TAL TECH

DOCTORAL THESIS

The Effect of Diet and Dietary Fiber on the Human Gut Microbiota *in vitro* and *in vivo*

Madis Jaagura

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TALLINN UNIVERSITY OF TECHNOLOGY
School of Science
Department of Chemistry and Biotechnology
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Supervisor: Kaarel Adamberg, PhD
Division of Food Technology
Tallinn University of Technology
Tallinn, Estonia

Co-supervisor: Professor Raivo Vilu, PhD
Department of Chemistry and Biotechnology
Tallinn University of Technology
Tallinn, Estonia

Opponents: Associate Professor Henrik Munch Roager, PhD
Department of Nutrition, Exercise and Sports
University of Copenhagen
Copenhagen, Denmark

Associate Professor Elin Org, PhD
Institute of Genomics
University of Tartu
Tartu, Estonia

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Madis Jaagura

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Toitumise ja kiudainete mõju inimese seedetrakti mikrobiootale *in vitro* ja *in vivo*

MADIS JAAGURA

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Contents

List of publications

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II Adamberg, K., Jaagura, M., Aaspõllu, A., Nurk, E., & Adamberg, S. (2020). The composition of faecal microbiota is related to the amount and variety of dietary fibres. International Journal of Food Sciences and Nutrition, 1–11.

III Jaagura, M., Viiard, E., Karu-Lavits, K., & Adamberg, K. (2021). Low-carbohydrate high-fat weight reduction diet induces changes in human gut microbiota. MicrobiologyOpen, 10(3), e1194.

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Contribution to the papers in this thesis are:

I Experimental work, analysis, interpretation

II Data analysis, interpretation, and manuscript drafting

III Data analysis, interpretation, and manuscript drafting

Introduction

Significant associations between metabolic health, food intake and gut microbiota structure indicate the potential of diet-based strategies for improving human well-being. Limited information about the effect of food composition on the microbial consortia advocates for the future research to form a basis for healthy diets.

Nutritional value of diets is characterized by total energy and the balance of micro- and macronutrients. Human digestive system can effectively extract energy from dietary proteins and fats, while a significant fraction of ingested carbohydrates can be extracted only by the cooperation of the intestinal bacteria. These mostly plant origin indigestible components, collectively called as dietary fiber (DF) together with host-derived components form a major source of energy for the gut microbiota.

Modern diet incorporates refined foods which are low in DF. It has been estimated that daily consumption of DF is on average only 50-75% of minimally recommended values (which is 25-35 g of fiber per day). Studies suggest that changes in dietary choices in recent 50-80 years could be the main reason for the low diversity of the gut microbiota and premise for the modern metabolic diseases.

Resident microorganisms outnumber the human cells: it has been estimated that approximately 10^{14} bacteria reside in and on the human body, collectively forming a human microbiota. Hundreds of species filling out the different niches of the body - specific resident microbes in the stomach, small, and large intestine. Compared to the upper gastrointestinal tract, the environment of the large intestine favors the growth of obligate anaerobic bacteria with low oxygen levels, slow transit, and mildly acidic conditions. A large bowel hosts the most diverse and populous bacterial community of a single individual. The major players of the adult gut include members of Bacteroidetes, Firmicutes, and Actinobacteria. Levels of the gut bacteria change with age, diet and differing ratios have been associated with significant effect on human metabolic health and disease. Thus, considerable research has been devoted to investigate how the human gut microbiota structure could be altered for the benefit of the host.

Synergistic processing, i.e., degradation and fermentation of polysaccharides by the gut microbiota yields significant amounts of short-chain fatty acids (SCFA-s). These bacteria derived metabolites can benefit the host mostly as an energy source, e.g., by feeding the gut epithelial cells in the case of butyrate, be metabolized by liver or reach other peripheral tissues in the case of propionate and acetate, respectively. On the other hand, surplus or imbalance of bacterial metabolites can lead to gastrointestinal discomfort and harm human health.

As gut microbiota harbors a complicated machinery to facilitate the degradation of complex carbohydrate molecules, a better understanding is needed of the processing of indigestible dietary components by the gut community. More specifically, which gut species react and respond to the variable intake of carbohydrates from different sources, and how these components are metabolized into health affecting substances. Advancements in sequencing technologies have expanded the possibilities for human microbiota research and allow for more detailed analysis providing a more complete picture of the gut habitat interactions and reactions to dietary components.

Literature overview of this thesis gives an introduction to human gut microbiota and its functions, elaborates the plant polysaccharide degradation, and SCFA production by the functional groups.

The results presented in this thesis describe the gut microbiota structure in a subgroup of Estonian population, the habitual dietary patterns regarding fiber consumption, the associations between food intake and growth of explicit microbial groups, and the *in vitro* metabolic activity of colon microbiota on various nondigestible carbohydrates.

Abbreviations

5C	five carbon sugars
6C	six carbon sugars
AG	arabinogalactan
AX	arabinoxylan
AXOS	arabinoxylan-oligosaccharide
CAZy	carbohydrate-active enzyme (database)
CAZymes	carbohydrate-active enzymes
CE	carbohydrate esterase
B-C	Bray-Curtis
BMI	body mass index
EMP	Embden-Meyerhof-Parnas (pathway)
DF	dietary fiber
DP	degree of polymerization
FOS	fructo-oligosaccharide
GH	glycoside hydrolase
GOS	galacto-oligosaccharide
GPCR	G-protein coupled receptors
HAG	high abundance group
HDL	how-density lipoprotein
HF	high fiber
HP	high polymerization (inulin)
HPLC	high-performance liquid chromatography
HSI	high soluble inulin (oligofructose)
IMC	isothermal microcalorimetry
LAG	low abundance group
LDL	low-density lipoprotein
LCHF	low-carbohydrate high-fat
Lev	levan
LF	low fiber
ND	not detected
NGS	next generation sequencing
NMDS	non-metric multidimensional scaling
PFL	pyruvate formate lyase (pathway)
PL	polysaccharide lyase
POS	pectic oligosaccharides
PPP	pentose phosphate pathway
PUL	polysaccharide utilization loci
rDNA	ribosomal DNA
RS	resistant starch

SCFA	short-chain fatty acid
WLD	weight loss diet
XOS	xylo-oligosaccharide

1 Literature overview

1.1 Human microbiota

The human body is inhabited by a diverse range of microorganisms, collectively called as human microbiota. These microorganisms are of bacterial, archaeal, fungal or viral origin and specific for a defined environment, e.g., different body sites and organs. The term "microbiota" has been in use for over 50 years (Prescott, 2017) and forms a basis for a more broad definition of "microbiome", which includes microorganisms and their genomes along with surrounding environmental conditions, while more strict understanding of the term "microbiome" includes only the collection of genes and genomes of a microbiota members (Marchesi & Ravel, 2015). Still, the terminology has not always been clear as these terms have been used interchangeably (Ursell et al., 2012).

Most populous environment in the human body is the intestinal tract which supports up to 10^{14} microbes (Ley, Peterson, et al., 2006; Sender et al., 2016). Bacterial levels increase along the gastrointestinal tract as lowest counts have been observed in the stomach and accordingly, highest in the distal colon (Payne et al., 2012).

Human gastrointestinal microbiota is mainly modulated by the transit time, pH, and nutrient availability (Gibson & Roberfroid, 1995) and more optimal ratio of these conditions favors the high colonic microbial growth up to 10^{12} cells per gram (Payne et al., 2012). The intestinal microbiota comprise the main fraction of fecal matter (Stephen & Cummings, 1980).

1.2 Gut microbiota functions

As the human gut microbiota has co-evolved with the host, it can provide multiple functions from digestion of complex dietary components and production of metabolites to immunomodulation and protection against pathogenic agents (Koh et al., 2016).

1.2.1 Nutrient and host metabolites metabolism

Intake of food introduces a diverse range of nutrients, some easily degraded by the host enzymes while other dietary components need to be broken down into smaller fragments to benefit the host. Carbohydrates that have escaped digestion by human enzymes are mostly degraded and metabolized by the colon bacteria and thus contribute to human energy intake in a significant manner. Similarly, undigested fats and proteins can reach the large intestine and be a substrate for colonic fermentation. As a result, short chain fatty acids (SCFAs), primarily butyrate, propionate and acetate are being produced to be absorbed and metabolized by the host. These major microbial products play an important role in maintaining human and gut health.

Aside from carbohydrates, plant-based foods contain a significant amount of polyphenols, and recent studies have shown the significant involvement of the gut microbiota in the breakdown process of these dietary components (Aravind et al., 2021). The contribution of microbial fermentation to human energy intake through the activities of intestinal microorganisms is significant (Krajmalnik-Brown et al., 2012). Aside SCFAs, important fermentation products include succinate, lactate, hydrogen, CO2, ethanol.

1.3 The effect of SCFAs on human health

SCFAs, a subgroup of fatty acids made of six or less carbons, and organic acids, succinate and lactate are derived from intestinal microbial fermentation. These metabolites are absorbed through the portal vein and then further metabolized by other tissues. Butyrate is also the important substrate for both colonocytes and enterocytes (Donohoe et al., 2011). The metabolic fate of succinate is different - it is either transformed to propionate by the intestinal microbiota or metabolized to glucose by the intestinal epithelial cells limiting its effects within the gut (De Vadder et al., 2016). Propionate is mainly metabolized in the liver, while acetate is the only SCFA with more systemic implications (Morrison & Preston, 2016). Lactate can be converted to acetate, butyrate or propionate by lactate-utilizing bacteria (Belenguer et al., 2006; Bourriaud et al., 2005; Morrison et al., 2006), while butyrate can be also derived from acetate (Duncan, Holtrop, et al., 2004).

SCFAs promote the secretion of mucus and exert immunomodulatory effects through G-protein coupled receptors (GPCRs) (Husted et al., 2017; Thorburn et al., 2014). Compared to other SCFAs, acetate may have obesogenic properties and enhance fat storage (Gao et al., 2016; Perry et al., 2016). Butyrate and propionate provide metabolic benefits by inducing intestinal gluconeogenesis through mediation of intracellular cAMP or interaction with the gut-brain axis, respectively (De Vadder et al., 2014). Furthermore, butyrate metabolism by the intestinal epithelial cells reduces the availability of oxygen and supports the growth of anaerobic bacteria (Roediger, 1980). But most importantly, butyrate can interact with transcription factors (Shakespear et al., 2011) by inhibiting histone deacetylases (Davie, 2003) resulting in enhanced gut barrier function (Kelly et al., 2015; Kelly & Colgan, 2016). Butyrate also induces antimicrobial peptide production (Zhao et al., 2018), cell proliferation (Park et al., 2016) and reduces inflammation (Chang et al., 2014).

1.4 Development and stability of the adult human gut microbiota

Change from less diverse infant microbiota to adult-like microbiota (Yatsunenko et al., 2012) with more complex structure coincides with establishment of enterotypes between 9 and 36 months of age (Bergström et al., 2014). Driven by species composition, enterotypes are loosely separated clusters of human gut communities, which are identified by their predominant bacteria from *Bacteroides*, *Prevotella* and *Ruminococcus* genera (Arumugam et al., 2011). Personal status of enterotypes is relatively robust (Roager et al., 2014).

Similarity of personal gut microbiota profiles in short-term is very high, indicating “fingerprint-like” properties, although, decreasing significantly over time (Faith et al., 2013). Uniqueness of the individual gut microbial consortia is further asserted by the high average beta-diversity between unrelated subjects (Goodrich et al., 2014) compared to low within-person metagenomic variation (Mehta et al., 2018).

Multiple factors from diet to genetic variation play a role in shaping gut microbiota composition and enterotype distribution as both have been correlated with geographical distance, which as a single parameter could collectively represent the effect of environmental and genetic factors (Mobeen et al., 2018). For example, a recent study that recruited participants from five European countries showed significant differences between the dietary intake of the residents, which were also reflected in the gut microbiota profiles (Ghosh et al., 2020).

It has been proposed that host genetics can have influence on metabolic phenotypes through shaping of the gut microbiota as family members have been shown to share gut microbes (Schloss et al., 2014). The association between host genetic variation and gut microbiome has been studied in more detail on twin-pairs by Goodrich and others (Goodrich et al., 2014). They showed that *Christensenellaceae*, *Methanobacteriaceae*, and *Bifidobacteriaceae* members are one of the most heritable taxa while Bacteroidetes members are mostly environmentally determined and not heritable (Goodrich et al., 2014). Interestingly, higher relevance of environmental effects on the human gut communities was suggested by a newer study from 2018, which showed the gut microbiome Westernization in individuals after long-term migration from Thailand to US (Vangay et al., 2018). Similarly, analysis of a large cohort of 1046 individuals and reanalysis of other datasets by Rothschild and others demonstrated that if corrected for environmental factors, microbiome similarity among relatives is not significant compared to the high variance explained by environmental features (Rothschild et al., 2018).

Microbiome association index calculated by Rothschild and others showed the more significant effect of blood lipid parameters, lactose consumption, and obesity status parameters (Rothschild et al., 2018). In addition to previously mentioned parameters, Falony and others showed that microbiome covaries more with stool consistency, red blood cell count, and blood uric acid concentration, but less with dietary intake (Falony et al., 2016). However, it is possible that modest effects of nutrition and dietary parameters are explained by the relative lack of high quality and resolution food intake data collected in the population studies together with concurrent fecal sampling. Based on the detailed dietary records, daily microbiome variation is related to food choices (Johnson et al., 2019). Supporting the significant effect of the dietary intake choices on the gut microbiota levels, rapid alteration of the microbial community structure has been observed by David and others, who showed the significant effect of the dietary extremes (David et al., 2014). For example, the animal-based diet was shown to support bile-resistant bacteria at the expense of plant polysaccharide degrading bacteria (David et al., 2014).

1.5 The core gut microbiota

The composition of adult gut microbiota and predominant species have been well characterized using 16S amplicon sequencing (Falony et al., 2016). Across all included datasets and based on 3948 samples, core microbiota was formed by *Lachnospiraceae unclassified*, *Ruminococcaceae unclassified*, *Bacteroides*, *Faecalibacterium*, *Blautia*, *Roseburia*, *Erysipelotrichaceae*, *Coprococcus*, *Dorea* (Falony et al., 2016). Recent re-analysis of 34 metagenomic datasets including 4347 stool samples showed six species that were prevalent in 90% of the samples: *Bacteroides ovatus (B. ovatus)*, *Bacteroides uniformis (B. uniformis)*, *Bacteroides vulgatus (B. vulgatus)*, *Faecalibacterium prausnitzii (F. prausnitzii)*, *Ruminococcus obeum*, and *Ruminococcus torques* (Gupta et al., 2020). Additional 307 species were prevalent in at least 1% of the samples.

In Estonia, only one study has characterized the fecal microbiota of eleven healthy adults with next generation sequencing (NGS) methods (Adamberg et al., 2015). In that study the dominant bacteria were listed as *Ruminococcaceae*, *Lachnospiraceae*, *Dialister*, *Faecalibacterium*, *Pseudobutyrivibrio*, *Subdoligranulum*, *Blautia*, *Prevotella*, *Bifidobacterium*, *Coprococcus*. A more recent study about adult Estonian population has shown dominance of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria phyla, and at family and genus level, *Clostridiales*, *Prevotellaceae*, *Finegoldia*, *Faecalibacterium*,

Bacteroides, *Streptococcus*, and *Campylobacter* in the rectal swab samples of pregnant women before and after labour (Drell et al., 2017). Additional previous studies carried out in Estonia characterize the gut microbiota of children and infants (Drell et al., 2014, 2015; Sepp et al., 2013). Only a very recent study published in an open archive has described core microbiota of 30 adult Estonian subjects (Krigul et al., 2021).

1.6 Prebiotics, probiotics, and synbiotics - definitions

Although prebiotic concept is already 25 years old, the most recent generally accepted definition for dietary prebiotics is from 2008: "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al., 2010). Prebiotics can be classified as compounds, which are: 1) resistant to acidic pH of the stomach, cannot be hydrolyzed by mammalian enzymes, and are not absorbed in the gastrointestinal tract; 2) fermented by the intestinal microbiota; 3) stimulate selectively the growth and/or activity of the intestinal bacteria, and as a consequence, improve host´s health (Gibson et al., 2010). Prebiotic status has been given to fructans (inulin and fructo-oligosaccharides (FOS)) and galacto-oligosaccharides (GOS), while starch and glucose-derived oligosaccharides, pectic oligosaccharide (POS), and non-carbohydrate oligosaccharides are considered as candidates (Davani-Davari et al., 2019; Lockyer & Stanner, 2019; Roberfroid et al., 2010).

Latest definition for synbiotics is from 2019: "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host", and they are separated into two subsets: complementary and synergistic (Swanson, Gibson, et al., 2020).

Latest definition for probiotics is from 2013, and it's an slightly updated version of the 2001´s: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). At the same time it was decided that human derived strains are probiotics and faecal microbiota transplants are not.

1.7 Food components available for microbial utilization

1.7.1 Microbiota accessible carbohydrates

Human food components can be divided into three categories: carbohydrates, proteins, and lipids. Carbohydrates consist mostly of pentoses or hexoses, and by molecule length are divided into mono- or disaccharides, oligosaccharides, and polysaccharides. Oligosaccharides are typically 3-9 monomer or longer molecules. Polysaccharides have molecule length equal or higher 10 monomers (Cummings & Stephen, 2007). Most abundant homopolymeric polysaccharides from plant sources are starch and cellulose. Larger portion of starches and most sugars (mono-, disaccharides) are easily digestible by humans and are not accessible to colonic microbiota, while celluloses are resistant to human enzymes. Starch fractions resisting digestion by human enzymes are called resistant starches (RSs). Xyloglucans, β-glucans, and homopolymeric xylans, mannans, galactans, arabinans, and their monomers comprise hemicelluloses and form approximately 25% of the plant cell wall polysaccharides (Scheller & Ulvskov, 2010). Significant fraction of the plant cell wall components include pectins, which are prevalent in peels of fruits and have the most complex structure of all plant carbohydrates (Mohnen, 2008; Ross et al., 1985).

Oligosaccharides mostly derived from plants include FOS; also called oligofructose or oligofructan, GOS; constituents of raffinose family oligosaccharides that are prevalent in legumes (J. Zhang et al., 2019), XOS, POS, etc. Most used synthetic oligosaccharide is polydextrose.

Non-saccharidic dietary components not digested by human enzymes include lignin, which are complex heteropolymeric noncarbohydrates significantly present in fruits and vegetables, and polyols, which are sugar alcohols present in certain fruits and vegetables (Rice et al., 2020).

Most polysaccharides are generally almost completely digested in the large intestine, but some (RS, cellulose, polydextrose) may escape complete degradation by the colonic microbiota (Cummings et al., 1996; Figdor & Bianchine, 1983), while, for example, lignin is not digested nor degraded by the gut microbes (Holloway et al., 1978).

1.7.2 Dietary fibers

Set by Codex Alimentarius and European Commission, DFs are lignin and carbohydrates with a degree of polymerization (DP) equal or higher than three, with one or more beneficial physiological effects if obtained from food raw material or have synthetic origin, and are not hydrolyzed by endogenous small intestinal enzymes (Howlett et al., 2010; Stephen et al., 2017).

Importantly, based on the water solubility, two categories of DFs exist: soluble fibers include pectins, fructans, resistant oligosaccharides, most hemicelluloses, while insoluble fibers contain cellulose, RS, chitin, lignin (Stephen et al., 2017). Insolubility along with packaging of polysaccharides limits the accessibility by bacterial enzymes due to less exposed surface area. This is crucial as insoluble carbohydrates and lignin form a significant proportion of overall DFs in human diet (Stephen et al., 2017), which means that bacteria need to employ multi-enzyme complexes to be able to succeed with carbohydrate degradation into smaller fractions.

1.7.3 Macronutrient metabolism

Lipids and proteins are mostly metabolized and absorbed in the small intestine, unless primary digestion is escaped by exceeding the rate of digestion (Hoyles & Wallace, 2010; Oliphant & Allen-Vercoe, 2019). They have a less significant role in manipulating the colonic processes directly due to lower availability and bacterial preference for carbohydrate degradation. Still, as carbohydrate levels decrease during the digestion, relative availability of residual lipids and protein increases leading to more considerable fermentation in the distal colon. Overall, human derived proteases have a more significant role in breaking down dietary proteins, thus providing the single amino acids for mostly anabolic use by the gut bacteria, as catabolic use is less energy efficient (Oliphant & Allen-Vercoe, 2019). In the context of colonic fermentation, aside positive effects from SCFA production, protein catabolism is associated with negative effects from production of the toxic compounds (Yao et al., 2016). Increased ammonia, phenols, and H_2S levels in the gut can negatively affect colonic epithelium structure and cause inflammation.

Correspondingly, increased lipid metabolism in the colon has been associated with major implications to human health due to microbially produced or modulated metabolites (Schoeler & Caesar, 2019). High dietary fat consumption can have a significant contribution to microbial community modulation by increasing bile acid release leading to increased levels of bile-tolerant microorganisms. Consumption of dietary fats can shift the hepatic conjugation of bile acids, from glycine to taurine

conjugates, which in turn can support hydrogen sulfide production by resident microbes from higher colonic availability of sulfur (Peck et al., 2019; Singh & Lin, 2015; Trefflich et al., 2020). Therefore, the variable intake of fibers and ratio of macronutrients in the diet can shift the fermentation type from carbohydrate utilization to proteolytic fermentation and vice versa (Diether & Willing, 2019; Neis et al., 2015).

1.8 Dietary fiber intake and sources in habitual diet

Current dietary recommendations suggest daily intake of 25-35 g of DFs for adults corresponding to 12.5 g/1000 kcal (Stephen et al., 2017). However, these values are not reached on a typical diet in European countries, which provide on average 16-24 g of fibers per day (Stephen et al., 2017). Main dietary fiber sources in habitual diet are grains and cereal products (one third to half of total DF intake), while second-third position is shared between vegetables or fruits, which provide less than quarter of total DFs (Stephen et al., 2017). Daily intake of over 4 g of RS has been also reported by several studies, but depending on the intake of the starch-containing foods, it can be up to 40 g per day and thus represent the major part of the fiber intake (Cummings & Macfarlane, 1991). Due to storage and cooking effects, exact amounts of RS in habitual diet could not be fully captured (Stephen et al., 2017). Data about the intake of specific fibers, e.g., arabinoxylans (AXs) or fructans are scarce. Recommended proportions of intakes of various food groups or specific fibers relies currently on the health claims assessed and approved by the European Food Safety Authority. Overall, intake of wholegrains, fruits and berries, vegetables, and legumes is recommended according to the Nordic Nutrition Recommendations 2012 (Øverby et al., 2013; Wirfält et al., 2013).

1.9 Degradation of specific dietary fibers by the gut microbiota

The following short overview presents the main sources, bacterial, and SCFA effects of all DFs used in the *in vitro* or *in vivo* experiments. Literature review covers human interventional trials and some animal model data, and *in vitro* experiments employing either pure cultures or fecal consortia from donors as inocula.

1.9.1 Galacto-oligosaccharides

GOS are prebiotics composed of a variable number of galactose units (Martins et al., 2019). Studies have shown that GOS is highly bifidogenic (Davis et al., 2010) at the expense of *Bacteroides* species (Davis et al., 2011). It has been also noted, that bifidogenic response to GOS in highly personal, but no specific other taxa or the initial levels of bifidobacteria have been associated with individual reaction (Davis et al., 2010, 2011). Still, GOS does not appear to have a synergistic effect to *Bifidobacterium animalis (B. animalis)* BB-12 or *Bifidobacterium adolescentis (B. adolescentis)* in the synbiotic applications (Krumbeck et al., 2018). GOS can support the butyrate generation after antibiotics course *in vivo*, but the data on the effect to the overall SCFA production is scarce (Ladirat et al., 2014).

1.9.2 Resistant starch

Starch fractions RSs can be divided into four categories (type I-IV) (Sajilata et al., 2006). Whole grains and legumes contain the RS1, which is entrapped in inaccessible matrix, whereas RS2 is present as native starch granules and can be sourced from raw potatoes, bananas or specifically from maize in high amylose form. RS3, a retrograded starch is formed during cooling of gelatinized starch, and can be obtained from cooked and cooled

rice, potatoes, etc., while RS4 designates chemically modified starches. Enrichment of *B. adolescentis*, *Eubacterium rectale (E. rectale)*, and *Ruminococcus bromii* (R. bromii) and a significant increase in fecal acetate and butyrate levels has been observed on RS2 (Abell et al., 2008; Alfa et al., 2018; Baxter et al., 2019; Flowers et al., 2019; Maier et al., 2017; Martínez et al., 2010; Venkataraman et al., 2016; Vital et al., 2018). Literature analysis highlighted a significant difference in the effects of high amylose maize RS2 compared to potato RS2, which differ in amylose and amylopectin ratio, as former is richer in less branched amylose and latter in amylopectin, which contains short sidechains. In most human studies, RS2 from maize supports the growth of *R. bromii*, while RS2 from potato predominantly increases the abundance of bifidobacteria. Therefore, it is possible that structurally different RS2 variants enhance different groups of colonic bacteria with distinct activities towards amylose and amylopectin. *In vitro* studies show that amylopectin is more accessible and more efficiently degraded by various fecal bacteria compared to high-amylose maize starch or amylose (Sheridan et al., 2016; Wang et al., 1999). Also, the bacterial community species structure could be important for RS metabolism, supported by the studies showing personalized response both *in vitro* and *in vivo* (Leitch et al., 2007; Martínez et al., 2010; Venkataraman et al., 2016). These studies show that either levels of ruminococci or bifidobacteria increase, depending on the initial levels of one or another. Overall, this suggests that individual communities promote different dynamics in the gut ecosystem. It is also possible that other host factors, e.g., availability of non-carbohydrate growth factors could determine/limit specific growth of a bacterial group. A recent review assessed the environmental, diet, host, and methodological factors to RS2-induced microbiome changes and came to similar conclusions about the RS2 source specific microbial modulation and metabolite production capacity (Bendiks et al., 2020). In general, RS2 consumption supports SCFA production *in vivo*, but importantly, there is more data about the significant effect on the butyrate production by amylopectin-rich potato RS2 (Abell et al., 2008; Alfa et al., 2018; Baxter et al., 2019; Venkataraman et al., 2016; L. Zhang et al., 2019). *In vitro* studies suggest that of bifidobacteria, *B. longum* and *B. infantis* cannot degrade amylose or amylopectin individually (Crociani et al., 1994).

1.9.3 Inulin and fructo-oligosaccharides

Inulin and FOS are prebiotics composed of β-(2,1)-linked fructose units (Martins et al., 2019). The bifidogenic properties of FOS and inulin have been studied extensively *in vitro* (Pompei et al., 2008; Rossi et al., 2005; Van Den Abbeele et al., 2013) and *in vivo* (Swanson, de Vos, et al., 2020). Importantly, species and strain specific effects of bifidobacteria have been characterized, as some strains can degrade longer molecules, while others are oriented for shorter ones, and depend on the hydrolytic activity of other primary inulin degraders (Rossi et al., 2005). For example, *B. adolescentis* strains have been shown to be able to degrade both FOS and inulin (Rossi et al., 2005; Scott et al., 2014), while *B. animalis* strains degrade preferentially FOS (Van Der Meulen et al., 2004). Overall, bifidobacteria degrade short-chain fructans more effectively than longer chains of inulin (Perrin et al., 2002; Rossi et al., 2005). Some *Bifidobacterium* strains possess extracellular hydrolytic activity of fructans, while others lacking this activity, have to facilitate the intake of FOS first followed by intracellular hydrolysis (Moens et al., 2016; Rossi et al., 2005). Therefore, it is possible that most bifidobacteria rely on keystone species that execute the primary degradation of longer molecules. Aside bifidobacteria, FOS supports the growth of lactobacilli (Pompei et al., 2008), and both FOS and inulin are degraded by *E. rectale* and *F. prausnitzii in vitro* (Duncan & Flint, 2008; Moens et al.,

2016; Moens & de Vuyst, 2017). Similarly, stimulation of growth of bifidobacteria and *F. prausnitzii* by inulin supplementation has been shown in human studies (Dewulf et al., 2013; Ramirez-Farias et al., 2009). An *in vitro* study showed that the degradation of long-chain inulin was carried out by multiple *Bacteroides* species (Rakoff-Nahoum et al., 2014), but this has not been supported by an *in vivo* study with mix of inulin and FOS (Dewulf et al., 2013).

In vitro experiments with fecal inocula have shown that major products of FOS fermentation are lactic and acetic acids, while on inulin, instead of lactate, significant amount of butyrate is produced (Pompei et al., 2008; Rossi et al., 2005). Butyrate production from FOS or long-chain inulin has been shown *in vivo* by *E. rectale*, and *F. prausnitzii* (Moens & de Vuyst, 2017). Somewhat contrastingly, a humanized rat study showed that a mixture of fructans dominated by FOS induces butyrate and propionate production, but in agreement with other *in vitro* and *in vivo* human studies, supports the growth of bifidobacteria, and butyrate producing bacteria: *E. rectale*, *Roseburia intestinalis (R. intestinalis)*, *Anaerostipes caccae (A. caccae)* (van den Abbeele et al., 2011). Still, Simulator of the Human Intestinal Microbial Ecosystem (SHIME) experiments suggest that inulin could possess stronger prebiotic properties, as its fermentation is delayed compared to shorter fructans, which could result in higher fermentation in distal colon (Van De Wiele et al., 2007).

1.9.4 Levan

Levan is a β-(2,6)-linked fructose polymer synthesized by a wide range of microorganisms and some plant species (Öner et al., 2016). An *in vitro* study demonstrated that among species of Bacteroidales, levan can be degraded and consumed only by *Bacteroides thetaiotaomicron (B. thetaiotaomicron)*, while several other *Bacteroides* species can utilize the degradation products of *B. thetaiotaomicron* (Rakoff-Nahoum et al., 2014). Meanwhile, another *in vitro* study showed that levan is more bifidogenic than inulin and supports also the growth of *E. rectale* (Liu et al., 2020). In a rat model, levan is bifidogenic (Bahroudi et al., 2020).

1.9.5 Arabinogalactan

Arabinogalactan (AG) from larch is a type-II AG and is used as a dietary supplement. Major degraders of AG have been shown to be members of *Bacteroides* (Fujita et al., 2019; Ndeh & Gilbert, 2018). Comparative *in vitro* analysis showed that *Bifidobacterium adolescentis* degrades AG main- and side-chains in equal manner, whereas *Bacteroides thetaiotaomicron* degrades preferentially the galactan backbone, and of both, *B. thetaiotaomicron* is significantly more adept at hydrolysis of AG (G. T. Macfarlane et al., 1995). According to another study, which investigated the degradation capacity of bifidobacteria on various polysaccharides, only *B. longum* strains were able to hydrolyze AG (Crociani et al., 1994).

1.9.6 Xylan (arabinoxylan)

Xylans have structural differences depending on the source. Xylan backbone is typically decorated with arabinoses (therefore called arabinoxylans), glucuronic acids, and acetyl groups. *Bacteroides* species are a major group of bacteria, which have capacity to preferentially degrade xylans and AXs due to the production of endoxylanases (Broekaert et al., 2011). Lack of sequencing based studies was observed for the AX, but a human intervention has reported increase of bifidobacteria on AX based on qPCR (Depeint et al., 2017). Only a single very recent human intervention reported that AX supports the

growth of *B. longum*, *Prevotella copri (P. copri)*, *Bacteroides plebeius (B. plebeius)*, *B. ovatus*, and others, but has no effect to fecal SCFAs, other than increase of propionate levels in responders (Nguyen et al., 2020). Batch-based *in vitro* studies suggest that AX is degraded by *Bacteroides* species, but not by bifidobacteria (Hopkins et al., 2003), while experiments carried out using an *in vitro* model of the colon suggest that specifically *B. longum* can be supported (Van Den Abbeele et al., 2013). Interestingly, an *in vitro* study showed that only *B. ovatus* and *B. vulgatus* are the utilizers of xylan, while other tested *Bacteroides* species were not able to grow even on the degradation products of xylan (Rakoff-Nahoum et al., 2014). A very recent study showed that several other *Bacteroides* species (*B. intestinalis*, *B. cellulosilyticus*, and *B. oleiciplenus*) can also degrade AXs (Pereira et al., 2021). Arabinose side chains have been shown to be hydrolyzed by some bifidobacteria in the case of arabinoxylan-oligosaccharides (AXOS-s) (Rivière et al., 2015), while individually, most bifidobacteria cannot degrade AX (Crociani et al., 1994), other than *B. longum* (Crittenden et al., 2002). Increase of *Bifidobacterium*, *Prevotella* and butyrate-producing bacteria on AXOS has been shown *in vivo* (Benítez-Páez et al., 2019). Also, for example, *E. rectale* can release XOS from AXOS (Rivière et al., 2015). It has been suggested that cooperation of multiple community members is needed to metabolize AX or xylan (Broekaert et al., 2011).

1.9.7 XOS

XOS has been shown to be bifidogenic in the human interventional studies using plate culturing (Chung et al., 2007; Finegold et al., 2014). Meanwhile, studies using 16S based community analysis have not proven significant support of XOS to bifidobacteria (Finegold et al., 2014; Yang et al., 2015). An *in vitro* study suggests that XOS is bifidogenic (Monteagudo-Mera et al., 2018), while several publications have shown that the growth of *Bifidobacteriu*m species on XOS mainly depends on the strain status. For example, one study proved the growth of *B. adolescentis* (Crittenden et al., 2002), but another did not (Hopkins et al., 1998). Several butyrate-producing bacteria, e.g., *E. rectale*, *Roseburia* (*R. hominis*, *R. intestinalis*, *R. faecis*, *R. inulinivorans*), and *A. caccae* can grow on XOS, but it is more selective on species of Firmicutes than FOS (Scott et al., 2014).

1.9.8 Pectin

Pectins have variable effects depending on the source, as the structure of the pectin varies, for example, between citrus or apple pectin. Higher levels of *Bacteroides* species has been observed on citrus pectin in rat colon (Dongowski et al., 2002). Apple pectin supports the growth of various *Bacteroides* species (*B. thetaiotaomicron*, *B. ovatus*, *B. vulgatus*, *B. fragilis*, etc.), *Eubacterium eligens*, and *F. prausnitzii in vitro* (Chung et al., 2016; Lopez-Siles et al., 2012). An *in vitro* study has shown that most bifidobacteria are not able to degrade pectin, while specifically *B. breve* strains have this capacity (Crociani et al., 1994). A survey carried out on 10 *Bacteroides* species showed that only *B. thetaiotaomicron*, *B. ovatus*, *B. fragilis* species, and some *B. vulgatus* strains were able to degrade pectin individually (Salyers et al., 1977). One of the most acknowledged pectin-degraders is *Lachnospira pectinoschiza* (*L. pectinoschiza)* (Bang et al., 2018; Cornick et al., 1994).

1.9.9 Polydextrose

Although polydextrose supports bifidobacteria *in vitro* and in mouse interventions (Probert et al., 2004; Raza et al., 2017), it is not proven to have bifidogenic properties based on studies, which analyze the total gut microbial community alterations in

humans. Several interventions indicate positive effect to *Parabacteroides* levels, and variable effect to other species (Hibberd et al., 2019; Holscher et al., 2015; Hooda et al., 2012). Reduction of bifidobacteria along with several butyrate producing bacteria, but increase of *F. prausnitzii* was observed in a single study (Hooda et al., 2012). More specifically, the polydextrose supplementation does not support the growth of *Bifidobacterium lactis* nor *Lactobacillus acidophilus* (*L. acidophilus)* (Hengst et al., 2009). It also does not have an positive effect on the fecal SCFA levels according to most studies (Costabile et al., 2012; Hengst et al., 2009; Vester Boler et al., 2011), other than one study showing increased fecal acetate and butyrate levels (Jie et al., 2000).

1.10 Dietary fiber degradation

The degradation of the dietary polysaccharides is carried out by glycoside hydrolases (GHs), polysaccharide lyases (PLs), and carbohydrate esterases (CEs), collectively called carbohydrate-active enzymes (CAZymes) (Lombard et al., 2014). GHs catalyze the hydrolysis of glycosidic bonds in glycans, while PLs can degrade uronic acid-containing polysaccharides via a β-elimination mechanism, and CEs hydrolyze the ester linkages of pectins.

Bacterial hydrolysis of the large polysaccharides takes place on the surface of the cell or in the periplasm (in the case of Gram-negative bacteria), which means that GHs and PLs require specific signal sequences for the extracellular/periplasmic transport and targeting. Aside from having better overall carbohydrate degradation capacity, members of Bacteroidetes have these signal sequences for most of the GHs and PLs, while species belonging to Firmicutes have those for less than quarter of GHs and PLs (Kaoutari et al., 2013). This is an important distinction between Gram-positive and Gram-negative bacteria, as lack of periplasmic space in the case of former means that longer polysaccharides can be degraded only extracellularly (Cockburn & Koropatkin, 2016).
Polysaccharide utilization loci (PUL) orchestrate the degradation of specific glycans. Due to high amount of discrete structures present in the DFs, it has been suggested similar number of PULs could be present that encode the enzymes needed for polysaccharide degradation (Hamaker & Tuncil, 2014), which has been confirmed by predictive studies (Terrapon et al., 2015, 2018). One example of a PUL is the starch utilization system (Sus) of *B. thetaiotaomicron* (Tancula et al., 1992). Even more complicated aggregates (enzyme structures) can be employed to carry out the degradation of less accessible polysaccharides. Similar to cellulosomes, which are present in rumen bacteria, amylosomes integrate various amylases into an unique organization that enable RS degradation in the case of *R. bromii*, a human gut bacterium (Ze et al., 2015).

DF degradation can be characterized in three distinct processes: 1) insoluble complex carbohydrate degradation by primary degraders; 2) soluble polysaccharide degradation by primary or secondary degraders; 3) oligosaccharide degradation and monosaccharide utilization mostly by secondary degraders or utilizers (Flint et al., 2007). Thus, as intestinal bacteria carry out subsequent degradation of DF, intermediate metabolites are formed and utilized by secondary fermenters leading to generation of diverse range of end-products.

Overall, this means that members of Bacteroidetes can be considered as generalists and Firmicutes species as specialists in glycan degradation, significantly influenced by their Gram-negative or Gram-positive status, respectively (Cockburn & Koropatkin, 2016). Also, it can be concluded that for many polysaccharides, Bacteroidetes can be considered as primary degraders, while only some Firmicutes can perform this role and

mainly serve as secondary degraders in cross-feeding (Rakoff-Nahoum et al., 2014). The diversity of the chemical structure of DFs, for example, different linkage types and various degree of polymerization affect their utilization by the gut microbiota.

1.11 Dietary fiber fermentation

Polysaccharide degradation produces monosaccharides, which enter into various metabolism pathways. Distinction can be made for the metabolism of fructans, GOS, RS, and levan, which are composed of hexoses (6C), while AG, AX, and pectin are mostly degraded into five carbon (5C) sugars. For bacteria, glycolysis is the dominant pathway for the catabolism of glucose and other 6C sugars (S. Macfarlane & Macfarlane, 2003). Most common type of glycolysis for intestinal microbes is the Embden-Meyerhof-Parnas (EMP) pathway (Miller & Wolin, 1996). On the other hand, all polysaccharides composed of 5C monosaccharides are metabolized through a pentose phosphate pathway (PPP). Many lactic acid bacteria employ a phosphoketolase pathway (bifid shunt) for the generation of pyruvate, while the ones that lack this pathway, use glycolysis.

Carbohydrate metabolism results mainly in production of acetate and butyrate, which are produced from acetyl-CoA, while propionate is derived from succinate or lactate through Succinate or Acrylate pathway, respectively (Figure 1). Succinate and lactate are typically derived from pyruvate. Overall, acetate production is carried out by a wide range of gut microbes, while propionate, succinate, lactate and butyrate production capacity are specific for a subset of the gut microbiota members (Koh et al., 2016).

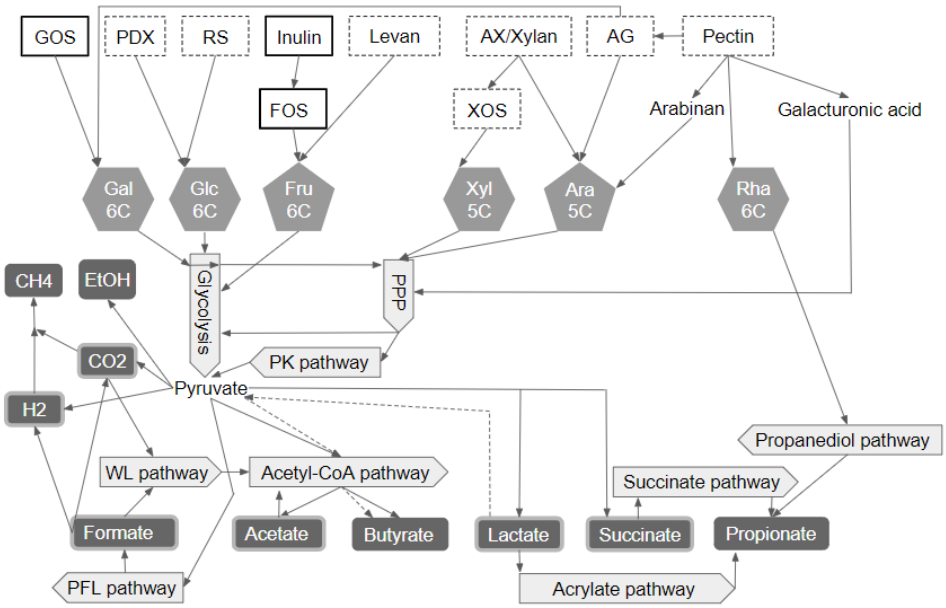

Figure 1. Overview of the major metabolites and pathways involved in the used polysaccharides. PPP - Pentose phosphate pathway, PFL - Pyruvate formate lyase (pathway), PK - Phosphoketolase (pathway), WL - Wood-Ljungdahl (pathway). Dashed line for butyrate production from lactate indicates the most well-known cross-feeding pattern observed between gut microbes. Prebiotic status (accepted prebiotics: bold line box, candidate prebiotics: dashed line box) according to Roberfroid et al., 2010.

Many butyrate-producing bacteria use acetate for butyrate generation, e.g., *R. intestinalis*, *E. rectale*, *F. prausnitzii*, while some can form butyrate from lactate, e.g., *Eubacterium hallii (E. hallii)* and *A. caccae* (Duncan, Louis, et al., 2004). *Anaerostipes hadrus (A. hadrus)* is a butyrate-producing bacteria that can utilize both acetate and lactate (Allen-Vercoe et al., 2012).

Formate production through pyruvate formate lyase (PFL) pathway is prevalent in many gut microbes, for example, in the *Bacteroides* species (Lehtiö & Goldman, 2004). A dominant methane-generating bacteria in the human gut is *Methanobrevibacter smithii*.

Although various microbiota members have different metabolite production capacity, in general, high functional redundancy exists among the gut microorganisms. This understanding is supported by the *in vivo* data, where similar metabolite patterns have been observed between different individuals. Approximate ratio of acetate, propionate, and butyrate in the human colon is 3:1:1 (Cummings et al., 1987). Overall, total SCFA concentrations are highest in the cecum, over 100 mM, while preceding illeal levels are approximately 10 fold lower (Cummings et al., 1987).

1.12 Cross-feeding mechanisms

Diverse communities in the human gut can support various modes of interrelationship between its members: from commensalism, where one species can support the growth of another, to competition, where two or more species depend on a limited substrate (Großkopf & Soyer, 2014). Various modes of cross-feeding have been proven by *in vitro* experiments (Table 1) (Smith et al., 2019). For example, metabolite cross-feeding as a form of commensalism predominantly occurs between lactic acid producing bacteria and butyrate-producers, e.g., *E. hallii* and *A. caccae* (Bourriaud et al., 2005; Duncan, Louis, et al., 2004). On the other hand, a more competitive setup is illustrated by substrate cross-feeding, e.g., *B. adolescentis* can degrade FOS and release shorter oligosaccharides, which are available for consumption for both butyrate producing *Roseburia sp.*, and to the primary degrader, *B. adolescentis* (Belenguer et al., 2006). In this case, lactate released by *B. adolescentis* was not consumed by the *Roseburia sp.* and it did accumulate in the environment. Due to the competitive nature of this interaction, substrate cross-feeding might even decrease the growth potential of the primary degrader itself as evidenced by the more significant growth of the secondary fermenters in co-cultures of both (Belenguer et al., 2006).

Cross-feeding modes can also occur in combinations, as shown by Moens and others, who showed that in a co-culture, either by production of lactose or by release of extracellular fragments of oligofructose, *L. acidophilus* can support the growth of *A. caccae*, which in turn can produce butyrate (Table 1) (Moens et al., 2017).

Overall, cross-feeding involves the aspect of keystone species, as even poor growth of a primary degrader, e.g., *B. adolescentis* can boost the growth of secondary fermenters/degraders, e.g., *E. hallii* or *A. caccae* (Belenguer et al., 2006). Another proposed keystone species, *R. bromii* can support the growth of *E. rectale* in a co-culture compared to its sole capacity to grow on RS individually (Ze et al., 2012). This can be explained by the degradation patterns of primary degraders, which can effectively hydrolyze longer polysaccharides into more accessible, e.g., more soluble fragments or shorten the polysaccharide backbone into smaller fragments available for intake by other members of the community (Moens et al., 2016; Rakoff-Nahoum et al., 2014; Ze et al., 2012).

Finally, it can be hypothesized that by specific modulation of these type of interactions, more effective production of beneficial metabolites can be supported, e.g., butyrate by complementing specific communities with individual substrate cocktails, e.g., various ratios of short or long-chain inulin or different types of resistant starches that will be degraded in anticipated manner through various cross-feeding modes in cooperation of the gut microbiota members.

Table 1. Cross-feeding modes by the gut microbes. Based on Smith et al., 2019.

Mode	Schema	*In vitro* examples
Metabolite cross-feeding (commensalism)	Waste metabolite from A is used by B	Soluble starch -> *B. adolescentis* -> lactate -> *E. hallii*/*A. caccae* (Duncan, Louis, et al., 2004) FOS -> *L. acidophilus* -> lactate -> *A. caccae* -> butyrate* (Moens et al., 2017) FOS/potato starch -> *B. adolescentis* -> lactate/acetate -> *E. hallii*/*A. caccae* -> butyrate* (Belenguer et al., 2006)
Substrate cross-feeding (competitive)	Substrate degradation products (via enzymatic action by A) are used by both A and B	FOS -> *L. acidophilus* -> fructose -> *A. caccae* (and *L. acidophilus*) -> butyrate* (Moens et al., 2017) Inulin -> *L. paracasei* -> FOS -> *B. longum* (and *L. paracasei*) -> acetate* (Moens et al., 2017) FOS/potato starch -> *B. adolescentis* -> oligosaccharides -> *Roseburia sp.* (and *B. adolescentis*) -> butyrate* (Belenguer et al., 2006) FOS -> *B. adolescentis* -> oligosaccharides -> *E. hallii*/*A. caccae* (and *B. adolescentis*) -> butyrate* (Belenguer et al., 2006) FOS -> *B. longum* -> fructose -> *A. caccae* -> acetate/butyrate* (Falony et al., 2006)
Mutual cross-feeding	Substrate degradation products of A and/or B or metabolism waste products of A and/or B are used by both A and B	AXOS -> *B. longum* -> acetate -> *E. rectale* -> butyrate & AXOS -> *E. rectale* -> xylose -> *B. longum* -> acetate (Rivière et al., 2015)

* - metabolite that was mainly supported by co-culturing

2 The aims of the thesis

This thesis aims to study the human intestinal consortia and to determine the diet-microbiota interactions by *in vitro* and *in vivo* methods. Specifically, following objectives were set:

- To apply isothermal microcalorimetry (IMC) for the investigation of the degradation and metabolism of various polysaccharide substrates by the fecal microbial consortia.
- To investigate the fecal consortia and explore the short-term variability in Estonian subjects by the next generation sequencing (NGS) methods.
- To analyze the dietary fiber (DF) intake and sources in Estonian subjects and compare dietary intake in the subgroups of low and high fiber consumption.
- To evaluate the associations of dietary intake of specific fiber and the gut microbiota based on the habitual intake and in the dietary interventions.

3 Materials and methods

3.1 Study cohorts

Overall, *in vitro* experiments produced 322 samples, of which 266 were analyzed for this thesis (Publication I). 228 samples were analyzed in the observational study, where two sequential samples from two time points were gathered (Publication II). Similarly, 76 samples were analyzed in the interventional weight-loss study (Publication III). Last, 162 samples were analyzed in the parallel randomized dietary intervention (unpublished data) (Table 2).

* - metabolite that was mainly supported by co-culturing

Table 2. Overview of the in vitro ja in vivo studies, and study cohorts involved regarding the results presented in this thesis.

Subjects	No of samples analyzed	Analytics, collected data	Main aims	Publication
Adults (N = 5, 3F, 2M, 28-48 years)	266 (14 slurries; 8 substrates + control; inocula, 0h and 48 h samples)	16S v3-4, HPLC, IMC heatflow	To investigate how different oligo- and polysaccharides are degraded by the human fecal microbiota	I
Adults (N = 59, 38F, 21M, 23-52 years)	228 (sequential samples in two time points)	16S v3-4, food intake diaries, and questionnaire	To analyse DF intake and sources in estonian population, and to study the fecal microbiota profiles in low and high fiber groups.	II
Overweight or obese adults (N = 19, 14F, 5M, 25-43 years)	76 (sequential samples in two time points)	16S v3-4, assigned diet data, questionnaire	To analyse the DF intake during a weight loss intervention, to evaluate the effect of the weight loss intervention to the fecal microbiota.	III
Adults (N = 81, 53F, 28M, 25-55 years)	162 (in two timepoints, parallel randomized study)	16S v4, and questionnaire	To investigate whether a consumption of fiber-enriched dairy product can support the growth of beneficial bacteria in the human gut.	Unpublished data

HPLC - high-performance liquid chromatography, IMC - isothermal microcalorimetry

3.2 Experimental setups and designs

In vitro experiments in the publication I were based on the fecal material from five individuals without chronic diseases. Overall, 14 fecal slurries with dilution of 10-100x were produced from frozen samples and used as inocula for IMC experiments (Figure 2). A detailed description of sample preparation, cultivation conditions, and analytics has been published previously (Adamberg et al., 2015). Briefly, the growth experiments were started at pH 7.2 using a defined growth medium with minerals, vitamins supplemented with individual carbohydrates (5 g/L). For IMC, sterile ampoules were filled with inoculated medium, closed hermetically, and incubated at 37 C h in an isothermal microcalorimeter. For 48 h, heat flow was recorded to yield a total accumulated heat, which correlates with the total biomass formation. Samples from the beginning and the end of the growth experiment were used for 16S rDNA sequencing and metabolite detection by high-performance liquid chromatography (HPLC).

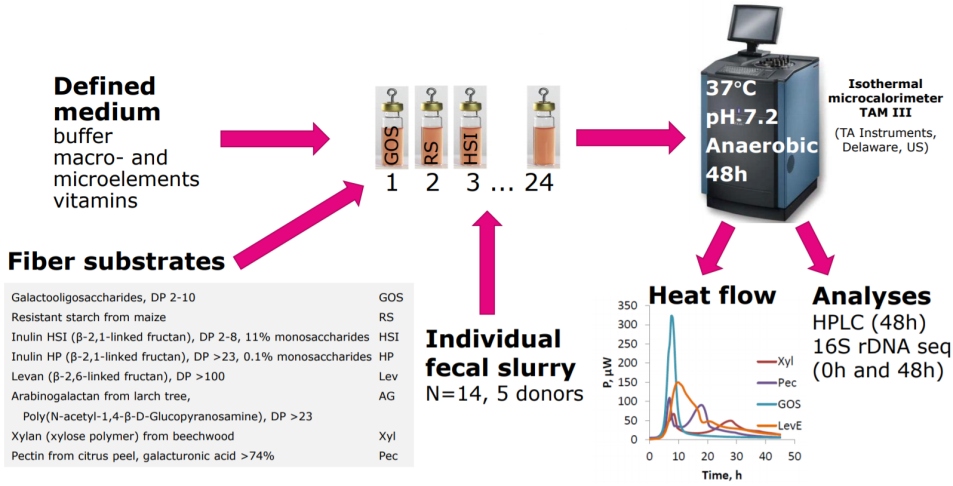

Figure 2. Experimental setup of isothermal microcalorimetry experiments (Publication I).

In the observational study (Publication II), 59 healthy Estonian subjects were recruited to analyze their habitual dietary intake (Figure 3). Exclusion criteria included food restrictions due to allergies, food intolerances, antibiotic intake within the last three months, chronic illnesses, pregnancy or breastfeeding, and travelling to subtropical or tropical regions within the last month. Dietary records were collected for at least 24 h, but up to 72 h before fecal sampling at two timepoints with 61 days (41-121 days) in between. Also, a secondary consecutive fecal sample was collected in the beginning and in the end of the study. Based on the average fiber intake over the period covering the provided dietary records low, medium, and high fiber intake subgroups were formed (DF intake < 9.4, 9.4 - 15.1, and > 15.1 g/1000 kcal, respectively).

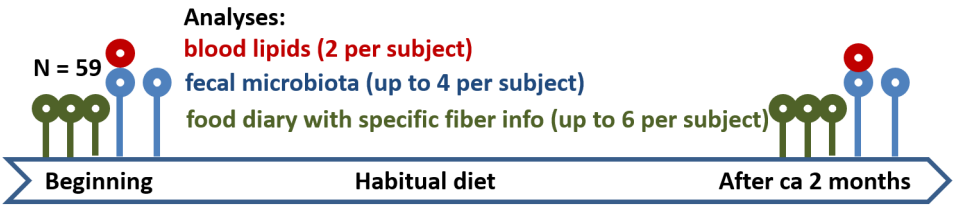

Figure 3. Schematic figure of the food consumption survey and sampling (Publication II).

The first interventional study (Publication III) was carried out in parallel with the observational study. Here, 19 overweight or obese subjects completed a four-week low calorie weight loss dietary regime accompanied with sequential fecal sampling before and after the intervention (Figure 4). Exclusion criteria were previous history of gastrointestinal disease, reported antibiotic use in preceding three months, taking of any medication known to alter bowel motility, history of food allergies and being on medications, and pregnancy or breastfeeding.

Dietary intake analysis for these studies was based on the Estonian food composition database (Nutridata v6-7, National Institute for Health Development, Tallinn, Estonia), food composition data from the literature, and custom R scripts (R Foundation for Statistical Computing, Vienna, Austria). Employed pipeline produced detailed information about the consumption of 53 nutrients in 72 food categories. Additionally, specific fiber intake of the following fiber compounds was calculated - AX, β-glucan, cellulose, fructans, lignin, and pectin.

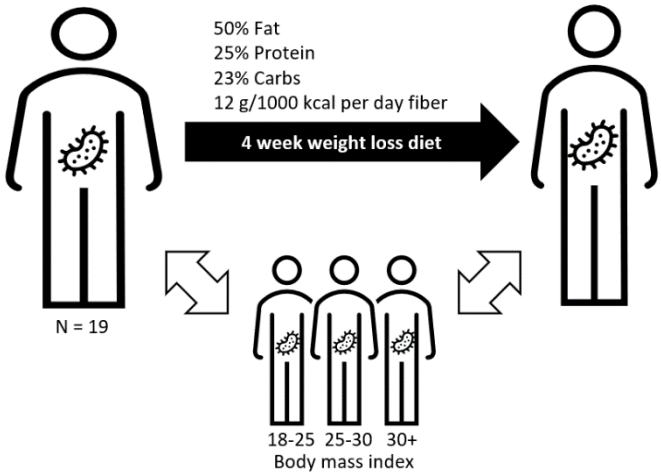

Figure 4. Schematic figure of the weight-loss intervention (Publication III).

The second interventional study (unpublished data) was a double blind randomized controlled trial. Here, 81 healthy subjects completed a two-week dietary intervention with either a control yoghurt or a test yoghurt supplemented with a mix of oligo- and polysaccharides (Figure 5). Inclusion criteria were as follows: aged between 25 and 55 years, ability to consume dairy products and willing to comply with the study procedures and sign the written informed consent. Exclusion criteria included antibiotic use three months before the study, any gastrointestinal and/or other severe chronic diseases, highly dosed probiotic supplement before starting and throughout the study, regular consumption of medication that might interfere with symptom evaluation, and pregnancy.

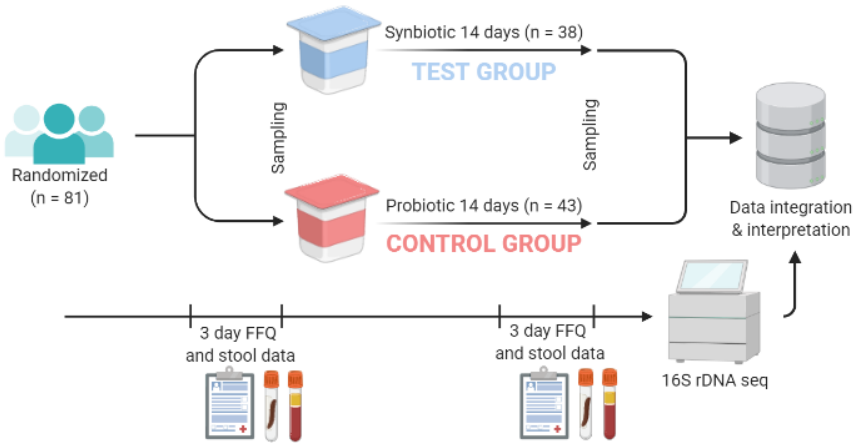

Figure 5. Schematic figure of the randomized controlled trial with synbiotic and probiotic yogurt (unpublished). Created with BioRender.com.

The study products consumed during the intervention were 200 g of unsweetened yogurt or the same yogurt supplemented with high amylose RS from maize (1.8 g/100 g), polydextrose (1.3 g/100 g), long-chain inulin (1.3 g/100 g), and XOS (0.2 g/100 g), thus leading to additional daily intake of 9.2 g of DFs in the test group.

All studies were approved by the Tallinn Medical Research Ethics Committee (TMEK No. 1631 and 2099) and informed consent was obtained from all subjects involved.

3.3 Microbiota profiling pipelines

All studies employed similar bacterial community analysis pipelines (Table 3). Shortly, in first studies (Publication I-III), DNA was extracted using Purelink Microbiome DNA extraction kit (Thermo Fisher Scientific, United Kingdom) according to the manufacturer's instructions followed by PCR amplification of the V3-4 hypervariable regions of the 16s rRNA genes with universal primers, library preparation, and sequencing using Illumina MiSeq 2x250 v2 platform (Estonian Genome Centre, University of Tartu, Estonia).

In the last study (unpublished data), DNA was extracted using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions followed by PCR amplification of the V4 hypervariable regions of the 16s rRNA genes with universal primers, library preparation, and sequencing using Illumina iSeq 100 2x150 platform (TFTAK, Estonia).

Table 3. DNA extraction, sequencing pipeline, and data analysis methods used in the in vitro ja in vivo studies.

DNA extraction	16S rRNA region	Sequencing apparatus and kit	Data analysis pipeline	Publication
PureLink Microbiome DNA extraction kit	V3-V4	MiSeq, 2×250 bp, v2	BION-meta, Silva S123	I-III
ZymoBIOMICS™ DNA Miniprep Kit	V4	iSeq 100, 2×150 bp	BION-meta, Silva S123	Unpublished data

DNA sequence data were analyzed using the BION-meta application according to the author´s instructions. Sequences were cleaned at both ends using a 99.5% minimum quality threshold for at least 18 of 20 bases for 5'-end and 28 of 30 bases for 3'-end, joined, followed by removal of shorter contigs than 350 bp or 150 bp (for V3-4 and V4 amplicons, respectively). Then, sequences were cleaned from chimaeras and clustered by 95% oligonucleotide similarity (k-mer length of 8 bp, step size 2 bp). Finally, consensus reads were aligned to the SILVA reference 16S rDNA database (v123) using word length of 8 and similarity cut-off of 90%.

3.4 Statistical analysis

Data analysis and statistical assessments were carried out using MS Excel or R software (R Foundation for Statistical Computing, Vienna, Austria). 'Vegan' package was used to calculate α-diversity, β-diversity, assess the similarity, and analyze the variance of groups for anthropometric, bacterial, and metabolite data. Figures were produced with MS Excel and 'ggplot2' package. Linear correlations were analyzed with Pearson and Spearman correlation coefficients and assessed for statistical significance. Both parametric (T-test) and non-parametric (Wilcoxon signed rank test) statistical tests were employed. Also, paired analysis was carried out where applicable. P-values were corrected for multiple comparisons using Benjamini-Hochberg procedure where applicable.

4 Results and discussion

4.1 Core gut microbiota of Estonian subjects (II p., unpublished data)

Previous research of the gut microbiota in Estonians is based on either low sample size (Adamberg et al., 2015), very specific cohort (Drell et al., 2015, 2017), non-NGS methods (Sepp et al., 2014) or was aimed at specific bacterial groups (Štšepetova et al., 2011). Thus, the human gut microbiota status in Estonians was elaborated by the NGS methods, which enable whole community analysis down to species levels. Gut microbiota levels of Estonians could be different from populations, which have followed the Western-type of diet for a longer period of time, and possibly supported by the wide variety of DFs from cereal grains resulting in specific selection of the primary degraders of the DF.

About 50 species formed the "core" fecal microbiota found in the samples of at least 80% of the subjects (Figure 6A). On average, species from 10 most abundant genera covered 51% of the sequences of all samples, which illustrates the high diversity present in the human gut communities (Figure 6B). Analysis of the most dominant genera showed similarly to other studies that most abundant were members of *Bacteroides* and *Prevotella*, which have been also considered as the major players associated with long-term dietary intake and are highly responsible for the gut microbiota variations (Arumugam et al., 2011; Wu et al., 2011).

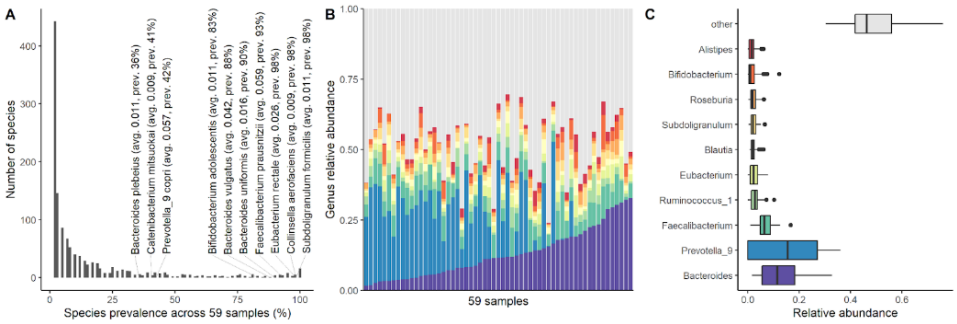

Figure 6. Core microbiota in subgroup of Estonians. A - Distribution of the prevalence across 59 samples (histogram) and most abundant species (avg. - average relative abundance, prev. - prevalence). B, C - Dominant genera present in Estonian subjects.

The most abundant species were unclassified species from *Prevotella* (average rel. abundance 0.096; prevalence 64%), *P. copri* (0.057; 42%), *F. prausnitzii* (0.059; 93%), *B. vulgatus* (0.042; 88%) and *E. rectale* (0.026; 98%). While most of the abundant bacteria were also prevalent, the colonization ratio of *Prevotella* was lower, but still quite high compared to Western populations (Asnicar et al., 2021). Besides *F. prausnitzii* and *E. rectale*, other dominant butyrate-producing bacteria included species of *Roseburia*, e.g., *R. faecis* (0.008; 95%), *R. inulinivorans* (0.004; 97%), and *R. intestinalis* (0.004; 76%). The diversity of the *Bacteroides* species was high, aside *B. vulgatus*, high prevalence was detected for *B. uniformis* (0.016; 90%), *B. ovatus* (0.005; 81%), *B. caccae* (0.005; 76%), while *B. plebeius* (0.011; 36%), *B. dorei* (0.006; 10%), and *B. cellulosilyticus* (0.004; 47%) were rarer. The abundance of *B. thetaiotaomicron* (0.002; 64%) and *Bacteroides xylanisolvens (B. xylanisolvens)* (0.001; 54%) was low. Although considered as one of the keystone species, levels of *Bifidobacterium* were mediocre, *B. adolescentis* as the most

abundant (0.011; 83%) followed by *B. longum* (0.004; 70%), and *B. pseudocatenulatum* (0.003; 32%). Of *Ruminococcus* species, *R. bromii* (0.007; 56%) and *R. bicirculans* (0.006; 80%) were more abundant. Core species from other genera included *Subdoligranulum formicilis* (0.011; 98%), *Alistipes putredinis* (0.008; 90%), *Blautia wexlerae* (0.005; 91%), and *Blautia faecis* (0.003; 100%).

Regarding the Bacteroides species, these results corroborate with the results of Kollarcikova and others, who showed the dominance of *B. vulgatus* and *B. uniformis* compared to other members of this genus (Kollarcikova et al., 2020). *In vitro* studies have shown that *Bacteroides* species have highly variable DF degradation or utilization capacity, e.g., *B. uniformis* functions as the primary degrader, while *B. vulgatus* could have a recipient status as an utilizer of intermediate degradation products (Porter & Martens, 2016).

Overall, this data shows that primary degraders, e.g., species of *Bacteroides*, *Bifidobacterium* are abundant but not necessarily prevalent. Disparity in their colonization ratio indicates that status of these bacteria is more personalized, possibly a consequence of the variable dietary habits in the population. However, secondary metabolizers, e.g., butyrate-producing bacteria are more prevalent signifying their general role in the gut community, which could be not specifically dependent on the specific diet but instead could be supported by the various metabolites produced by the other community members. It is important to note that due to high effects of the methodologies used for gut community analysis, e.g., DNA extraction, or the choice of primers for the amplification of the 16s rDNA variable region, multiple variables are introduced which could mainly affect the species relative abundance.

Also, differential prevalence of specific gut microbiota members could be considered in the design of the experiments as statistical significance could be only reached for the species with high prevalence.

4.2 Unique microbiota “fingerprints” and short-term stability (II p, III p, unpublished data)

To explore and understand daily variability of the gut microbiota of a single individual, a series of samples were collected. Sequential sampling in two separate occasions enabled investigation of the day-to-day fluctuations while samples taken on average after two months allowed to gather insights into short-term stability of the gut microbiota.

Based on the samples collected from 59 subjects (reference) and 19 subjects before and after the weight-loss diet (WLD) intervention, strong inter-individual variations in the gut microbial communities were detected (Figure 7AB). The majority of the variance in the bacterial community composition was explained by the individual status in both reference and WLD datasets (83% and 79% respectively, $p < 0.001$, PERMANOVA). In two months, fecal communities changed significantly in the reference group (0.4%, $p = 0.047$, PERMANOVA). As expected, the impact of four-week WLD was more significant (2.1%, $p = 0.018$, PERMANOVA). These results corroborate with results of other studies, which also show that variance of the bacterial community compositions is predominantly explained by the individual (Jian et al., 2021).

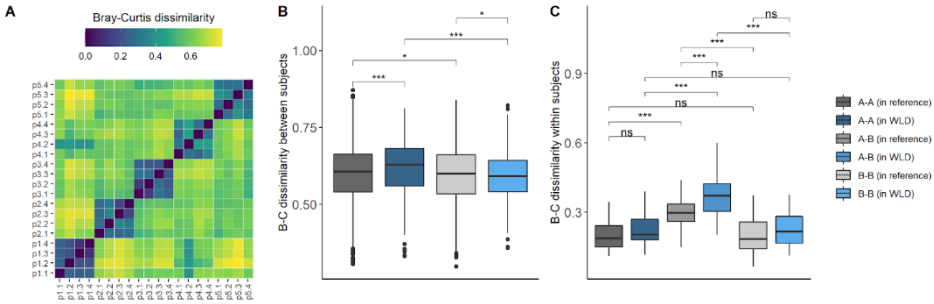

*Figure 7. Fecal microbiota similarity in sequential samples (.1 vs .2 and .3 vs .4) and over 2 months (.1, .2 vs .3,.4). A - beta-diversity matrix shown for 5 subjects (out of 59 subjects without dietary intervention). B - beta-diversity between subjects in the beginning (A-A) or in the end of the study period (B-B). C - beta-diversity within subjects between consecutive samples in the beginning (A-A) or in the end of the study period (B-B), between initial samples and retrospective samples taken after 2 months (reference) or 4 week WLD intervention (A-B). Statistical significance was evaluated based on the Bray-Curtis (B-C) dissimilarity values at species level and Wilcoxon signed rank test. Lower B-C values indicate higher similarity. *, p < 0.05; **, p < 0.01; ***, p < 0.001.*

The difference between the microbial composition of a single individual to all other samples (between subjects beta-diversity) was much higher (B-C values ~0.6, A-A or B-B, on average) than similar values obtained from comparison of the samples of a single individual (within subject beta-diversity) taken sequentially (B-C values ~0.2, A-A or B-B) or after two month interval (B-C values ~0.3, A-B) (Figure 7BC, in gray). These results indicate that daily variability is already quite high compared to short-term variability over two months. Similar outcome was observed in the case of the WLD study (Figure 7BC, in blue), but notably, higher similarity between the subjects was seen after the dietary intervention by WLD, which suggests that similar dietary approach can effectively reduce the inter-individual variation. As expected, the effect of the low-carbohydrate high-fat WLD was much more significant (B-C values ~0.4, A-B) than supported by the habitual diet in the other study group over a similar timeframe, where dietary alterations could have been more random (Figure 7C). These results support the idea of a relative long-term stability of the gut microbiota if lifestyle or eating habits are not specifically altered (David et al., 2014; Faith et al., 2013).

Overall, clear subject-wise distribution of samples was observed indicating the presence of a characteristic “fingerprint” across all samples from a single individual.

4.3 Dietary fiber intake profile of the Estonian’s diet (II p., unpublished data)

To link the gut microbiota profiles of Estonians with dietary nutrient consumption, dietary intake of foods was analyzed based on the electronic food diary data. To determine the sources of DF, a database of 46 food categories was formed, where each dietary component was classified under a certain category. Based on the literature data, we also calculated the average specific fiber composition of each category to assess the quantity of specific type of fiber in the habitual diet. This is a unique procedure to evaluate the exact fiber intake profiles from dietary records about complete food intake. Also, dietary intake was further analyzed in the low and high fiber subgroups (LF, HF).

Based on the food records of 59 subjects, it was apparent that the habitual diet of Estonians is high on fat (median intake 41.3 g/1000 kcal, 37% of total energy intake),

decent on protein (40.2 g/1000 kcal, 16%E), but slightly low on digestible carbohydrates (106.0 g/1000 kcal, 42%E). As carbohydrate-rich foods contain also dietary polysaccharides, low DF intake was observed (median intake 12.0 g/1000 kcal). This result indicates that more than half of the subjects consumed less fiber than recommended (> 12.5 g/1000 kcal). Median daily fiber intake was 24 g, which is on par with values reported by other studies (Stephen et al., 2017).

Major DF sources were cereals (average intake 4.8 g/1000 kcal, 39% of DF intake), vegetables (3.3 g/1000 kcal, 27%DF), and fruits and berries (2.4 g/1000 kcal, 19%DF) (Figure 8A). This data is in accordance with other studies, which show that in Europe principal food sources of DF in adults include grain products (34-49% of total DF), potatoes (6-23%), vegetables (14-21%), and fruits (8-23%) (Stephen et al., 2017). More specifically, dominant DF fractions were cellulose (3.3 g/1000 kcal), AX (2.4 g/1000 kcal), pectin (1.9 g/1000 kcal), and fructans (1.7 g/1000 kcal) (Figure 8D).

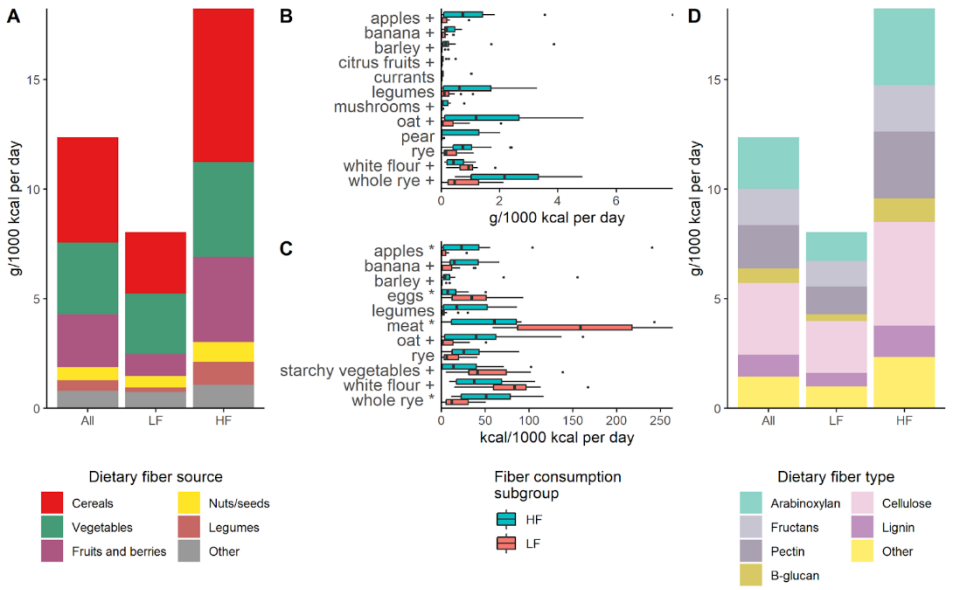

*Figure 8. Average DF intake in Estonian subjects. A - main DF sources; B - DF intake across food categories; C - energy intake across food categories; D - specific DF intake. All values are g or kcal per 1000 kcal per day. Results are presented for all subjects (All, N = 59), and low fiber intake (LF, N = 15, < 9.4 g/1000 kcal/day) and high fiber (HF, N = 14, > 15.1 g/1000 kcal/day) intake subgroups. Detailed food intake data was recorded for at least one day in two separate occasions two months apart (on average). Significant differences (Wilcoxon signed-rank test) after false discovery rate correction between LF and HF subgroups are designated with + ($p < 0.1$) or * ($p < 0.05$).*

When subjects were divided into two subgroups based on low fiber (LF, 8.1 g/1000 kcal) and high fiber (HF, 18.2 g/1000 kcal) consumption, notable differences were observed. Mainly, compared to LF, subjects in HF subgroup consumed significantly more DF from fruits and berries (3.9 vs 1 g/1000 kcal), cereals (7 vs 2.8 g/1000 kcal), legumes (1.1 vs 0.2 g/1000 kcal), while intake of DF from vegetables and nuts/seeds was more similar (Figure 8A). Going into more detail, LF members consumed more fibers from refined cereals, e.g., white flour, however, DF intake from all other food categories was lower than in the HF subgroup (Figure 8B). The primary source of DF in HF were cereals (rye,

oats, wheat), which are rich in AX, beta-glucan and fructans. Importantly, regarding energy intake, compared to HF, diets of LF subjects were enriched with food products from meat category, eggs, and starchy vegetables (potatoes) (Figure 8C). Most remarkable differences in specific fiber consumption in HF vs LF subgroups was observed for beta-glucan (1.1 vs 0.3 g/1000 kcal, respectively) and AX (3.5 vs 1.3 g/1000 kcal, respectively), while intake of other fiber types was proportional to the differences in the total DF intake (Figure 8D). High proportion of cereal-based DFs in the HF group emphasizes the importance of consumption of cereal grain-based foods, which can significantly boost both the total fiber intake and the diversity of the microbiota accessible carbohydrates.

It would be important to also evaluate the intake of RS, but this was not included due to the limited information available about the food processing carried out by the subjects, and similarly because of the disparate data present in the literature. Still, based on the population data it has been estimated that on average 5 g of RS per day could be accessible for the colon microbiota (Stephen et al., 2017).

Overall, these results indicate that DF consumption and sources in Estonian habitual diet are similar to typical intake in other European countries. It is important to note that data collected from a small cohort is not fully representative of a total population habitual food intake but gives valuable insights about how dietary habits relate with total DF intake and sources, and provides a basis for the study of diet-microbiota interactions.

Due to high diversity of the polysaccharide molecules (structure and monomer composition), more detailed food composition data could be used to interpret food intake records (Hamaker & Tuncil, 2014). Analytical methods for DF quantification have improved significantly in recent decades and nationwide agreements have been made to standardize DF determination (Stephen et al., 2017). Still, our results show that significant differences in specific DF composition were observed by applying food intake composition analysis at more general, food category level.

4.4 Diet-microbiota interactions (II p., III p.)

To evaluate the associations of dietary intake of specific fiber and the gut microbiota, the DF intake profile of 59 Estonian subjects was correlated with the fecal microbiota composition data from several time points analyzed by 16S rRNA sequencing (publication II). Additionally, a dietary intervention was carried out to assess the effect of the low-carbohydrate high-fat (LCHF) WLD on the gut microbial communities of 19 obese/overweight subjects (publication III). Furthermore, observed changes were analyzed in the context of the reference group (N = 59) data split into three subgroups based on body mass index (BMI) status.

When α-diversity or species richness were compared between LF or HF subgroups, no significant differences were observed (data not shown). This was unexpected as earlier studies have shown the positive correlation between DF intake and microbiome diversity (Menni et al., 2017), but could be explained with small sample size or short duration of the intervention. Similarly, the four-week interventional study with WLD did not affect species richness or diversity (Publication III, Figure A2). Body weight change has not been associated with changes in the microbial diversity but with changes in the abundance of specific taxa (Ley, Turnbaugh, et al., 2006).

Various levels of DF intake were associated with specific microbial signatures (Figure 9). For example, *B. pseudocatenulatum* was more prevalent in the HF subgroup (present in 8 subjects out of 14) compared to the LF subgroup (3/15). Opposite situation was observed

for *Bacteroides coprocola* and *Allisonella histaminiformans (A. histaminiformans)*, which were much more prevalent in the LF subgroup (6/15 and 8/15, respectively) compared to the HF subgroup (1/14 and 3/14, respectively).

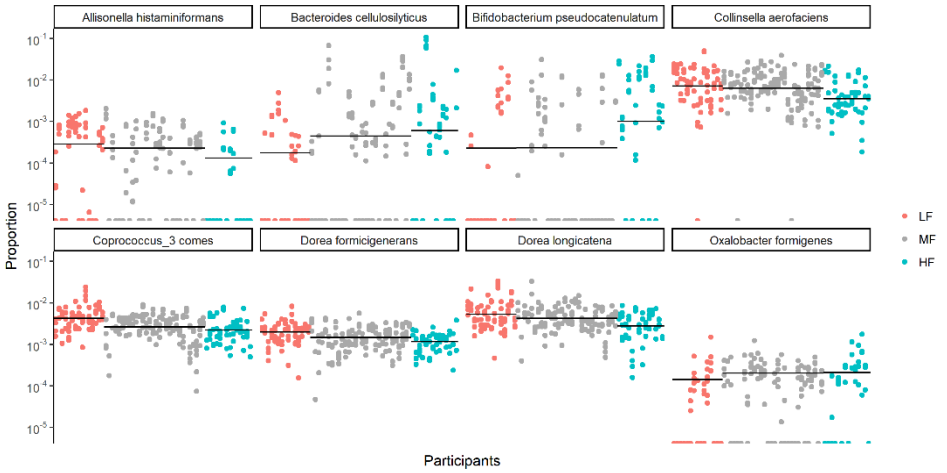

Figure 9. Species abundance of selected fecal bacteria, which were significantly different between the low fiber intake (LF, N = 15, < 9.4 g/1000 kcal/day, red) and high fiber (HF, N = 14, > 15.1 g/1000 kcal/day, green) intake subgroups. Values for medium fiber (MF) intake are shown in gray. Participants are ordered in decreasing fiber intake from left to right. Horizontal lines indicate group average relative abundance (log-transformed). Significant differences (Wilcoxon signed-rank test) after false discovery rate correction, p < 0.05.

B. pseudocatenulatum encompasses high number of carbohydrate-active enzyme (CAZy) genes (Wu et al., 2017), has been associated with a high fiber diet rich in whole grains (Zhang et al., 2015), and an *in vitro* screening suggests that *B. pseudocatenulatum* strains possess capacity to degrade xylan (Crociani et al., 1994). Specifically, the participants in the HF subgroup consumed much more AX compared to the subjects in the LF subgroup. Higher abundance of *B. cellulosilyticus* in the HF group could be related to its specific capacity to degrade cellulose among other Bacteroides members (Robert et al., 2007), but also to its overall status as a versatile carbohydrate utilizer adept at wheat xylan degradation (Centanni et al., 2017; McNulty et al., 2013; Pereira et al., 2021). Higher prevalence of *A. histaminiformans* could be linked with higher meat product consumption in the LF subgroup as it is a histidine utilizer (Garner et al., 2002). Lower DF intake in the LF subgroup was associated with higher abundance of *Coprococcus comes*, *Dorea formicigenerans (D. formicigenerans)*, and *Dorea longicatena (D. longicatena)*, which have been found to be enriched in obese individuals (Liu et al., 2017). BMI comparison in the DF intake subgroups showed that lower values were associated with higher DF intake and vice versa (Publication II, Table 1).

Several results from the dietary WLD intervention study (publication III) corroborated with associations observed previously in the Estonian subjects (publication II). For example, WLD, which contained moderate levels of DF, but was specifically low in cereal fibers (AX, β-glucan) decreased the abundance of *Collinsella aerofaciens (C. aerofaciens)*, *D. formicigenerans*, *D. longicatena*, and *Bifidobacterium species* (Figure 10).

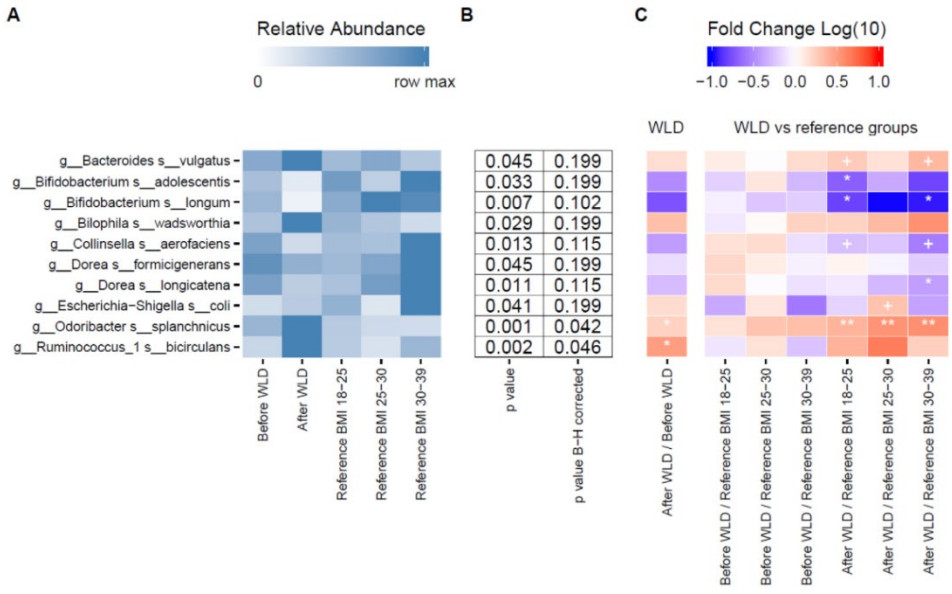

*Figure 10. Altered taxa in response to WLD and comparison with reference groups. A - average abundance of altered taxa, B - uncorrected and corrected p values between before and after samples, C - logarithmic fold change values for the study group and compared to reference group abundances. Benjamin-Hochberg corrected: +, p < 0.1; *, p < 0.05; **, p < 0.01.*

C. aerofaciens has been shown to decrease in a weight-loss intervention compared to RS-rich weight-maintenance intervention (Salonen et al., 2014). Negative correlation of DF intake and *C. aerofaciens* levels observed by Gomez-Arango and others further supports the results of the observational and interventional study presented here (Gomez-Arango et al., 2018) (Figure 9, Figure 10). Although quantitative RS content in the WLD was not calculated, due to low consumption of cereals and starchy vegetables, it could be presumed that intake of RS type II and III (retrograded starch) was low, thus potentially being the crucial factor for the decrease of the previously mentioned species. On the other hand, observed increase of *R. bicirculans*, a xyloglucan-utilizer, could be due to high consumption of fruits and vegetables, which are rich in xyloglucans compared to cereal grains (Wegmann et al., 2014). Despite the relatively high cellulose content in the WLD, relative abundance of *B. cellulosilyticus*, a cellulose degrading bacterium, did not increase, but increased prevalence was observed (Publication III, Supplementary Figure 5).

Bacterial levels before and after WLD were compared to reference subgroup levels to verify and validate the observed changes as this study was carried out in a single arm without randomized control or crossover design (Figure 10C). These comparisons asserted that other than some bacteria, e.g., lower levels of *Christensenellaceae* or higher levels of *Dorea* in the overweight/obese subjects compared to normal weight reference subgroup, no significant differences were found (Publication III, Figure 4). Also, most of the taxa, which were significantly shifted in response to WLD, also exhibited distinct relative abundances in post-WLD samples compared to bacterial levels in the reference subgroups.

Last, the effect of the initial bacterial levels to the WLD outcome was assessed for species, which were significantly increased or decreased during the intervention ($p < 0.05$). Three subgroups were formed based on the initial bacterial abundance: not detected,

low and high abundance groups (ND, HAG, LAG, respectively). According to this analysis, levels of bacteria increased more in the subjects of LAG and decreased more in the subjects of HAG (Figure 11). These individualized reactions to the changes in the dietary intake could be due to variations in the habitual diet, which could be confirmed by the analysis of food intake before the intervention and by application of baseline diet, which should produce the same effects observed here. Furthermore, it could indicate that some communities exhibit more responsive properties to dietary alterations. Still, these results are preliminary and need confirmation by prospective studies, possibly admitting participants based on their microbiota status.

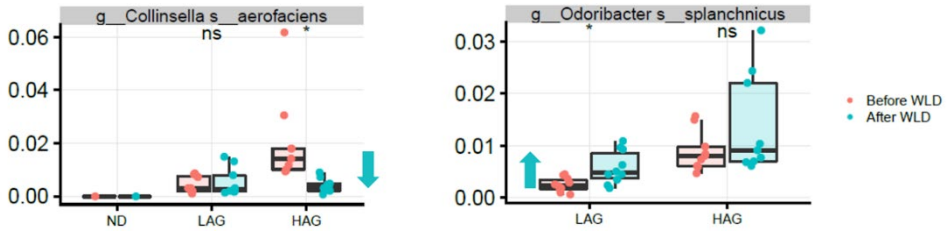

Figure 11. Relative abundance C. aerofaciens and O. splanchnicus before and after WLD separated into 2-3 groups based on the initial bacterial levels. ND - not detected (Before WLD rel. abund. = 0), LAG - low abundance group (Before WLD rel. abund. < 50 percentile), HAG - high abundance group (Before WLD rel. abund. > 50 percentile).

As presented results were based on the relatively small sample size, further studies would be needed to provide more in-depth analysis, also supported by whole genome sequencing of the gut communities to analyze strain-specific effects. Notably, this work demonstrates that analysis of dietary records or assigned diets down to specific fiber content or sources can be valuable to gain insights into the diet-microbiota interactions and particular microbial changes elicited by dietary intervention.

4.5 Combinatory effect of pre- and probiotics on the fecal consortia (unpublished data)

To explore the combinatory effect of pre- and probiotics on human gut microbiota a controlled human dietary intervention was carried out. In the control group, a non-prebiotic but a probiotic yogurt was ingested for 14 days. Subjects in the test group consumed the same yogurt supplemented with a mix of fibers consisting of high amylose RS from maize, polydextrose, long-chain inulin, and XOS.

The most dominant genera in subjects were *Bacteroides*, *Faecalibacterium*, *Blautia*, and *Prevotella_9* with average relative abundance over 6% in all groups before and after the intervention (Figure 12A). Neither of the treatments introduced significant differences in the relative abundance of the dominant genera (Figure 12A). High relative abundance of prevailing genera suggests that due to the general role of these bacteria, they could be less dependent on the availability of specific substrates introduced by the intake of synbiotic yogurt in the test group. Important substrate for gut microbiota is mucin, which is also degraded by many Bacteroides species (Crouch et al., 2020). High variability in the metabolic capacity of the distinct strains of abundant genera, e.g., ability to scavenge polysaccharides, could be an additional factor why no significant shifts were observed in the dominant genera. For example, members of the genus Bacteroides exhibit high diversity in the capacity of glycan degradation (Briliūtė et al., 2019).

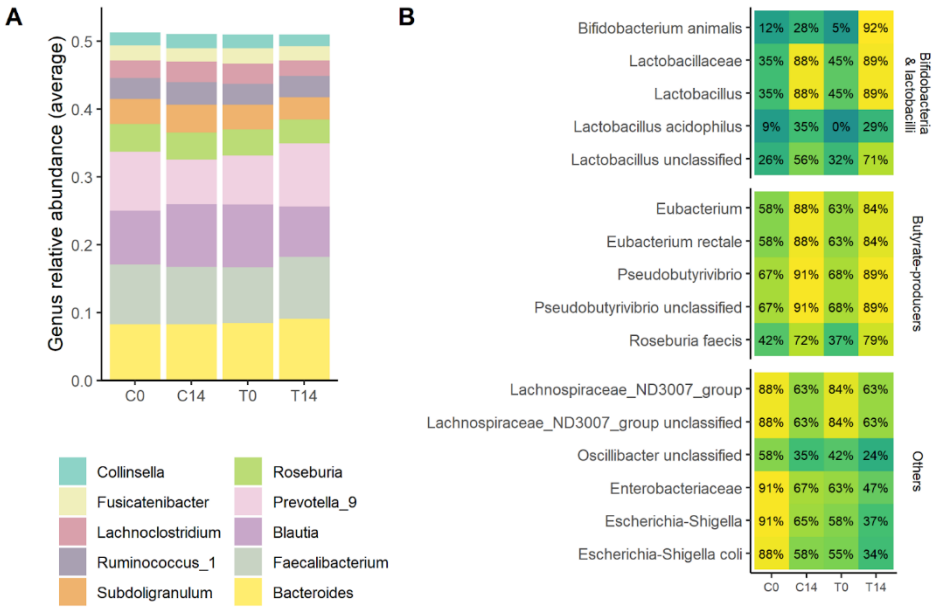

Figure 12. The composition of fecal microbiota in the study groups. A - Average relative abundance of TOP10 genera in both interventional groups. B - Prevalence of most significantly altered bacteria at family, genus, and species levels in the control and the test treatment arm before and after intervention. Percentage in the tile shows the proportion of subjects harboring the bacteria (>0.0001 relative abundance). C0; T0: control and test group before intervention, C14; T14: control and test group after intervention.

Intake of both probiotic and synbiotic yogurts increased the prevalence of several butyrate-producing bacteria (*E. rectale*, *Pseudobutyrivibrio*, *R. faecis*) and both prevalence and relative abundance of lactobacilli (*L. acidophilus*) ($q < 0.001$, Figure 12B, Figure 13A). Of added probiotic bacteria, *B. animalis* was only increased with the intake of fiber-enriched yogurt ($q < 0.001$, Figure 12, Figure 13). Butyrate-producers serve as consumers of intermediate products, which could suggest that either substrate or metabolite cross-feeding was altered by the intake of probiotic strains (Baxter et al., 2019). Previous studies suggest that acetate and lactate produced or oligosaccharides released by lactobacilli or bifidobacteria can support the growth of members of *Roseburia* and *Eubacterium in vitro* (Belenguer et al., 2006).

We observed the decrease in prevalence of *Escherichia* in both groups and negative correlation between *B. animalis* and Escherichia in the test group (Spearman, $p = 0.03$, $r = 0.48$, Figure 13B), which indicates that ingestion of yogurt could reduce the levels of *Escherichia* in subjects with more significant response and increase in relative abundance of *B. animalis*. Still, no difference in relative abundance of *Escherichia* was found within or between groups ($q > 0.1$). Inhibitory activity of *B. animalis* BB-12 against *E. coli* has been shown by *in vitro* assay (Martins et al., 2009) and in simulated gastrointestinal conditions (Verruck et al., 2020). In the study by Verruck and others, an improved antagonistic effect of BB-12 was observed *in vitro*, when inulin was used as an encapsulating agent. Supplementation with prebiotic mix also supported the significant increase in relative abundance of other resident gut microbes (Figure 13). Interestingly, streptococci and lactobacilli were significantly increased in both groups ($q < 0.1$, Figure

13A), but relative abundance of bifidobacteria remained unchanged in both groups (q > 0.1, data not shown). This could be explained by the relatively high proportion of the endogenous bifidobacteria compared to streptococci and lactobacilli.

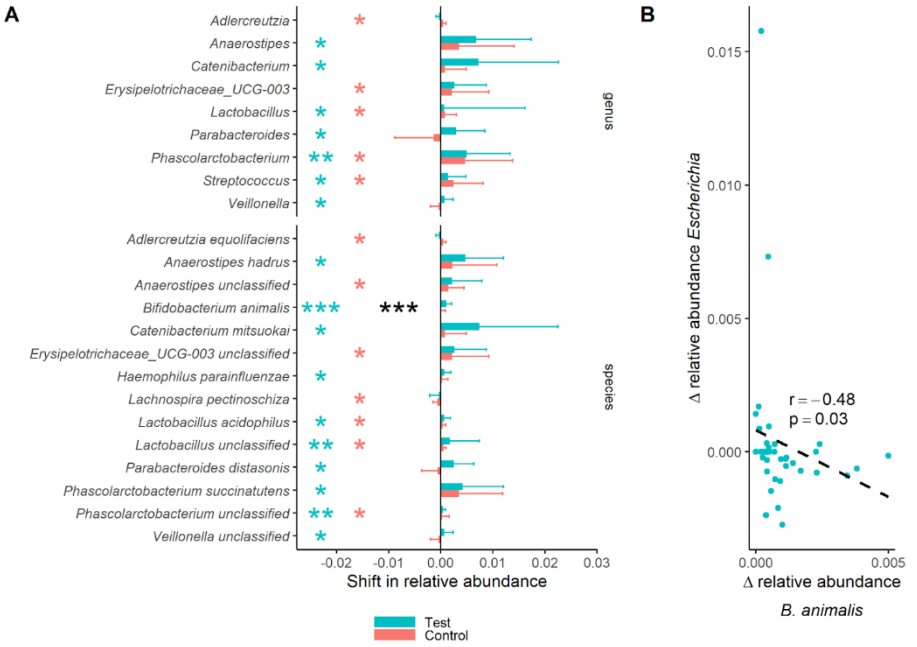

*Figure 13. The significant alterations in the fecal microbiota introduced by the interventions. A - Changes of relative abundance of significantly altered fecal bacteria at genus and species level. Corrected p-values are shown by asterisks: within test (green) or control (red) group before and after treatment and between test and control group after treatment (black). Statistical significance was assessed by Wilcoxon test (within-group comparisons) or by Mann-Whitney test (between-group comparisons). *, q < 0.1; **, q < 0.01; ***, q < 0.001. B - Increases of B. animalis correlates with decrease of Escherichia in the test group. Spearman correlation corrected for multiple comparisons.*

4.6 Variable gut microbiota growth on dietary oligo- and polysaccharides *in vitro* (I p., unpublished data)

To characterize the growth profiles of human fecal consortia on different DF substrates an *in vitro* study using isothermal microcalorimetry, 16S rDNA sequencing, and HPLC analysis was performed. This *in vitro* setup enabled the direct comparison of microbial growth and metabolite production capacity of the various commercially available DFs.

Heat flow curves from the calorimetry experiments showed inter-individual variation in the heat evolution patterns (Figure 14). Still, clear trends for the substrates were observed. Starting with the maximum heat flow, which indicates degradation rate of the substrates, the highest values were for GOS, RS, high soluble inulin (HSI), and pectin and lowest for AG and xylan (Table 4, Table 5). This in accordance with the data from Englyst and others, which showed incomplete and slow degradation of xylan and AG *in vitro* compared to pectin (Englyst et al., 1987). Also, total heat evolution on xylan, and AG was lowest of all other substrates (Table 5).

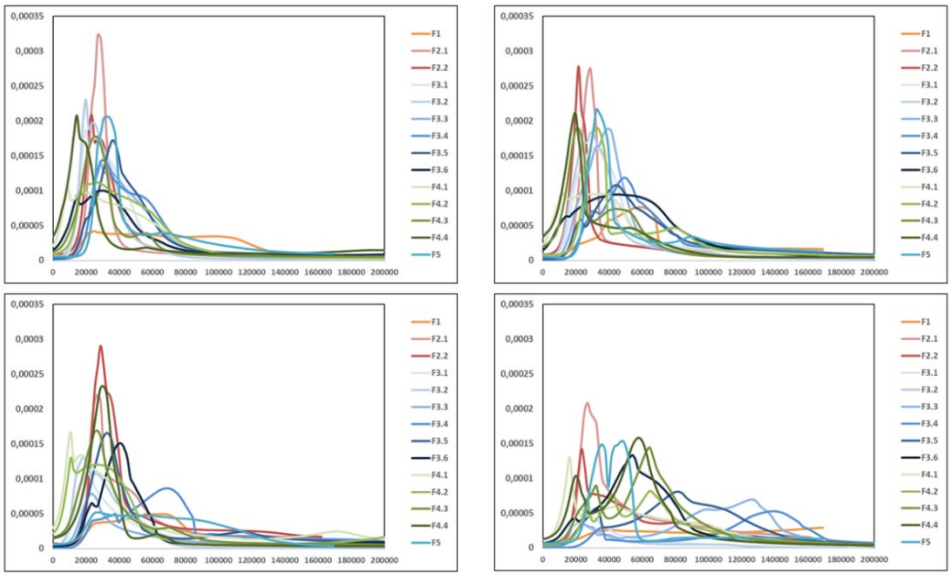

Figure 14. Heat flow curves for GOS, RS (top row), and HSI, HP (bottom row). X-axis - time (s), Y-axis - heat flow (µW). "Fa.b" designates an inoculum, where "a" is a donor and "b" is a number of the sample. GOS - galacto-oligosaccharides, RS - resistant starch, HSI - high soluble inulin (oligofructose), HP - high polymerization (inulin).

Continuing with the P-max times, which designate peak degradation timestamps of the substrates, we noted lower values (indicating quick growth/short lag phase) for GOS, HSI, RS, xylan, and pectin and higher values (indicating delayed growth) for AG, levan, and high polymerization (HP) inulin (Table 5). Again, this corroborates the findings from Englyst and others, where the highest AG consumption occurred much later in the experiment than that of a xylan, and pectin (Englyst et al., 1987). They also investigated the intra- and extracellular polysaccharidase activity in the *in vitro* experiments, which was not detectable for AG in the beginning of the experiment, while for xylan and pectin, significant cell-associated polysaccharidase activity was observed at zero time. These results provide a clear explanation for our results regarding some of the substrates, as AG degradation in our system was delayed.

Table 4. Heat generation profiles, metabolites, and microbial signatures of investigated substrates. Major SCFA-s in uppercase (median > 10 mM), minor SCFA-s in lowercase (median > 0 mM). Oligo- and polysaccharides have been ordered according to the growth profiles, metabolite production and bacterial growth patterns.

Substrate	Heat generation profile observed for the most cases for each substrate	Major and minor SCFA-s	Relative comparison of average bacterial genus levels and produced SCFA-s compared to other substrates
GOS	**Single early peak, quick degradation**	A, L, s, f	Highest Bifidobacterium, lowest Bacteroides, lowest propionate
RS	**Single early peak, quick degradation**	A, L, s, f, p	Highest Bifidobacterium, Blautia, Collinsella, Lactobacillus, lowest Bacteroides, lowest butyrate, propionate

HSI (oligo-fructose)	**Single early peak, quick degradation**	A, L, s, f, b	Highest Enterococcus.
HP (inulin)	Multiple delayed peaks, slow degradation	A, L, s, p, b, f	Highest Collinsella, highest formate.
Levan	**Single delayed peak, slow degradation**	A, L, s, p, b, f	Highest Collinsella, lowest Bifidobacterium, highest succinate, lactate, formate
AG	Multiple delayed peaks, slow degradation	A, p, s, l, b	Highest Bacteroides, highest propionate
Xylan	Multiple early peaks, quick degradation	A, p, l, s, b	Highest Bacteroides
Pectin	Multiple early peaks, quick degradation	A, s, p, b, f	Highest Lachnospira, Roseburia, Escherichia, lowest Bifidobacterium, lowest butyrate, lactate

A/a – acetate, B/b – butyrate, f – formate, L/l – lactate, p – propionate, s – succinate.
GOS - galacto-oligosaccharides, RS – resistant starch, HSI - high soluble inulin (oligofructose), HP - high polymerization (inulin), AG – arabinogalactan.

Table 5. Maximum heatflow (P-max), time of the maximum heatflow (Time P-max), total heat, and specific growth rate (μ) at first part of the fermentation of the investigated substrates.

	Substrate	P-max Avg, ± SD (μW)	Time P-max Avg, ± SD (h)	Total heat ± SD (J/ml)	Average μ 0-50% of total heat ± SD (1/h)
a	GOS	166.9 ± 68.0^{DEFG}	6.9 ± 2.0^{BdEFgh}	5.79 ± 0.66^{Fg}	0.57 ± 0.20
b	RS	170.0 ± 64.4^{DEFG}	9.7 ± 3.2^{AEF}	6.03 ± 0.87^{FG}	0.52 ± 0.16
c	HSI	144.1 ± 70.0^{FG}	8.4 ± 4.5^{EF}	5.52 ± 1.68	0.48 ± 0.14
d	HP	102.5 ± 57.0^{AB}	18.2 ± 13.3^{a}	5.32 ± 1.96	0.34 ± 0.16
e	Levan	105.5 ± 50.3^{ABFg}	18.9 ± 7.9^{ABCGh}	5.68 ± 1.67	0.26 ± 0.10
f	AG	60.3 ± 12.8^{ABCEH}	18.4 ± 8.5^{ABCg}	4.67 ± 0.97^{AB}	0.24 ± 0.07
g	Xylan	71.1 ± 30.3^{ABCeH}	11.5 ± 6.9^{af}	4.79 ± 1.12^{aB}	0.27 ± 0.11
h	Pectin	124.8 ± 50.4^{FG}	12.6 ± 7.2^{a}	5.68 ± 1.05	0.32 ± 0.13

Significantly different from: a - GOS, b - RS, c - HSI, d - HP, e - levan, f - AG, g - xylan, h - pectin. Uppercase - $p < 0.05$, lowercase - $p < 0.1$. Wilcoxon sign rank test corrected for multiple comparisons (Benjamin-Hochberg).
GOS - galacto-oligosaccharides, RS – resistant starch, HSI - high soluble inulin (oligofructose), HP - high polymerization (inulin), AG – arabinogalactan.

Overall, growth profiles on the included substrates could be divided into two groups: 1) single growth phase occurring early (GOS, RS and HSI) or slightly delayed response (levan) observed in most cases for each substrate; 2) multiple subsequent growth phases, with maximal growth occurring early (pectin, xylan) or delayed (AG, HP) (Table 5).

Multiple growth phases seen from the heat evolution curves indicate that either the growth of some members of the community was delayed by lag phase, or cross-feeding of the produced metabolites or degradation products of substrates enabled the growth of the other bacteria and subsequent production of secondary metabolites.

Remarkable similarities between the growth profiles of GOS and RS was observed (Figure 14). Analysis of the metagenomic and metabolite data also confirmed that across samples these fibers yield similar fermentation capacity mainly affected by inter-individual factors (Table 4, Table 5).

NMDS of the relative abundance data at genus level revealed clear clustering of the samples based on the donor and inoculum status (Figure 15). This clearly illustrates that *in vitro* cultivation of the gut samples is highly dependent on the initial community. Contrastingly, metabolite profiles provided closer clustering of the samples based on the substrate used and a considerably smaller effect of the inoculum was seen (Figure 16). These observations suggest that metabolite production is mainly determined by the specific substrate, not by the inoculum/donor status and resulting final microbial community profiles.

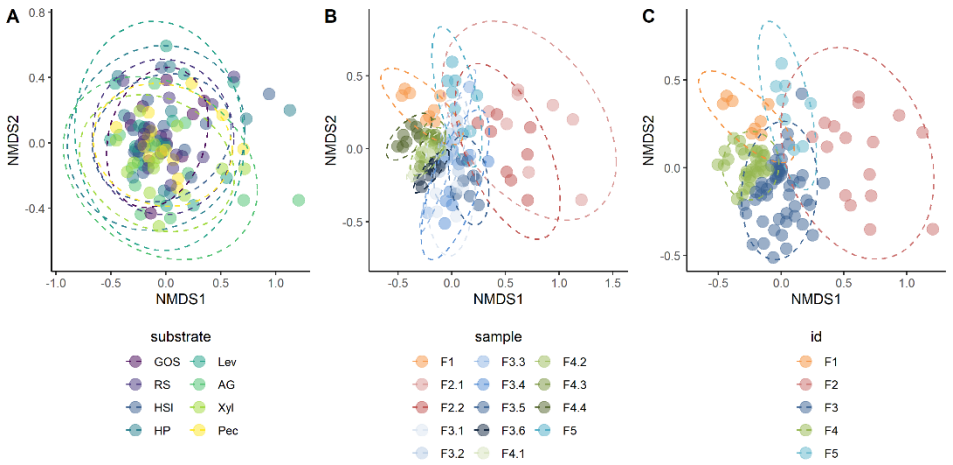

Figure 15. Non-metric multidimensional scaling (NMDS) ordination plot based on Bray–Curtis distances between 16S metagenomic data of samples in the end of the cultivation (48h) at genus level shows that: a) stratification based on substrates produces significantly different distributions of end-point bacterial profiles according to PERMANOVA (r2 = 0.218, p < 0.001) and among- and within-group dissimilarities differ by a great margin (ANOSIM r = 0.189, p < 0.001); b) stratification based on inocula produces significantly different distributions of end point bacterial profiles according to PERMANOVA (r2 = 0.145, p < 0.001) corrected for donors and among- and within-group dissimilarities differ by a great margin (ANOSIM r = 0.413, p < 0.001); c) stratification based on donors produces significantly different distributions of end-point bacterial profiles according to PERMANOVA (r2 = 0.310, p < 0.001) corrected for inocula and among- and within-group dissimilarities differ by a great margin (ANOSIM r = 0.458, p < 0.001). Each point corresponds to a sample. GOS - galacto-oligosaccharides, RS - resistant starch, HSI - high soluble inulin (oligofructose), HP - high polymerization (inulin), Lev - levan, AG - arabinogalactan, Xyl - xylan, Pec - pectin.

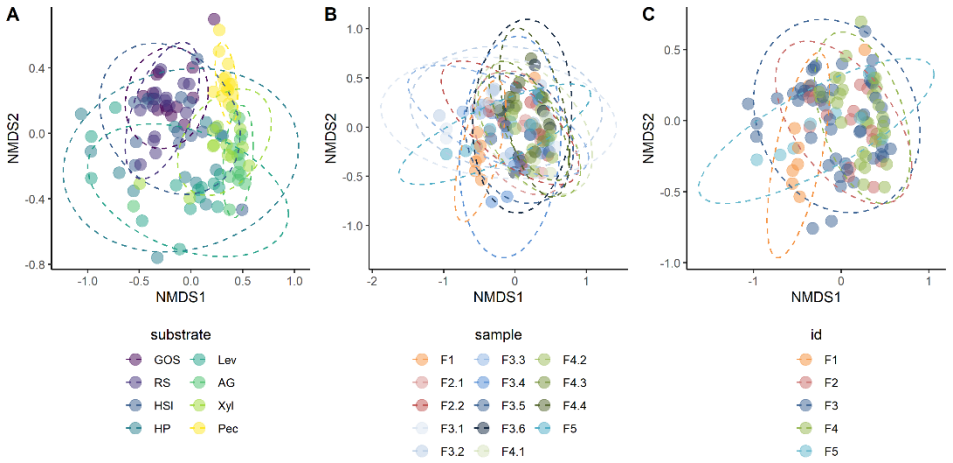

Figure 16. Non-metric multidimensional scaling (NMDS) ordination plot based on Bray–Curtis distances between final SCFA profiles of samples shows that: a) stratification based on substrates produces significantly different distributions of end-point bacterial profiles according to PERMANOVA (r2 = 0.429, p < 0.001) and among- and within-group dissimilarities differ by a great margin (ANOSIM r = 0.430, p < 0.001); b) stratification based on inocula produces significantly different distributions of end-point bacterial profiles according to PERMANOVA (r2 = 0.055, p = 0.005) corrected for donors and among- and within-group dissimilarities differ significantly (ANOSIM r = 0.124, p < 0.001); c) stratification based on donors produces significantly different distributions of end-point bacterial profiles according to PERMANOVA (r2 = 0.212, p < 0.001) corrected for inocula and among- and within-group dissimilarities differ significantly (ANOSIM r = 0.151, p < 0.001). Each point corresponds to a sample. GOS - galacto-oligosaccharides, RS - resistant starch, HSI - high soluble inulin (oligofructose), HP - high polymerization (inulin), Lev - levan, AG - arabinogalactan, Xyl - xylan, Pec - pectin.

Since the significant effect of the inoculum was observed, the initial abundance of the species was correlated with final levels at the end of the experiment. Out of the 10 most abundant species in the end of the experiment, we saw significant correlation between 0 h and 48 h bacterial levels in the case of *B. adolescentis* (Spearman, $p < 0.001$, $r = 0.57$), *F. prausnitzii* (Spearman, $p < 0.001$, $r = 0.42$), *Ruminococcaceae unclassified* (Spearman, $p < 0.001$, $r = 0.46$). However, the negative correlation was observed for *C. aerofaciens* (Spearman, $p < 0.001$, $r = -0.44$). Aside from those, we also investigated the correlation between the initial and endpoint relative abundances of *Prevotella*, which showed strong positive association (Spearman, $p < 0.001$, $r = 0.36$). Erratic representation of *Prevotella* illustrates the donor specific effects regarding the less prevalent bacteria, which were not present in all the initial consortia used as inocula for *in vitro* cultivation experiments.

Enhanced growth of multiple bacteria could be associated with specific substrates, e.g., members of *Lachnospira* and *Roseburia* were supported by pectin (Table 4). These specific effects were expected as, for example, *L. pectinoschiza* has the capacity to ferment pectin (Bang et al., 2018; Cornick et al., 1994).

Based on the IMC growth profiles, it would be possible to associate the variable end-point microbial patterns with the differential growth observed. As experiments were carried out with single end-point data collection, it was not possible pinpoint at specific bacteria growing in the first growth phase, but based on metagenomic data from the GOS, RS, and HSI samples, it is possible to deduce that fastest batch of bacteria

included members of *Bacteroides*, *Bifidobacterium*, *Blautia*, *Collinsella*, *Enterococcus*, *Escherichia*, and *Lactobacillus* (Table 4).

Overall, more significant growth of *Bacteroides* was observed for the substrates that produced delayed growth along with multiple growth phases (Table 4, Figure 18AB). *Bifidobacterium* and *Blautia* species were supported by the quickly degraded substrates as indicated by the single growth phase observed in the most cases on the mentioned substrates (Table 4).

Metabolite profile characterization by HPLC revealed significant differences between samples (Publication I, Figure 4). All substrates supported the production of high levels of acetate, but more significant differences were observed in the levels of butyrate, formate, propionate, succinate and lactate (Table 4). Acetate and lactate were the main metabolites on GOS, RS, HSI, HP, and levan. Significantly lower levels of lactate were detected on AG, xylan, and pectin (Table 4). Lactate levels were highest on substrates that provided a single growth peak and were quickly degraded. The only exception here was levan, which supported a delayed time of P-max compared to other substrates, but this substrate also exhibited divergent bacterial end-profile, where abundance of *Bifidobacterium* and *Collinsella* were lowest and highest, respectively, compared to other observed substrates. Earlier study by our group has shown that levan degradation results in significant production of both acetate and lactate, which was observed here too (Adamberg et al., 2015).

Highest succinate levels were observed in the case of HP, Lev, and AG, which coincided with delayed time of P-max. Succinate levels were lowest on GOS and RS along with earliest time of P-max (Table 4, Table 5). Similar patterns were observed for propionate. In the case of substrates, which resulted in the highest P-max values (GOS, RS), there was a tendency toward higher lactate, formate, and lower propionate levels compared to substrates with lowest P-max (AG, xylan).

Strongest positive correlations between produced metabolites and bacterial growth across all substrates were observed for *Bacteroides* genus and propionate (Pearson, $r = 0.69$, $p < 0.001$), acetate (Pearson, $r = 0.61$, $p < 0.001$) and succinate (Pearson, $r = 0.57$, $p < 0.001$) (Figure 17). However, negative correlations between produced metabolites and bacterial growth were weak: between *Bifidobacterium* genus and propionate (Pearson, $r = -0.32$, $p = 0.004$), and succinate (Pearson, $r = -0.30$, $p = 0.01$) and between *Lachnoclostridium* and lactate (Pearson, $r = -0.27$, $p = 0.03$) (Figure 17). Notably, there was positive correlation between butyrate and butyrate-producing bacteria: *Lachnoclostridium* (Pearson, $r = 0.33$, $p < 0.001$), *Eubacterium* (Pearson, $r = 0.29$, $p = 0.02$), *Faecalibacterium* (Pearson, $r = 0.25$, $p = 0.04$) (Figure 17).

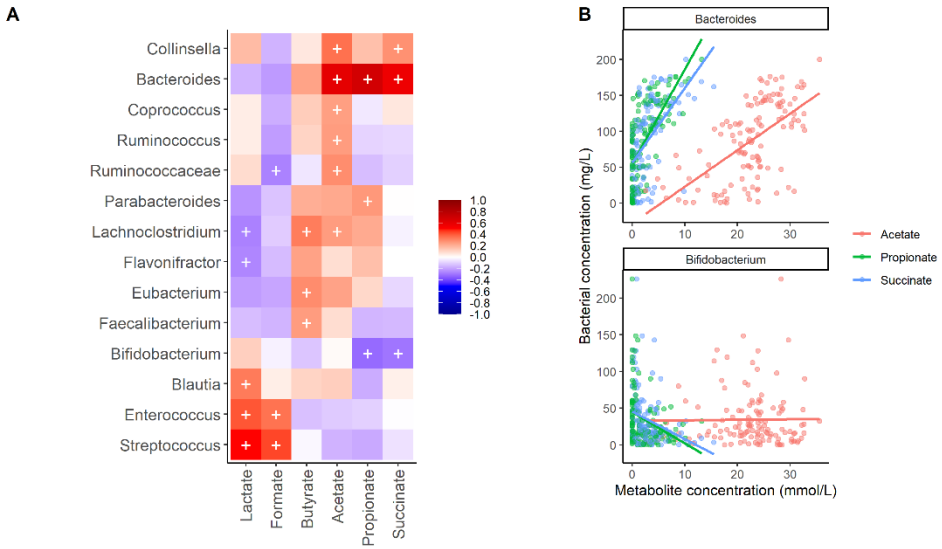

Figure 17. Correlation analysis of the genera and metabolites in in vitro experiments. A - Significant Pearson correlations (+ indicates multiple comparison corrected p-value < 0.05 between SCFAs, succinate, lactate and genera. The p-values were adjusted for multiple comparisons. B - Scatter plots of the significant correlations with metabolites of Bacteroides and Bifidobacterium: x-axis - metabolite concentration (mmol/L), y-axis - bacterial concentration (mg/L).

Compared to other fibers, highest production of butyrate and propionate were observed on xylan and AG (but not statistically significant). Still, while investigating the levels of known butyrate-producing bacteria, e.g., *Faecalibacterium*, *Roseburia*, *Eubacterium*, *Coprococcus*, higher levels were not observed other than for *Faecalibacterium* in the case of xylan. Instead, in the case of xylan and AG, highest levels of *Bacteroides* species were observed, which would indicate that potentially, on these substrates, butyrate production was supported by exogenous acetate produced by *Bacteroides* species. Surprisingly, compared to RS, low production of butyrate on xylan and AG has been shown by Englyst and others, which does not agree with our results (Englyst et al., 1987). Unexpectedly, high abundance of *Faecalibacterium* on Pec was not accompanied with higher butyrate levels compared to other substrates. Pectin is the only polysaccharide that *F. prausnitzii* can degrade outside the cell, as it is more oriented to consumption of smaller carbohydrates (Heinken et al., 2014). Low production of butyrate on pectin has been observed in other *in vitro* studies (Duncan, Holtrop, et al., 2004; Englyst et al., 1987).

Overall, compared to other SCFA-s, butyrate production in this experimental system on selected substrates was modest, as butyrate levels did not exceed 10 mM for any of the substrate/inoculum combinations. Relatively low levels of butyrate and butyrate-producing bacteria can be explained by neutral initial pH levels, but based on the other experiments from our group, where pH were monitored, a considerable decrease of pH during the cultivation would be expected (Adamberg et al., 2015). Results from the Reichardt and others show that low pH induces higher butyrate production due to shift in fermentation stoichiometry of butyryl-CoA:acetate CoA-transferase route, which favors greater butyrate production and acetate consumption (Kettle et al., 2015; Louis & Flint, 2017; Reichardt et al., 2018). Another explanation for low butyrate generation

was potentially the depletion of simple sugars. It has been shown by Duncan and others that butyrate-producing species require glucose supply to cover for the reducing power needed in butyrate generation from acetyl-CoA (Duncan, Holtrop, et al., 2004).

It is important to note that with pH measurements from the end of the experiment, more evident interpretations could be made about the effect of acidity on the outcome of the cultivations. Lower, slightly acidic pH (5.5) benefits the growth of SCFA producing Gram-positive bacteria while almost neutral pH (6.2-6.7) supports the growth of Gram-negative *Bacteroides* and *E. coli* (Duncan et al., 2009; Reichardt et al., 2018; Walker et al., 2005). Also, our experiments were started without initial SCFA present in the culture medium, which could be crucial for the growth of SCFA sensitive strains. A recent study has shown that, especially, *Bacteroides* species flourish without physiological levels of SCFAs (Tramontano et al., 2018).

Higher levels of *Bifidobacterium* on HSI compared to HP shows the high degradability of FOS by the colonic bifidobacteria. This is in agreement with other *in vitro* studies, which show diverse range of inulin-degradation capacity of bifidobacteria (Rossi et al., 2005). As growth rates of bifidobacteria on fructose are lower than on FOS, it could be presumed, that oligosaccharide portion of the HSI could have been the main substrate for the bifidobacteria (Rossi et al., 2005).

Degradation of least utilized DFs xylan and AG could be inhibited by the relative insolubility of these substrates. Since the water solubility of xylan and AG depends on the presence and the structure of the side chains, it is possible that degradation patterns of primary degraders significantly affect the potential utilization rate for other microbes (Saeed et al., 2011).

Altogether, these experiments show that *Bifidobacterium* was the bacterium that grew fast with low lag phase on GOS, RS, and HSI, while *Bacteroides* was supported by all conditions and only outcompeted on GOS and RS. Specifically, the prerequisite for the high growth of *B. adolescentis* were high initial levels, as only inocula from the donors with highest *B. adolescentis* levels supported growth capacity to outcompete the *Bacteroides* species. Contrarily, initial levels of *B. longum* did not correlate with the relative abundance observed in the end of the experiment. But in the case of donor F1, we noticed high growth of *B. longum* on all substrates instead of *B. adolescentis*, which was not present in the inoculum. In this case, a significant growth of *B. longum* on AG was observed (Figure 18, Figure 19). *B. longum* is the only *Bifidobacterium* species with significant capacity to degrade AG (Crociani et al., 1994). It can be presumed that utilization of AG by *B. longum* was supported by *Bacteroides* species, which were dominant bacteria on AG using inocula from other donors. An *in vitro* study has shown that the growth capacity of *B. longum* on AG is supported in a co-culture with *B. caccae* (Wang & Lapointe, 2020).

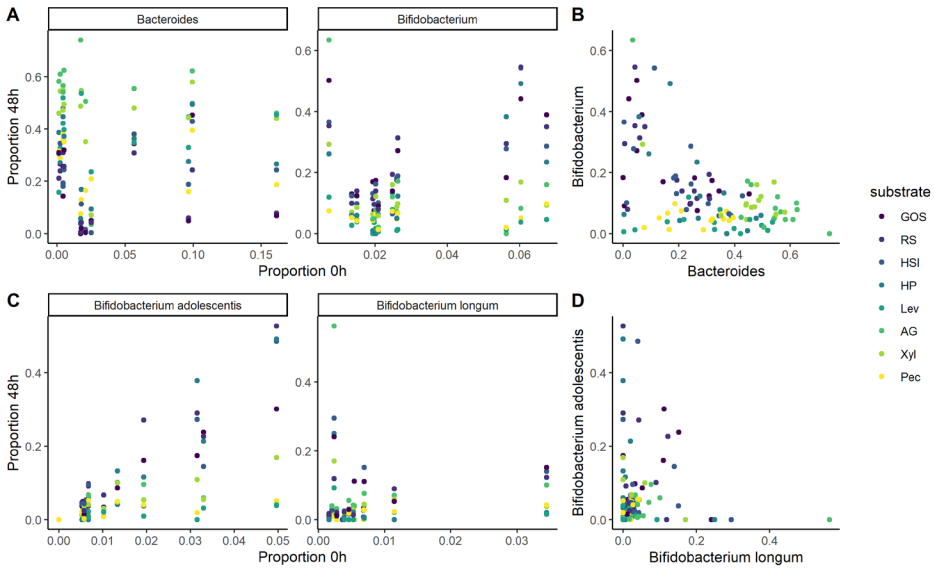

Figure 18. Initial vs endpoint relative abundance of (a) Bacteroides and Bifidobacterium, and (c) B. adolescentis and B. longum. Correlation between end-point relative abundance of (b) Bacteroides and Bifidobacterium (Pearson, $p < 0.05$), and (d) B. adolescentis and B. longum (Pearson, $p < 0.05$). GOS - galacto-oligosaccharides, RS - resistant starch, HSI - high soluble inulin (oligofructose), HP - high polymerization (inulin), Lev - levan, AG - arabinogalactan, Xyl - xylan, Pec - pectin.

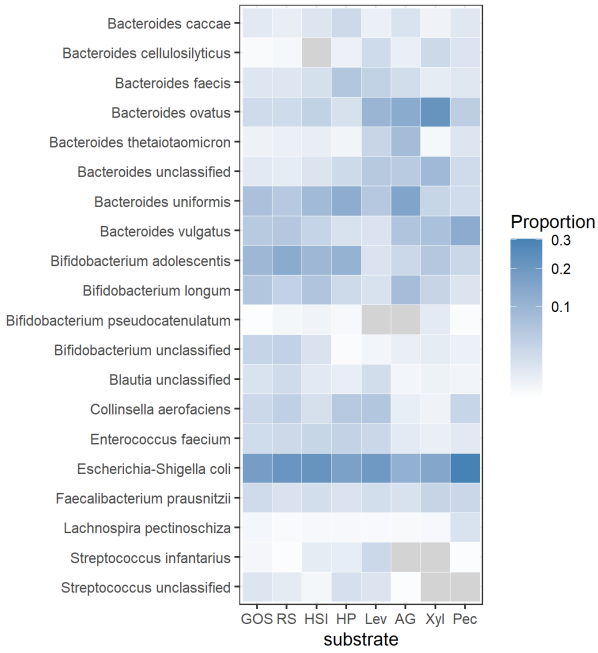

Figure 19. The most dominant species in the endpoint samples. Each tile represents the species average relative abundance of 14 experiments with individual inocula. GOS - galacto-oligosaccharides, RS - resistant starch (type-2), HSI - fructo-oligosaccharides, HP - inulin, Lev - levan, AG - arabinogalactan, Xyl - xylan, Pec - pectin.

Comparing *in vitro* results presented here with the data collected from human interventions, a meta-analysis from 2018 declared that fructans and galacto-oligosaccharides, which are classified as accepted prebiotics, and RS, classified as a candidate prebiotic, had more bifidogenic properties compared to non-prebiotic general fibers (So et al., 2018). Similar results were produced by the IMC experimental system showing high concordance with *in vivo* data.

5 Conclusions

For normal gut and body function, the diet should contain a variety of DFs. Unique selection of indigestible but microbiota accessible carbohydrates in each food can support the growth of specific gut microbes. For example, cereal grains are rich in arabinoxylan, which can be used to increase acetate and propionate producing bacteria such as *Bacteroides ovatus*, while inulin, oligosaccharides or amylopectin-rich resistant starch preferentially supports the growth of bifidobacteria.

To get more insight of behaviors of complex microbiota, an *in vitro* approach using isothermal microcalorimetry (IMC) was implemented. Combined with analytical methods, IMC was shown to be an effective method for screening the impact of specific DFs on functional changes in fecal microbiota. Overall, *in vitro* analysis of the modulation capacity of polysaccharides showed significant differences in the bacterial growth profiles. Specific growth curve patterns were associated with metabolite production and bacterial growth. Significant effect of donor status was identified in the fecal consortia after cultivation, whereas metabolite production was more dependent on the used fiber substrates.

As each food has its own nutritional profile and chemical composition, characteristics of foods should be analyzed in more detail, especially in regard to components, which can modulate the gut microbiota. In this work, a database of DF content in 46 food categories was formed to analyze the composition of assigned diets and dietary records. This analysis revealed that high-fat weight-loss diet rich in cellulose and pectin but low in cereal fibers (arabinoxylan, β-glucan) supported the growth of bile resistant bacteria (*Alistipes*, *Odoribacter splanchnicus*), *Ruminococcus bicirculans* and *Butyricimonas*, and reduced the levels of bacteria associated with inflammation such as *Collinsella* and *Dorea* in parallel with decrease of BMI.

Similarly, low fiber habitual diets were associated with higher consumption of meat and white flour products and increased levels of *Collinsella*, *Coprococcus*, and *Dorea*. At the same time, high fiber intake correlated with higher consumption of cereals, fruits and berries, and elevated levels of *Roseburia*, *Bacteroides cellulosilyticus* and *Oxalobacter formigenes*.

We also explored the effect of probiotic and synbiotic yogurts on the human gut microbiota. Intake of fiber-enriched yogurt specifically increased the levels of a probiotic strain (*Bifidobacterium animalis*) and several resident microbes. In the synbiotic group, *Escherichia* was correlated negatively with *Bifidobacterium animalis*, which supports the findings shown by previous *in vitro* studies.

Presented results demonstrate the effect of foods on gut microbiota and suggest that specific modulation can be realized based on the careful variation and enrichment of DF sources.

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Abstract

The effect of diet and dietary fiber on the human gut microbiota *in vitro* and *in vivo*

The incidence of lifestyle-related diseases is constantly increasing, which has been associated with modern eating habits. Consumption of high-fiber foods has declined significantly in recent decades, which may be one of the reasons for the increased risk of diseases. Significant changes in the intestinal microbiota, especially in the large intestine, have also been associated with the Western diet.

The effects of diet on the human microbiota can be studied in laboratory conditions as well as in nutrition studies, where it is crucial to determine the amount and composition of the consumed foods. Analysis of habitual diets requires collection of high-quality food intake data and specific information about the composition and distribution of fiber in food components. As the knowledge about the associations between specific food groups, fiber intake and intestinal microbiota is not exhaustive, further explorations are needed.

The aim of this doctoral dissertation was i) to investigate the fecal consortia and explore the short-term variability in Estonian subjects by the next generation sequencing methods, ii) to analyze the dietary fiber intake and sources in Estonian subjects and compare dietary intake in the subgroups of low and high fiber consumption, iii) to evaluate the associations of dietary intake of specific fiber and the gut microbiota based on the habitual intake and in the dietary interventions, iv) to investigate how different oligo- and polysaccharides are degraded by the human fecal microbiota *in vitro*.

Approximately 50 dominant species were identified in the gut consortia of the subgroup of Estonians, which accounted for half of the relative share. The predominant bacterial families were *Bacteroides* and *Prevotella*, which have been associated with long-term eating habits. It was also showed that the prevalence of primary fiber degraders was lower than that of secondary fermenters, e.g., butyrate producers in the fecal consortia.

The analysis of the Estonian dietary intake showed that the fiber consumption was lower than recommended for more than half of the participants. The main sources of fiber were cereals, vegetables and fruits, which supported the high proportion of the cellulose, arabinoxylan, pectin and fructans. Low fiber intake was associated with a meat-rich and refined grain diet, while higher fiber intake was associated with a whole grain-rich diet.

In the intestines of individuals with low fiber intake, there were more bacteria associated with obesity and inflammation, such as *Collinsella aerofaciens*, *Dorea longicatena* and less primary degraders, such as *Bacteroides cellulosilyticus*.

A weight loss diet introduced significant changes to the colonic microbiota, where a high-fat regime rich in cellulose and pectin but low in cereal fibers arabinoxylan and β-glucan supported the growth of bile-resistant bacteria (*Alistipes*, *Odoribacter splanchnicus*) and reduced the levels inflammation-associated bacteria (*Collinsella* and *Dorea*) and bifidobacteria. Lower counts of the former bacteria were also found in the higher fiber intake group in the above-mentioned habitual diet study.

Consumption of both regular yogurt and a yoghurt enriched with fiber increased the prevalence of lactobacilli, streptococci and butyrate-producing bacteria, and decreased levels of *Escherichia coli*. A specific positive effect of fiber-enriched yogurt on the proportion and frequency of *Bifidobacterium animalis*, a bacterium added to the dairy

product was also identified. A negative correlation was found between the change in the proportion of *B. animalis* and *E. coli*. Thus, additional proof was presented about the synbiotic fiber-enriched probiotic product.

The microcalorimetry cultivation platform was successfully employed to deliver the growth curves of the microbial consortia, and to identify quickly degraded bifidogenic fibers (resistant starch, galacto-oligosaccharides and short-chain inulin) or more slowly degraded primarily acetogenic fibers (long-chain inulin, levan, arabinogalactan, xylan, and pectin) supporting the growth of *Bacteroides* species. This *in vitro* study was unique in terms of the number of fibers compared and evaluated on the fecal inocula from various donors. These inocula from different donors supported personalized response in terms of the fiber degradation and bacterial growth. However, the SCFA production was more strongly associated with the fiber substrate used.

Collectively, these results showed the significant effect of diet and dietary fiber on the human gut microbiota.

Lühikokkuvõte
Toitumise ja kiudainete mõju inimese seedetrakti mikrobiootale *in vitro* ja *in vivo*

Elustiiliga seotud haiguste esinemissagedus on pidevas kasvus, milles nähakse olulist rolli kaasaegsetel toitumisharjumustel. Viimaste aastakümnete jooksul on langenud olulisel määral kiudainerikaste toiduainete tarbimine, mis võib olla üheks haiguste riski kasvu põhjuseks. Samuti on tuvastatud nn lääne dieedi korral ka olulised muutused soolestiku, eelkõige jämesoole mikrobiootas.

Toitumise mõju inimese mikrobiootale uuritakse nii laboritingimustes kui ka toitumisuuringutes, kus on väga oluline määratleda ära tarbitud toidu kogus ja koostis. Selle jaoks on eelkõige tavatoitumise analüüsimisel kriitilised nii kvaliteetsed toitumisandmed kui ka toidukomponentide koostise andmed kiudainete sisalduse ja jaotuse osas. Kuna spetsiifiliste toidugruppide ja kiudainete tarbimise ning soolestiku mikrobioota seosed pole lõplikult selged, siis on vajalikud jätkuvad uuringud.

Käesoleva doktoritöö eesmärgiks oli kaardistada eestlaste soolekoosluses leiduvaid baktereid, kirjeldada soole mikrobioota lühiajalist stabiilsust tavapärase toitumise ja kaalulangusdieedi korral, uurida spetsiifiliste kiudainete sisaldust tavatoitumises ning leida seoseid kiudainete tarbimise ja jämesoole mikrobioota vahel põhinedes tavapärase toitumise ja sekkumiskatsete andmetel. Samuti uuriti inimese soolekoosluste võimet lagundada erinevaid kiudaineid ja toota metaboliite *in vitro*.

Uuritud eestlaste jämesoole koosluses tuvastati umbes poolsada domineerivat liiki, mis kirjeldasid keskmiselt poole suhtelisest osakaalust. Domineerivad bakteriperekonnad olid *Bacteroides* ja *Prevotella*, kelle arvukust seostatakse pikaajaliste toitumisharjumustega. Samuti ilmnes, et primaarsete kiudainelagundajate esinemissagedus on madalam kui sekundaarsetel lagundajatel või fermenteerijatel, nt butüraaditootjatel.

Uuritud eestlaste toitumispäevikute analüüs näitas, et kiudainete tarbimine jäi alla soovitusliku üle pooltel osalejatel, põhilised kiudainete allikad olid tera-, köögi- ja puuviljad ning tarbitud toitudes olid kiudainetest peamiselt tselluloos, arabinoksülaan, pektiin ja fruktaanid. Madal kiudainete tarbimine seostub liharikka ning rafineeritud teravilju sisaldava toidu söömisega ning kõrgem kiudainete tarbimine täisteravilja-rikka dieediga.

Madala kiudainete tarbimisega indiviidide soolestikus on rohkem ülekaalu või põletikega seostatud baktereid, nt *Collinsella aerofaciens*i, *Dorea longicatena*t ning vähem kiudainete esmaseid lagundajad, nt *Bacteroides cellulosilyticust*.

Kaalulanguse sekkumisuuringus tuvastati oluline mõju seedetrakti mikrobiootale, kus kõrge rasvasisaldusega tselluloosi- ja pektiinirikas, kuid vähese teraviljasisaldusega ja arabinoksülaani ning β-glükaanivaene dieet toetas sapiresistentsete bakterite (*Alistipes, Odoribacter splanchnicus*) osakaalu kasvu ning põletikega seostatud bakterite (*Collinsella* ja *Dorea*) ja bifidobakterite osakaalu kahanemist. Esimeste bakterite madalam arvukus tuvastati ka kõrgema kiudainetarbimisega rühmas eelpool toodud tavatoitumise uuringus.

Nii tavalise jogurti kui ka kiudainetega jogurti tarbimine toetasid laktobatsiilide, streptokokkide ja butüraati tootvate bakterite esinemissageduse kasvu ning *Escherichia coli* kahanemist. Samuti tuvastati spetsiifiline, kiudainetega rikastatud jogurti positiivne mõju piimatootesse lisatud *Bifidobacterium animalis* osakaalule ning esinemissagedusele. *B. animalis* ning *E. coli* osakaalu muutuse vahel leiti negatiivne seos.

Seega leidis kinnitust, et kiudainetega rikastatud probiootilise toote tarbimisega on võimalik saavutada sünbiootiline mõju.

Kasutusel olnud IMC kultiveerimisplatvormiga määrati bakterite kasvukõverad ning tuvastati kiiremini lagundatud bifidogeensed kiudained (resistentne tärklis, galakto-oligosahhariidid ja lühikeseahelaline inuliin) ja aeglasemalt lagundatud eelkõige atsetaati tootvate bakterite nagu *Bacteroides* kasvu toetavad kiudained (pikaahelaline inulin, levaan, arabinogalaktaan, ksülaan ja pektiin). Antud töö oli unikaalne võrreldud kiudainete ja kaasatud doonorite arvu osas, kus nähti ka personaalsest, koosluse algsest mustrist sõltuvat mõju kiudainete lagundamisvõimekusele ja bakterite kasvule. Samas oli toodetud orgaaniliste hapete muster tugevamas seoses tarbitud kiudainete tüübiga.

Kokkuvõtvalt, esitletud tulemused näitasid toitumise ja kiudainete olulist mõju inimese seedetrakti mikrobiootale.

Appendix

Publication I

Adamberg, K., Kolk, K., Jaagura, M., Vilu, R., & Adamberg, S. (2018). The composition and metabolism of faecal microbiota is specifically modulated by different dietary polysaccharides and mucin: An isothermal microcalorimetry study. Beneficial Microbes, 9(1).

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The composition and metabolism of faecal microbiota is specifically modulated by different dietary polysaccharides and mucin: an isothermal microcalorimetry study

K. Adamberg[1,2*], K. Kolk[1,2], M. Jaagura[1,2], R. Vilu[1,2] and S. Adamberg[1]

[1]Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia, [2]Competence Center of Food and Fermentation Technologies, Akadeemia tee 15A, 12618 Tallinn, Estonia; kaarel.adamberg@ttu.ee

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RESEARCH ARTICLE

Abstract

The metabolic activity of colon microbiota is specifically affected by fibres with various monomer compositions, degree of polymerisation and branching. The supply of a variety of dietary fibres assures the diversity of gut microbial communities considered important for the well-being of the host. The aim of this study was to compare the impact of different oligo- and polysaccharides (galacto- and fructooligosaccharides, resistant starch, levan, inulin, arabinogalactan, xylan, pectin and chitin), and a glycoprotein mucin on the growth and metabolism of faecal microbiota *in vitro* by using isothermal microcalorimetry (IMC). Faecal samples from healthy donors were incubated in a phosphate-buffered defined medium with or without supplementation of a single substrate. The generation of heat was followed on-line, microbiota composition (V3-V4 region of the 16S rRNA using Illumina MiSeq v2) and concentrations of metabolites (HPLC) were determined at the end of growth. The multiauxic power-time curves obtained were substrate-specific. More than 70% of all substrates except chitin were fermented by faecal microbiota with total heat generation of up to 8 J/ml. The final metabolite patterns were in accordance with the microbiota changes. For arabinogalactan, xylan and levan, the fibre-affected distribution of bacterial taxa showed clear similarities (e.g. increase of *Bacteroides ovatus* and decrease of *Bifidobacterium adolescentis*). The formation of propionic acid, an important colon metabolite, was enhanced by arabinogalactan, xylan and mucin but not by galacto- and fructooligosaccharides or inulin. Mucin fermentation resulted in acetate, propionate and butyrate production in ratios previously observed for faecal samples, indicating that mucins may serve as major substrates for colon microbial population. IMC combined with analytical methods was shown to be an effective method for screening the impact of specific dietary fibres on functional changes in faecal microbiota.

Keywords: 16S rDNA, *in vitro* experiments, non-starch polysaccharides, short chain fatty acids, isothermal microcalorimetry

1. Introduction

Humans have co-evolved with symbiotic bacteria in the gut complementing the metabolic apparatus of the host with a diverse set of polysaccharide-degrading enzymes, which are not encoded in the mammalian genome. Microbiota-accessible carbohydrates (MACs) present in dietary fibre comprise major substrates for colon bacteria. In general, dietary fibre participates in appetite regulation through increased food volume and decreased fluctuation in blood sugar level; it alleviates constipation and may reduce the risks of various health problems, such as cardiovascular diseases, diabetes and colorectal cancer, according to reviews by several authors (Fuller *et al.*, 2016; McRorie, 2015; Roberfroid, 2007). Compared with plant-based diets (vegetables, cereals, fruits, berries and nuts, with supplements of milk, fish, eggs and meat), a typical Western diet contains more fat, simple carbohydrates and significantly less fibre (Sonnenburg and Sonnenburg, 2014). About 90% of the population of developed countries (as estimated in the US and Europe) consume on average only 15 g/d of dietary fibres (McRorie, 2015). Dietary fibres that are intrinsic in fruits, vegetables, legumes and whole grains are widely recognised as beneficial for

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health when consumed at recommended levels (25 g/d for adult women, 38 g/d for adult men, according to the Nordic Nutrition Recommendations 2012; Norden, 2014). Notably, in mice a chronic lack of dietary fibre reduces the diversity of bacteria in the gut; this increases in severity over multiple generations and is not fully reversed when fibre is reintroduced (Martens, 2016; Sonnenburg *et al.*, 2016). Fermentable dietary fibres form an important source of energetic substrates for gut bacteria and regulate intestinal pH through the fermentation of carbohydrates to organic acids and gases. As more diseases are linked to the Western type of gut microbiota, reprogramming is necessary to develop strategies that incorporate dietary MACs, as well as taxa not currently present in the Western gut (Sonnenburg *et al.*, 2016).

Dietary fibres comprise an extremely heterogeneous group of oligo- and polysaccharides, from short to long, linear to branched chain molecules, mono- to multimeric composition, and often protein or lipid-conjugated (Koropatkin *et al.*, 2012). Depending on the composition, structure and solubility, every carbohydrate molecule possesses specific functions in the body (e.g. a laxative effect), as well as impacts on gut microbiota through fermentation. Indeed, certain fibre types are degraded and fermented by specific bacteria, leading to directed modulation of gut microbiota. The elucidation of relationships between the nutrients and microorganisms at a detailed molecular level is essential to more completely understand the forces shaping microbial ecology and the genomic evolution of the gut (Martens *et al.*, 2014), as well as the peculiarities of the interaction of microbiota with human physiology.

Galactooligosaccharides (GOS, mostly 2-8 monomers), usually produced enzymatically from lactose, are soluble and fermentable galactose polymers that have been used as food ingredients in Japan and Europe for at least 30 years. The positive effect of GOS on gut microbiota, including their bifidogenic effect, has been shown in many studies (Belorkar and Gupta, 2016; Macfarlane *et al.*, 2008). Inulin and levan (Lev) are soluble fructose polymers (β-2,1 and β-2,6 linked, respectively) selectively fermented by several gut bacteria. The bifidogenic effect of inulin has been proven in many *in vitro* and human studies, while the effect of Lev on gut bacteria has only been modestly investigated so far (Adamberg *et al.*, 2015; Porras-Domínguez *et al.*, 2014; Visnapuu *et al.*, 2015). Resistant starch (RS) is a modified starch from various plant sources, not degraded by human intestinal enzymes but supporting the growth of different bacteria, such as *Ruminococcus, Eubacterium* and *Bifidobacterium* (Hald *et al.*, 2016; Walker *et al.*, 2011). Arabinogalactan (AG) is a plant-derived dietary fibre shown to increase the production of butyrate and propionate, and to decrease the generation and absorption of ammonia in the colon (Englyst *et al.*, 1987; Kelly, 1999). Xylans (Xyls) are pentose-containing polymers found in plant cell walls and contain predominantly β-D-xylose units linked as in cellulose. These may additionally contain arabinose and/or proteins. Pectins (Pec), also known as pectic polysaccharides, are rich in galacturonic acid and may have branching chains of various saccharides, such as galactose, rhamnose, xylose or arabinose. Pec have been widely used as dietary supplements to improve digestion and as therapeutic agents in several diseases (Dorokhov *et al.*, 2015). Chitin is a long-chain polymer of an *N*-acetylglucosamine and is a characteristic component of fungal cell walls, exoskeletons of arthropods such as crustaceans and insects, and the radulae of molluscs. In addition to diet-derived MACs, there are carbohydrate sources originating from the intestinal tract itself, such as mucins: high molecular weight, heavily glycosylated proteins produced by goblet cells. Along with diet derived MACs, mucins act as important indigenous carbon sources and supplementary substrates providing carbohydrates and amino acids. Several species of colon microbiota belonging to such genera as *Akkermansia* and *Bacteroides* have been shown to degrade mucins (Derrien *et al.*, 2004; Salyers *et al.*, 1977).

There is only limited information about how responses of human organisms are related to fibre composition and structure. In particular, non-digestible dietary carbohydrates have enormous potential for modifying gut microbiota, but these modifications occurring at the level of individual strains and species are not easily predicted *a priori.* The metabolism of such dietary fibres as fructooligosaccharides (Sghir *et al.*, 1998), inulin (Aguirre *et al.*, 2016; Chung *et al.*, 2016; Jung *et al.*, 2015), Pec (Chung *et al.*, 2016; Gómez *et al.*, 2016), RS (Sharp and Macfarlane, 2000), Xyl (Chassard and Bernalier-Donadille, 2006), arabinoxylan (AX) oligosaccharides (Sanchez *et al.*, 2009), AG (Aguirre *et al.*, 2016), AXs from different cereals (Rumpagaporn *et al.*, 2015) and other dietary fibres (Aguirre *et al.*, 2014; Velázquez *et al.*, 2000) by faecal microbiota have been studied in complex basal media.

For the development of efficient synbiotics, it is necessary to identify which species and which respective metabolic activities are likely to respond to a given dietary manipulation (Chung *et al.*, 2016). *In vivo* feeding experiments cannot readily distinguish the effects mediated via the gut environment (the pH or changes in the rate of gut transit) from the direct selective effects caused by supplemented substrate. Furthermore, *in vivo* nutritional studies do not provide precise control over the substrates available to microbiota, since endogenous substrates, such as mucin and other food components, are always present.

In the current microcalorimetry study, different polysaccharide substrates – GOS, inulin (high soluble inulin (HSI) and long-chain inulin (HP)) Lev, RS, AG, Xyl, Pec, chitin and mucin – were tested to elucidate their effect on

the growth, composition and metabolism of adult faecal microbial consortia. A growth medium with a defined composition, instead of a rich medium containing yeast extract and peptones, was supplemented with a single substrate (a fibre or mucin) in each experiment.

2. Materials and methods

Faecal inocula and culture media

Fourteen faecal samples donated by five Estonian adult subjects (age 28-48 years) without chronic diagnoses were tested. The samples were collected and processed as described previously in Adamberg *et al.* (2015). Faecal samples were collected aseptically into plastic bag where anaerobic environment was generated (GasPak EZ Anaerobe Pouch System with indicator, Becton Dickinson, Franklin Lakes, NJ, USA) and kept at -20 °C until further processing. The storage cultures were prepared aseptically from the molten faecal material under nitrogen environment by continuous flushing with nitrogen gas (Captair Pyramid Glove Bag®, Erlab, Köln, Germany) and diluted five times in sterile anaerobic phosphate-buffered saline (PBS) (mM): NaCl (160), KCl (3), Na_2HPO_4 (8), NaH_2PO_4 (1), containing 5% dimethylsulfoxide (DMSO) (v/v) as protectant, pH 7.2, supplemented with fresh filter sterilised (0.2 µm, FP 30/0.2 CA-S, Whatman, Maidstone, UK) cysteine-hydrochloride (Cys-HCl) (0.5 g/l in final solution). Aliquots of the diluted samples to start a cultivation experiments were stored at -80 °C.

The impact of poly- and oligosaccharides with different monomeric contents, structures and properties (solubility, fermentability) were studied. Culture medium without any carbohydrates added served as negative control and porcine mucin as an indigenous gut polymer (Table 1).

Growth experiments

The growth experiments were performed as described in Adamberg *et al.* (2015). Briefly, the faecal inocula were mixed with defined growth medium containing: 0.05 M potassium phosphate buffer made from 1 M stock solutions (ml/l): K_2HPO_4 (35.85) and KH_2PO_4 (14.15); mineral salts (mg/l): $MgSO_4 \cdot 7H_2O$ (36), $FeSO_4 \cdot 7H_2O$ (0.1), $CaCl_2$ (9), $MnSO_4 \cdot H_2O$ (3), $ZnSO_4 \cdot 7H_2O$ (1), $CoSO_4 \cdot 7H_2O$ (1), $CuSO_4 \cdot 5H_2O$ (1), $MgCl_2$ (2), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1), NaCl (527), NH_4Cl (400); and 10 ml/l hemin-vitamin K_1 solution (hemin – 5 mg/l and vitamin K_1 0.5 mg/l in final medium); and one of the carbohydrates (5 g/l) listed in Table 1; pH 7.2. Substrates that cannot be filter-sterilised, such as mucin, AG, Pec, chitin and RS were sterilised by autoclaving at 110 °C/3 min (Systec V-100; Systec GmbH, Linden, Germany). The heat sensitive solutions of Lev, GOS, and inulin were filter-sterilised (0.2 µm, FP 30/0.2 CA-S, Whatman) to avoid degradation.

Before inoculation, the growth media were pre-reduced by supplementing with stock solutions of freshly prepared and filter sterilised Cys-HCl (concentration in the medium 0.5 g/l) and autoclaved sodium thioglycolate (concentration in the medium 0.5 g/l) and incubated overnight in an anaerobic jar with GasPak EZ Anaerobe Pouch System (Oxoid Inc., Basingstoke, UK). The media were inoculated with aliquots prepared from frozen faeces to obtain approximately 10-100 times dilution of the original sample that was found to be optimal in our previous experiments (Adamberg *et al.*, 2015).

The sterile ampoules with total volume of 3.3 ml were filled with 2 ml of the inoculated medium, closed hermetically and incubated at 37 °C in a 24-channel isothermal microcalorimeter TAM III (TA Instruments, New Castle, DE, USA) as described by Kabanova *et al.* (2009). The heat flow (P, µW) was recorded and total accumulated heat

Table 1. Carbohydrate substrates added to the base medium in cultivation experiments.

Substrate	Abbreviation	Supplier
Galactooligosaccharides, DP 2-10	GOS	Friesland Campina, Wolvega, the Netherlands
Inulin HSI (β-2,1-linked fructan), DP 2-8, 11% monosaccharides	HSI	Beneo Orafti, Oreye, Belgium
Inulin HP (β-2,1-linked fructan), DP >23, 0.1% monosaccharides	HP	Beneo Orafti
Levan (β-2,6-linked fructan), DP >100	Lev	Synthesised by Lsc3 protein of *Phytophthora syringae* pv. tomato; kindly provided by Dr. T. Alamäe (University of Tartu, Estonia)
Resistant starch	RS	Cargill Deutschland Gmbh, Malchin, Germany
Arabinogalactan from larch tree, Poly(N-acetyl-1,4-β-D-Glucopyranosamine), DP >23	AG	Sigma-Aldrich, St. Louis, MO, USA
Xylan (xylose polymer) from beechwood	Xyl	Sigma-Aldrich
Pectin from citrus peel, galacturonic acid >74%	Pec	Sigma Aldrich
Chitin from shrimp shells, (N-acetylglucosamine polymer)	Chitin	Sigma-Aldrich
Mucin from porcine stomach, type III	Muc	Sigma-Aldrich

(Q, J) which is proportional to the biomass formation was calculated by integration of the heat flow data.

Analytical methods

Samples were taken at the beginning and the end of the growth experiments for microbial 16S rDNA sequencing and metabolite determination. The samples were centrifuged (21,000×*g*, 10 min, 4 °C) and kept at -20 °C until use. Bacterial DNA was analysed from cell pellets. For chromatographic analysis, 10% sulfosalicylic acid (1:0.25; v/v) was added to the supernatant and centrifuged (21,000×*g*, 15 min, 4 °C, Thermo MicroCL R21; Thermo-Fisher Scientific, Waltham, MA, USA). The supernatants were filtered through 0.20 µm PTFE syringe filters (Millex filters SLLGH13NK; Merck-Millipore, Billerica, MA, USA) and additionally ultra-filtered using AmiconR Ultra-10K Centrifugal Filter Devices, cut-off 10 kDa (Merck-Millipore).

The concentrations of organic acids (succinate, lactate, formate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate), glycerol and ethanol were determined by high-performance liquid chromatography (HPLC, Alliance 2795 system; Waters, Milford, MA, USA), using a BioRad HPX-87H column (Hercules, CA, USA) with isocratic elution of 0.005 M H_2SO_4 at a flow rate of 0.6 ml/min and at 35 °C. Refractive index (RI) (model 2414; Waters) and UV (210 nm; model 2487; Waters) detectors were used for quantification of the substances. Detection limit for the HPLC method was 0.1 mM.

DNA extraction and amplification

DNA was extracted from all samples using PureLink Microbiome DNA extraction kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Universal primers S-D-Bact-0341-b-S-17 Forward 5'TCGTCG GCAGCGTCAGATGTGTATAAGAGACAGCCTACGG GNGGCWGCAG F and S-D-Bact-0785-a-A-21 Reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTATCTAATCC were used for PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA genes (Klindworth *et al.*, 2013). The amplified region was about 450 bp and on average 24,660 reads per sample were obtained. The mixture of amplicons was sequenced using Illumina MiSeq 2×250 v2 platform (Microsynth AG, Balgach, Switzerland).

Taxonomic profiling of faecal samples

Sequence data was analysed using BION-meta (http://www.box.com/bion), a yet unpublished open source program, according to author´s instructions. First, the sequences were cleaned at both ends using a cut-off for minimum quality of 95%, then joined, followed by removal of shorter reads than 350 bp. Second, identical sequences were merged to one while keeping track of original read counts and then cleaned from chimeras. Last, consensus reads were aligned to the SILVA reference 16S rDNA database (v123) using word length of 8 and similarity cut-off of 90% with taxonomical classification of top 3% of hits (Simrank2; DeSantis *et al.*, 2011). Samples were rarified to an even depth of 1,395 reads.

Calculation of the residual faecal energy

Based on the heat released during growth without added substrates the residual faecal substrate concentrations in hexose equivalents were calculated taking into account the standard molar enthalpy of formation of acids ($\Delta_fH^0_{acetate}$=-484.3 kJ/mol, $\Delta_fH^0_{propionate}$=-510.7 kJ/mol, $\Delta_fH^0_{butyrate}$=-533.8 kJ/mol, $\Delta_fH^0_{lactate}$=-694.1 kJ/mol, $\Delta_fH^0_{succinate}$=-940.5 kJ/mol, $\Delta_fH^0_{formate}$=-425 kJ/mol, $\Delta_fH^0_{CO_2}$=-393.5 kJ/mol, $\Delta_fH^0_{hexose}$=-1,273 kJ/mol (Haynes, 2012) and using the formula:

$$n_{hexose} = Q_{exp}/Q_{hexose} \text{ [mol]} \quad (1)$$

where n_{hexose} is the faecal carbohydrate content (in hexose equivalents = 1 mol), Q_{exp} is the total heat evolved during the calorimetry experiment, Q_{hexose} is the theoretical heat released according to the fermentation pattern observed during the experiment calculated by formula:

$$Q_{hexose} = \Sigma n_{prod\_i} \times \Delta H^0_{f\_prod\_i} - \Sigma n_{hexose} \times \Delta H^0_{f\_glucose} \text{ [kJ/mol]} \quad (2)$$

where n_{prod_i} is the number of moles of a metabolite_i (acetate, butyrate, formate, lactate, propionate, succinate and carbon dioxide) formed per one mole of hexose.

Energy extraction

The potential energy that could be obtained by colon epithelial cells from the oxidation of the organic acids was calculated based on stoichiometry of oxidation:

$$R\text{-}COOH + x \times O_2 \rightarrow y \times CO_2 + z \times H_20$$

where acetate, butyrate, lactate, propionate or succinate are expressed as R-COOH and the enthalpy is calculated by using the formula:

$$\Delta H = \Sigma n_{prod\_i} \times \Delta H^0_{f\_prod\_i} - \Sigma n_{subst} \times \Delta H^0_{f\_subst} \text{ [kJ/mol]} \quad (3)$$

which give ΔH values 874 kJ/mol for acetate, 1,527 kJ/mol for propionate 2,183 kJ/mol for butyrate, 1,343 kJ/mol for lactate and 1,491 kJ/mol for succinate.

Statistical analysis

For correlation and multivariate analysis, first the relative data of bacterial abundances from 16S rDNA sequencing analysis were converted to quantitative values (X_i [g/l] where i illustrates bacterial taxa i by formula: $X_i = X_t \times A_i$, where X_t is the dry weight of total biomass of bacteria (g/l) and A_i is the relative abundance of bacterial taxa i in the sample. Biomass concentration was calculated from the power-time curves taking into account heat accumulated (Q) and heat generation coefficient 20 kJ per gDW of biomass produced (Russell, 1986). Quantitative data of all experiments (X_i values, specific growth rate, concentration of metabolites, and maximal power values from calorimetric data) were merged into a matrix table. Partial least squares discriminant analysis (PLS-DA) and correlation analysis of the data was performed using web-based software MetaboAnalyst 3.0 (Xia *et al.*, 2015). For diversity analysis Shannon index (S_i) was calculated using formula: $S_i = \Sigma p_i \times \ln(p_i)$, where p_i is the relative abundance of bacterial taxa i in the sample.

Ethics statement

During a preparatory consultation interview, the volunteers were informed about the scope of research and all the subjects gave written informed consent. The study was approved by Tallinn Medical Research Ethics Committee, Estonia (protocol No. 554).

3. Results

Microbiota composition of the initial faecal samples

Samples donated by five volunteers showed donor-characteristic composition of the faecal microbiota. Based on 16S rDNA sequence analysis, 398 operational taxonomic units (OTUs) were identified with 175 taxa of relative abundance over 0.1% in at least one out of fourteen faecal samples. All faecal consortia were dominated by species from the phylum *Firmicutes* (abundance over 50%). The major taxa represented in the samples belonged to *Dialister*, *Faecalibacterium, Eubacterium, Christensenellaceae, Bacteroides, Ruminococcus, Prevotella*, *Akkermansia, Subdoligranulum, Coprococcus* and *Lachnospiraceae* (Figure 1, Supplementary Table S1), while the taxa observed in all samples were *Faecalibacterium, Eubacterium, Ruminococcus, Lachnospiraceae, Subdoligranulum* and

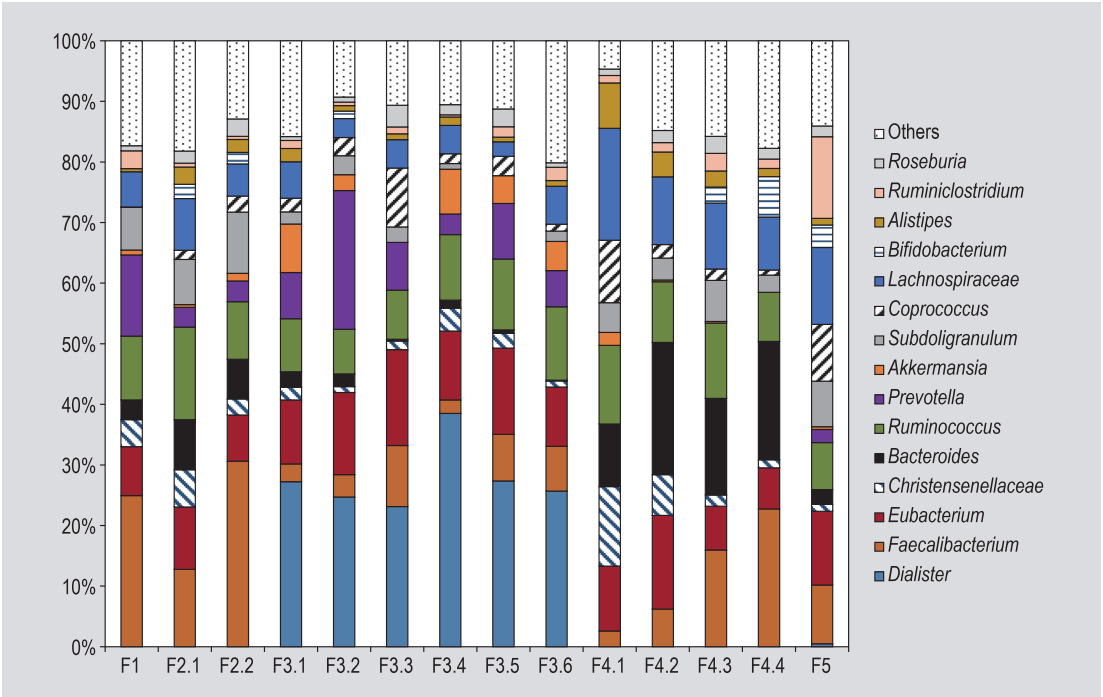

Figure 1. Distribution of the dominating bacterial genera (family if genera is not identified) (% with abundance of 0.015) in fourteen faecal samples tested. Samples (F) are named with the donor number (first place) and sample number (second place, e.g. F2.1). Single samples were donated by donors 1 and 5. The order of appearance of the genera in the bars is the same as that of the legend.

Bacteroides. Genus *Faecalibacterium* dominated in samples from all donors (3-23%), except for the sample F4.1. Abundant taxa in sample F1 included other *Ruminococcaceae* (*Subdoligranulum, Ruminiclostridium* and *Ruminococcus*), *Prevotella* and some *Lachnospiraceae*. Samples F2.1 and F2.2 can be characterised as a diverse mixture of different species with abundance up to 5%, and the most common were *Eubacterium oxidoreducens*, *Subdoligranulum formicilis*, *Bacteroides vulgatus*, *Bacteroides uniformis* and *Christensenella* spp. *Dialister invisus* was a very dominant species in all samples form a donor no 3 (F3.1, F3.2, F3.3, F3.4, F3.5 and F3.6), followed by *Akkermansia muciniphila* and *E. oxidoreducens*. Samples F4.1, F4.2, F4.3 and F4.4 were rich in *Bacteroides* species (over 10%, with *B. vulgatus* and *B. uniformis* as the most abundant), followed by *Christensenella*, *E. oxidoreducens* and several unknown *Ruminococcaceae*. Characteristic taxa in sample F5 were *Ruminiclostridium, Coprococcus eutactus* and several other *Ruminococcaceae,* such as *Eubacterium coprostanoligenes* and *S. formicilis,* and *Lachnospiraceae,* such as *Eubacterium rectale*, *Blautia luti* and *Anaerostipes hadrus*.

Growth dynamics of faecal microbiota based on power-time curves

The faecal microbial consortia are extremely complex. Already minor fluctuations in bacterial proportions and metabolism (cross-feeding, etc.) would influence the population dynamics and heat evolution patterns remarkably, thus the calorimetry experiments (heat evolution patterns) are not reproducible in detail. However, the substrate-specific fermentation duration (until 75% of total energy evolved) and metabolite profiles are better reproducible. The total heat generated during the growth of faecal consortia without additional substrates was used to calculate the amount of residual substrates in the faeces. The values of the residual energy ranged from 0.24 to 1.12 J/ml, which corresponds to the estimated amount of 7-26 mg/g faeces expressed as C6 carbohydrates. Based on the heat production and metabolite formation data, more than 70% of all substrates (5 g/l), except chitin, were fermented by all faecal consortia (Figure 2). Despite the great inter-individual variation of faecal microbiota, substrate-specific heat evolution patterns and metabolic products for most of the samples were observed. On the basis of heat generation profiles the carbohydrate substrates formed two groups with linear, and/or short homopolymer hexoses (GOS, HSI, RS) as readily accessible and long-chain and complex (branched, heteropolymers, including pentosans) as hard to digest substrates for faecal consortia. The multipeak (multiphase) heat flow curves were characterised by the number and width of peaks but not by exact time of occurrence that is dependent on bacterial concentration of the inoculum. The period between two sequential heat peaks was defined as phase (Figure 2), whilst the overlapped peaks were taken as one peak. In most cultures, fermentation of GOS, HSI, RS were the fastest generating single or overlapped heat peaks comprising over 75% from total heat generated before 24 hours of growth (Supplementary Figure S1). This rapid fermentation was accompanied by the highest heat evolution rate (P_{max}, 150±79 μW) (Figure 2), and the biggest amount of total heat generated (up to 8 J/ml). In different, prolonged heat evolution on GOS was observed in samples F1, F4.1 and F4.2 (Supplementary Figure S1). In addition, fermentation of RS by samples F3.4, F3.5 and F3.6 and that of inulin HSI by samples F1, F3.4 and F5 was extended.

In the case of multiphasic heat profiles, we presumed that the first heat peak corresponded to the fast fermentation of the easily accessible carbohydrates yielding acetate and lactate, while the later peaks reflected the gradual degradation of complex poly- and oligosaccharides, secondary fermentation of lactate and acetate end formation of propionate and butyrate (Figure 3). This was supported by the product analysis, as butyrate was found in media containing Xyl, AG and Lev but not those of rapidly degraded HSI, GOS and RS. Fermentation of inulin HP exceeded 40 hours in cultures of F1, F3.3, F3.4, F3.5 and F3.6, showed specific multiphasic behaviour in samples F4.1, F4.2, F4.3, F4.4 and F5, but was comparable to inulin HSI in cultures of F2.1, F2.2, F3.1, F3.2. This extended heat evolution can be explained by the continuing release of monosaccharides from long-chain polymers. Prolonged heat evolution without distinct phases in most of the cases was also observed for Lev possibly by the presence of only a few bacterial taxa, such as *Bacteroides thetaiotaomicron* able to degrade the high molecular polymer (>20 kDa, Adamberg *et al.*, 2014).

The pentose containing Xyl and AG were fermented slowly (typically over 40 h), characterised by several distinct growth phases and low heat evolution rates (below 100 μW; Figure 2, Supplementary Figure S1). Such sequential heat evolution data may indicate either a low abundance of Xyl and arabinan-degrading bacteria, such as *Bacteroides, Prevotella* and *Bifidobacterium,* or xylose/arabinose metabolising bacteria in the consortia.

Mucin, a heavily glycosylated protein, was included as representative of an indigenous substrate for the colon bacteria. In contrast with most hexoses, mucin was fermented in several phases (excluding samples F3.6, F4.1 and F4.2) up to 50 h, with only 3.9 to 6.4 J/ml heat generated and about two times lower P_{max} compared to GOS or RS (82±29 vs 150±79 and 130 μW, respectively). According to the heat evolved during the fermentation and enthalpy of the reactions of the acid formation (Haynes, 2012), it was calculated that mucin contained 30-60% of the fermentable carbohydrates available for bacteria.

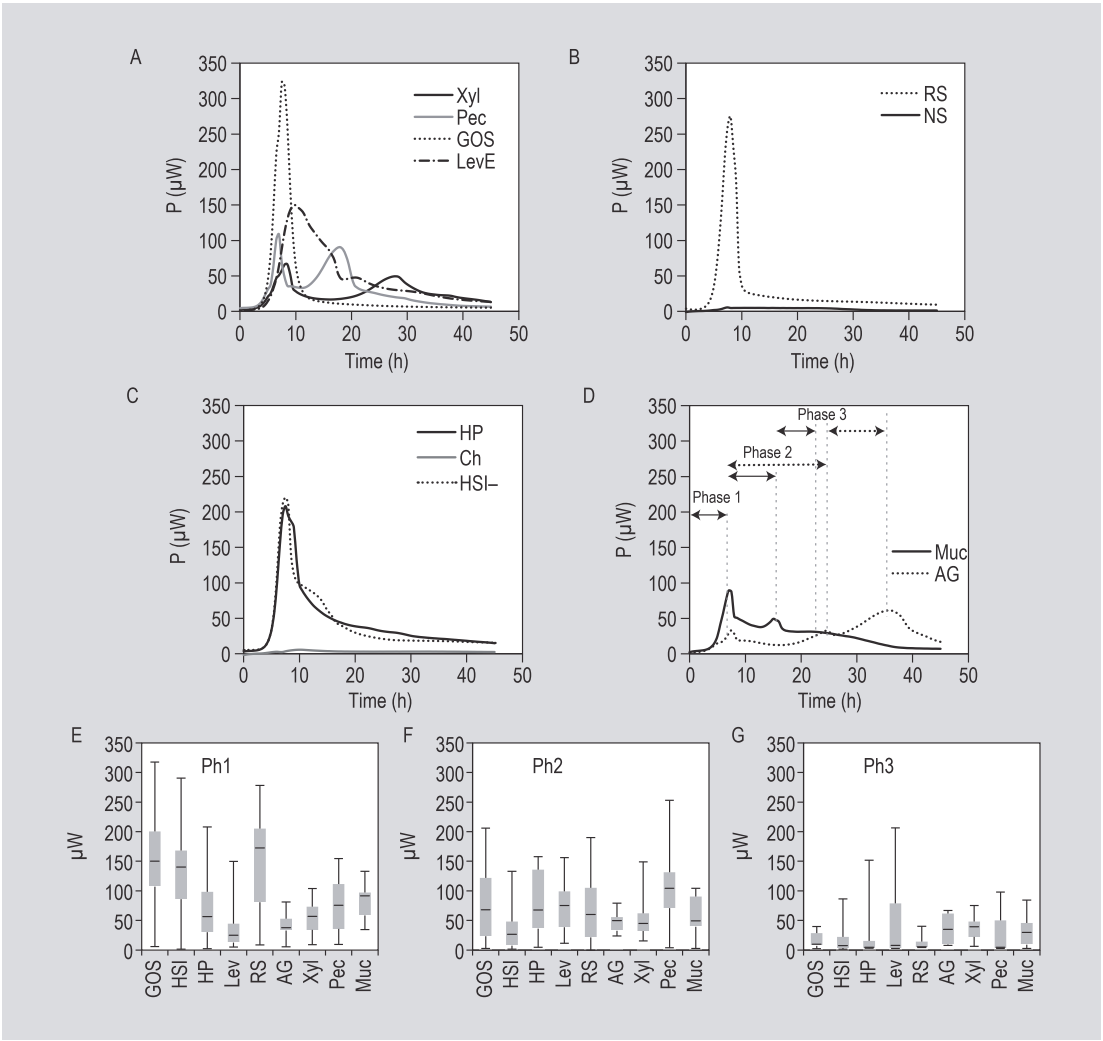

Figure 2. (A-D) Examples of heat flow during the growth of faecal consortia in defined media supplemented with arabinogalactan (AG), chitin (Ch), galactooligosaccharides (GOS), inulin HP (HP), inulin HSI (HSI), levan (Lev), no substrate (NS), mucin (Muc), pectin (Pec), resistant starch (RS) or xylan (Xyl). (D) Separation of the growth phases, which are defined as the time periods between the two local heat flow (P) peak values. (E-G) Box plots illustrate the maximum power values (Pmax) in growth phases no 1 (Ph1), 2 (Ph2) and 3 (Ph3), respectively. All heat flow data are given in the Supplementary Figure S1.

Relationship between bacterial composition changes and metabolite profiles in response to different fibres and mucin

The most abundant metabolite (calculated as the difference between the end and start of the experiment) on all substrates was acetate, a typical fermentation end-product in the colon. The proportion of acetate from total organic acids ranged from 45% (in the case of Lev and HP) up to 80% (Pec) (Figure 4). The highest production of acetate with small amounts of propionate and succinate from Pec was in accordance with the prevalence of the *Bacteroides* species, especially *B. vulgatus,* but also *B. ovatus*, *B. uniformis*, *Bacteroides faecis* and *Bacteroides caccae* (Figure 5). Similarly, Chung *et al.* (2016) found enrichment of *B. vulgatus/dorei* on Pec, in addition to *Bacteroides stercoris*, *Bacteroides eggerthii, Bacteroides cellulosilyticus*/*intestinalis* and *B. thetaiotaomicron*. The second major metabolite in media containing GOS, HP, HSI, Lev and RS was lactate (25-35% of total acids), which together with acetate formed over 90% of the total acids produced from these substrates (Figure 4). This metabolite profile was accompanied by a higher proportion of bifidobacteria and lactobacilli (up to 77% and 22% of the total population in extreme occasions,

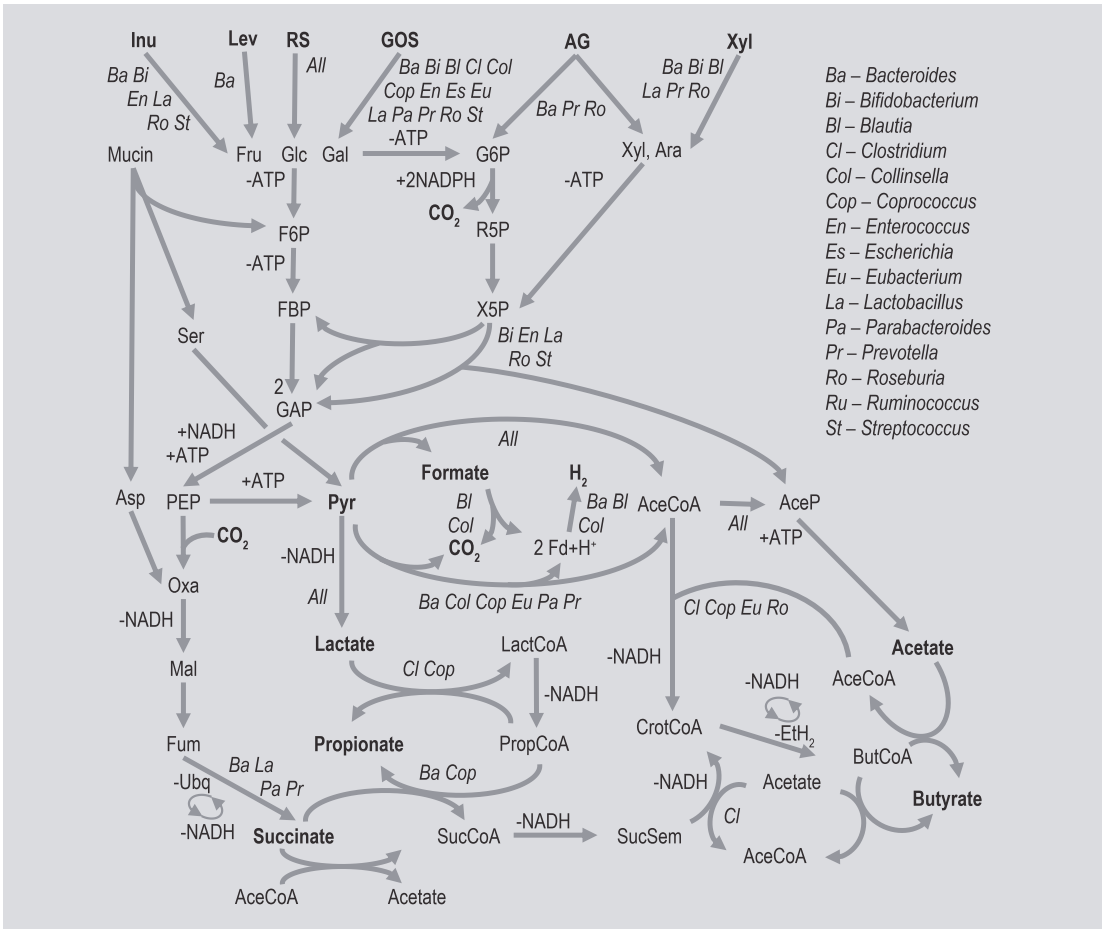

Figure 3. Metabolic pathways of production of organic acids. Fibre degradation pathways were estimated based on MetaCyc database (see details in Supplementary Table S3).

respectively) (Figure 5). It has been shown that RS supports the growth of *Bifidobacterium* but also *Bacteroides* and *Fusobacterium/Butyrivibrio* (*Faecalibacterium*) (Macfarlane and Englyst, 1986), which is in accordance with our results. Enrichment of bifidobacteria up to 60% was observed in case of higher (over 2% from total bacteria, samples F2.1, F2.2, F4.3, F4.4, Table S1) and lower (below 0.01% in samples F3, F4.1, F4.2) initial amounts of bifidobacteria. The species from genus *Bacteroides* were enriched up to 37% from the total population (samples F3 and F4.1 and F4.2). Generally, no lactate was detected in media containing Muc and Pec, while only trace amounts of lactate were produced from AG and Xyl. Instead, the fermentation of Muc, AG and Xyl resulted in the highest amounts of propionate and succinate (7-15% of total acids), along with a notable increase in *Bacteroides* species (62% and 58% of AG and Xyl media, respectively). In contrast to the dietary fibres, mucin supported the growth of species from genera *Clostridium, Lachnoclostridium* and *Parabacteroides*. Based on the fermentation patterns, the theoretical energy extraction can be calculated from the acids formed (Aguirre *et al.*, 2016). The energy extraction values were similar for most substrates, but the highest and lowest values differed significantly (377±24 kJ/C-mol on HP and 320±6 kJ/C-mol on Pec, respectively).

4. Discussion

A large part of the dietary fibres that reach the colon are fermented by the concerted action of complex microbiota. A variety of oligo- and polysaccharides that differ in molecular mass, monomeric composition, linkage type and general structures serve as substrates for specific members of the colon microbiota. There are two *in vitro* approaches to studying fermentations in the colon, either by using pure cultures or faecal microbial consortia, and in both cases either single or mixed carbohydrate substrates can be supplemented. Obviously, the results from *in vitro* growth

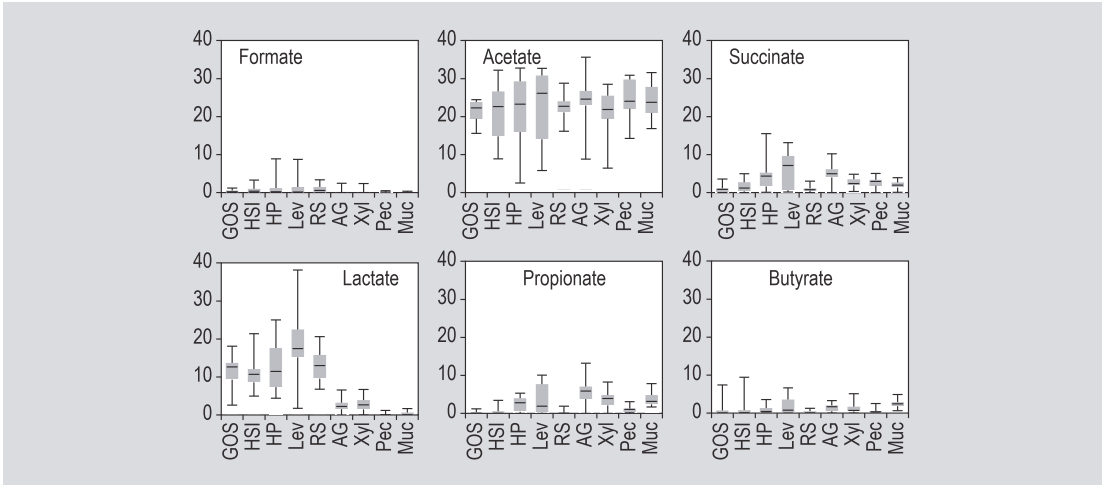

Figure 4. Box plots of concentrations (mM) of acetate, butyrate, formate, lactate, propionate and succinate produced during the growth of 14 faecal samples on different carbohydrate substrates.
AG = arabinogalactan; GOS = galactooligosaccharides; HP = long chain inulin; HSI = high soluble inulin; Lev = levan; Muc = mucin; Pec = pectin; RS = resistant starch; Xyl = xylan. Detailed data about metabolic products and bacterial compositions to the end of experiment is given in Supplementary Table S2.

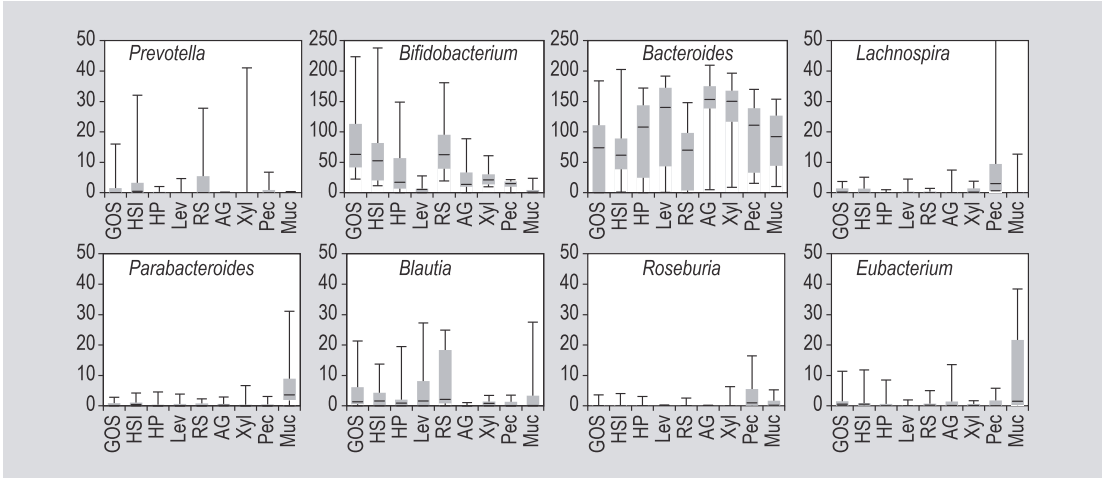

Figure 5. Increase of selected bacteria (mg/l) during the growth of 14 faecal samples on different carbohydrate substrates. Box plots of other bacterial genera are given in the Supplementary Figure S2. Abbreviations as shown in Figure 4. Detailed data about metabolic products and bacterial compositions to the end of experiment is given in Supplementary Table S2.

experiments under defined conditions (batch mode, defined medium etc.) are not directly comparable to acid profiles and bacterial composition of real faeces. However, the *in vitro* trials provide evidence of how the colon microbiota and its metabolism can be manipulated by introducing specific dietary fibres.

The fermentation of plant cell polysaccharides from beets, wheat, apples and soy by strains of *Bifidobacterium*, *Bacteroides*, *Clostridium* and *Klebsiella* of faecal origin was shown by Van Laere *et al.* (2000). All of the tested substrates were fermented to some extent by one or more of the species tested. *Bacteroides* and *Bifidobacterium* spp. were able to utilise most of the plant cell-wall derived oligosaccharides whilst *Clostridium* spp., *Klebsiella* spp., and *Escherichia coli* fermented only some of the substrates. Stimulation of faecal bifidobacteria (*Bifidobacterium longum* by long-chain AX and other bifidobacteria by inulin) was demonstrated by using two different dynamic *in vitro* gut models (Van den Abbeele *et al.*, 2013). AX and inulin specifically

increased the production of propionate and butyrate, respectively, by faecal cultures, which was also confirmed in experiments with humanised rats (Van den Abbeele *et al.*, 2011). Among several probiotic and gut bacteria from the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacteroides*, *Clostridium* and *Escherichia*, only *Clostridium beijerinckii* was shown to grow on oat spelt Xyl, and with much lower growth yields than in the case of glucose, whilst xylooligosaccharides were fermented by almost all strains tested. Strains of *B. longum*, *B. adolescentis*, *Bacteroides fragilis*, *B. thetaiotaomicron, B. vulgatus* and *C. beijerinckii* also fermented rye AX (Crittenden *et al.*, 2002). In our experiments, AG enhanced the growth of several health-promoting genera, such as *Bifidobacterium*, *Bacteroides*, *Coprococcus* and *Lachnoclostridium*, supporting the proposed re-definition of prebiotics as compounds that enhance ecological biodiversity rather than stimulating single species (Van den Abbeele *et al.*, 2013) through the degradation of complex polysaccharides.

Xylan and arabinogalactan promote the growth of acetate-, butyrate- and propionate- producing bacteria

The fermentation of Xyl and AG resulted in the production of 1.3-3.6 (an average of 2.5) times more butyrate and 2 to 23 times more propionate compared to other fibres tested (Figure 4). These data indicate the symbiotic growth of polysaccharide-degrading and butyrate- and propionate-producing bacteria on these substrates. Indeed, based on the MetaCyc database, Xyl-degrading enzymes have been reported in several species of genera *Bacteroides* and *Parabacteroides,* and only in a few species of *Bifidobacterium* (e.g. *B. longum*), *Prevotella* and *Roseburia,* which may explain the clear prevalence of bacteroidia in the medium with Xyl as a single substrate. AG-degrading enzymes have been found in even less bacteria among the butyrate producers *Roseburia hominis* and *A. hadrus*, in propionate-producing *Bacteroides,* and in the succinate-producing *Prevotella salivae* and *Prevotella copri*. Amongst the major fermentation products (acetate and lactate), more succinate than lactate was produced from AG and Xyl, which is in accordance with the abundance of *Prevotella* and *Bacteroides* in the respective consortia.

Several butyrate producers such as *Roseburia inulinivorans, Roseburia intestinalis* and *E. rectale* have been shown to degrade RS (reviewed by Birt *et al*., 2013), however, only marginal growth of *Roseburia* was observed in this study (below 0.01 in all samples) that is consistent with very low butyrate production (less than 1.3 mM) on RS. The butyrate producing species *E. rectale* and *Faecalibacterium prausnitzii* were most probably outcompeted by *E. coli* and bacteroidia in defined medium. Thus, the data after *in vitro* incubation under defined conditions (batch mode, defined medium etc., Figure 5) are not directly comparable with acid profiles in real faecal samples but rather indicate the potential of a certain fibre compound to support certain bacterial groups and fermentation profile. The real diet contains multiple fibre substrates and the processes are also interrelated to the host responses.

Estimated gas formation

Excessive gas formation by gut microbiota is associated with several forms of inconvenience and health concerns. Thus, the potential for gas production is considered an important parameter for a dietary fibre used either in pre- or synbiotic preparations. In fermentation processes, low gas production is a trade-off for ATP production, meaning that high ATP production (at least 4 mol/mol-hexose) requires CO_2 delivery from pyruvate, followed by NAD+ regeneration via H_2 production (Figure 3). Xyls and arabinans are built up of pentoses, which can be degraded to acids with low yields of gas formation, as no NADH through the concomitant production of hydrogen is accompanied by the acetyl-P formation from xylulose-5P in the phosphoketolase pathway. If the number of hydrogen producers is negligible, lactic acid and ethanol fermentation (redox-balanced fermentations with ATP yield 2 mol/mol-hexose), or mixed acid fermentations yielding acetic, propionic or succinic acids are favoured (Figure 6). Our results showed a considerable variation in fermentation patterns between different dietary fibres, ranging from almost homoacetic fermentation (in the case of Pec) to mixed acid fermentation (in the case of HP). The classical bifidus pathway pattern (3:2, acetate:lactate) resulting in roughly three moles of ATP and three moles of gases per one mole of hexose metabolised was observed in the case of HSI, GOS and RS. The fermentation of GOS was shown to produce the least volume of gas compared to other prebiotic oligosaccharides (Rycroft *et al.*, 2001).

Our results showed the lowest gas formation in the case of long-chain fructans Lev and inulin HP, with over 30% lactate and around 10% succinate as products. In a real gut environment, both acids (lactate and succinate) can be either converted to acetate (with accompanying gas formation) or absorbed by colon cells and used for oxidation in the respiratory chain. In contrast, the fermentation of Xyl and AG yielded significant amounts of gas, which can be explained by the predominance of acetic acid fermentation and low proportions of phosphoketolase-possessing microbes growing on these substrates. The phosphoketolase pathway is present in lactobacilli, bifidobacteria and *Roseburia,* whose growth was observed in this study but not in bacteroidia (*Bacteroides* and *Prevotella*), the dominant bacteria (over 60% of total consortia) growing in Xyl and AG media. Hence, the phosphoketolase pathway might not have been the major route for pentose fermentation in our study, which was also supported by a labelled substrate experiment where the bifidus pathway contributed 21% of total acetate production (Miller and Wolin, 1996). Still,

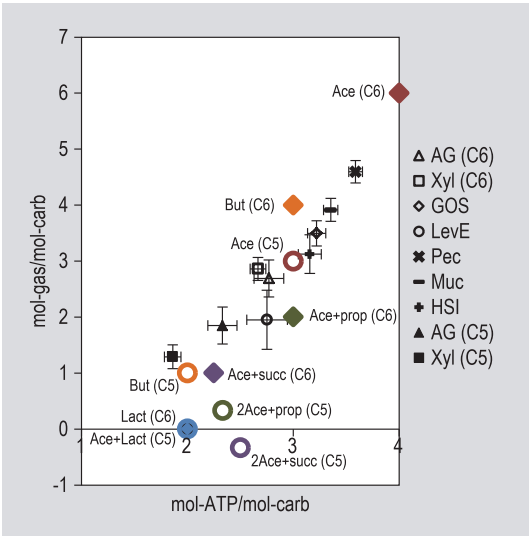

Figure 6. Molar yields of ATP and gases from growth of faecal consortia on different dietary fibres. Abbreviations as shown in Figure 4. Molar yields are calculated based on metabolic reaction stoichiometries given in the Supplementary Table S4. Error bars indicate confidence interval (95%). Data of resistant starch and levan are not shown in the figure because of high similarity to galactooligosaccharides and long-chain inulin, respectively. In comparison theoretical yields of ATP and gases from hexose (C6) and pentose (C5) fermentations to acetate (ace), lactate (lact), propionate (prop), succinate (succ) or butyrate (but) or combinations of these acids are given. Only fermentation patterns giving balanced redox reactions are shown. Pentose fermentation assumes degradation through phosphoketolase pathway.

Xyl and AG obviously resulted in lower amounts of gas compared to RS and GOS, which should be taken into consideration and tested in practice when planning diets for gas-sensitive people (e.g. those having irritable bowel syndrome).

Contribution of mucin in the formation of butyric acid

It is estimated that only about 1% of colonic microbiota possess the mucin degrading enzymes (e.g. glycosidases and sulfatases) to degrade the oligosaccharide chains (Hoskins and Boulding, 1981) and thus provide carbon and energy for these bacteria. However, excessive degradation of the protective mucus layer may expose the GI tract cells to pathogens (Derrien *et al.*, 2004). The degradation of mucin has been shown in isolates from the genera *Enterobacteria*, *Eubacteria*, *Ruminococcus*, *Bacteroides* (*B. thetaiotaomicron*, *B. uniformis*), *Bifidobacterium* (*B. longum*, *B. bifidum*), *Clostridium* (*C. clostridiiforme*, *C. malenominatum*) and *A. muciniphila* (Derrien *et al.*, 2004; Salyers *et al.*, 1977). In our study, fermentation of mucin by faecal microbiota led to the highest Shannon indexes (data not shown) compared to the other substrates, suggesting the importance of mucins in contributing to diverse colon microbiota. The highest amounts of butyrate and considerable amounts of propionate were formed from mucin. The observed ratios of short chain fatty acids produced from mucin were similar to those described in human faeces (Cummings, 1981). The role of mucins as important carbon sources for gut bacteria is hard to measure in practice. The contribution of mucin can be estimated based on the consumption of dietary fibres, the composition and amount of colonic bacteria and the efficacy of anaerobic fermentation. Sender *et al.* (2016) have estimated the total number of bacterial cells in the colon as on average 9×10^{10} cells/g, with a coefficient of variation of 46%. According to Burkitt *et al.* (1972) the faecal output of white British people ranges from 39 to 248 g/day, or an average of 150 g/day (Stephen and Cummings, 1980), giving a range of daily bacterial output from 1.9 to 33×10^{12} cells. Taking into account the average mass of one bacterial cell of 1 pg, this corresponds to the evacuation of 1.9 to 33 g cells per day, with a dry weight (30%) of 0.6 to 10 grams. Assuming the typical fermentation pattern of acetate:propionate:butyrate as 3:1:1 in the colon (Stephen and Cummings, 1980) and an ATP yield per acetate, propionate and butyrate 2, 1, 3 mol-ATP/mol-acid, respectively, a total of 20.6 mmol-ATP/g-fibre can be drawn (1 g of fibre equals 6.17 mmol hexose equivalents, yielding 6.17 mmol acetate, 2.06 mmol propionate and 2.06 mmol butyrate and correspondingly $2\times6.17+2.06+3\times2.06=20.6$ mmol ATP). Elia and Cummings (2007) have estimated an average dietary fibre consumption of 17 to 56 g/day (correlating to faecal output by Burkitt *et al.* (1972) of which 70% is fermentable; hence an anaerobic metabolism yielding of 10-20 gDW of bacteria per mol of ATP can support the formation of 1.5 to 10 g of bacterial biomass per day (details in Supplementary Table S3)). This is in the range of bacterial output through defecation as shown above (0.6-10 gDW/day). However, the typical Western diet contains less than 25 grams of dietary fibres per day, supporting less than half of the energetic requirements for bacterial growth estimated (up to 5 gDW/day). The rest of the energy needed must be synthesised from other sources, such as mucins secreted by the epithelial cells and dead epithelial and microbial cell material, or from residual proteins. In the future, we plan to combine several substrates assayed in the current study to mimic natural multi-substrate conditions in the gut.

5. Conclusions

The major taxa represented in the faecal samples belonged to the phylum *Firmicutes* and genera *Dialister*, *Faecalibacterium*, *Eubacterium*, *Christensenellaceae*, *Bacteroides*, *Ruminococcus*, *Prevotella*, *Akkermansia*, *Subdoligranulum*, *Coprococcus* and *Lachnospiraceae*. Substrate-specific growth patterns of the faecal microbiota samples can be drawn from the microcalorimetry

experiments by combining heat evolution, carbohydrate metabolism and sequencing data. More than 70% of AG, Xyl, inulin, Lev, Pec and mucin were fermented by faecal microbiota. Different metabolite profiles observed on hexose and pentose polymers and mucin were in accordance with the development of the faecal microbial consortia. The study showed that different fibre types support different metabolic routes and the growth of specific bacterial groups, suggesting that, besides the amount of dietary fibre, also the variety of fibres is crucial to support the healthy biodiversity of gut microbiota by meandering gut microbiome through cross-feeding. The proposed metabolic scheme presents some cross-feeding possibilities. Significant amounts of butyrate were formed from slowly degraded Xyl and AG but not from fast degraded fructans, GOS or RS. The fermentation of mucin, AG and Xyl resulted in the highest amounts of propionate and succinate. Mucin supported the growth of species from the genera *Clostridium, Lachnoclostridium* and *Parabacteroides* and resulted in the highest amounts of butyrate.

Apparently biodiversity is a key to healthy microbiota. Only a diverse spectrum of glycans in the diet can contribute to diverse microbiota of the host. The synthesis and degradation of the mucin glycoproteins has to be well balanced to provide optimal protection and energy generation to the host. Data from *in vitro* experiments can be applied in the development of fibre-specified diets to modulate gut microbiota.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2016.0198.

Table S1. Relative abundances of bacterial taxa in the initial faecal samples.

Table S2. Concentrations of organic acids and increment of bacteria at the end of microcalorimetry experiment.

Table S3. Estimation of the requirements for dietary fibre by colon bacteria.

Table S4. Metabolic reactions used to calculate the redox equivalent and ATP balance.

Figure S1. Heat flow patterns during the growth of all faecal consortia in defined media supplemented by arabinogalactan, chitin, galactooligosaccharides, inulin HP, inulin HSI, levan, no substrate, mucin, pectin, resistant starch or xylan.

Figure S2. Box plots of increment of bacterial genera after the microcalorimetry experiments.

The 16S rRNA amplicon sequencing data from this study is available from the European Nucleotide Archive (ENA) database (http://www.ebi.ac.uk/ena): accession number ERP017494.

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RESEARCH ARTICLE

The composition of faecal microbiota is related to the amount and variety of dietary fibres

Kaarel Adamberg[a,b], Madis Jaagura[a,b], Anu Aaspõllu[c], Eha Nurk[c,d] and Signe Adamberg[b]

[a]Center of Food and Fermentation Technologies, Tallinn, Estonia; [b]Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia; [c]National Institute for Health Development, Tallinn, Estonia; [d]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

ABSTRACT

For normal gut and body function, the diet should contain variety of dietary fibres. To elucidate the links between food intake, especially the variety of dietary fibres, faecal microbiota, body mass index and content of blood lipids, 59 healthy subjects on common Estonian diet were enrolled. The dietary records were analysed at nutrient level while seven categories of fibres were characterised to differentiate variety of fibre profiles consumed. The data of the high fibre (HF) intake (>15.1 g/1000 kcal) and the low fibre (LF) intake (<9.4 g/1000 kcal) groups were comparatively evaluated. LF diets associated with *Collinsella*, *Coprococcus* and *Dorea*, and higher consumption of meat and white flour products while HF diet with *Roseburia*, *Bacteroides xylanisolvens* and *Oxalobacter formigenes*, and arabinoxylan and pectin rich cereals and vegetables. Based on the results, modulation of the colon microbiota can be suggested by careful variation and enrichment of dietary fibre sources.

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Faecal microbiome; dietary fibre; pectin; arabinoxylan

Introduction

Non-digestible carbohydrates are the primary energy source for various gut microbes, while others depend on the by-products or metabolites of the primary degraders through cross-feeding mechanisms (Falony et al. 2006; Belenguer et al. 2007). The diversity of gut microbiota is also greatly influenced by luminal pH, host secretions, and gut transit rate (Roager et al. 2016). Prolonged consumption of fibre-deficient diets decrease the diversity of the colon microbiota and alter its metabolism that is associated with aetiology of several chronic diseases (Arora and Bäckhed 2016). Non-starch polysaccharides (NSP) are the main fraction of dietary fibre to control the bowel function. Low amounts of NSP would lead to constipation and can be expressed as low Bristol stool scores (BSS) (Cummings et al. 2004). Furthermore, constipation is associated with elevated abundance of bacteria like Ruminococcaceae, *Akkermansia*, *Methanobrevibacter* in the faecal microbiota, while loose stools dominate by *Prevotella* (Vandeputte et al. 2015).

The typical recommended daily intake of total dietary fibre ranges from 25 to 35 g/2000 kcal/day, however, this requirement is poorly met in many Western countries (Stephen et al. 2017). During about 50 years, the diet in developed countries has continuously changed towards extensively processed and refined foods, along with the decrease in dietary fibre to less than 20 g/day on average (Stephen et al. 2017). Among other functions, dietary fibres are important for bowel physiology such as peristalsis and transit rate by increasing the faecal volume through water binding (Stephen and Cummings 1980). Furthermore, BSS and gut transit time (Lewis and Heaton 1997; Vandeputte et al. 2015; Roager et al. 2016) can be used as indicators of the microbial and metabolic markers. Better understanding of these relationships at personal level may have significant implications for gastrointestinal health and disease prevention.

The effect of diet as a major factor driving the composition and metabolism of the colonic microbiota and health markers has been extensively investigated in recent years (Dominianni et al. 2015; Verbeke et al. 2015; Zeevi et al. 2015). Epidemiological evidence is consistently showing that fibre-rich diets associate with a reduced risk of chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer (Dahl and Stewart 2015). There has been growing proof that the

CONTACT Kaarel Adamberg kaarel.adamberg@taltech.ee Department of Chemistry and Biotechnology, Tallinn University of Technology, 12618 Tallinn, Estonia

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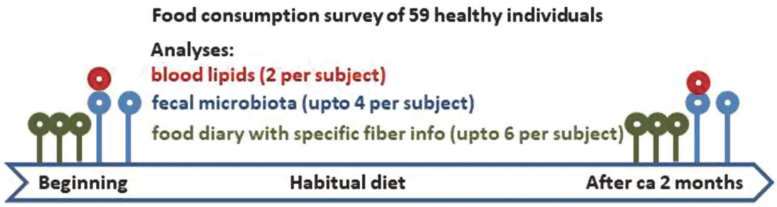

Figure 1. Schematic figure of the experimental set up of food consumption survey.

microbial metabolites, in addition to influences on host physiology, also play an integral role in the host immune system and metabolism through a complex array of chemical interactions and signalling pathways (Hooper et al. 2012; Nicholson et al. 2012).

Aim of the current study was to categorise the typical Estonian foods, evaluate the common nutrition patterns of healthy Estonian adults, with focus on the amount and variety of dietary fibres, and elucidate their relationship with faecal microbiota, and related health markers.

Materials and methods

Study design and food composition analyses

The study design is presented in Figure 1. Fifty-nine healthy Estonian participants (38 females and 21 males) aged 23–52 years were enrolled in this study.

Exclusion criteria comprised antibiotic treatment within last three months; food restrictions due to allergies, any kind of food intolerance, veganism/vegetarianism or other extremes in food consumption practices; the absence of chronic illnesses; current pregnancy or breastfeeding and travel to subtropical or tropical regions within one month before the study. The participants mostly continued their habitual diet including some season-related variations (e.g. more local fruits and vegetables in summer period). The participants were instructed to record their food intake for at least 24 h, preferably for 72 h before collecting the faecal samples with recorded BSS data on two consequent defecations at the beginning and at the end of study (April–June 2017 and June–August 2017, respectively). In addition, bowel habit characteristics such as BSS, defaecation frequency and abdominal symptoms were asked to record by the subjects. All participants signed written informed consent forms. The study was approved by the Tallinn Medical Research Ethics Committee (TMEK No. 1631).

The nutrition data were analysed at nutrient level according to the Estonian food composition database and all foods were categorised based on the fibre content and variety.

The participants were grouped into three groups according to the average dietary fibre intake per person calculated from all food records two days before faecal samplings at the beginning and end of the study (in total 2–4 faecal samples and nutrition data per subject): low fibre intake (LF $<$ 9.4 g/1000 kcal, first quartile of fibre intake), $N = 15$; medium fibre intake (MF, fibre intake between 9.4 and 15.1 g/1000 kcal, second and third quartiles), $N = 30$; and high fibre intake (HF $>$ 15.1 g/1000 kcal fourth quartile), $N = 14$, groups.

Based on average BSS score, the participants were divided into three groups according to quartile distribution (three persons did not mark the BSS score): low BSS $<$ 3 (firm stools, $N = 14$), medium BSS (two middle quartiles) between 3–5 (normal stools, $N = 28$), and high BSS $>$ 5 (very soft, watery stools, $N = 14$).

Analysis of the foods and diet records

Detailed daily food intakes (including amounts of each item) were recorded as precisely as possible by the participants using the in-house software NutriData pro (National Institute for Health Development, Tallinn, Estonia). The software allowed to enter the foods and beverages directly from the Estonian Food Composition Database (tka.nutridata.ee/en/) and to calculate the contents of 53 nutrients. Accordingly, all foods were categorised. In the absence of specific information on a certain food, similar plant foods were grouped together (into the same category). For instance, the fibre composition for all root vegetables was calculated based on the data on carrot and turnip. In addition, the amounts of seven most abundant fibre compounds – arabinoxylan, β-glucan, cellulose, inulin, lignin, pectin and resistant starch – were calculated based on the published data on raw materials (Herranz et al. 1981; Bengtsson and Aman 1990; Karppinen et al. 2000; Holtekjølen 2006; Dodevska

et al. 2013, 2015; Kalala et al. 2017, and the food database at nutritional-software.at). The processing may change the amount of dietary fibres slightly, resistant starch being the most affected, however, changes of other dietary fibres during ordinary cooking are small. As the specific data are not available, the effect of cooking was not taken into account. Meat and milk-based products, if not mixed with cereals, fruit or vegetables, were considered as insignificant sources of dietary fibres. Thereafter all foods were divided into 72 categories. From those, 46 food categories contained dietary fibres and foods in these categories were grouped based on their fibre patterns to cover similar products in the same category (e.g. flours, grains and flakes of the same cereal were considered as one category). The food data also contained information about energy, protein, fat, carbohydrate and micronutrient (microelements and vitamins) contents. The contribution of dietary fibres to the energy pool was compared between the LF and HF intake groups.

Faecal sampling and sequencing analyses

The subjects were asked to collect faecal samples immediately after defaecation by sterile swab and to suspend these in a buffer containing ammonium sulphate (40% solution), EDTA (16 mM) and sodium citrate (20 mM). The samples were transported at room temperature to the laboratory and stored at −20 °C before DNA extraction.

DNA was extracted from the cell pellets using PureLink Microbiome DNA extraction kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions. Universal primers: S-D-Bact-0341-b-S-17 Forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 Reverse (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used for PCR amplification of the V3–V4 hypervariable regions of the 16S rRNA genes (Klindworth et al. 2013). Sequencing libraries were prepared with Nextera XT DNA Library Preparation Kit v2 (Illumina). Prepared libraries were quantified using Qubit™ dsDNA HS Assay Kit (quantitation range 0.2–100 ng; Thermo Fisher Scientific). If the DNA quantity turned out to be too high, the sample was diluted and re-measurement was performed with Qubit™ dsDNA BR Assay Kit (quantitation range 2–1000 ng; Thermo Fisher Scientific). All reagent kits were handled in accordance with manufacturer's instructions. Pooled libraries were sequenced using Illumina MiSeq 2x250 v2 platform (in Estonian Genome Centre, Estonia). The amplified region was 390–410 bp long and on average 60,000 reads per sample were obtained.

DNA sequence data were analysed using BION-meta (www.box.com/bion), currently unpublished open source programme, according to authors' instructions. Detailed description of the methods used can be found in McDonald et al. (2016, Supplementary methods S1–S3). First, sequences were cleaned at both ends using a 99.5% minimum quality threshold for at least 18 of 20 bases for 5′-end and 28 of 30 bases for 3′-end, then joined, followed by removal of contigs shorter than 350 bp. The sequences were cleaned from chimaeras and clustered by 95% oligonucleotide similarity (k-mer length of 8 bp, step size 2 bp). Lastly, consensus reads were aligned to the SILVA reference 16S rDNA database (v123) using word length of 8 and similarity cut-off of 90. Raw sequencing data can be requested from the National Institute for Health Development.

Anthropometric data and health markers

For anthropometric measurements, calibrated SECA 878 digital medical scales and Tanita Leicester HR 001 portable vertical height boards were used. Height was measured to the nearest 0.1 cm and weight to the nearest 0.1 kg. Blood samples from the study subjects were drawn at the beginning and end of the study and concentrations of total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides were determined in ISO 15189 accredited laboratories. Body mass index (BMI) was calculated at the beginning and end of the study using the formula: BMI = weight/height2 [kg m^{-2}].

A single fasting blood sample from the study subjects was drawn at the beginning and end of the study. Concentrations of total cholesterol, HDL- and LDL-cholesterol, and triglycerides were measured by colorimetric enzymatic method.

Statistical analyses

Statistical analyses were performed on R using public and in-house packages. Non-paired *t*-tests with non-equal variances of food consumption, nutrient amounts, bacterial abundances in microbiota and health markers between LF and HF groups were performed. Single parametric *t*-test was used to compare the differences between 16S rDNA or food consumption analyses and fibre intake or BSS groups. All *t*-tests were corrected for multiple testing using Benjamini–Hochberg approach if not stated otherwise. Results were considered significant at false discovery

Table 1. Main parameters of the study subjects.

Parameter	Total	LF intake <9.4 g/1000 kcal	MF intake 9.4–15.1 g/1000 kcal	HF intake >15.1 g/1000 kcal
Male/Female, *N*	21/38	8/7	10/20	3/11
Age, years: median (range)	36 (23–52)	36 (26–51)	42 (23–52)	38 (24–49)
BMI, kg m^{-2}: median (range)	24.6 (18.5–38.9)	27.3 (19.4–38.9)	24.1 (18.6–31.2)	21.9 (19.2–36.1)[a]
Carbohydrates, g: median (range)	106.0 (69.7–139.1)	90.8 (69.7–121.7)	106.0 (57.4–136.4)	117.7 (83.1–139.1)
Fibre intake, g: median (range)	12.0 (6.6–23.1)	8.1 (6.6–9.4)	12.0 (9.4–14.8)	18.2 (15.1–23.1)
Fats, g: median (range)	41.3 (27.1–58.6)	46.4 (38.7–58.6)	41.4 (30.2–56.8)	36.8 (27.1–42.9)
Protein, g: median (range)	40.2 (30.3–63.2)	42.2 (34.2–63.2)	41.4 (28.6–59.3)	39.4 (30.3–56.9)
Number of subjects with average BSS[b]				
Low BSS < 3, firm stools	14	4	8	2
Medium BSS 3–5, normal stools	28	5	15	8
High BSS > 5, very soft, watery stools	14	4	6	4
Blood lipids, mM: median (range)				
Cholesterol	5.1 (2.8–7.4)	5.2 (4.1–7.4)	5.0 (3.3–6.7)	4.7 (2.8–5.8)[a]
HDL-cholesterol	1.6 (1.0–2.9)	1.6 (1.0–1.8)	1.8 (1.0–2.9)	1.4 (1.0–2.1)
LDL-cholesterol	2.9 (1.0–5.3)	3.5 (2.0–5.3)	2.8 (1.0–4.1)	2.8 (1.3–4.0)[a]
Triglycerides	1.0 (0.5–5.2)	1.1 (0.5–3.1)	1.0 (0.5–5.2)	0.9 (0.5–1.5)

[a]*p* value < 0.05 between low fibre (LF) and high fibre (HF) groups. MF indicates medium fibre intake group.
[b]Three persons did not mark the BSS score.

rate <5% (unless specified otherwise). Graphics were built using R Studio and ggplot plots.

Results

General composition of the diets and specific fibre intake

Consumption of macronutrients (fat, protein, carbohydrates and fibres) based on the food records were analysed in three fibre intake groups (Table 1). Compared to the subjects with LF intake, the subjects with HF intake consumed less fats (median intake 37 vs. 46 g/1000 kcal, respectively) and more carbohydrates (median intake 118 vs. 91 g/1000 kcal, respectively), and thus, obtained less energy from fats (Figure 2). Such eating pattern (less fat and more fibre) was reflected also in a healtier blood lipid profile, whilst in the LF group, higher values of the total cholesterol and LDL-cholesterol were detected (Table 1). Moreover, on average 5.4 units, lower values of the BMI were observed in the HF group compared to those in the LF group.

In a more detailed view, notable differences in the diets of the LF and HF groups were found. Apples, legumes, oat and rye (rich in arabinoxylan, β-glucan and pectin) formed the main fibre sources in the habitual diet and were more prevalent in the diets of the HF intake group (Figure 3). Whole-grain cereals formed an important energy source in the HF group (in the most cases more than 15% from total energy) whilst remained below 5% from total energy in the LF group, assumed as 2 kcal per gram of dietary fibre. Rye and oat were the main fibre sources of cereal origin providing 16 and 10% of the total dietary fibre in the HF group, respectively. The most important

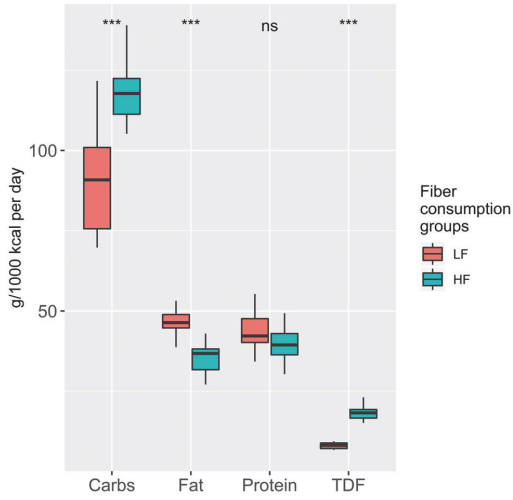

Figure 2. Intake of macronutrients (g/1000 kcal/day) by study subjects of low fibre (LF: < 9.4 g/1000 kcal/day, $N = 15$) and high fibre intake (HF: > 15.1 g/1000 kcal/day, $N = 14$). TDF – total dietary fibres. Dietary fibres are excluded from the carbohydrates (Carbs). ns – not significant, *** indicates significant difference between LF and HF groups.

energy sources in the LF group comprised white flour and meat products that jointly provided 16 and 8% (median) of total energy in the LF group, respectively, whilst only 10% in the HF group. It appeared that the consumption of whole-grain products, legumes and vegetables by the HF intake group accompanied also with higher amounts of vitamins (B5, C, K, folates, biotin) and minerals (K, Mg) than that of the LF intake group. For example, the intake of vitamin C was more than two times higher in the HF group than that in the LF group (107 and 40 mg/1000 kcal,

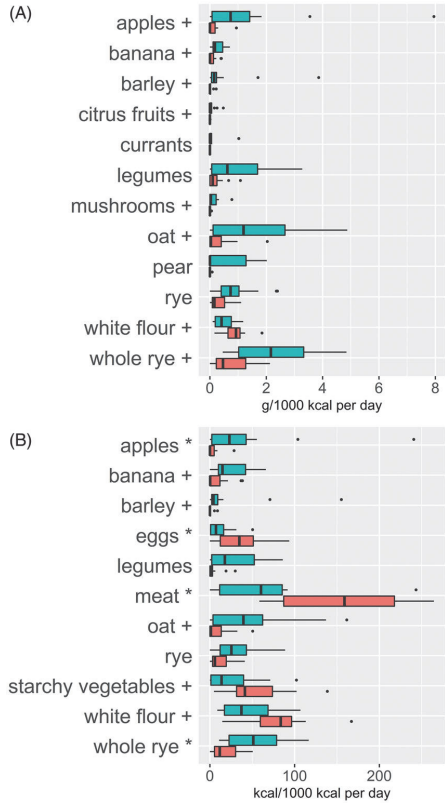

Figure 3. Dietary fibre (A) and energy (B) sources per 1000 kcal of daily meal in the low fibre (red bars, LF < 9.4 g/1000 kcal/day, $N = 15$) and the high fibre (blue bars, HF > 15.1 g/1000 kcal/day, $N = 14$) intake groups. Detailed food data were recorded two days before each faecal sampling in the beginning and end of the study period. Food categories of significant differences are presented. "+" sign indicates significant difference after false discovery rate correction. All food categories are shown in Supplementary Figure S1.

respectively, $p = 0.008$). Significant differences were also observed in the supply of pantothenic acid (3.3 and 2.8 mg/1000 kcal, respectively, $p = 0.03$), folate (181 and 107 µg/1000 kcal, respectively, $p = 0.001$) and biotin (25 and 19 µg/1000 kcal, respectively, $p = 0.02$). The intake of potassium and magnesium in the HF group exceeded those of the LF group by approximately 1.5 times (2.1 and 1.6 g/1000 kcal, $p = 0.002$ for K, and 228 and 156 mg/1000 kcal, $p = 0.003$, for Mg, respectively). Our results also showed that the HF intake group obtained 3.9 g fibre/1000 kcal from vegetables while LF group only 2.5 g fibre/1000 kcal ($p = 0.03$).

According to the composition analysis, the most abundant dietary fibres in the habitual diet were arabinoxylan (from rye products such as bread and porridge) and cellulose (from leafy and root vegetables) (Figure 4). Median values of the total fibre consumption per 1000 kcal differed more than twice between the HF and LF intake groups (18.2 and 8.1 g/1000 kcal/day, respectively) with the most pronounced differences in fructan, β-glucan and arabinoxylan consumption. The fructans composed 2.1 and 1.2 g/1000 kcal/day from total dietary fibre consumption in HF and LF intake group, respectively, indicating only 1.7 times difference between these two groups. In contrast, the intake of β-glucan differed nearly four times – 1.1 vs. 0.3 g/1000 kcal/day, in the HF vs. LF intake groups, respectively. Remarkable differences were observed also in arabinoxylan consumption between HF and LF intake groups (3.6 vs. 1.3 g/1000 kcal/day, respectively) while the intake of pectins and cellulose remained proportional to the total dietary fibre providing 2.9 vs. 1.2 g/1000 kcal/day for pectin, and 4.8 vs. 2.4 g/1000 kcal/day for cellulose, respectively in the HF and the LF intake groups.

Relationships between composition of the faecal microbiota, the Bristol stool score, dietary fibre intake and health markers

Significant inter-individual variations in the faecal microbiota structure were observed. The composition of the faecal microbiota from the same person with two months interval always showed the closest β-diversity (value below 0.25, the diagonal) making a characteristic "fingerprint" ("microbe-print") (Supplementary Figure S3). β-diversity between the samples of different subjects always exceeded 0.5. About 50 species formed the "core" faecal microbiota found in the samples of at least 80% of the subjects. The most abundant species were *Faecalibacterium prausnitzii* (median 6.6%), *Bacteroides vulgatus* (2.5%) and *Eubacterium rectale* (2.1%) and *Prevotella* was present in 64% of the subjects. Higher fibre intake was associated with the increased abundances of *Roseburia hominis*, *Bacteroides xylanisolvens*, *Bifidobacterium pseudocatenulatum* and *Oxalobacter formigenes* (Figure 5). The fibre-deficient diets were associated with *Allisonella histaminiformans*, *Bacteroides coprocola*, *Collinsella aerofaciens*, *Coprococcus comes*, *Dorea formicigenerans*, *Lachnoclostridium faecis*, *Romboutsia ruminantium* and others.

Interestingly, no clear correlation was seen between the fibre intake and the BSS (Table 1). Also, no significant differences in the consumption of carbohydrates, proteins and fats were found between the three BSS groups. However, high BSS values were recorded

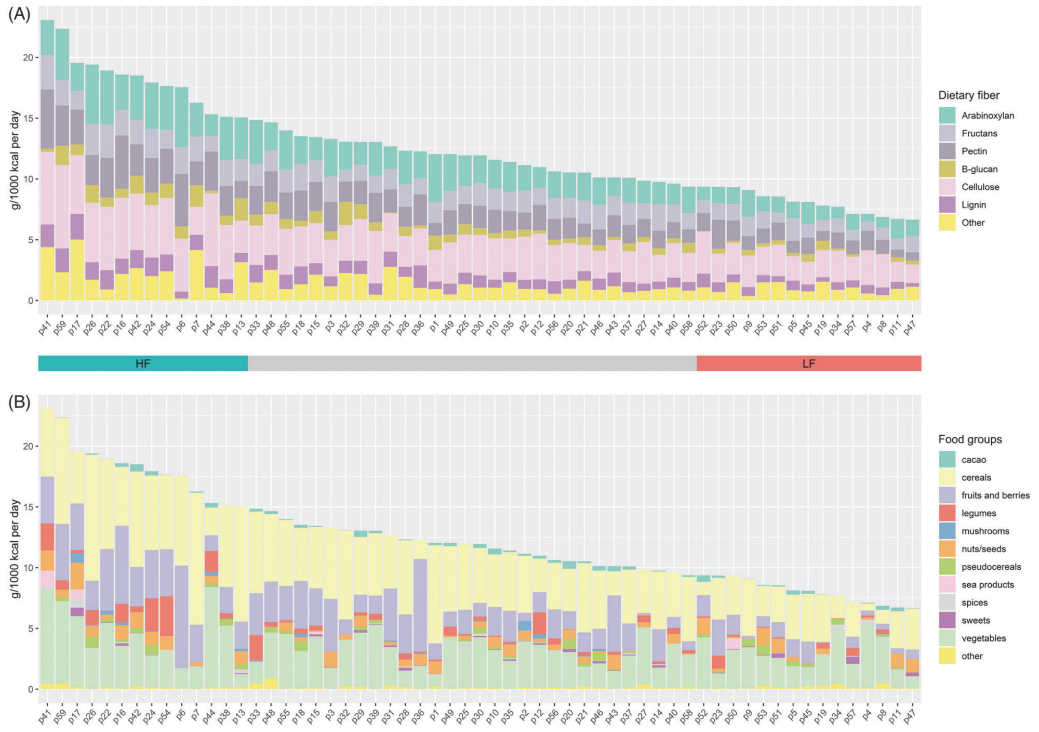

Figure 4. Distribution of the average fibre intakes of 59 study subjects (g/1000 kcal per day) presented as fibre types (A) and food categories (B). Detailed food data were recorded two days before each faecal sampling in the beginning and end of the study period. Details of each dietary fibre types by proportions are shown in Supplementary Figure S2.

by the subjects with HF intake obtaining most of the dietary fibres from whole-grain cereals (rye, oat) and root vegetables while the subjects having LF intake and high BSS (BSS $>$ 5) gained the fibres mainly from white flour and apples. The only genus significantly more abundant in the high BSS group was *Prevotella* although similar tendencies were observed also for other taxa (Supplementary Figure S5). *Prevotella* was found in 64% of the study subjects but in the high BSS group, its proportion was 71% and in the low BSS group 46%. In eight subjects out of 14 having regular BSS above 5, the abundance of *Prevotella* was higher than 20% from total bacterial population while only one such subject from 13 having 20% of *Prevotella* in low BSS group was observed. Increased abundances of *Methanobrevibacter*, *Romboutsia*, *Ruminiclostridium*, Ruminococcaceae UCG-014 were characteristic to the low BSS group. In contrast, the genera such as Lachnospiraceae UCG-001, Ruminococcaceae NK4A214 and Ruminococcaceae UCG013 were more prevalent in the high BSS group.

The blood lipid content did not correlate with the BSS but some associations between the BMI and faecal bacteria were observed. For instance, the elevated BMI was associated with *Coprococcus*, and *Dialister*. The genus *Dialister* was below detection limit 0.01% in all subjects with BMI below 22 kg m^{-2} ($N = 14$) while the median abundance of this bacterium in the high BMI subjects (BMI $>$ 28 kg m^{-2}, $N = 14$) was 1.9% (range 0–6.8%, while 0% for three participants, $p = 0.01$). Also, the significantly higher abundance of *Coprococcus* was observed in the high BMI group compared to that of the low BMI group (0.5 and 0.2%, respectively, $p = 0.004$).

Discussion

Novel approach to categorise the foods and fibres

The key modulator of the colon microbiota is dietary fibre – extremely heterogeneous group of molecules, different in their monomeric composition, structure, solubility and bound compounds such as phytochemicals (Sawicki et al. 2017). To our knowledge, such

Figure 5. Species of faecal bacteria significantly different and false discovery rate corrected between low fibre (<9.4 g/1000 kcal/day, $N = 15$, red boxes) and high fibre (>15.1 g/1000 kcal/day, $N = 14$, blue boxes) intake groups. Each box corresponds to one study subject with 2–4 faecal samples (two sequential samples in the beginning and two in the end of the study period). Species significantly different before false discovery rate correction are shown in Supplementary Figure S4 and abundances of all detected species are given in Supplementary Table S1.

detailed analysis of dietary fibre intake based on food diaries was performed first time. All foods and fibre sources were categorised based on their composition, energetic and nutritional values. Detailed fibre contents in habitual diets were analysed in a nutrition study with 59 participants. Considering the median values of fibre intake, consumption of cellulose, lignins and pectins were proportional to the total fibre intake, while that of fructans were proportionally lower and β-glucan and arabinoxylan higher in the HF intake group compared to that in the LF intake group. The smallest difference between the HF and LF intake groups was seen in fructan intake that can be explained by the fact that endosperm of wheat (white flour) as well as rye is rich in fructo-oligosaccharides or fructans and these cereals were the main fibre sources for the LF consumers. In contrast, more wholegrain cereals were consumed in the HF group, increasing the proportion of arabinoxylan. Wheat bran as a source of pentose polymers (arabinoxylan) is an important factor for gut function through improving BSS and increasing the volume of faeces, which is reflected by shorter colonic transit times (Cummings 1984). Besides the general trends, BSS also depends on several physiological, lifestyle and metabolic factors of the host, overall diet composition, fibre types and amount of liquid consumed. For example, consuming plenty of whole-grain rye bread without vegetables, water nor physical activity, may also lead to constipation. The associations between transit time or/and BSS with faecal microbiota have been studied by Roager et al. (2016) and Vandeputte et al. (2015), however, these studies lack the diet data. In the current study, we did not find clear correlation between the BSS and consumption of total dietary fibre. Within the whole range of fibre intake (6.6–23.1 g/1000 kcal) the BSS varied from low to high values as was also reported in a children study by Berding et al. (2018). However, subject-specific effects are most probably related to colon microbiota and should be elucidated in controlled dietary intervention trials. Analysing the effect of foods on faecal microbiota we found that food categories such as apples, root vegetables, barley and oat together determined about 8% total of all variabilities of the faecal microbiota. Therefore, by food choices and diet planning, we can modify the colon microbiome and consequently the bowel habits. Combined analyses of food composition and consumption data revealed also that the LF intake associated with higher intake of fat, while the diets with higher fibre contents provided up to two times more vitamins (C, K, folates, biotin) and minerals (K, Mg). The latter can be explained by the fact that vitamins and minerals are partly bound to fibre molecules in plant foods such as rye, oat, fruits and vegetables.

Links between diet, faecal microbiota and faecal consistency

Adult faecal microbiota tends to be rather stable in a long-term period established by lifestyle and eating habits (Burkitt et al. 1972; David et al. 2014). The β-diversity of the faecal samples from the same subject taken with 2 months interval clearly differentiated from those of all other subjects (Supplementary Figure S3) confirming the overall stability of individuals faecal microbiota despite the moderate dietary variations. However, diet change may shift the colon microbiota from one stable state to another (David et al. 2014). We showed that amounts and variety of several faecal bacteria of the HF and LF intake persons differ significantly. Although the relationship between dietary fibre intake and chronic diseases of Western lifestyle has been shown already several decades ago (Burkitt et al. 1972), the current analytical capacity enables to analyse these effects on the level of molecular markers and microbial species. Lower concentrations of blood triglycerides have been associated with higher abundance of several species such *Alistipes shahii*, *Alistipes putredinis*, *Roseburia hominis* and *Coprococcus catus* in faecal microbiota and higher consumption of fibre-rich foods such as fruits and vegetables (Zhernakova et al. 2016). Improved blood lipid profile has been observed also in case of higher abundance of *Akkermansia* in faecal microbiota (Falony et al. 2016). It has been shown that whole-grain foods reduced fasting plasma glucose concentration and body weight increase in pre-diabetic subjects (Hjorth et al. 2017). The same group showed recently that the increased ratio of *Prevotella*/*Bacteroides* in the faecal microbiota is a good basis for bodyweight management in obesity treatment using fibre-enriched diets (Hjorth et al. 2018). In another study, high BMI was associated with *Dorea longicatena*, *Coprococcus comes* and *Collinsella aerofaciens* (Liu et al. 2017). These species were also associated with higher BMI and LF intake in our study (data not shown), showing that those species could be manipulated by dietary fibres.

Strong associations of stool consistency with gut microbiota richness, composition and bacterial growth rates were first shown by Vandeputte et al. (2015) and confirmed in a large cohort study ($n = 1125$) by Tigchelaar et al. (2015). In accordance with these

studies, we found that around 5% of variabilities in faecal microbiota can be predicted by BSS. Within the *Roseburia-Bacteroides* enterotype, *Methanobrevibacter* and *Akkermansia* positively correlated with colon transit time (Vandeputte et al. 2015). Also, the decreased abundance of *Bilophila* following inulin consumption was associated with softer stools (higher BSS) and a favourable change in constipation-specific quality-of-life measures (Vandeputte et al. 2017). According to Stephen et al. (1987), the mean BSS correlated well with the whole gut transit time ($r = -0.77$, $p < 0001$). We cannot compare our results to these studies directly due to the lack of transit time data, however, we can assume that persons recording loose stools had shorter transit times (faster transit rates). This assumption is also supported by the bacterial composition data, for instance significantly higher abundances and prevalence of *Prevotella* in case of high BSS (>5).

The two genera of the phylum Bacteroidetes – *Bacteroides* and *Prevotella* – have different properties and functions in the colon. It has been shown that dietary fibre-induced improvements in post-prandial blood glucose and insulin were positively associated with the abundances of *Prevotella* (Kovatcheva-Datchary et al. 2015). Similarly, the fibre-rich diets have been shown to support the dominance of *Prevotella*, whereas *Bacteroides* has been shown to dominate with the diets rich in protein and animal fat (Arumugam et al. 2011). Nevertheless, closely related species may harbour different genetic pools. De Filippis et al. (2016) showed that some *Prevotella*oligotypes were significantly associated with the plant-based diets but others with animal-based nutrients, and the same applied for *Bacteroides*. Therefore, an indiscriminate association of Bacteroidetes genera with specific dietary patterns may lead to an oversimplified vision that does not take into account the possible different responses to dietary components.

The question is which dietary patterns will lead to the domination of *Prevotella* in case of the LF diet. It might be related to white flour product consumption in the LF intake group and rye product consumption in the HF intake group as both of these are rich in fructans as discussed above. Whole rye and wheat are also rich in arabinoxylans that support the growth of *Prevotella*. *In vitro* studies have shown that xylose containing oligosaccharides and fructans can selectively enrich *Prevotella* (Fehlbaum et al. 2018; Van den Abbeele et al. 2018), however, also the contradictory effect of fructans has been reported (Fehlbaum et al. 2018). These results suggest that persons having *Prevotella* enterotype may possibly modulate the bowel habits and supposedly health characteristics by changing the fructan content in the diet. This assumption, however, needs further investigations.

Conclusions

According to our survey, the average amount of dietary fibres in the habitual Estonian diet in most cases remained below 13 g/1000 kcal/day that is the lowest quantity recommended. The biggest gap between the HF and LF intake groups were seen in the amounts of arabinoxylan, cellulose and pectin. Diets of the HF intake group provided also double amounts of several vitamins (C, K, folates, biotin) and minerals (K, Mg) compared to those of the LF intake group.

Samples of the same person taken within two sequential days in two months interval formed a characteristic „microbe-print", different from all other subjects. In general, *Faecalibacterium prausnitzii* and *Bacteroides vulgatus* were the most prevalent faecal species. *Prevotella* was observed in 64% of the study subjects and it is suggested as indicator of the colon habits in terms of BSS. The faecal BSS > 5 were recorded by the subjects with the HF intake obtaining most of the non-digestible polysaccharides from whole-grain cereals (rye, oat) and root vegetables. In different, the subjects having the LF intake gained the NSP mainly from white flour products and apples. To support the balanced and well-functioning gut microbiota (for example generating personal diets), knowledge of specific type of fibres in foods and their degradation products by bacteria are crucial along with gut microbiota at species level. For example, arabinoxylan-, pectin- or inulin-rich foods promote the growth of several health-related gut bacteria. To prove the causal associations, further *in vitro* and *in vivo* studies are needed, taking into account the gut transit rate and colon microbiome composition, specific information on the dietary fibres and eating habits, lifestyle and environmental data.

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Disclosure statement

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ORCID

Kaarel Adamberg http://orcid.org/0000-0002-7203-925X

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Low-carbohydrate high-fat weight reduction diet induces changes in human gut microbiota

Madis Jaagura[1,2] | Ene Viiard[1] | Kätrin Karu-Lavits[1] | Kaarel Adamberg[1,2]

[1]Center of Food and Fermentation Technologies, Tallinn, 12618, Estonia

[2]Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, 12618, Estonia

Correspondence
Kaarel Adamberg, Competence Center of Food and Fermentation Technologies, Tallinn 12618, Estonia.
Email: kaarel.adamberg@ttu.ee

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Abstract

Obesity has become a major public health problem in recent decades. More effective interventions may result from a better understanding of microbiota alterations caused by weight loss and diet. Our objectives were (a) to calculate the fiber composition of a specially designed low-calorie weight loss diet (WLD), and (b) to evaluate changes in the composition of gut microbiota and improvements in health characteristics during WLD. A total of 19 overweight/obese participants were assigned to 20%–40% reduced calories low-carbohydrate high-fat diet for four weeks. Protein and fat content in the composed diet was 1.5 times higher compared to that in the average diet of the normal weight reference group, while carbohydrate content was 2 times lower. Food consumption data were obtained from the assigned meals. Microbial composition was analyzed before and after WLD intervention from two sequential samples by 16S rRNA gene sequencing. During WLD, body mass index (BMI) was reduced on average 2.5 ± 0.6 kg/m^2 and stool frequency was normalized. The assigned diet induced significant changes in fecal microbiota. The abundance of bile-resistant bacteria (*Alistipes*, *Odoribacter splanchnicus*), *Ruminococcus bicirculans*, *Butyricimonas*, and *Enterobacteriaceae* increased. Importantly, abundance of bacteria often associated with inflammation such as *Collinsella* and *Dorea* decreased in parallel with a decrease in BMI. Also, we observed a reduction in bifidobacteria, which can be attributed to the relatively low consumption of grains. In conclusion, weight loss results in significant alteration of the microbial community structure.

KEYWORDS
16S rRNA gene, bifidobacteria, BMI, *Collinsella*, microbiome, obesity, weight loss

1 | INTRODUCTION

A rise in obesity has become a major public health problem over the past three decades (Mitchell et al., 2011) due to associated chronic diseases such as type 2 diabetes (T2D), hypertension, and dyslipidemia, among others (Bays et al., 2013; Landsberg et al., 2013; Ng et al., 2014). Diet has been recognized as one of the major contributing factors in maintaining weight homeostasis (Carels et al., 2008; Keränen et al., 2009), and various dietary approaches have been described to both treat obesity and manage weight loss (Botchlett & Wu, 2018; Sacks et al., 2009).

Popular eating trends have also changed during this time with a rise in popularity of low-carbohydrate high-fat (LCHF), paleo, vegan, and vegetarian diets. LCHF is also very popular for the treatment of obesity due to its effectiveness when losing weight (Hall et al., 2015; Mansoor et al., 2016). To better understand the relationship between

diet and human health and their associations with obesity, scientists have extensively studied the gut microbiome in recent years. Despite much work, it remains unclear how our diet and gut microbiome influence weight management and its association with obesity status.

Obesity has been linked with altered gut microbiota by additional energy harvest (Turnbaugh et al., 2006). Diet is one of the main factors that modulate gut microbiota because changes in bacterial abundance can occur very rapidly after the digested food has reached the colon (David et al., 2014). Although plant-based diets have a significant effect on gut microbiota, animal-based diets have been shown to have a greater impact (David et al., 2014). Nutritional profiles of the dietary patterns differ greatly in total energy and the content of macronutrients and dietary fiber (DF). Bacterial fermentation of DF has a major influence on intestinal function—the production of organic acids influences glucose and lipid metabolism, the immune system, and hormone secretion (Chambers et al., 2018). While caloric and macronutrient intake has been implicated in microbiome modulation, the effect of fiber content in weight reduction diets on the gut microbiota has been less extensively studied (Table A1). Weight reduction studies have demonstrated disparate results with regard to changes in the composition of the microbiota, which may be partially due to differences in the amount and choice of fiber in the diet. Thus, evaluating DF-associated effects during weight loss is crucial because reducing the daily caloric intake can also lead to a reduction in DF intake (Brinkworth et al., 2009).

Generally, interventional studies that investigate the relationship between food intake and the gut microbiome are often performed by supplementing extracted and purified fiber within a regular diet, however, baseline consumption of foods can remain different (Baxter et al., 2019; Dewulf et al., 2013). To circumvent this problem, uniform diets can be designed using prepared foods for all participants, and however, it can be costly to manage food delivery and the variety of foods that can be prepared in this way is too restrictive for the subjects. Alternatively, more subject-friendly diets can be formulated based on whole food components. To use this approach, a database of whole foods with detailed nutrient information is required, which would enable one to fine-tune diets with specified amounts of selected nutrients. With regard to microbiota, fiber composition should be determined in detail because fibers (type and amount) drive the modulation of gut microbiota (Dewulf et al., 2013; Walker et al., 2011).

We aimed to characterize the specific fiber content and sources in both weight loss and habitual diets, to analyze the fecal microbial composition of obese and normal weight subjects, and to investigate the effect of WLD on fecal microbial consortia.

2 | MATERIALS AND METHODS

2.1 | Study design

A group of overweight/obese participants began a voluntary low-calorie low-carbohydrate high-fat WLD interventional program to follow the appointed regime for at least four weeks. Inclusion criteria for participants included BMI of >28 kg/m^2 in the intervention arm (maximum 25% in BMI range 28–30 kg/m^2), BMI of >18 kg/m^2 in the reference group, no previous history of gastrointestinal (GI) disease, no reported antibiotic use in preceding 3 months, or taking any medication known to alter bowel motility, no history of food allergies and not taking medications, not pregnant nor breast-feeding, no specific dietary choices, and with ability to adhere to an omnivorous diet. Participant recruitment for the intervention arm was carried out in April and May 2017, and the 4-week WLD was carried out in June 2017. Reference group recruitment was carried out in March–April 2017, and sampling in April–August 2017, described in detail previously (Adamberg et al., 2020).

2.2 | Diet design for WLD

Assigned diet plans included 3 main meals (30 ± 5% E each) and 1 dessert (10% E) per day. Subject-specific daily energy intake was calculated based on daily expenditure, which accounted for body weight, activity, and energy restriction of 30 ± 10%. Meals in the diet were randomly selected from specifically designed recipes, which consisted mostly of animal-based foods (meat and dairy products) as a protein source, supplemented with vegetables, fruits, and a limited amount of cereals. Participants were provided with individual daily meal plans based on their energy needs. Subjects were asked to confirm that they had eaten the prescribed meals and they received personal assistance via phone calls if they were having any difficulties adhering to the diet. Diet plan meals were prepared by the subjects, and no additional food intake was allowed. None of the subjects consumed pre- or probiotics as supplements.

2.3 | Diet analysis in the WLD and reference groups

The subjects in the reference group were instructed to record their food consumption for at least one day before sampling, described in detail previously (Adamberg et al., 2020). For calculations, meals were decomposed to ingredients based on dietary instructions in the WLD group and based on consumed foods in the reference group. Assigned WLD and reference group consumption data were analyzed for energy, macronutrient, and total DF content based on the NIHD (The National Institute for Health Development) food composition database Nutridata v6/7.

2.4 | Diet quantification

Nutritional data were analyzed and normalized to 1,000 kcal caloric intake and presented as the mean of a group ± *SD*. Analysis of the food and diet records was carried out as previously described (Adamberg et al., 2020). Shortly, all food components were

decomposed into 35 primary and 72 secondary food groups, of which 46 dietary fiber-containing foods groups were characterized based on the fiber patterns in raw food materials to cover similar products within the same category. Meat and milk-derived products were considered as negligible sources of DF if not containing cereals, fruit, or vegetables. Specifically, the content of arabinoxylan, β-glucan, cellulose, inulin, lignin, and pectin was calculated for each category based on the literature data on raw foods (Bengtsson et al., 1990; Dodevska et al., 2013, 2015; Herranz et al., 1981; Holtekjølen et al., 2006; Kalala et al., 2018; Karppinen et al., 2000; nut.s, 2020). Analysis of food consumption data was carried out using custom R scripts.

2.5 | Fecal sampling and anthropometric data collection

The subjects were asked to collect fecal samples immediately after defecation by sterile swab and to suspend the collected material in a buffer containing ammonium sulfate (40% solution), EDTA (16 mM), and sodium citrate (20 mM). With each fecal sampling, a Bristol stool scale score (BSS) was also recorded by the subjects. The samples were transported at room temperature to the laboratory and stored at −20°C before DNA extraction. For anthropometric measurements, body weight and height were measured before and after the intervention period. Height was measured to the nearest 0.5 cm and weight to the nearest 0.5 kg. BMI was calculated at the beginning and end of the study using the formula: BMI = Weight/Height^2 [kg/m^2]. Two fecal samples were collected sequentially before intervention and four weeks later. Participants were provided stool collection swab kits, which contained saturated ammonium sulfate solution and EDTA-citrate buffer. Samples were delivered to the laboratory every three days and were stored at +2–6°C until extraction. Reference group samples were collected similarly, but the interval between two sampling time points varied between 41 and 121 days, on average 61 days (Adamberg et al., 2020).

2.6 | DNA extraction and 16S rRNA gene library preparation

Fecal DNA was isolated with the use of a PureLink Microbiome DNA Isolation Kit (Thermo Fisher Scientific, US, CA, Carlsbad) according to the manufacturer's instructions. Universal primers: S-D-Bact-0341-b-S-17 (5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used for PCR amplification of the 16S rRNA gene V3-4 region (Klindworth et al., 2013). The mixture of amplicons was sequenced on Illumina MiSeq with the use of v2 reagents yielding 2 × 250 bp paired-end reads (Estonian Genome Centre, Estonia). On average, 84,324 reads per sample were obtained for the intervention group and 82,639 reads per sample for the reference group.

2.7 | Taxonomic profiling of the sequencing data

DNA sequence data were analyzed using BION-meta according to the author's instructions (https://box.com/v/bion). First, sequences were cleaned at both ends using a 99.5% minimum quality threshold for at least 18 of 20 bases for 5′-end and 28 of 30 bases for 3′-end, then joined, followed by removal of shorter contigs than 350 bp. Sequences were cleaned from chimeras and clustered by 95% oligonucleotide similarity (k-mer length of 8 bp, step size 2 bp). Lastly, consensus reads were aligned to the SILVA reference 16S rDNA database (v123) using a word length of 8 and a similarity cut-off of 90%. All mapped taxa with relative abundance <0.0001 were discarded and considered as potential noise.

2.8 | Statistical analysis

Based on sample size calculations, we estimated that with 17 participants, the study would have more than 80% power to detect a significant difference among weight loss study groups, assuming a mean BMI reduction by 3 kg/m^2, with a mean BMI and standard deviation of 35 and 3.2 kg/m^2, respectively, at an alpha level of 5%.

Statistical analysis included bacteria with average colonization frequency >70% and average abundance >0.001. Analysis of data was carried out in the R statistical programming language, version 3.5.0 (R Core Team, 2020). The resulting *p*-values were corrected for multiple comparisons for each phylogenetic level using Benjamini–Hochberg correction (FDR). A corrected *p*-value <0.1 was considered statistically significant. Unless stated otherwise, corrected *p*-values are shown in the text.

Pairwise comparisons were evaluated using Wilcoxon signed-rank test, for the comparison of test and reference groups Kruskal–Wallis test was applied.

To control for within-subject variability, we used the subsequent sample pairs as within-subject controls and compared β-diversity before and after the intervention. This was also applied to reference group samples. The following cutoffs were used: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$.

2.9 | Agglomerative hierarchical clustering

Ward's agglomerative hierarchical clustering on a distance matrix was generated from a species by sample Bray-Curtis distance matrix. The method produces a dendrogram by treating each sample as a singleton cluster, merging pairs of clusters until all clusters have been merged into one big cluster containing all samples. Ward's agglomeration method minimizes the total within-cluster variance.

3 | RESULTS

A total of 27 overweight/obese participants were enrolled in a WLD regime. Of those, one participant did not enter the study. By the

end of the study, three participants were lost due to low compliance or incomplete baseline measurements, three canceled for unknown reasons, and one discontinued the weight loss program due to an unexpected antibiotic course. Thus, a total of 19 overweight/obese participants (14 females, 5 males, aged 25 to 43) with BMI ranging between 28.9 and 44.4 kg/m^2 successfully finished. A TREND flow diagram is displayed in Figure 1.

Additionally, 59 subjects (39 females, 20 males, aged 23–52 years) were recruited into a reference group, which was divided into three subgroups based on BMI: 18–25, 25–30, 30–39 kg/m^2 (N = 33, N = 16, and N = 10, respectively) (Adamberg et al., 2020). Characteristics of both the WLD and reference groups are depicted in Table 1.

3.1 | Assigned diet was high in fat, reduced BMI, and normalized bowel habits

The diet used for all participants was rich in fat (55.6 ± 2.1 g / 1,000 kcal, 50% from total energy), protein (62.7 ± 3.2 g / 1,000 kcal, 25%), and low in carbohydrates (56.5 ± 3.5 g / 1,000 kcal, 23%, Figure 2). Normalized intake values per 1,000 kcal were used because the caloric intake of the participants varied greatly due to highly dissimilar body weights. Both fat and protein were mainly sourced from animal-based foods (Figure A1). DF content was moderate (11.6 ± 1.1 g / 1,000 kcal), but it was comparable to levels in an average Estonian diet in an obese reference group (9.8 ± 4.8 g / 1,000 kcal). DF composition analysis based on food categories showed that most of the DF was cellulose and pectin (3.8 ± 0.4 and 2.4 ± 0.2 g / 1,000 kcal, respectively), which was higher than these in the obese reference group (2.5 ± 1.4 and 1.5 ± 1.0 g / 1,000 kcal, respectively). On the other hand, consumption of arabinoxylan and β-glucan were relatively low (1.4 ± 0.2 and 0.3 ± 0.1 g / 1,000 kcal, respectively), yet a similar intake was observed in the obese reference group (1.8 ± 1.1 and 0.5 ± 0.4 g / 1,000 kcal, respectively).

DF source analysis showed that vegetables were the main source of DF (5.7 ± 0.6 g / 1,000 kcal), while intake of DF originating from cereals was very low in the WLD group (1.4 ± 0.5 g /

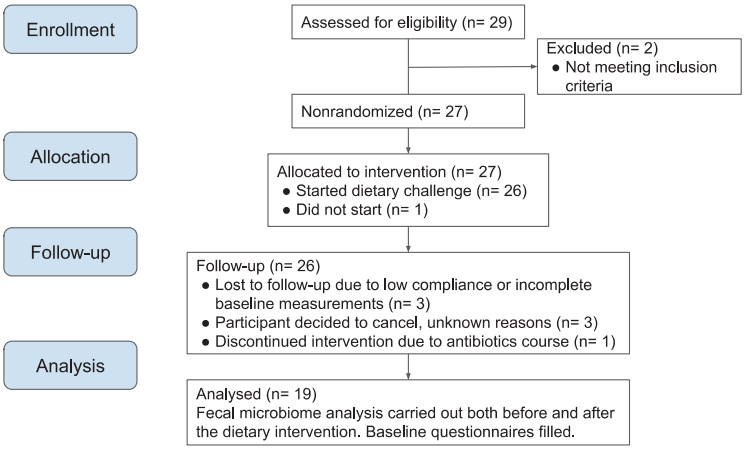

FIGURE 1 TREND flow diagram

TABLE 1 Participant characteristics of the WLD and reference group at baseline

	WLD group		Reference group	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Gender				
Female	14	73.7	39	66.1
Male	5	26.3	20	33.9
Age				
18–24			3	5.1
25–34	11	57.9	23	39.0
35–44	8	42.1	21	35.6
45–54			12	20.3
BMI				
18–25			33	55.9
25–30	1	5.2	16	27.1
30+	18	94.8	10	16.9

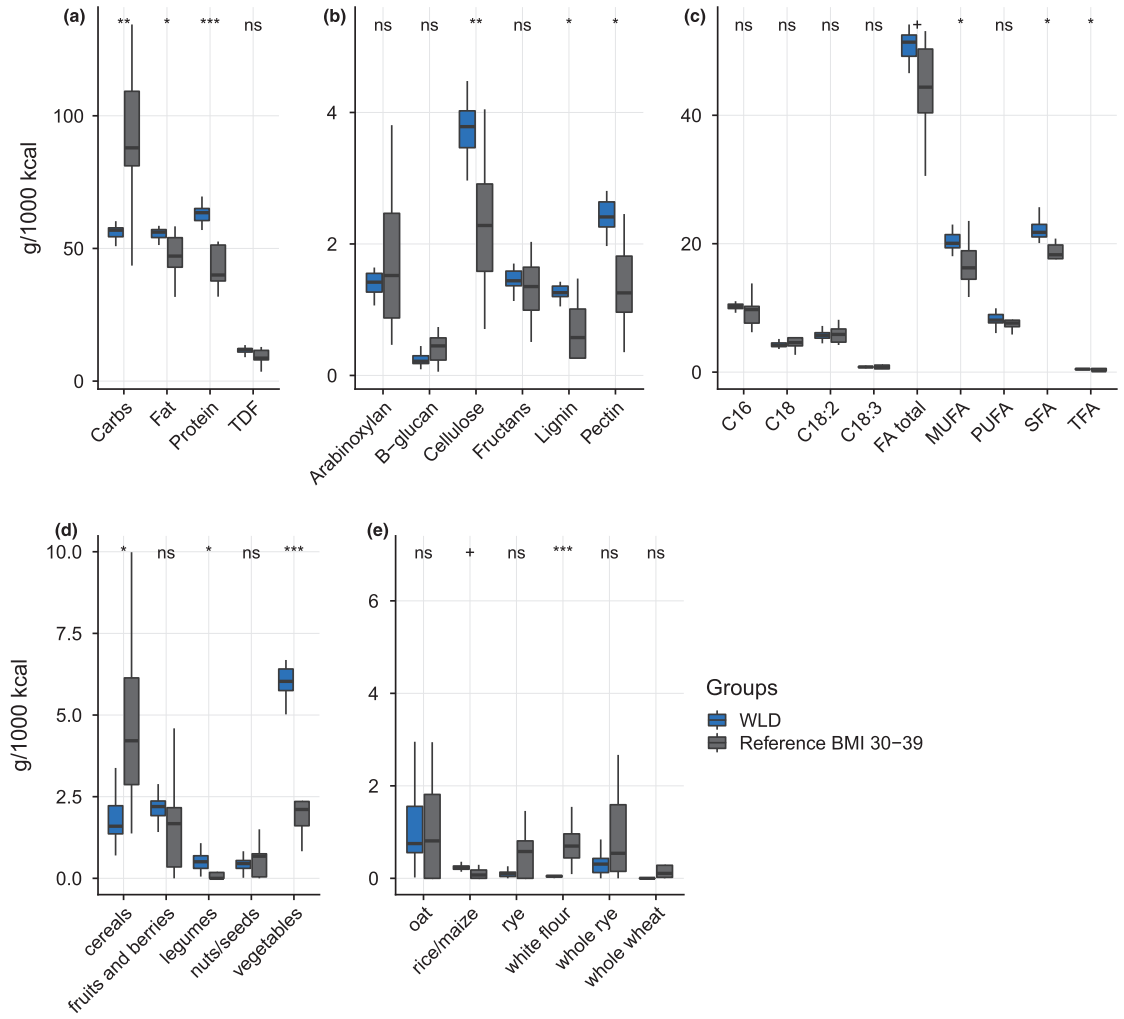

FIGURE 2 Nutritional and food intake in the WLD, and obese reference group. (a) macronutrients and total DF; (b) specific DF categories; (c) fatty acid profile; (d) main total DF sources; (e) main cereal DF sources. All values are g per 1000 kcal per day. Outliers are not shown

1,000 kcal) compared to the reference group (2.0 ± 1.2 and 3.5 ± 2.0 g / 1,000 kcal, respectively). Specifically, consumption of DF from wheat, rye, and barley during WLD was low (0.5 ± 0.4 g / 1,000 kcal). Due to limited information about food processing, the content of resistant starch (RS) was eliminated from our analysis. Low DF intake from cereals (1.4 ± 0.5 g / 1,000 kcal), starchy vegetables (0.5 ± 0.2 g / 1,000 kcal), legumes (0.6 ± 0.4 g / 1,000 kcal), and no detected increase of RS fermenting bacteria (Venkataraman et al., 2016; Ze et al., 2012) suggest that RS levels in the WLD group were low.

Analysis of fatty acids revealed that WLD was rich in saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) (22.0 ± 1.7 and 20.5 ± 1.7 g / 1,000 kcal, respectively) and contained similar levels of polyunsaturated fatty acids (PUFAs) (8.2 ± 1.0 g / 1,000 kcal) compared to the obese reference group (18.0 ± 4.5, 16.7 ± 3.8, and 9.0 ± 4.6 g / 1,000 kcal, respectively).

During the intervention, the BMI and body weight were significantly reduced on average by 2.5 ± 0.6 kg/m^2 and 7.7 ± 2.5 kg, respectively ($p < 0.0002$ for both) (Figure 3a,b).

Out of 14 participants, who filled questionnaires about gastrointestinal (GI) disturbances before and after WLD, not a single participant reported daily constipation or diarrhea and only one subject reported daily flatulence at the end of the intervention (before intervention: 1, 1, 7 subjects, respectively). A detailed description of bowel habits before and after the intervention can be found in Table A2. THE mean BSS value before WLD was 3.9 ± 1.5 and decreased to 3.4 ± 0.8 after the intervention, but in general BSS values stabilized (Figure 3c).

3.2 | Changes in microbiota associated with food consumption and health parameters

Statistically significant changes were not observed in α-diversity (Shannon: 3.5 ± 0.2 and 3.6 ± 0.2, before and after WLD, respectively) or species richness (135.3 ± 15.3 and 135.4 ± 14.5, before and after WLD, respectively) (Figure A2).

Bray–Curtis (B-C) distances of species composition within subjects reveal that intervention resulted in significantly altered microbial profiles in the WLD group. Similarly, within two months, changes in the microbiota in the reference group were also significant but not as extensive as in the test group (Figure 4b). Over the study period, change in β-diversity was observed between participants in the test and reference group (Figure A3). Overall, between-subject dissimilarities in the test group decreased more significantly, potentially due to a similar dietary regime with comparable nutrient composition. Hierarchical clustering revealed that most of the samples before and after intervention paired together (Figure A7).

Although within-subject B-C distances changed significantly, both samples from the same participant before and after WLD clustered together in most cases (Figure 4a), and only one of the sample pairs switched from a *Bacteroides*-dominated cluster to a *Prevotella*-dominated cluster (Figure 4a).

Starting first from higher taxonomic levels, the relative abundance of members of the families Enterobacteriaceae, Rikenellaceae, and Desulfovibrionaceae increased significantly after WLD (Figure 5, Figure A4), while the abundance of Bifidobacteriaceae was reduced.

From the dominant genera, the abundance of *Prevotella* decreased, while the abundance of *Bacteroides* increased, yet both changes were statistically insignificant ($p \geq 0.1$ for both, data not shown). A more pronounced response was associated with an increase in *Odoribacter* (only species is *O. splanchnicus*) and *Ruminococcus_1* (only species *R. bicirculans*). Additionally, the relative abundance of *Alistipes* and *Butyricimonas* increased, while the abundance of *Bifidobacterium*, *Collinsella*, and *Dorea* decreased.

Among the Enterobacteriaceae, WLD supported the growth of *Escherichia*, mainly *E. coli* (uncorrected $p < 0.05$). Another bacteria that was supported during WLD was (Figure 5, Figure A4) *Bilophila wadsworthia*. No other taxonomic groups within the *Desulfovibrionaceae* family showed significant differences.

Additionally, the abundance of Christensenellaceae, Porphyromonadaceae, *Lachnospira*, and *Bacteroides vulgatus*

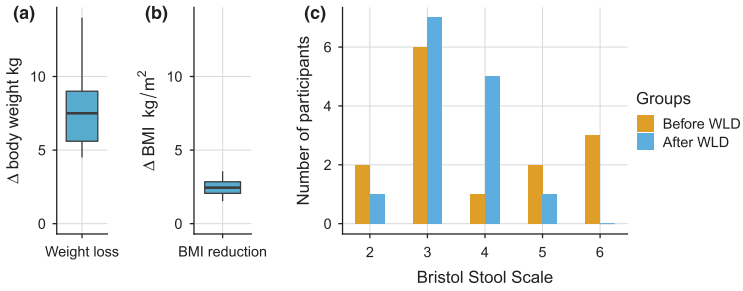

FIGURE 3 Changes in body mass (a), BMI (b), and BSS before and after the intervention (c) in the WLD group

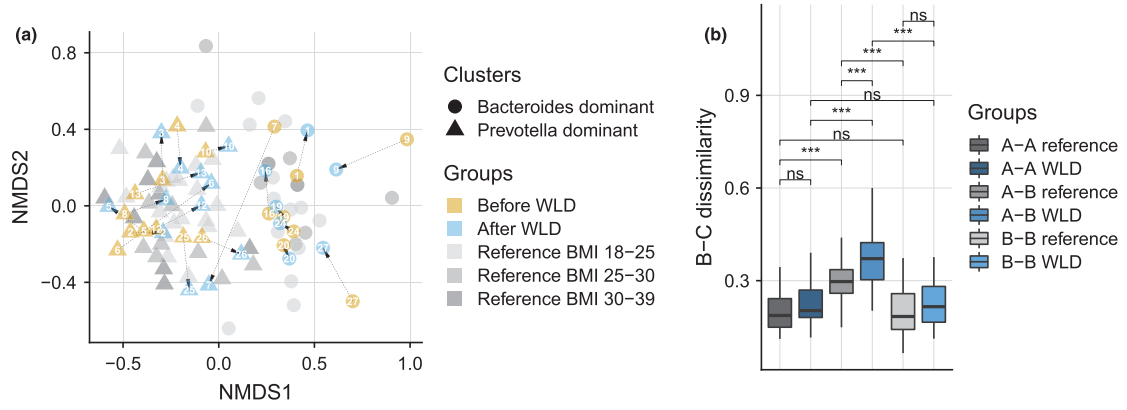

FIGURE 4 Impact of WLD on microbiota composition. A non-metric multidimensional scaling (NMDS) ordination displaying before and after WLD samples, and reference group samples ($N = 59$). Ward's agglomerative hierarchical clustering was used to cluster all samples into two clusters, dominated by *Bacteroides* or *Prevotella* species. (a) NMDS; (b) within-subject β-diversity of WLD and reference samples was compared at baseline (A-A), after intervention in the WLD group, and after a similar timeframe in the reference group (B-B) and between two time points (A-B). Reference—all BMI groups

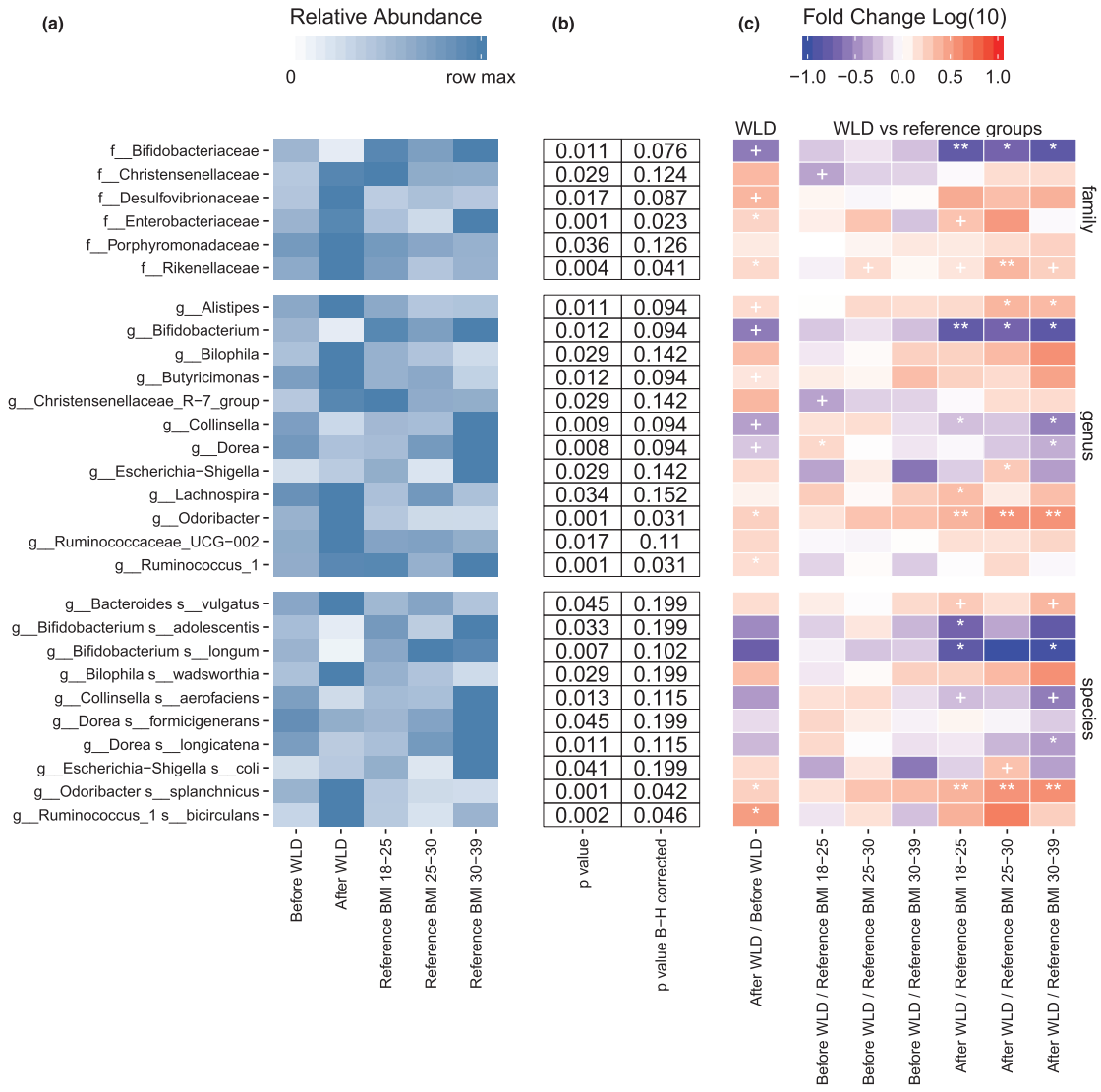

FIGURE 5 Altered taxa in response to WLD and comparison with reference groups. Statistical analysis was carried out at the family, genus, and species levels. (a) Average abundance of altered taxa. (b) uncorrected and corrected *p*-values between before and after samples from the same subject in the WLD group. (c) Fold change after WLD vs before study and reference group abundances. The last six columns indicate the logarithmic fold change by the colors ranging from dark blue to red. B-H corrected $p < 0.1$ (+), <0.05 (*), and <0.01 (**). See Figure A4 for full data on subject level abundances

increased, but not significantly, after correcting for multiple comparisons ($p < 0.05$, uncorrected $p > 0.1$). At the species level, changes were less pronounced but still noteworthy, for example, the abundance of *B. adolescentis*, *B. longum*, *C. aerofaciens*, *D. formicigenerans*, and *D. longicatena* was reduced after WLD but not significantly ($p < 0.05$, uncorrected $p > 0.1$).

Analysis of presence/absence data at the species level showed that several bacteria were less or more frequent after WLD. The most striking example was observed in the case of cellulose-degrading *B. cellulosilyticus*, which was more prevalent after the intervention and detected (>0.01%) in 12/19 samples after WLD compared to 4/19 samples before the intervention (Figure A5). Additionally, the colonization rate of *A. muciniphila*, *B. wadsworthia*, *E. coli*, *O. formigenes*, and *T. sanguinis* increased, while the prevalence of *B. adolescentis* and *B. longum* decreased after WLD.

Participants in the WLD group were divided into three subgroups based on their initial abundance of significantly altered taxa: not detected, low, and high abundance groups (ND, HAG, and

LAG, respectively). This analysis revealed that changes in bacterial levels depend on starting levels which means that bacteria in the LAG group increased more than in the HAG group. For example, abundances of Porphyromonadaceae, Rikenellaceae (*Alistipes*), *Butyricimonas*, Ruminococcaceae_UCG−002, *Ruminococcus_1*, and *Odoribacter splanchnicus* were significantly increased after WLD in the LAG group compared to insignificant changes in the HAG group (Figure A6). In the case of bacteria, which decreased after WLD, reduction of *Bifidobacterium*, *Collinsella aerofaciens*, and both *Dorea* species was significant in the HAG group, while significant changes were not observed in the LAG group (Figure A6).

3.3 | Comparison of pre- and post-intervention time points to normal weight, overweight, and obese reference groups

To investigate how WLD altered bacteria compared to the wider population, pre-intervention and post-intervention time points were compared with normal weight, overweight, and obese reference groups. Further analysis included only taxa, which were significantly altered in the intervention. No significant difference in bacterial abundances was detected between the obese reference group and the WLD group (Figure 5c, Figure A4). On the other hand, compared to the normal weight reference group, the abundance of *Christensenellaceae* and *Dorea* were lower and higher, respectively, in the WLD group. Overall, most of the taxa, which were significantly shifted in response to WLD, also exhibited distinct relative abundances in post-WLD samples compared to bacterial levels in reference groups. Most coherent differences between post-WLD samples and reference groups were detected among *Rikenellaceae* and *O. splanchnicus*, which were significantly increased compared to their populations in all reference groups. Similarly, a decline in the abundance of Bifidobacterium was observed in post-WLD samples compared to that in all reference groups. In addition, post-interventional levels of *B. vulgatus* and *Enterobacteriaceae* were higher than those in the normal weight reference group. The abundance of *B. vulgatus* was also higher in the post-WLD group compared to that in the overweight reference group. In contrast, the abundance of *C. aerofaciens* was significantly lower after WLD compared to that in the normal weight and overweight reference group. Although abundances of Porphyromonadaceae, *Bilophila*, *Butyricimonas*, Ruminococcaceae_UCG-002, *B. wadsworthia*, *D. formicigenerans*, and *R. bicirculans* were elevated after WLD, their abundances remained similar to those observed in all other reference groups.

4 | DISCUSSION

This study evaluated the effect of a low-carbohydrate high-fat weight loss diet (WLD) on the intestinal microbiota of overweight/obese subjects. We specifically analyzed the macronutrient, fatty acid, and dietary fiber composition of appointed WLD to elaborate the effect of weight loss on fecal microbiota and gastrointestinal (GI) symptoms. Another goal of this study was to compare microbiota between WLD and reference groups (normal weight, overweight, and obese individuals).

Consumption of dietary fiber (DF) in a habitual diet has been well characterized by food sources (O'Neil et al., 2012), but weight reduction diets are poorly defined and only provide information about total DF, non-starch polysaccharides, or resistant starch (RS) content (David et al., 2014; Fava et al., 2013; Santacruz et al., 2009; Walker et al., 2011). To date, the most detailed dietary analysis of fiber intake in a habitual diet was conducted by Munch Roager et al. (2019), who analyzed the effects of wholew grain and refined grain diets on adult human fecal microbiota. They measured the RS, arabinoxylan, and monosaccharide composition of whole-grain and refined grain products in the diets utilized in the study. In another study, the same group investigated the effects of a low-gluten diet on fecal microbiota and analyzed the carbohydrate composition of DF in representative meals of the diets utilized in the study (Hansen et al., 2018). They observed that arabinoxylan-rich cereals were important to keep sufficient levels of bifidobacteria in the fecal microbiota (see below).

In our study, a diet analysis revealed that DF content was moderate and slightly below the recommended value of 12.6 g/1000 kcal (Øverby et al., 2013) and is comparable with the DF content in the reference group with a non-statistically significant upward trend. Because plants contain a wide variety of DFs, we formed 11 main categories and 45 subcategories of foods to characterize the specific fiber composition of foods. From the main categories, the most abundant fiber source was vegetables, which provided approximately 50% of the total DF intake and subsequently determined the cellulose-rich nature of WLD. The second richest source of DF was fruits, which significantly increased the amount of pectin and lignin content in WLD. Because cereal consumption was low in WLD, it resulted in a low-to-moderate amount of arabinoxylan and β-glucan in the diet.

In our study, before introducing the WLD regime, the GI symptoms of subjects varied from low Bristol Stool Scale Score (BSS) to high BSS, and many reported frequent flatulence. After the four-week intervention, these conditions normalized thereby demonstrating a positive effect of this WLD on GI symptoms. These effects can be explained by the high amount of vegetable fibers, for example, cellulose, pectin, and lignin in the WLD plan.

Previous weight-loss interventions have shown subject-specific deviations in community composition and considerable alterations in specific bacterial abundances (Ott et al., 2017). In our study, using NMDS analysis, we show that the microbial communities within participants drifted during the intervention and in some cases displayed large changes indicating more significant alterations in the microbial communities. According to the hierarchical clustering, our WLD intervention did not hamper subject-wise clustering, which has been also shown by Salonen et al. (2014).

Although baseline fecal microbiota had similar abundance profiles compared with reference groups, we observed a lower

abundance of *Christensenellaceae* and a higher abundance of *Dorea* compared to the normal weight reference group. These bacteria are known to correlate with BMI (Goodrich et al., 2014). After four weeks of WLD intervention, the enterotype status and α-diversity were mostly unchanged. However, specific changes in the microbiota were observed, for example, a decrease in the number of *Collinsella*, *Coprococcus*, and *Dorea* species. These results correspond with results from other weight loss studies and dietary interventions, where the α-diversity (Ott et al., 2017) or enterotype status (Wu et al., 2011) were not affected by short-term calorie reduction yet an increase in α-diversity has been reported after long-term weight loss (Liu et al., 2017).

Our study corroborates previous findings concerning the reduction of bifidobacteria on carbohydrate-limited hypocaloric diets (Duncan et al., 2007; Salonen et al., 2014; Santacruz et al., 2009). However, an increased abundance of *Bifidobacterium* has been observed on a moderate carbohydrate and fiber-rich weight reduction diet (Ott et al., 2017). This could be explained by contrasting the macronutrient profiles of the applied diets because the WLD intervention in our study was limited in fiber and carbohydrate content compared to the dietary regime applied by Ott et al. (2017). In studies where diets are aimed to maintain body weight, supporting effect to increase the abundance of bifidobacteria by high-carbohydrate diets has been shown in comparison with high-fat diets (Fava et al., 2013). However, a decline in bifidobacteria has been observed also in weight loss intervention on a macronutritionally balanced diet (Santacruz et al., 2009), gluten-free diet (Palma et al., 2009), and low-gluten intervention diet (Hansen et al., 2018). Thus, the reduction of *Bifidobacterium* levels after WLD intervention can be attributed to the low intake of cereal grains and starchy vegetables. There is some evidence to support that bifidobacteria are supported by arabinoxylan oligosaccharides, although only a few studies show that long-chain arabinoxylan is bifidogenic (Hopkins et al., 2003; Monteagudo-Mera et al., 2018; Truchado et al., 2017). The growth of bifidobacteria is supported by dietary fructans (Dewulf et al., 2013), which are enriched in the wheat endosperm. In WLD, the intake of arabinoxylan and fructan was low-to-moderate but comparable to reference group values, and the nature of food components suggests that RS intake could have been low, thus potentially limiting the growth of bifidobacteria. Studies that compare the microbiota of normal weight and obese subjects have shown contrasting results regarding *Bifidobacterium*, which has been associated with both high (Selma et al., 2016; Sepp et al., 2013; Verdam et al., 2013) and low BMI (Ignacio et al., 2016; Korpela et al., 2017; Santacruz et al., 2010) in adults and children. Our study supports the idea that the abundance of bifidobacteria is not conditionally dependent on weight or weight loss and is rather related to fiber type and carbohydrate content in WLD.

Another interesting shift after WLD intervention is an increase in *R. bicirculans*. This trend has been also observed in a high-protein low-fat weight reduction diet (Salonen et al., 2014). It has been suggested that *R. bicirculans* selectively utilizes certain hemicelluloses, especially β-glucans and xyloglucan (XyG) (Wegmann et al., 2014). Vegetables, especially leaves are rich in XyG, which could also explain the increase in the prevalence of *B. cellulosilyticus* on vegetable-rich low-cereal WLD (McNulty et al., 2013; Williams et al., 2017).

Collinsella and *Dorea* species, which were both reduced in WLD, have been associated with metabolic diseases (Candela et al., 2016; Duvallet et al., 2017; Gomez-Arango et al., 2018; Goodrich et al., 2014; Lahti et al., 2013; Liu et al., 2017; Zupancic et al., 2012). *C. aerofaciens* levels have been shown to decrease on a high-protein low-fat weight reduction diet (Walker et al., 2011). Diet-specific effects on *C. aerofaciens* have not yet been elucidated: an increase in prevalence has been observed after a high-cereal-grain diet (Foerster et al., 2014) and reduced abundance after vegetable and whole-grain fiber-rich fruit-free diet (Candela et al., 2016). Nutritional studies have shown that low-gluten intervention reduces the abundance of *Dorea* (Hansen et al., 2018), which agrees with our results because consumption of DF from wheat, rye, and barley during WLD was minimal. Biochemical tests have shown that both *Collinsella* and *Dorea* species exhibit low-carbohydrate fermentation while the latter species can consume sugars derived from arabinoxylan or fructose (Kageyama et al., 1999; Taras et al., 2002).

Enrichment of *Butyricimonas* and *Rikenellaceae* in lean subjects, negative correlation with BMI and triglyceride levels indicates that these taxa may promote health or contribute to the prevention of obesity (Goodrich et al., 2014; McNulty et al., 2013). Our study supports this idea because these taxa increased after WLD intervention. Furthermore, a high abundance of butyric-acid-producing *Butyricimonas* has been associated with normal weight and diets high in animal protein and saturated fats (Garcia-Mantrana et al., 2018).

High-fat diets have been previously associated with increased bile release (Cummings et al., 1978; David et al., 2014), while weight reduction diets can reduce serum bile acid (BA) (Biemann et al., 2016; Jahansouz et al., 2016; Straniero et al., 2017) and total fecal BA concentration (Kudchodkar et al., 1977). On the other hand, fecal BA concentrations were not altered during dietary weight loss therapy (Damms-Machado et al., 2015) but were reduced with a low-fat hypocaloric diet supplemented by high fiber (Reddy et al., 1988). Even though we did not analyze the BA concentrations in feces or plasma, the abundance of several bile-tolerant bacteria increased during WLD such as *Rikenellaceae* (*Alistipes*), *Odoribacter splanchnicus*, and *Bilophila wadsworthia*, which indicates that bile concentration may have increased in the GI tract. An increase of *B. wadsworthia* on a high-fat diet has also been observed in other studies (David et al., 2014).

5 | CONCLUSIONS

This study investigated changes in fecal microbiota during significant weight loss on a high-fat diet. In contrast with most weight loss studies, we characterized the DF sources and estimated the specific DF intake in the diets used which provides an additional layer of

data to link microbiota alterations with diet. To our knowledge, this is the first publication that characterizes specific fiber intake and DF intake quantitatively by food subcategories in a weight loss study based on food composition data. WLD intervention both reduced BMI and improved GI symptoms. High vegetable intake increased the levels of cellulose and low-cereal intake reduced the levels of arabinoxylan and β-glucan content in the diet, which were accompanied by shifts in microbiota such as a reduction in the abundance of bifidobacteria. WLD supported the growth of bile-resistant bacteria, while the abundance of bacteria associated with inflammation was reduced. We conclude that the dietary intake of different fibers and the initial abundance of bacteria in the microbiota (low or high abundant groups) should be taken into account when analyzing the impacts of a weight reduction diet.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Madis Jaagura: Conceptualization (supporting); Data curation (equal); Methodology (supporting); Software (lead); Validation (equal); Visualization (lead); Writing-original draft (lead); Writing-review & editing (equal). **Ene Viiard:** Conceptualization (equal); Writing-review & editing (supporting). **Kätrin Karu-Lavits:** Data curation (equal). **Kaarel Adamberg:** Conceptualization (lead); Funding acquisition (lead); Methodology (lead); Supervision (lead); Validation (equal); Writing-review & editing (equal).

ETHICS STATEMENT

The study protocol was approved by the Tallinn Medical Research Ethics Committee (TMEK no 1631). Informed consent was obtained from all subjects involved in the study.

DATA AVAILABILITY STATEMENT

All data are provided in full in this paper and its appendices. The raw sequences obtained were demultiplexed and uploaded to the European Nucleotide Archive (ENA) under the accession number PRJEB35687: https://www.ebi.ac.uk/ena/browser/view/PRJEB35687

ORCID

Madis Jaagura https://orcid.org/0000-0003-2329-0242

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APPENDIX

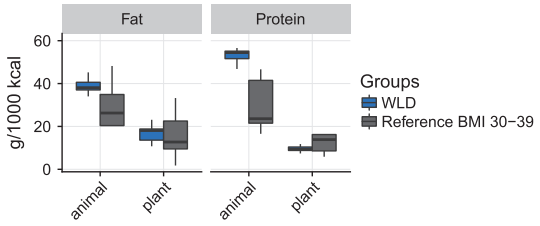

FIGURE A1 Origin of fat and protein in WLD and obese reference group

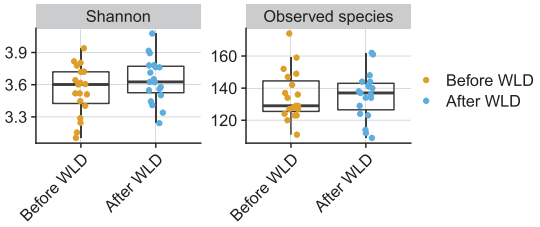

FIGURE A2 Alpha diversity and species richness

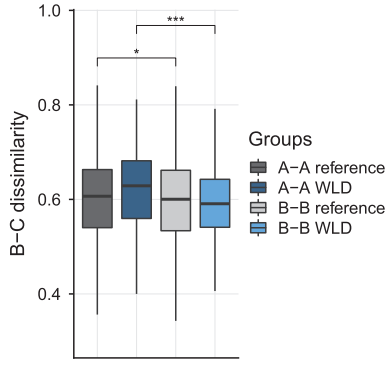

FIGURE A3 Between-subject β-diversity of WLD and reference samples

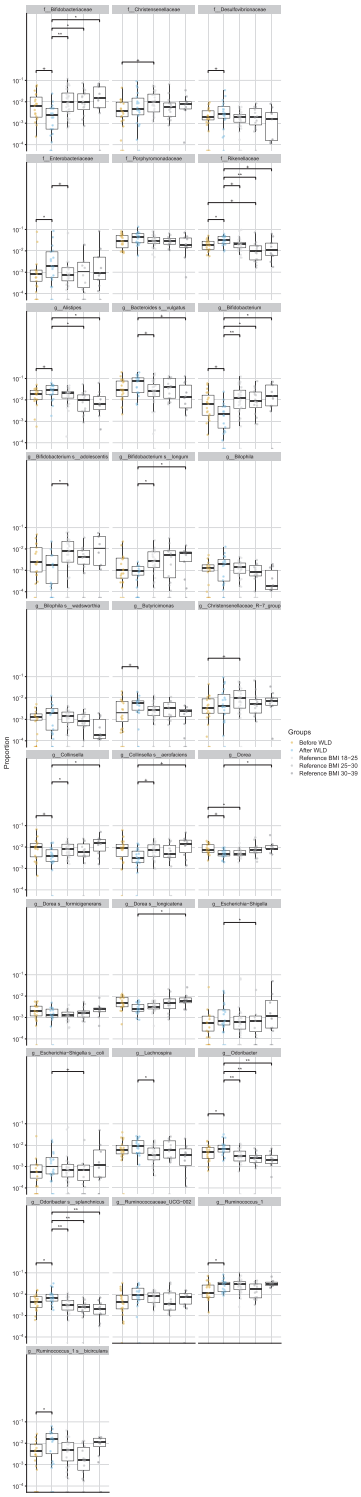

FIGURE A4 Abundance of significantly altered (uncorrected $p < 0.05$) taxa after WLD in comparison with reference groups

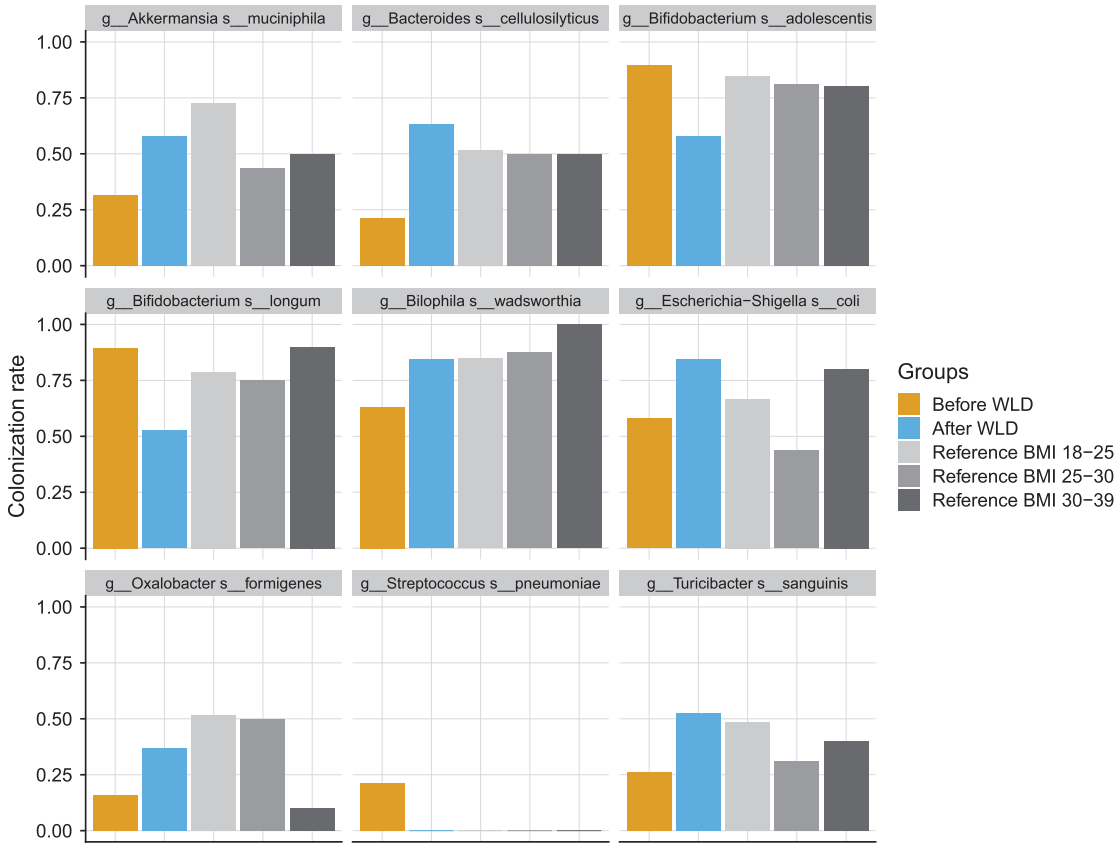

FIGURE A5 Prevalence of species (|delta prevalence| >3 between before and after WLD) in the test and reference groups

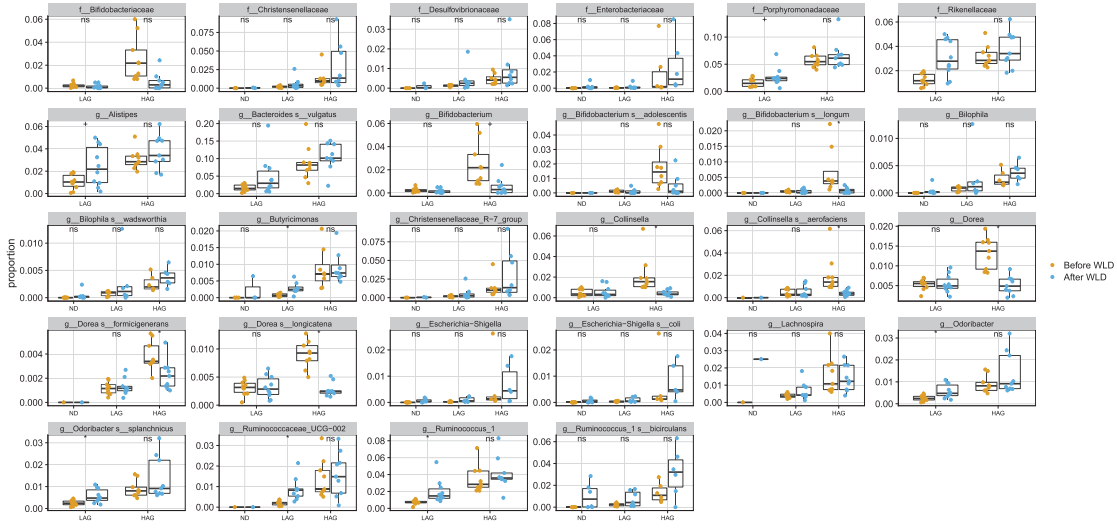

FIGURE A6 Abundance of significantly altered (uncorrected $p < 0.05$) taxa before (beg) and after (end) WL diet

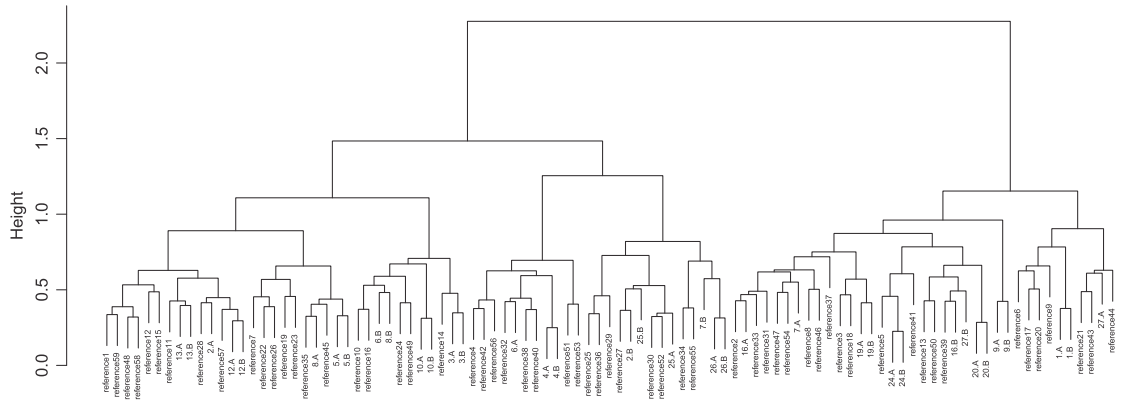

FIGURE A7 Ward's agglomerative hierarchical clustering on a Bray–Curtis distance matrix

TABLE A1 Literature overview of weight loss interventions

Tested diets	Main TDF sources	Main results	Reference
M—energy maintenance diet (~3100 kcal, ~28 g NSP/day, 52% C, 12% P, 35% F) 3 days **HPMC**—ad libitum (~1800 kcal/day, ~12 g NSP/day, 35% C, 30% P, 35% F) 4 weeks **HPLC**—ad libitum (~1700 kcal/day, ~6 g NSP/day, 4% C, 30% P, 66% F) 4 weeks.	M/HPMC/HPLC: not available/unknown	HPMC and HPLC (vs M): fecal acetate↓, butyrate↓, total SCFA↓, Roseburia/E.rectale↓, bifidobacteria↓ HPLC (vs HPMC): fecal butyrate↓	Duncan et al., (2007) *N* = 19
LC—ad libitum (~1600 kcal/day, ~13 g TDF/day, 5% C, 35% P, 58% F) 8 weeks **HC**—ad libitum (~1500 kcal/day, ~32 g TDF/day, 46% C, 24% P, 28% F) 8 weeks	LC: >2.5 cups green vegetables, 40 g nuts HC: 40 g bran cereal, 35 g whole grain bread, 300 g fruit, >2.5 cups green vegetables, potato, pasta, rice, bean lentils, 20 g nuts	LC (vs HC): fecal output↓, defecation frequency↓, fecal butyrate↓, total fecal SCFA↓, bifidobacteria↓	Brinkworth et al., (2009) *N* = 91
M—energy maintenance diet (~2800 kcal/day, ~22 g NSP/day, 51% C, 12% P, 37% F) 4 weeks **HPMC**—(~2000 kcal/day, ~13 g NSP/day, 36% C, 28% P, 37% F) 4 weeks **HPLC**—(~1900 kcal/day, ~9 g NSP/day, 4% C, 30% P, 66% F) 4 weeks.	M/HPMC/HPLC: varied	HPMC (vs M): fecal isovalerate↑, isobutyrate↑ HPLC (vs M): fecal butyrate↓, total SCFA↓, isovalerate↑, isobutyrate↑, *Roseburia/E.rectale*↓, *Bacteroides*↓	Russel et al. (2011) *N* = 17
M—maintenance diet (~3300 kcal/day, ~33 g TDF/day, 52% C, 13% P, 35% F) 1 week **WL**—weight loss diet (~2000 kcal/day, ~28 g TDF/day, 41% C, 30% P, 29% F) 3 weeks	M: not available/unknown (RS 5 g/day, soluble NSP 4 g/day, insoluble NSP 23 g/day) WL: not available/unknown (RS 3 g/day, soluble NSP 3 g/day, insoluble NSP 22 g/day)	WL (vs M): *C. aerofaciens*↓, *Roseburia*↓, *Oscillibacter*↑ (Walker) WL (vs M): fecal acetate↓, propionate↓, butyrate↓, succinate↓, *Bifidobacterium*↓, *P. cinnamivorans*↓, *B. vulgatus*↑ (Salonen)	Walker et al., (2011)[a] Salonen et al., (2014)[a] *N* = 14
Before intervention (~2100–2400 kcal/day, ~17–18 g TDF/day, 42–43% C, 19% P, 38–40% F) 10 weeks **WL**—weight loss diet (~1400–1500 kcal/day, ~18–22 g TDF/day, 45–47% C, 22–23% P, 32–34% F) 10 weeks	WL: Vegetables, cereals, fruits, and legumes	*B. fragilis*↑, *Lactobacillus*↑, *C. coccoides*↓, *B. longum*↓, *B. adolescentis*↓	Santacruz et al., (2009) *N* = 36
Before intervention (~1700 kcal/day, ~15 g TDF/day, 45% C, 17% P, 38% F) **VLCD**—(~800–900 kcal/day, ~15 g TDF/day, 51% C, 26% P, 23% F) 4 weeks	Not available/unknown, supplementary vegetables	HDL↓, LDL↓, TG↓, CRP↓, LBP↓, gut paracellular permeability↓, *A. rectalis*↓, *R. faecis*↑, *Bifidobacterium* sp.↑, *Blautia* sp.↑, *A. hadrus*↑	Ott et al., (2017) *N* = 20

TDF (total dietary fiber) includes both non-starch polysaccharides (NSP) and resistant starch (RS).

Abbreviations: %E, Percentage from energy; C, carbohydrates; F, fat; P, protein.

[a]Other tested diets not described

TABLE A2 GI disturbances frequency and intensity

	Before WLD						After WLD					
	Co	D	B	F	Cr	S-P	Co	D	B	F	Cr	S-P
Frequency of gastrointestinal symptoms												
Daily	1	1	2	7	0	0	0	0	1	1	0	0
Weekly	3	3	4	6	0	0	3	2	3	8	0	1
Monthly	6	5	4	1	2	5	6	5	3	3	1	3
<10 × year	4	5	3	0	2	4	4	6	5	1	3	4
Never	0	0	1	0	10	5	1	1	2	1	10	6
Intensity of gastrointestinal symptoms												
Strong[a]	1	0	1	0	0	0	0	0	0	0	0	0
Moderate	3	3	3	8	1	1	2	2	2	6	2	2
Mild[b]	8	9	7	6	2	6	10	9	8	7	2	4
No symptoms	2	2	3	0	11	7	2	3	4	1	10	8

Abbreviations: B, bloating; Co, constipation; Cr, cramps; D, diarrhea; F, flatulence; S-P, stomach pain.

[a]Disturbs considerably.

[b]Does not disturb.

Curriculum vitae

Personal data

Name: Madis Jaagura
Date of birth: 10.12.1987
Place of birth: Estonia
Citizenship: Estonia

Contact data

E-mail: madisjaagura@gmail.com

Education

2015-2021	Tallinn University of Technology, PhD
2011-2015	Tallinn University of Technology, gene technology, MSc
2008-2011	Tallinn University of Technology, gene technology, BSc
2006-2008	Arhus Tekniske Skole, chemical and biotechnical science, AP
1999-2006	Tallinna Tehnikagümnaasium

Language competence

Estonian	native speaker
English	fluent

Professional employment

2020-…	Center of Food and Fermentation Technologies LTd, project manager
2015-2019	Center of Food and Fermentation Technologies LTd, researcher
2011-2015	Nova Vita Clinic Ltd, embryologist
2008-2010	Tallinn University of Technology, engineer

Elulookirjeldus

Isikuandmed

Nimi:	Madis Jaagura
Sünniaeg:	10.12.1987
Sünnikoht:	Eesti
Kodakondsus:	Eesti

Kontaktandmed

E-post:	madisjaagura@gmail.com

Hariduskäik

2015-2021	Tallinna Tehnikaülikool, PhD
2011-2015	Tallinna Tehnikaülikool, Geenitehnoloogia, MSc
2008-2011	Tallinna Tehnikaülikool, Geenitehnoloogia, BSc
2006-2008	Arhus Tekniske Skole, Keemia ja biotehnoloogia teadused, AP
1999-2006	Tallinna Tehnikagümnaasium

Keelteoskus

Eesti keel	emakeel
Inglise keel	kõrgtase

Teenistuskäik

2020-…	AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus, projektijuht
2015-2019	AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus, teadur
2011-2015	AS Nova Vita Kliinik, embrüoloog
2008-2010	Tallinna Tehnikaülikool, insener

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