.40 | -0.99 | -0.84 | -0.22 | 1.44 | -0.41 | 2.50 | 0.33 | 1.88 | 0.24 | 0.98 |
| 361811 | p_Firmicutes; f_Ruminococcaceae; g_; s_ | 0.3521 | 1.29 | -0.92 | -0.84 | 0.02 | 0.21 | -0.35 | 1.86 | 0.08 | 0.23 | 0.10 | 0.51 |
| 564806 | p_Firmicutes; f_Lachnospiraceae; g_; s_ | 0.0272 | 1.48 | -0.95 | -0.88 | 0.06 | 0.63 | 0.26 | 1.45 | -0.24 | 1.96 | -0.04 | 0.78 |
| 571178 | p_Firmicutes; o_Clostridiales; f_; g_; s_ | 0.0110 | 1.32 | -0.98 | -0.88 | 0.00 | 0.01 | 0.07 | 0.50 | 0.06 | 0.27 | -0.11 | 0.67 |
| 351659 | p_Firmicutes; f_Lachnospiraceae; g_; s_ | 0.0987 | 1.41 | -0.96 | -0.90 | -0.12 | 1.19 | 0.20 | 0.81 | 0.18 | 1.05 | -0.04 | 0.59 |
| 364824 | p_Firmicutes; g_Blautia | 0.0077 | 1.58 | -0.99 | -0.91 | -0.31 | 2.18 | 0.09 | 0.90 | 0.10 | 0.90 | 0.27 | 1.29 |
| 580008 | p_Firmicutes; f_Erysipelotrichaceae; g_; s_ | 0.2837 | 1.37 | -0.93 | -0.92 | -0.18 | 1.69 | 0.00 | 0.28 | 0.28 | 2.06 | -0.04 | 0.70 |
| 107098 | p_Firmicutes; f_Lachnospiraceae; g_; s_ | 0.0055 | 1.42 | -0.97 | -0.94 | 0.00 | 0.40 | 0.23 | 1.17 | -0.09 | 0.73 | -0.04 | 0.13 |
| 193769 | p_Firmicutes; f_Ruminococcaceae; g_; s_ | 0.0063 | 1.59 | -0.95 | -1.03 | -0.08 | 0.67 | -0.08 | 0.74 | 0.12 | 0.72 | 0.17 | 0.90 |
| 573110 | p_Firmicutes; f_Lachnospiraceae; g_; s_ | 0.0671 | 1.62 | -0.90 | -1.07 | -0.18 | 1.95 | -0.01 | 0.77 | 0.04 | 0.81 | 0.22 | 0.88 |
| 583398 | p_Firmicutes; f_Lachnospiraceae; g_[Ruminococcus]; s_ | 0.5781 | 1.88 | -0.95 | -1.09 | -0.26 | 1.63 | 0.06 | 0.38 | 0.12 | 0.87 | 0.21 | 1.18 |
| 324236 | p_Firmicutes; f_Lachnospiraceae; g_Dorea; s_ | 0.0216 | 1.90 | -0.95 | -1.13 | -0.24 | 2.03 | 0.02 | 0.18 | 0.14 | 1.17 | 0.26 | 1.44 |
| 844006 | p_Firmicutes; f_Ruminococcaceae; g_; s_ | 0.0081 | 1.80 | -0.97 | -1.16 | -0.14 | 0.85 | -0.08 | 0.68 | 0.04 | 0.22 | 0.29 | 1.15 |
| 372348 | p_Firmicutes; f_Ruminococcaceae; g_Ruminococcus; s_ | 0.5814 | 2.07 | -0.94 | -1.27 | -0.07 | 0.88 | -0.24 | 1.43 | 0.11 | 1.02 | 0.21 | 1.07 |
| 523140 | p_Firmicutes; f_Ruminococcaceae; g_Ruminococcus; s_ | 0.0113 | 2.06 | -0.99 | -1.34 | -0.06 | 0.49 | -0.25 | 1.56 | 0.02 | 0.34 | 0.27 | 1.17 |
| 4422039 | p_Firmicutes; f_Lachnospiraceae | 0.0220 | 2.08 | -0.96 | -1.39 | -0.22 | 1.95 | -0.06 | 0.47 | 0.11 | 0.88 | 0.34 | 1.63 |

*NR - New.ReferenceOTU

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**Online Resource 5.** Alpha diversity analysis of the intervention design using Mixed Effects Model (ANOVA).

| Alpha diversity metrics | Observed OTUs | ChaoI | PD_whole_tree |
|---|---|---|---|
| Subject | 0.004 | 0.004 | 0.002 |
| Treatment | 0.748 | 0.886 | 0.686 |
| Week | 0.125 | 0.146 | 0.322 |
| Period | 0.507 | 0.427 | 0.623 |
| Treatment x Week | 0.236 | 0.385 | 0.282 |

# Electronic Supplementary Material

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**Online Resource 6** SCFA baseline concentrations before and after washout[1]

| | **Baseline concentrations** | | |
|---|---|---|---|
| | **Before washout** | **After washout** | ***p* value** |
| Total SCFA (mmol/kg) | 62.70 (51.91-78.62) | 62.67 (49.17-82.95) | 0.91 |
| Acetic acid (mmol/kg) | 36.00 (30.59-43.23) | 36.71 (26.47-41.15) | 0.39 |
| Propionic acid (mmol/kg) | 11.02 (8.15-15.69) | 11.06 (6.89-15.99) | 0.98 |
| Butyric acid (mmol/kg) | 9.21 (7.29-16.20) | 11.95 (8.49-15.03) | 0.44 |

[1]Data are median ($25^{th}$-$75^{th}$ percentils). Wilcoxon Signed Rank Test. Significant differences in bold

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https://doi.org/10.1007/s00394-020-02314-0

CORRECTION

# Correction to: Prebiotic effect of inulin-type fructans on faecal microbiota and short-chain fatty acids in type 2 diabetes: a randomised controlled trial

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**Correction to: European Journal of Nutrition**
**https://doi.org/10.1007/s00394-020-02282-5**

The original version of this article unfortunately contained a mistake. The presentation of Fig. 4 was incorrect.

The corrected Fig. 4 is placed in the following page.

The original article can be found online at https://doi.org/10.1007/s00394-020-02282-5.

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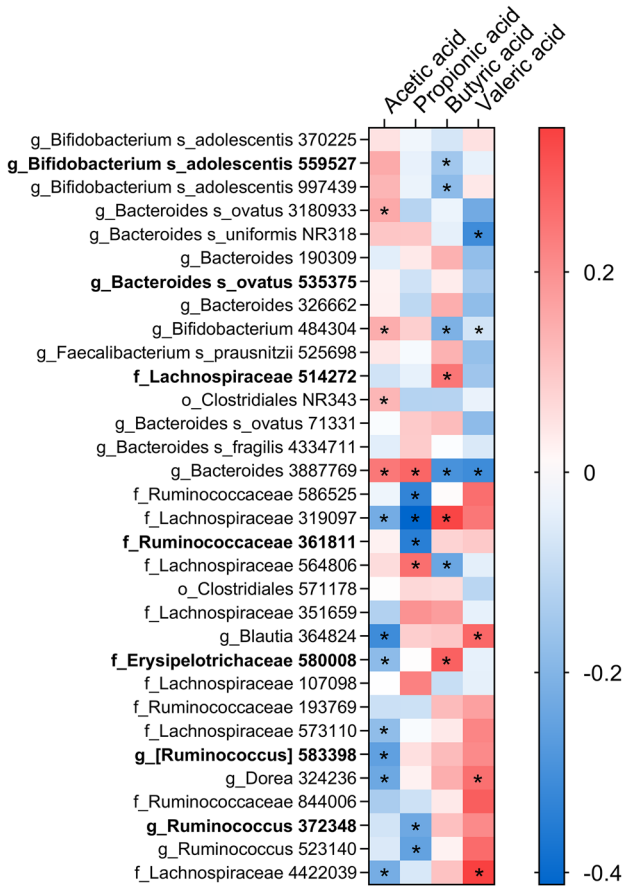


**Fig. 4** Heatmap of OTUs related to SCFA by PLS regression. Only OTUs affected by the prebiotic intervention are presented and sorted by their effect sizes (as in Fig. 2). Correlation is estimated with Spearman's rho coefficient, where red is a positive and blue is a negative relation. Asterisk indicates significant relationship (VIP > 1.2). Dominating OTUs (> 0.1%) are indicated in bold