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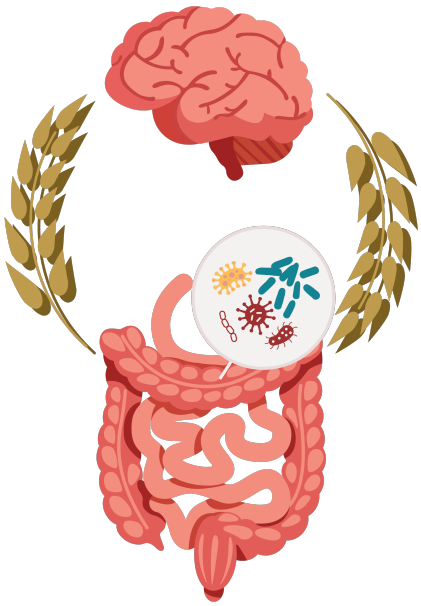

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Abstract

Whole grain (WG) is rich in dietary fiber, other nutrients, and phytochemicals, and has potential effects on the brain through the gut-brain axis and non-microbiota-related routes. This thesis explored the interplay between gut microbiota, dietary fiber, and microbiota-derived metabolites during *in vitro* fermentation of WG oat bread, WG rye bread, and refined bread. The effects of WG rye bread on the gut-brain axis were studied in a three-week dietary intervention study.

Differences in short-chain fatty acid (SCFA) production and dietary fiber degradation were observed between two donors with different fecal microbiota composition and between different breads during the 24-h *in vitro* fermentation experiments. High levels of butyrate were observed especially in fermentation samples with rye substrate in one donor, where a high relative abundance of *Subdoligranulum* genus was also observed. The metabolite profile of fermentation samples with WG rye differed from that of fermentation samples with WG oats and refined bread. Metabolites showing significant changes included several suggested microbiota-derived metabolites.

The three-week intervention with high intake of WG rye bread did not reveal any significant effects on fecal microbiota, SCFA levels, blood markers related to the gut-brain axis, intestinal permeability, stress responses, cognitive performance, or perceived long-term stress and well-being in healthy subjects. However, the abundance of two butyrate-producing taxa, *Anaerobutyricum hallii* and *Eubacterium ventriosum* group increased within the group consuming WG rye bread.

Altogether, these results indicate that WG rye may have butyrate-promoting effects depending on gut microbiota composition, but effects on the gut-brain axis were not observed.

Keywords: whole grain, gut microbiota, gut-brain axis, dietary fiber, short-chain fatty acids, butyrate, rye, oats

Fullkorn och tarm-hjärna-axeln. Rollen av mikrobiotasammansättning, kostfiber och metaboliter

Abstrakt

Fullkorn är rikt på kostfibrer, andra näringsämnen och fytokemikalier, och kan ha effekter på hjärnan genom tarm-hjärna axeln och icke-mikrobiotarelaterade mekanismer. Denna avhandling undersökte samspelet mellan tarmmikrobiota, kostfiber och mikrobiota-producerade metaboliter under *in vitro*-fermentering av fullkornshavre-, fullkornsråg- och raffinerat bröd. Effekterna av fullkornsrågbröd på tarm-hjärna axeln studerades också i en tre veckor lång kostinterventionsstudie.

Skillnader i produktion av kortkedjiga fettsyror (SCFA) och nedbrytning av kostfiber observerades mellan två donatorerna med olika fekal mikrobiotasammansättning och mellan olika bröd under 24-timmars *in vitro*-fermentering. Höga nivåer av butyrat observerades särskilt i proverna med råg hos en av donatorerna, där den relativa mängden av *Subdoligranulum* också var hög. Metabolitprofilen för fermenteringsprover med fullkornsråg skiljde sig från den för prover med fullkornshavre och raffinerat bröd. Metaboliter som visade signifikanta förändringar inkluderade flera föreslagna mikrobiota-producerade metaboliter.

Den tre veckor långa interventionen med högt intag av fullkornsrågbröd visade inga signifikanta effekter på fekalmikrobiota, SCFA-nivåer, tarm-hjärna axel relaterade blodmarkörer, tarmpermeabilitet, stressresponser, kognitiv prestation eller upplevd långvarig stress och välbefinnande hos friska människor. Den relativa mängden av två butyratproducerande taxa *Anaerobutyricum hallii* och *Eubacterium ventriosum* grupp ökade inom gruppen som konsumerade fullkornsrågbröd.

Sammantaget indikerar dessa resultat att fullkornsråg kan ha butyratfrämjande effekter beroende på mikrobiotans sammansättning, men effekter på tarm-hjärna axeln observerades inte.

Nyckelord: fullkorn, tarmmikrobiota, tarm-hjärna axeln, kostfiber, kortkedjiga fettsyror, butyrat, råg, havre

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Laura Pirkola, Johan Dicksved, Jussi Loponen, Ingela Marklinder, Roger Andersson (2023). Fecal microbiota composition affects *in vitro* fermentation of rye, oat, and wheat bread. Scientific reports, 13, 99.

II. Laura Pirkola, Ville Koistinen, Anton Klåvus, Johan Dicksved, Jussi Loponen, Roger Andersson. Metabolomics analysis of *in vitro* fermentation of oat, rye, and wheat bread – The effect of fecal microbiota composition on gut-brain metabolites (submitted manuscript)

III. Laura Pirkola, Annalena Kamm, Jonas Persson, Roger Andersson, Tatiana Marques, Rebecca Wall. Impact of whole grain rye bread on modulating microbiota-gut-brain axis in healthy subjects (manuscript)

The contribution of Laura Pirkola to the papers included in this thesis was as follows:

I. Participated in planning the study, compiled the ethical application, recruited study subjects, conducted the experiments including preparation of the fermentation substrate, carried out sample preparation and dietary fiber analysis of all samples, conducted the statistical analysis, participated in interpretation of the results, and had the main responsibility for writing and revising the manuscript.

II. Participated in planning the study, participated in the statistical analysis and interpretation of the results, and had the main responsibility for writing and revising the manuscript.

III. Participated in planning the study and compiling the ethical application, recruited study subjects, planned and conducted the intervention visits, conducted the blood sample analysis, analyzed data from the multi-sugar permeability test, planned and conducted the analysis of physiological data, conducted the analysis of cognitive test data, analyzed the questionnaire data, conducted the statistical analysis, participated in interpretation of the results, and had the main responsibility for writing and revising the manuscript.

Abbreviations

ANOVA	Analysis of variance
ANS	Autonomic nervous system
AR	Alkylresorcinol
AUC	Area under the curve
AX	Arabinoxylan
BDNF	Brain-derived neurotrophic factor
BG	Beta-glucan
BMI	Body mass index
CD14	Cluster of differentiation 14
CNS	Central nervous system
DF	Dietary fiber
ECG	Electrocardiogram
EDA	Electrodermal activity
ELISA	Enzyme-linked immunoassay
ENS	Enteric nervous system
FFAR	Free fatty acid receptor
GBA	Gut-brain axis
GC-MS	Gas chromatography-mass spectrometry
GI	Gastrointestinal

GLP	Glucagon-like peptide
hMPP	3-(3-hydroxyphenyl)propionic acid
HPA-axis	Hypothalamic-pituitary-adrenal axis
HRV	Heart rate variability
IFN-γ	Interferon gamma
IL	Interleukin
LBP	Lipopolysaccharide binding protein
LC-MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
MAST	Maastricht acute stress test
PCA	Principal component analysis
PYY	Peptide YY
SCFA	Short-chain fatty acid
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor alpha
UHPLC-QTOF-MS	Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
WG	Whole grain

1. Background to the thesis

Interest in gut microbiota and the gut-brain axis and awareness of their role in health and disease have increased considerably during the past decade. Whole grain is rich in dietary fiber, vitamins, minerals, and phytochemicals, and has the potential to affect the brain either directly or indirectly through the microbiota-gut-brain axis. This thesis examined the effects of whole grain on gut microbiota, microbiota-derived metabolites, and the gut-brain axis.

1.1 Whole grain

According to the Healthgrain Forum definition, whole grain (WG) consists of the intact, ground, cracked, or flaked kernel after removal of inedible parts, such as hull and husk, and includes starchy endosperm, germ, and bran in the same relative proportions as in the intact kernel (van der Kamp *et al.* 2014). Cereal grains from the *Poaceae* family (most importantly wheat, rye, oats, barley, rice, maize, sorghum, millet, and triticale), and the pseudo-cereals amaranth, buckwheat, and quinoa are covered by this definition. Whole grain is rich in dietary fiber (DF) and contains vitamins, minerals, and phytochemicals (Fardet 2010). Most of these nutrients are present in the bran and germ (Figure 1), and thus WG cereals are more nutrient-rich and health-beneficial than refined grains, as the bran and germ are removed from refined grains.

Whole grain intake has been associated with lower all-cause, cancer, and stroke mortality, and lower incidence of type 2 diabetes (T2D) and coronary heart disease (Reynolds *et al.* 2019). In addition, WG interventions have been demonstrated to lower body weight and cholesterol levels (Reynolds *et al.* 2019). Low intake of WG is estimated to be the leading dietary risk factor

for deaths (3 million) and disability-adjusted life-years (82 million), both globally and in several individual countries (GBD 2017 Diet Collaborators 2019). The health benefits of WG are likely connected to its high DF content, as the health effects of DF are similar, but the micronutrients and phytochemicals in WG can have health benefits beyond those of DF (Fardet 2010). WG is also an important source of sustainable plant-based protein (Poutanen *et al.* 2022).

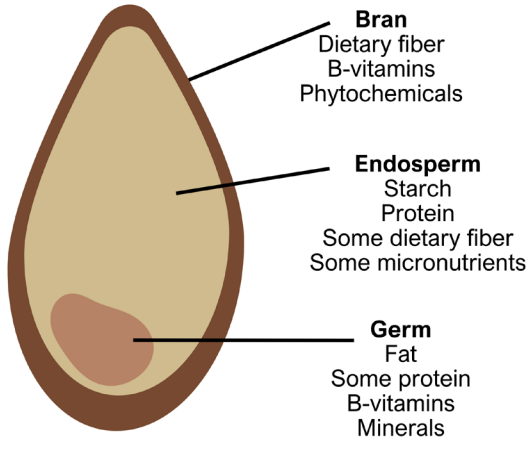

Figure 1. A simplified presentation of anatomical components of the kernel and their key nutrients. The bran and germ are present in whole grains and are removed from refined grains (modified from Edgar *et al.* 2022).

It is worth mentioning that WG contains some undesirable compounds with potential negative health effects, such as cadmium, arsenic, and mycotoxins, but the health benefits of WG intake are estimated to outweigh the risks related to these compounds (Nugent & Thielecke 2019). According to the Swedish Food Agency, increased cadmium intake is the most relevant health risk associated with WG, but the benefits of increased WG intake outweigh the risks of cadmium exposure (Edgar *et al.* 2022). WG contains certain antinutritional compounds, such as phytate, that can decrease absorption of essential minerals such as iron, zinc, and calcium, which is especially problematic in low-income countries (Fredlund *et al.* 2006).

In 2010, the average global WG intake in adults was estimated to be 38 g/day (1.3-334 g) (Micha *et al.* 2015), and in many countries, only a small proportion of the population meets the recommended WG intake (Kyro &

Olsen 2021). In the most recent Nordic Nutrition Recommendations, the recommended intake of WG is a minimum of 90 g per day (Blomhoff *et al.* 2023). In Europe and the USA, wheat is the most widely consumed WG cereal. In Scandinavia and Finland, daily WG intake is approximately 40-60 g, which is high compared with that in many other Western countries (Kyro & Olsen 2021; Tammi *et al.* 2021). Consumption of traditional cereals oats and rye, commonly used as WG, may partly explain the higher WG intake in the Nordic region than in other Western countries.

1.2 Dietary fiber

According to the *Codex Alimentarius* definition, DF refers to edible carbohydrate polymers with ≥10 monomeric units that are not hydrolyzed by human enzymes in the small intestine (Codex Alimentarius Commission 2021). Carbohydrates including 3-9 monomers, *i.e.*, oligosaccharides, are included in the definition depending on local regulations. Dietary fiber includes carbohydrate polymers that occur naturally in food as-consumed or that have been obtained from food raw materials by physical, enzymatic, or chemical means or have been created synthetically (Codex Alimentarius Commission 2021). If DF is obtained from raw material or synthesized, it needs to have demonstrated and authorized physiological health benefits. When derived from plant origin, DF may contain fractions of compounds associated with plant cell wall polysaccharides, but these compounds are not included in the definition of DF if isolated and re-introduced into food (Codex Alimentarius Commission 2021). It has been argued that DF oligosaccharides should be included in the CODEX definition, since they are not digested in the small intestine and have known health benefits, and since their exclusion leads to different definitions of DF (Jones 2014). In the European Union, oligosaccharides are included in the DF definition.

Dietary fiber comprises cell wall and non-cell wall polysaccharides and oligosaccharides of plant or algae origin (Dhingra *et al.* 2012; Mudgil & Barak 2013). Most importantly, these include cellulose, hemicelluloses (*e.g.*, arabinoxylan (AX) and β-glucan (BG)), pectin, resistant starch, fructo-oligosaccharides, galacto-olicosaccharides, modified cellulose, and polydextrose (Dhingra *et al.* 2012; Mudgil & Barak 2013). Moreover, lignin, a polymer containing oxygenated phenylpropane units, is defined as DF even though it is not a polysaccharide (Dhingra *et al.* 2012). The main dietary

sources of DF are WG products, fruits, vegetables, legumes, nuts, and seeds. In several European countries and the USA, grains are the largest source of DF (Stephen *et al.* 2017).

Dietary fiber varies structurally, consisting of different monomers (*e.g.*, glucose, arabinose, xylose, and mannose) with varying degrees of polymerization and molecule branching (Mudgil & Barak 2013). Moreover, DF differs in physiochemical properties, such as water solubility, water-holding capacity, viscosity, fermentability, and bulking ability. In terms of DF health effects, fermentability is a key property. As DF is not digested or absorbed in the small intestine by the host, it can be fermented by gut microbiota. Fermentability differs between DF types and is affected mainly by molecular structure and solubility (Williams *et al.* 2019). In most cases, soluble DF is more readily fermentable than insoluble DF, but several forms of insoluble DF can be fermented to some extent. The main DF types in cereal grain, their structural components, and selected properties are presented in Table 1.

Table 1. Main types of dietary fiber in cereal grains, their structural components, and selected properties (adapted from Stephen et al. 2017; Williams et al. 2019).

DF	Structural components	Solubility	Viscosity	Fermentability
Cellulose	β-(1,4) glucose	Insoluble	None	Partial
Arabinoxylan	Xylose, arabinose	Partly soluble	High[1]	High[1]
β-glucan	β-(1,3) and β-(1,4) glucose[2]	Mostly soluble	High	High
Fructan	D-fructose residues	Soluble	Low	High
Resistant starch	Amylose, amylopectin	Insoluble	None	Partial
Lignin	Phenylpropane	Insoluble	None	None

[1]Soluble arabinoxylan. [2]Mixed linkages.

In the human gastrointestinal (GI) tract, soluble and viscous DF increases the viscosity of the intestinal contents and reduces glycemic response and cholesterol absorption (Mudgil & Barak 2013). Insoluble, non-fermentable DF increases fecal bulk and decreases intestinal transit, including gastric emptying (Mudgil & Barak 2013). Fermentable DF can also contribute to fecal bulk through increased bacterial mass resulting from bacterial growth

(Binns 2013). The health benefits of DF are well established and result from the direct physiological effects of DF, such as cholesterol binding, satiety, and fecal bulking, and from the beneficial effect that DF has on gut microbiota (Dhingra *et al.* 2012).

1.3 Oats and rye

The main focus of this thesis was on the WG cereals oats (*Avena sativa* L.) and rye (*Secale cereale* L.), with emphasis on the latter. Oats and rye differ in terms of DF composition and certain phytochemicals, *e.g.*, WG rye contains a high amount of DF, consisting of AX, fructan, BG, cellulose, and lignin (Jonsson *et al.* 2018). Arabinoxylan is the main DF in rye, which contains both soluble and insoluble AX. Among different cereals, rye has the highest content of fructan (Karppinen *et al.* 2003). Oat DF consists mainly of BG, AX, and cellulose, and contains more soluble than insoluble DF (Manthey *et al.* 1999). Oat BG has high solubility and high molecular weight, resulting in high viscosity (Wood 2010). Oats contain the phytochemicals avenanthramides, saponins, flavonoids, lignans, and phenolic acids, while rye is rich in alkylresorcinols (AR), benzoxazinoids, betaines, lignans, and phenolic acids (Koistinen & Hanhineva 2017).

Several health benefits have been associated with both oat and rye consumption, resulting from the physiological effects of DF, micronutrients, and phytochemicals present in these cereals. Rye intake has consistently been associated with beneficial effects on insulin metabolism, satiety, weight management, inflammation, and blood lipids (Jonsson *et al.* 2018). Interestingly, rye bread has been shown to induce lower insulin response compared with other WG products, without affecting the glucose response (so-called 'rye factor') (Iversen *et al.* 2022b). Oats have proven health effects on blood glucose regulation, blood cholesterol levels, and satiety, effects mainly connected to properties of BG (Paudel *et al.* 2021).

1.4 Gut microbiota

The human gut microbiota is a collection of trillions of microorganisms (bacteria, viruses, archaea, and eukarya) that inhabit the GI tract, mainly the colon (Thursby & Juge 2017). During the past two decades, research on the role of gut microbiota and human health has increased rapidly, and gut

microbiota has been linked to all common non-communicable diseases, such as obesity, T2D, cancer, cardiovascular disease, and allergy (Butler *et al.* 2019). Moreover, emerging knowledge about the gut-brain axis (GBA) has highlighted the role of gut microbiota in mental health, and in neurodegenerative diseases and psychological disorders.

Gut microbiota has several physiological functions in the host, such as regulation of the immune system, protection against pathogens, and providing energy and vitamins (Thursby & Juge 2017). Gut microbiota develops during infancy and early childhood, and its composition is shaped by genetics, maternal microbiota, birth mode, breastfeeding, antibiotic use, diet, and other environmental factors (Rodríguez *et al.* 2015). After establishment, microbiota composition remains relatively stable throughout adult life but can be altered by aging, infections, pharmaceutical usage, long-term or drastic dietary changes, and other lifestyle factors (Rodríguez *et al.* 2015). An overview of the factors affecting gut microbiota composition is provided in Figure 2. There is a great inter-individual variation in gut microbiota composition, but the factors governing this variation are not well understood (Gilbert *et al.* 2018). High microbial richness and diversity are generally considered to be characteristics of a healthy microbiota (Rinninella *et al.* 2019). Alpha diversity is often used in the context of gut microbiota, where it refers to the number of species, *i.e.*, species richness, within the gut community.

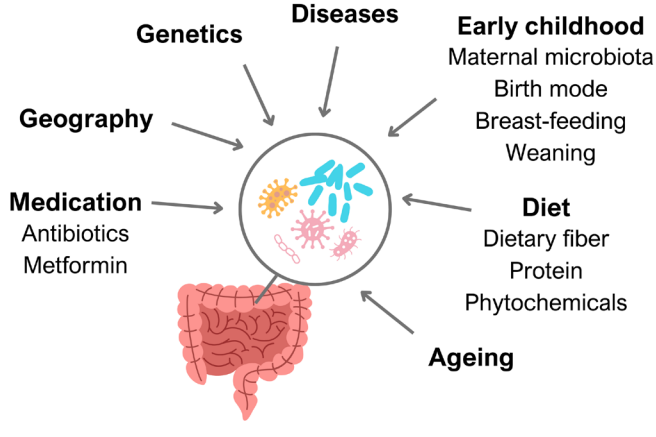

Figure 2. Key factors affecting gut microbiota composition (adapted from Rodríguez *et al.* 2015).

As gut microbiota is very complex, efforts have been made to group the human population based on microbiota composition, in order to understand the connection between microbiota and diet and health. The concept of enterotypes refers to three different types of microbiome community clusters in the human gut, driven by different bacterial genera (*Prevotella*, *Bacteroides*, or *Ruminococcus*) (Arumugam *et al.* 2011). Enterotypes are suggested to be complex and not explained by other properties, such as age, gender, body mass index (BMI), or geography. The enterotypes concept has attracted interest but also criticism since being proposed. Numerous studies have confirmed the findings, but identification of enterotypes depends on data structure and clustering method, and thus enterotyping method should be standardized (Koren *et al.* 2013; Costea *et al.* 2018). Moreover, the *Prevotella* and *Bacteroides* enterotypes seem to be more consistent than the *Ruminococcus* enterotype. The enterotypes have been linked to long-term diet (Wu *et al.* 2011), and different DF fermentation patterns have been observed between the enterotypes in previous *in vitro* studies (Chen *et al.* 2017; Wu *et al.* 2017; Yu *et al.* 2021).

Diet can have a major effect on gut microbiota composition and function through direct and indirect means (Zmora *et al.* 2019). Nutrients can directly affect the growth of microbes, with differences in the capability to extract energy from varying nutrient sources favoring microbes with suitable metabolic abilities. Moreover, microbial cross-feeding, *i.e.*, use of microbiota-derived compounds between different microbes, can shape microbiota composition, as members of gut microbiota interact with, and affect, the growth of other microbes (Culp & Goodman 2023). Diet can also affect host metabolism and the immune system which, in turn, affects microbiota indirectly (Zmora *et al.* 2019). Probiotics, defined as living microbes that confer documented health benefits to the host, and prebiotics, selectively fermented ingredients that result in specific changes in microbiota composition or function and confer health benefits to the host, are also dietary factors that affect gut microbiota directly and indirectly (Binns 2013). Probiotics are often specific microbial strains consumed as supplements or added to food products.

Gut microbiota can metabolize nutrients and other food-derived compounds that are not absorbed by the host, producing a wide variety of metabolites, many of which have physiological and health effects on the host. Dietary fiber is the most important energy source for gut microbiota, and its

interplay with microbiota will be discussed later in this thesis. Fermentation of DF produces short-chain fatty acids (SCFA) that have various effects on the host. Unabsorbed protein following excess protein intake or intake of plant proteins with lower digestibility can also be metabolized by gut microbiota in the colon (Wu *et al.* 2022). Moreover, only a small proportion of food phytochemicals is absorbed in the small intestine and can thus be metabolized by gut microbiota (Roager & Dragsted 2019). Examples of metabolites formed by gut microbiota, besides SCFAs, include branched-chain fatty acids, indole and indole derivates, secondary bile acids, trimethylamine and trimethylamine-N-oxide, gas, hippuric acid, and enterolactone (Roager & Dragsted 2019; Feng *et al.* 2022).

1.5 The interplay between dietary fiber and gut microbiota

Fermentation of DF by gut bacteria generates SCFAs, and DF is the major substrate for SCFAs produced by microbiota. Acetate, propionate, and butyrate are the main SCFAs produced during DF fermentation by gut bacteria. Some SCFAs, predominantly butyrate, serve as an energy source for colonocytes and other gut epithelial cells (O'Riordan *et al.* 2022). The remaining SCFAs are transported to the portal circulation and minor fractions reach the systemic circulation, while acetate and propionate are used as energy sources in peripheral tissues (O'Riordan *et al.* 2022). Besides being an energy source, SCFAs can regulate *e.g.*, satiety, insulin secretion, and body weight (Anachad *et al.* 2023). They are also considered key microbial metabolites in the gut-brain axis, as discussed later.

As mentioned in connection with DF, fermentability varies between different DF types. Moreover, DF utilization by gut microbiota varies depending on microbial ability to cleave and break down molecular linkages in DF to obtain simple sugars that can be used as fermentation substrates (Hamaker & Tuncil 2014). This ability depends on the genetic properties of a microbe to produce specific carbohydrate-active enzymes for cleavage and linkage, carbohydrate-binding proteins, and transporters. Some microbes can utilize only a few different DFs and are so-called specialists, whereas generalists can break down several DF structures (Hamaker & Tuncil 2014). Presence of different DFs thus gives a competitive advantage to microbes with suitable metabolic ability, and DF has been shown to change gut

microbiota composition, at least to some extent (Hamaker & Tuncil 2014; Simpson & Campbell 2015). DF interventions, especially involving fructan and galacto-oligosaccharides, have been shown to increase the abundance of *Bifidobacterium* and *Lactobacillus* spp., but not to affect the alpha diversity of gut microbiota (So *et al.* 2018).

The capability to form SCFAs also differs between bacteria. The SCFA-producing bacteria most commonly found in the human gut include *e.g.*, the genera *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, *Lactocaseibacillus*, *Ruminococcus*, *Blautia*, *Bacteroides*, *Roseburia*, *Prevotella*, *Eubacterium*, *Faecalibacterium*, *Enterococcus*, *Clostridium*, and *Coprococcus* (O'Riordan *et al.* 2022). Most anaerobic bacteria can produce acetate, but only certain bacteria can produce butyrate and propionate (Louis & Flint 2017). Examples of bacterial species found in fecal microbiota that produce butyrate from carbohydrates include *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii*, and *Subdoligranulum variabile*, whereas propionate is produced by *e.g.*, *Bacteroides uniformis*, *Prevotella copri*, *Alistipes putredinis*, and *Akkermansia muciniphila*. Moreover, the gut environment, such as pH, intestinal gases, and available micronutrients, affect butyrate and propionate production (Louis & Flint 2017).

1.6 The gut brain-axis

The GBA is a bi-directional communication system between the brain and the gut, including neural, endocrine (hormonal), and immune pathways (Mayer *et al.* 2022). Gut microbiota, the gut-associated immune system, the autonomic nervous system (ANS), the enteric nervous system (ENS), and the enteroendocrine system are included in the GBA. Moreover, the hypothalamic-pituitary-adrenal axis (HPA-axis) is part of the GBA. The key communication pathways of the GBA are shown in Figure 3. So far, most existing knowledge about modulation of the GBA derives from animal studies or *in vitro* models.

Gut microbiota can signal with the central nervous system (CNS) both directly and indirectly. Direct signaling occurs via microbiota-derived circulating signaling molecules, such as SCFAs, lipopolysaccharides (LPS), and tryptophan metabolites (Fung *et al.* 2017; Mayer *et al.* 2022). Microbiota can also produce and regulate the production of the neurotransmitters serotonin, gamma-aminobutyric acid, dopamine, and norepinephrine.

Indirect signaling occurs by microbiota interaction with intestinal cells, such as enteroendocrine cells and mucosal immune cells, which release signaling molecules such as cytokines and hormones that can interact with the receptors of vagal and spinal afferent neurons, leading to ANS and CNS signaling. In turn, the CNS modulates the sympathetic and parasympathetic branches of the ANS and regulates HPA-axis activation. This affects GI functions, including gut environment and intestinal permeability, stress responses, and the immune system (Fung *et al.* 2017; Mayer *et al.* 2022).

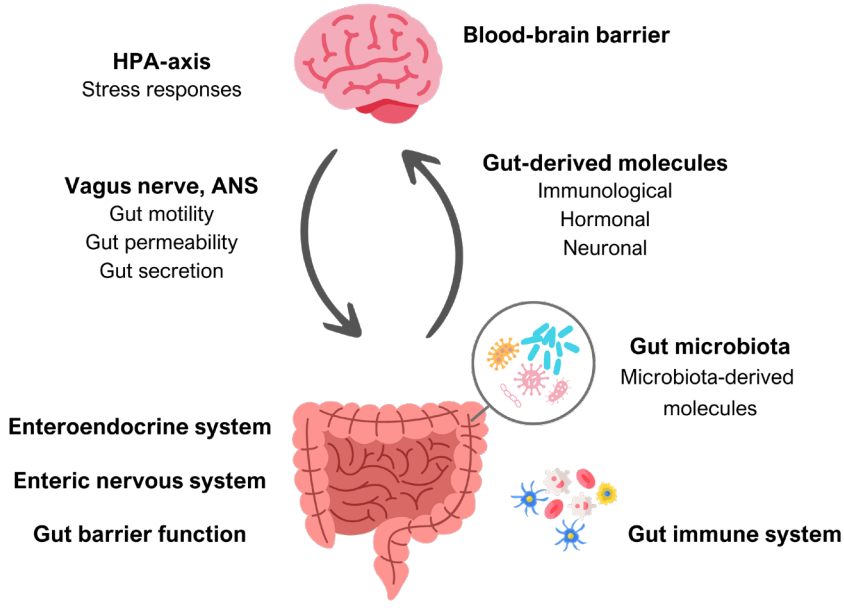

Figure 3. Key communication pathways in the gut-brain axis (adapted from Mayer *et al.* 2022) (ANS, autonomic nervous system; HPA-axis, hypothalamic-pituitary-adrenal axis).

Intestinal permeability is regulated by the gut barrier function. The gut barrier maintains the balance between selective permeability to nutrients and water from the intestinal lumen to the systemic circulation, and protection from pathogens and harmful compounds (Di Vincenzo *et al.* 2023). The integrity of the gut epithelium is supported by junctional proteins, such as tight junctions and desmosomes, that form a physical barrier and connect adjacent epithelial cells, together with the lamina propria. Gut barrier

function is influenced by both gut microbiota and the activity of intercellular connections, which are regulated by hormones, nutrients, inflammatory mediators, and the ENS. Disrupted gut barrier function can lead to the release of bacterial metabolites and endotoxins, such as LPS, into circulation, which in turn will result in immune system activation (Di Vincenzo *et al.* 2023).

The HPA-axis is a collection of structures that mediate the stress response (Smith & Vale 2006). Activation of the HPA-axis is a strictly controlled process that involves several neuronal and endocrine systems. Stress stimulates the release of corticotrophin-releasing hormone from the hypothalamus, which in turn stimulates the release of adrenocorticotrophic hormone into the circulation from the anterior pituitary gland. In the adrenal gland, adrenocorticotrophic hormone stimulates the synthesis and secretion of cortisol, a steroid hormone that regulates metabolic, cardiovascular, immunological, and behavioral processes. Cortisol also regulates the duration and magnitude of HPA-axis activation, through negative feedback (Smith & Vale 2006). Both physical and psychological stress can disrupt gut barrier function, possibly via corticotrophin-releasing hormone and cortisol (Vanuytsel *et al.* 2014; Varanoske *et al.* 2022). Stress can also cause blood-brain barrier dysfunction that impairs cognitive performance and mood state, and the effect may be enhanced by a simultaneous increase in intestinal permeability (Varanoske *et al.* 2022).

Alterations in the microbiota-GBA have been linked to several conditions, such as irritable bowel syndrome (Shaikh *et al.* 2023), clinical depression (Irum *et al.* 2023), and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Khatoon *et al.* 2023). The gut-brain axis is also relevant in the context of understanding how cognitive functions are affected by dietary factors. There is interest in assessing how cognition is affected by nutrition from early neurodevelopment to cognitive decline caused by neurodegeneration, and how nutrition can improve cognitive performance in the normal range of cognition (de Jager *et al.* 2014). Cognitive functions include several domains, such as executive function, memory, learning, attention, perception, and decision-making. Cognitive tests can assess global or domain-specific cognitive performance, but there are differences between available measures of specific cognitive functions with respect to their sensitivity to measuring nutrition-induced changes (de Jager *et al.* 2014).

1.7 Diet-derived microbial metabolites and the gut-brain axis

Several microbiota-derived metabolites formed from dietary compounds can participate in gut-brain communication, but SCFAs are considered to be the key microbial metabolites in the GBA (Dalile *et al.* 2019; O'Riordan *et al.* 2022). Various other diet-derived metabolites, such as serotonin, indole, and indole derivates also play a role in the GBA, as they can act as neurotransmitters and neuromodulators, and can affect *e.g.*, neurodevelopment, neuroinflammation, and blood-brain barrier integrity (Ahmed *et al.* 2022). The most relevant metabolite groups in the context of this thesis are discussed below.

1.7.1 Short-chain fatty acids

A vast body of evidence highlights the role of SCFAs in the GBA, and SCFAs have been associated with *e.g.*, GI function and intestinal permeability, gut hormone secretion, and immunoregulation (Dalile *et al.* 2019; O'Riordan *et al.* 2022). Moreover, SCFAs have been shown to directly modulate the levels of neurotransmitters and neurotrophic factors and to modulate the HPA-axis. Abnormal fecal SCFA levels have been linked to several disorders with altered brain physiology and neurodegenerative diseases, such as Parkinson's disease, autism spectrum disorder, chronic stress, and depression, in both clinical and pre-clinical studies (O'Riordan *et al.* 2022).

Short-chain fatty acids can interact with the GBA signaling pathways through several immune, endocrine, neural, and humoral routes (Dalile *et al.* 2019). In addition, SCFAs can activate several G-protein coupled receptors, of which two free fatty acid receptors (FFAR2 and FFAR3) are the most studied (Dalile *et al.* 2019). These receptors are expressed in several different cells, such as enteroendocrine cells, immune cells, and several cellular systems, *e.g.*, the colon and sympathetic nervous system. Some SCFA receptors have also been found in the peripheral neurons and the CNS. Moreover, SCFAs have been shown to cross the blood-brain barrier and modulate its integrity (O'Riordan *et al.* 2022). However, uptake of SCFAs into the brain appears to be minimal (Dalile *et al.* 2019), indicating that the impact of SCFAs on the brain is mediated through different signaling pathways, rather than directly.

Short-chain fatty acids can influence the secretion of the gut hormones glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), which regulate appetite and food intake (O'Riordan *et al.* 2022). In addition, SCFAs can influence the metabolic hormones leptin, ghrelin, and insulin (Dalile *et al.* 2019). They can also influence gene expression through inhibition of histone deacetylases, although the evidence is mainly preclinical (Dalile *et al.* 2019). Histone deacetylases are involved *e.g.*, in brain development and several neuropsychological diseases.

Short-chain fatty acids also regulate local and systemic immune responses and inflammation (Rooks & Garrett 2016). For example, they can inhibit pro-inflammatory activity and regulate T-cell-related immunity. In addition, SCFAs maintain gut immunity by enhancing intestinal barrier function (Rooks & Garrett 2016). In a recent animal study, high intake of pectin was shown to decrease hippocampal levels of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interferon gamma (IFN-γ), and to increase the levels of brain-derived neurotrophic factor (BDNF) (Church *et al.* 2023). Moreover, acetate was observed to be a strong mediator of high pectin content in the diet in increasing BDNF production.

Butyrate is a SCFA of specific interest, as it can play a key role in microbiota-host cross-talk. Butyrate can also regulate the immune system and the release of serotonin and gut hormones in the ENS, which stimulates the vagus nerve, regulates hormonal signaling, and can further affect the CNS (Stilling *et al.* 2016). In addition, butyrate can regulate tight junction protein expression, and thereby regulate intestinal permeability (Dalile *et al.* 2019).

1.7.2 Tryptophan metabolites

As mentioned earlier, unabsorbed proteins and amino acids can reach the colon and be metabolized by gut microbiota. The essential amino acid tryptophan can be metabolized by several gut microbes into indole compounds such as indole, indolepropionic acid, indoleacetic acid, and indolealdehyde (Roager & Licht 2018). Indole compounds have been shown *e.g.*, to suppress pro-inflammatory activity by binding on the transcription factor aryl hydrocarbon receptors in immune cell astrocytes, to decrease intestinal permeability, to regulate secretion of gastrointestinal hormones and gut motility, and possibly to modulate gut microbiota composition (Roager

& Licht 2018). For example, 3-indolepropionic acid has been shown to decrease neuroinflammation in preclinical and clinical studies, improve cognitive functions and neuronal energy metabolism, and decrease neuronal apoptosis and oxidative stress in preclinical studies (Ahmed *et al.* 2022). Additionally, 3-indoleacetic acid has been shown to have potential anti-depressive effects and to attenuate HPA-axis hyperactivity and increase BDNF expression in a preclinical study (Chen *et al.* 2022).

Tryptophan also acts as a substrate for biosynthesis of serotonin (5-hydroxytryptamine), a key signaling molecule in the ENS and the CNS (O'Mahony *et al.* 2015). The majority of serotonin is found in the gut, where it is synthesized in the enterochromaffin cells of the host. There is also some evidence that certain bacteria can produce serotonin from tryptophan. Moreover, gut microbiota can regulate tryptophan metabolism and serotonin biosynthesis (O'Mahony *et al.* 2015).

1.7.3 Phytochemical metabolites

Phytochemicals are bioactive compounds present in all plant-based foods, and WG is an important source of phytochemicals in the diet (Koistinen & Hanhineva 2017). Some phytochemicals can be metabolized by gut microbiota and may affect microbiota composition and further influence SCFA production (Catalkaya *et al.* 2020). However, knowledge of their role in the GBA is limited.

Only small proportions of food polyphenols, a group of phytochemicals found *e.g.*, in WG, vegetables, and fruit, are absorbed in the small intestine, and thus gut microbiota can metabolize them into compounds contributing to potential health effects (Roager & Dragsted 2019). Bioavailability of polyphenol metabolites may also be higher than that of their precursor molecules (Catalkaya *et al.* 2020). The benzoic acid metabolite hippuric acid has been positively associated with microbiota diversity and metabolic benefits (Roager & Dragsted 2019). Hippuric acid has also been linked to fetal neurodevelopment (Ahmed *et al.* 2022). Ferulic acid, a phenolic acid found in all cereals (Koistinen & Hanhineva 2017), has been linked to neuroprotection in preclinical studies (Ahmed *et al.* 2022). A metabolite of ferulic acid, dihydrocaffeic acid (Koistinen *et al.* 2017), has been associated with lower production of pro-inflammatory cytokine IL-6 in an animal model (Wang *et al.* 2018). Moreover, the ferulic acid metabolite dihydroferulic acid can be metabolized further to 3-(3-hydroxyphenyl)propionic acid (hMPP)

(Koistinen *et al.* 2017), which has been shown to interfere with β-amyloid aggregation in a preclinical model of Alzheimer's disease (Wang *et al.* 2015).

1.8 Whole grain and the gut-brain axis

Whole grain cereals are rich in DF, micronutrients, and phytochemicals, and thus have several potential mechanisms by which they can affect gut microbiota and the GBA. Besides the effects on gut microbiota and SCFA production, DF can affect the brain independently from microbiota-related mechanisms through modulation of the immune system, and by lowering blood cholesterol levels and blood pressure (La Torre *et al.* 2021). Moreover, vitamins and minerals present in WG have been shown to have direct effects on cognition and psychology (Tardy *et al.* 2020). So far, only a few studies have investigated the effect of DF on cognition, while most studies have focused on DF intake and mental health (Berding *et al.*). Moreover, current evidence of the effects of WG on cognition and mood is limited, with some evidence to suggest a positive effect of WG on mood and anxiety disorders but inconclusive findings on the overall effect on cognition (Ross *et al.* 2023).

The SCFA-promoting effect of cereal DF supplementation has been associated especially with wheat and rye arabinoxylan oligosaccharides (Bai *et al.* 2021). A number of intervention studies have demonstrated that WG rye intake can increase SCFA levels or alter fecal microbiota composition (Table 2). However, such effects have not been observed in other studies. Increased levels of the gut hormone PYY have been observed following a rye evening meal (Sandberg *et al.* 2016; Sandberg *et al.* 2017; Sandberg *et al.* 2018). However, no effects on cognition or plasma BDNF levels were observed following a rye evening meal in the latter study (Sandberg *et al.* 2018). Another study testing the effects of rye evening meals found that *Prevotella* was positively associated with plasma BDNF levels (Prykhodko *et al.* 2018). The SCFA- and in particular butyrate-promoting effect of rye might be associated with the high amount of fermentable AX (Knudsen & Lærke 2010).

Table 2. Summary of findings in human intervention studies on the effect of rye intake on short-chain fatty acid (SCFA) levels or fecal microbiota. (d, day; MeS, metabolic syndrome; w, week; WW, white (refined) wheat).

Study design	Study subjects	Diet	Effect on SCFAs	Effect on microbiota	Reference
Parallel, 12-w weight-loss intervention	Overweight and obese adults (n=207)	WG rye vs WW, energy standardized amount of different products	↑ Plasma butyrate ↓ Plasma acetate	↑ *Agathobacter,* UCG-003, *Haemophilus* ↓ *Ruminococcus torques*, *Eubacterium ventriosum*, *Anaerofilum,* H*oldemania*	Iversen *et al.* 2022a
Parallel, 12-w intervention	Adults with *Helicobacter pylori* infection (n=84)	Fermented rye bran vs. WW, different products	Higher acetic acid vs RF at w 12; intervention effect not reported	↑ *Romboutsia* ↓ *Bilophila*	Liu *et al.* 2021
Cross-over, 8-w intervention periods	Adult men with MeS risk (n=40)	WG rye bread with 4-w lignan supplement vs. WG wheat bread	↑ Fecal butyrate	↑ *Bifidobacterium* ↓ *Clostridium*	Eriksen *et al.* 2020
Cross-over, single-day, and 3-d consecutive day trials	Healthy, lean adults (n=19)	Rye kernel bread vs. WW bread evening meals	↑ Plasma total SCFA, acetate, propionate, and butyrate	↑ P*revotella, Faecalibacterium* ↓ *Bacteroides*	Prykhodko *et al.* 2018; Sandberg *et al.* 2016

Cross-over, 3-d intervention periods	Healthy, normal/slightly overweight adults (n=38)	Rye kernel bread supplemented with resistant starch vs WW bread on 3 meals/d	↑ Plasma total SCFA, acetate, and butyrate	n.a.	Sandberg *et al.* 2018
Parallel, 6-w	Healthy, normal/overweight adults (n=70)	WG rye vs. WG wheat vs. WW, different products ad libitum	Fecal butyrate differed from WW (both ↓)	No effect	Vuholm *et al.* 2017
Cross-over, 4-w intervention periods	Healthy, normal weight, overweight and obese adults (n=21)	WG rye bread vs. WW bread containing processed rye bran;	↑ Plasma propionate and butyrate vs. WW	n.a.	Lappi *et al.* 2014
Parallel, 12-w	Adult MeS patients (n=51)	High DF rye vs. WW bread	n.a.	No effect	Lappi *et al.* 2013
Cross-over, 4-w intervention periods	Overweight adult men (n=28)	High DF rye vs. high DF wheat vs. low DF foods	↑ Fecal butyrate	n.a.	McIntosh *et al.* 2003

1.9 Summary

Whole grain is rich in DF and other nutrients and phytochemicals that can be metabolized by the gut microbiota, and it can affect the brain by interacting with the GBA or by non-microbiota-related routes (Figure 4). Fermentation of DF produces SCFAs, which are speculated to be key microbiota-derived metabolites in the GBA. However, microbiota composition affects DF breakdown and production of metabolites, as different microbes have different metabolic abilities. Conversely, diet affects gut microbiota composition by providing a competitive advantage to microbes that have the genetic ability to metabolize the dietary compounds provided. So far, there is limited evidence of WG effects on gut microbiota composition and functioning, and on the GBA, besides those of DF. Whole grain rye is interesting in the context of the GBA, due to its potential butyrate-promoting effect, but no comprehensive studies on the long-term effects of rye on the GBA have been published to date.

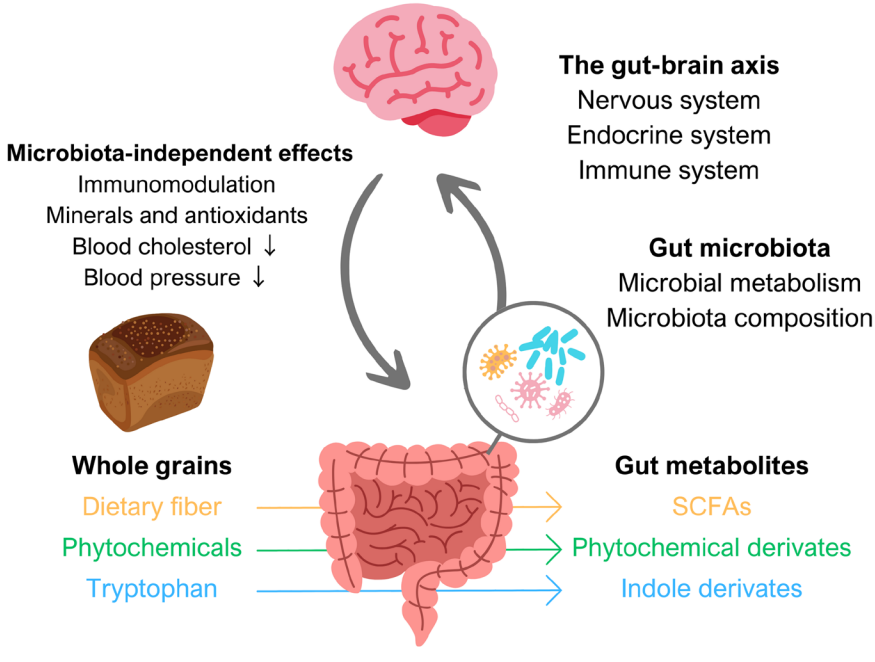

Figure 4. Potential effects of whole grain consumption on the brain through the gut-brain axis and *via* non-microbiota-related routes suggested in this thesis (SCFAs, short-chain fatty acids).

2. Aims

The overall aim of this thesis was to explore the interplay between DF, gut microbiota, and microbiota-derived metabolites during *in vitro* fermentation of different breads, and to study the potential beneficial effects of WG rye bread on the microbiota-gut-brain axis.

Specific objectives were to:

- Investigate the effect of two different fecal microbiota compositions on *in vitro* fecal fermentation of WG oat, WG rye, and refined bread in terms of DF utilization, SCFA levels, and changes in the microbiota composition (Paper I).

- Examine the effects of *in vitro* fecal fermentation and microbiota composition on the metabolite profile and the fate of precursor molecules present in WG oat, WG rye, and refined bread (Paper II).

- Study the effects of WG rye bread on gut microbiota composition and associated SCFAs, and its impact on GBA markers, stress responses, and cognitive performance in a dietary intervention study with healthy subjects (Paper III).

3. Overview of methods

An overview of the experimental settings used in this thesis is given below. Detailed descriptions of materials and methods can be found in Papers I-III.

3.1 *In vitro* fermentation study

Gut fermentation of WG oat, WG rye, and refined bread was studied using an *in vitro* model (Papers I and II). The *in vitro* model included fermentation substrate derived from three breads, and fecal material obtained from two donors on separate occasions. Four 24-hour fermentation experiments, two per donor, resulted in four replicates of each donor-substrate combination. The study outline is presented in Figure 5.

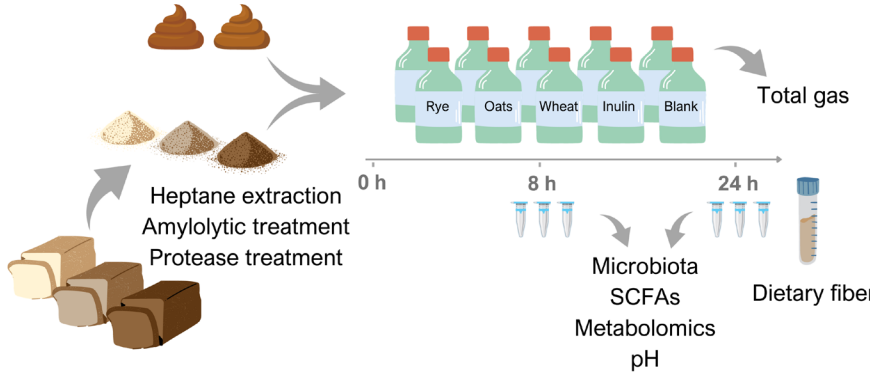

Figure 5. Outline of the *in vitro* fermentation study (Papers I and II). In total, four 24-hour experiments were conducted (SCFAs, short-chain fatty acids).

3.1.1 Bread samples and fermentation substrate preparation

A commercial WG oat bread, a commercial WG rye bread, and a refined wheat bread containing oat endosperm flour (25% of flour) were used in the fermentation experiments (the last is referred to hereafter as wheat bread). To simulate upper GI tract digestion, all breads were pre-treated to prepare fermentation substrate. Lipids were removed with heptane extraction, available starch was removed with amylolytic treatment, and protein was removed with the Savinase enzyme. Fructan was extracted with ethanol and restored to the substrate. The nutritional composition of the breads and derived substrates, as analyzed before the experiments, is shown in Table 3. Fermentation substrate preparation increased the proportional amount of DF in all samples, and over 80% of the DF in bread was recovered. Starch was almost completely removed from the samples and the amounts of protein and lipids decreased during the process, but they were not completely removed.

Table 3. Nutritional content (% of dry matter) of breads and fermentation substrates derived from the breads used in Paper & II, analyzed in duplicate samples (modified from Paper I).

	Bread			**Substrate**		
Nutrient	Oats	Rye	Wheat	Oats	Rye	Wheat
Protein	14.8	10.5	11.9	28.5	19.6	35.2
Lipids	14.0	1.9	3.4	7.9	1.1	5.4
Starch	35.8	47.7	57.9	0.3	0.3	0.1
Sugars total	4.9	1.7	2.9	13.3	8.0	12.4
Dietary fiber[1]	11.3	17.3	5.2	33.3	46.5	23.8
Insoluble DF	7.0	12.1	3.2	18.3	31.8	13.8
Soluble DF	4.3	5.2	2.0	15.0	14.7	10.0
Fructan	0.1	1.5	0.4	0.2	2.4	1.2

[1]Calculated as the sum of fructan (AOAC Method 999.03) and DF analyzed as described in Theander *et al.* 1995 with modifications (Andersson *et al.* 1999) (modified AOAC Method 994.13).

The amount of fermentation substrate used in the experiments was standardized for all breads, based on the ratio of DF to energy content. The calculated DF content in the actual amount of substrate used was 0.55 g in oats, 1.09 g in rye, and 0.25 g in wheat.

3.1.2 Study subjects

Healthy study subjects (n=10) as prospective donors for the fermentation experiments were recruited and screened according to exclusion and inclusion criteria. The study protocol was approved by the Swedish Ethical Review Authority. Study subjects collected screening fecal samples, which were analyzed using terminal restriction fragment length polymorphism (T-RFLP), and two donors were selected to provide samples for the *in vitro* experiments. Previous studies have reported differences in fermentation capacity between human enterotypes, and thus the relative abundance of *Bacteroides* and *Prevotella* genera in feces was used as a measure to find donors with contrasting microbiota composition. Donors with clear enterotypes were not found, but the two donors selected (Donor I and Donor II) had differing fecal microbiota composition (Figure 6).

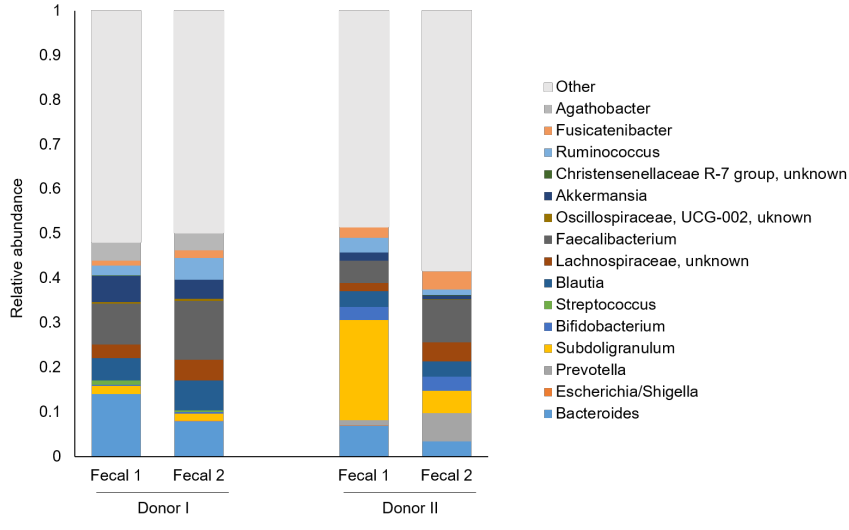

Figure 6. Microbiota composition in fermentation fecal samples analyzed using Illumina 16S rRNA gene sequencing. Samples used in the fermentation experiments are shown separately for the two experiment occasions (Fecal 1, Fecal 2) for Donor I and Donor II. Unknown indicates an unknown bacterial genus (modified from Paper I).

3.1.3 *In vitro* fermentation experiments

The donors collected fecal samples shortly before each experiment. Fecal inoculate for fermentation was produced by mixing a fecal sample with buffer to obtain a 1% (w/v) dispersion. Substrates were mixed in separate

bottles in 50 mL of buffer. In addition, positive control samples (1.00 g of inulin) and samples without substrate (blanks) were included in the experiments. A 50 mL aliquot of fecal inoculate was added to the bottles immediately after filtering. The bottles were then closed and incubated at 37 °C for 24 h with motor stirring.

Gas production was measured throughout the experiment. At 8 h and 24 h, 5 mL of liquid were collected from each bottle and divided into aliquots to analyze microbiota composition, SCFA concentrations, and metabolites. In addition, pH was measured. After 24 h of fermentation and sample collection, the fermentation residue material was centrifuged, the supernatant liquid was separated from the pellet, and both fractions were autoclaved and freeze-dried. All samples were stored at -20 °C until analysis.

3.1.4 Analysis of fermentation samples

Microbiota composition was analyzed using Illumina 16S rRNA gene sequencing. Data on relative abundance on genus level, with a cut-off value of 0.9%, were used for the analysis. Concentrations of SCFAs were analyzed as described previously (Uden & Sjaunja 2009). Content and composition of DF were analyzed from fermentation residue material. The pellet was analyzed to estimate insoluble DF and the supernatant was analyzed to estimate soluble DF. To estimate DF utilization during fermentation, the amount of each insoluble and soluble sugar residue in fermentation samples was calculated as a percentage of total sugar residues (sum of insoluble and soluble) in the substrate.

3.1.5 Metabolomics analysis

Metabolomics, *i.e.* large-scale study of metabolites, is one of the key approaches to identifying small molecules produced by gut microbiota. In Paper II, non-targeted metabolomics analysis of the breads, fermentation substrates, and *in vitro* fermentation samples was performed using ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). In brief, metabolites were extracted with methanol, filtered, and analyzed by LC-MS as described previously (Klåvus *et al.* 2020). Peak picking was performed on MS-DIAL (Tsugawa *et al.* 2015). Molecular features were retained only if they met the defined quality metrics. Drift correction was applied to the data based on signal intensities in the quality control samples. In non-targeted

metabolomics, global metabolite profiling is performed to identify the metabolites that discriminate sample groups. The chromatograph and mass spectrogram of significantly different molecular features were compared with entries in an in-house standard library and publicly available databases. The abundance of WG phytochemicals was investigated using a semi-targeted approach.

3.2 Dietary intervention study

A three-week double-blinded, controlled, parallel intervention study was conducted to study the effects of WG rye bread on gut microbiota composition, fecal and blood SCFA levels, gut peptides, BDNF, intestinal permeability, immune responses, stress responses, and cognitive performance in healthy subjects (Paper III).

3.2.1 Study design

The study comprised pre- and post-intervention visits 21 days apart. Sample collection, tests, and questionnaire-based surveys were conducted during the visits at the university or home, both before and after the visits (Figure 7). In the evening before the visit, study subjects consumed a standardized evening meal, and blood samples were collected after overnight fasting.

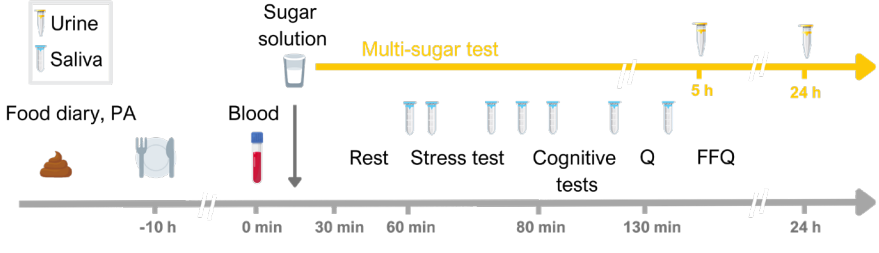

Figure 7. Outline of the intervention visits (FFQ, food frequency questionnaire; PA, physical activity questionnaire; Q, questionnaires).

Subjects were randomized to consume 180 g (six slices) of WG rye bread or control bread daily for three weeks. The nutritional content of these breads is presented in Table 4. Calculated DF intake from 180 g of rye bread was 19.4 g, and 8.8 g from the control bread, respectively.

Table 4. Nutritional content (per 100g of fresh product) of the breads used in the intervention study. Energy, protein, fat, carbohydrates, and salt content were calculated from ingredients, while dietary fiber (DF) content was analyzed.

Nutrient /100g	Rye	Control
Energy (kcal)	230	285
Protein	6.7	9.3
Fat	1.9	2.1
Carbohydrates	41	54
Dietary fiber[1]	10.8	4.9
Insoluble DF	7.4	3.5
Soluble DF	3.4	1.4
Salt	1.2	1.2

[1]Calculated as sum of fructan (AOAC Method 999.03) and DF analyzed as described in Theander *et al.* 1995 with modifications (Andersson *et al.* 1999) (modified AOAC Method 994.13).

The control bread was prepared to resemble WG bread, for blinding purposes (Figure 8). The control bread contained refined wheat flour and oat flour. Both breads were given to study subjects frozen in transparent plastic bags.

Figure 8. (a) Control bread and (b) whole grain rye bread.

3.2.2 Study subjects

Healthy, 18-44 years old study subjects were recruited through advertisement and screened according to exclusion and inclusion criteria. The study protocol was approved by the Swedish Ethical Review Authority. The number of study subjects (n=34) was determined with a power calculation based on a change in salivary cortisol levels. In total, 30 study

subjects completed both visits (male n=24, female n=6; age = 27.8 (±6.4) years; BMI = 24.0 (±2.3) kg/m^2). Data from one study subject in the WG rye group were excluded due to low compliance.

3.2.3 Sample analysis, tests, and questionnaires

Fecal samples were analyzed for microbiota composition using Illumina 16S RNA gene sequencing. Fecal and plasma SCFA concentrations were analyzed using a LC-MS method. Blood samples were analyzed for BDNF, gut hormones GLP-1 (total and active), PYY, and GLP-2, and proinflammatory cytokines IFN-γ, IL-1β, IL-6, and TNF-α, using the immunoassay methods ELISA (enzyme-linked immunoassay) and MSD Multiplex. Alkylresorcinol concentrations were analyzed using a Gas chromatography-mass spectrometry (GC-MS) method. Intestinal permeability was assessed using a multi-sugar urinary recovery test (van Wijck *et al.* 2013), and permeability markers lipopolysaccharide-binding protein (LBP) and CD14 (cluster of differentiation 14) in blood were analyzed with ELISA.

Stress responses were studied using the Maastricht acute stress test (MAST) (Smeets *et al.* 2012) combined with saliva sample collection at seven time points, a visual analog scale questionnaire on perceived stress, and electrocardiogram (ECG) and electrodermal activity (EDA) measurements. The ECG and EDA data were analyzed for heart rate variability (HRV) and skin conductivity measures, to assess the effects of acute stress on ANS. Saliva samples were analyzed for the stress markers cortisol and alpha-amylase, using chemiluminescence immunoassay and the enzyme kinetic method, respectively. Area under the curve (AUC) was calculated for salivary cortisol, alpha-amylase, and perceived stress measures and the AUC values were used for statistical analysis.

Cognitive performance was assessed by measuring cognitive control and working memory. Cognitive control was measured using the Eriksen flanker task (Eriksen & Eriksen 1974), and working memory performance was measured using a modified N-back task (Marklund & Persson 2012) and a recent-probes item-recognition task (Sternberg task, Sternberg 1966).

Dietary intake was measured with a three-day food diary and an online food frequency questionnaire. In addition, physical activity, stool consistency, GI symptoms, long-term perceived stress and anxiety, and well-being were measured with questionnaires.

3.3 Data analysis

3.3.1 Univariate analysis

Linear model and analysis of variance (ANOVA) were used to compare SCFA levels, DF utilization, and pH between substrates and donors, to determine differential metabolic features between the substrates, and to compare the levels of selected metabolites between the donors (Papers I and II). In Paper III, a repeated measure ANOVA with a general linear model was used to assess the effects of the intervention on blood and fecal biomarkers, intestinal permeability, stress responses, cognitive performance, and questionnaire responses between the groups. Analysis of differentially prevalent microbial taxa between and within groups and within-group analysis of biomarkers and questionnaire responses were performed using pairwise testing. Analytical results were adjusted for multiple comparisons with Tukey's HSD in Paper I and with Benjamini-Hochberg's false discovery rate in Papers II and III.

3.3.2 Multivariate analysis

Principal component analysis (PCA) was used to analyze microbiota profiles of the different sample groups in Paper I. In Paper III, multivariate analysis of microbiota data was conducted using permutational multivariate analysis of variance. In Paper II, PCA and t-distributed stochastic neighbor embedding were used to reduce dimensionality in metabolomics data and analyze metabolite profiles of the sample groups.

4. Results and discussion

Effects of WG oat, WG rye, and refined bread on microbiota composition and SCFA levels during *in vitro* fermentation were studied in Paper I, while effects of WG rye bread on microbiota composition and plasma and fecal SCFA levels were studied in a dietary intervention study in Paper III. Dietary fiber utilization during *in vitro* fermentation was also investigated in Paper I, while the effects of the three different breads on metabolic profiles and the fate of precursor molecules during *in vitro* fermentation were investigated in Paper II. The effects of WG rye bread on GBA markers, stress responses, and cognitive performance were studied in Paper III.

The same commercial WG rye bread product was used in all studies. Commercial WG oat bread was used in Papers I and II. The refined bread in Papers I and II contained refined wheat and oat endosperm flour, and was used since it was similar to the control bread containing refined wheat and oat flour used in Paper III.

4.1 Effects of WG bread on microbiota composition

In Paper I, the microbiota profile of the fermentation samples with rye, oat, and wheat substrate and of blank samples was studied using PCA. In the PCA score plot, the microbiota profile was separated for the two donors on the first principal component (Figure 9), demonstrating clear differences in fermentation sample microbiota composition between the donors. The samples collected at the two different time points (8 h and 24 h) were not separated, which indicates that donor had a stronger effect on microbiota profile than time point. Moreover, the blank samples were separated from the samples with substrates, and separation between the donors among the blank samples was observed on the second principal component (Figure 9).

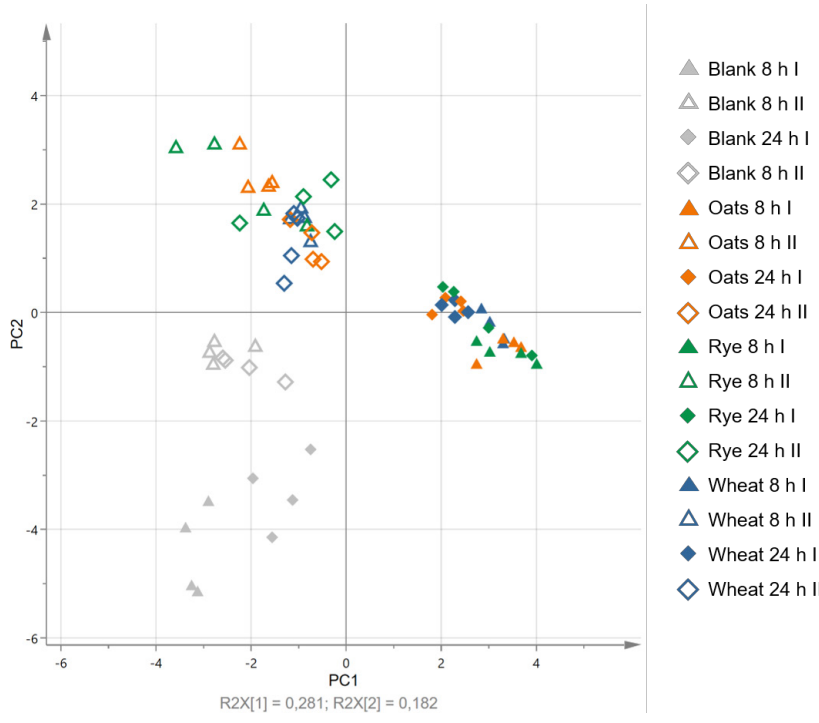

Figure 9. Principal component analysis (PCA) score plot of microbiota composition (the 20 most abundant bacterial genera) in fermentation samples with different substrates (rye, wheat, oat) and blank samples (filled and hollow markers for Donor I and II, respectively) (PC1, the first principal component; PC2, the second principal component) (modified from Paper I).

Shifts in microbiota composition were observed during the 24h *in vitro* fermentation in Paper I. In Donor I fermentation samples, microbiota composition was similar between all three substrates at 8 h and 24 h (Figure 10). The relative abundance of *Bacteroides* increased from 8 h to 24 h, while the relative abundance of genera *Escherichia/Shigella* and *Streptococcus* decreased. The relative abundance of the latter two genera was low in the fecal samples but increased greatly during fermentation. In Donor II, fermentation samples with rye substrate differed from oat and wheat fermentation samples, especially after 24 h of fermentation, with higher relative abundance of genera *Subdoligranulum* and *Bifidobacterium* in the rye samples compared with the other two substrates. Increased abundance of *Subdoligranulum* in connection with rye has not been reported previously, but increased relative abundance of *Bifidobacterium* after rye

intake has been observed previously (Eriksen *et al.* 2020). The Donor II samples with oat and wheat substrates had a higher relative abundance of *Prevotella* at 24 h compared with rye substrates from that donor. Similar, but less pronounced, differences between the substrates were observed already at 8 h. Interestingly, there were no prominent differences in microbiota composition between the three substrates in Donor I, whereas in Donor II rye differed from the other two substrates.

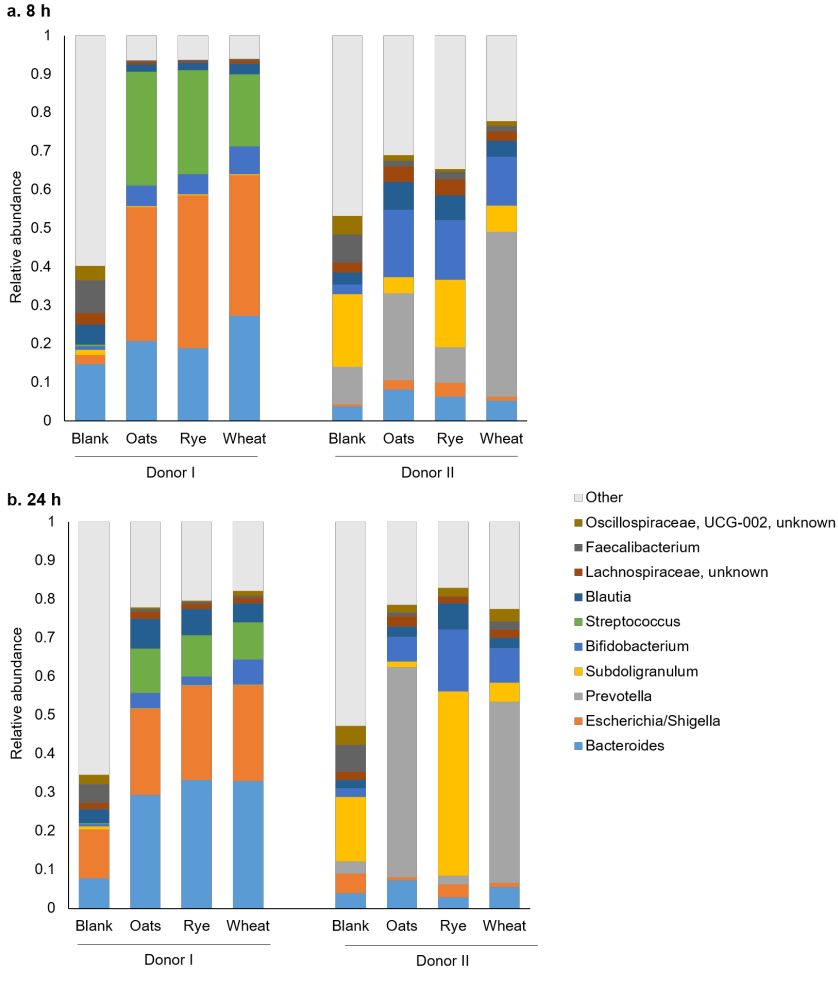

Figure 10. Microbiota composition in the different fermentation samples (blank, oats, wheat, rye) from Donors I and II at (a) 8 h and (b) 24 h. 'Unknown' indicates an unknown bacterial genus (modified from Paper I).

In Paper III, analysis of overall microbiota composition of the samples did not reveal any broad-scale changes after the intervention. No taxa were significantly changed between the intervention groups, but there were some indications that the WG rye bread had a more pronounced effect on the gut microbiota than the control bread. Within the rye group, the abundance of several taxa was significantly different on comparing samples from before and after the intervention. For example, the abundance of *Anaerobutyricum hallii* and *Eubacterium ventriosum* group increased in the rye group (Figure 11). Both taxa are known butyrate producers, and *Anaerobutyricum hallii* can produce propionate (Barcenilla *et al*., 2000; Engels *et al*., 2016). Although the abundance of these taxa increased after rye intake, their relative abundance was low (<1%). Whole grain rye has been reported to alter microbiota composition in previous studies, but other studies have observed no effect on microbiota following rye intake (Lappi *et al*., 2013; Vuholm e*t al*., 2017). Contrary to these findings, a decreased abundance of *Eubacterium ventriosum* has been observed after a 12-week rye intervention (Iversen *et al*., 2022a).

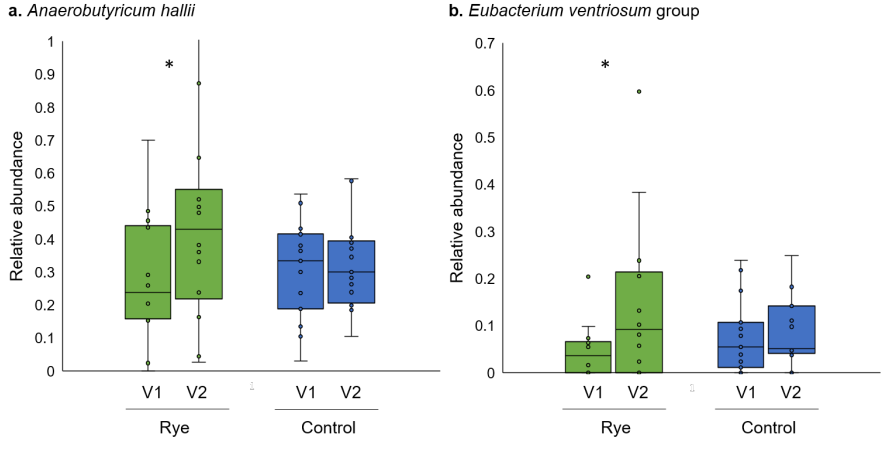

Figure 11. Relative abundance of (a) *Anaerobutyricum hallii* and (b) *Eubacterium ventriosum* group in microbiota before (V1) and after (V2) the three-week intervention (n=29) with high intake of whole grain rye bread (*p<0.01).

Alpha diversity, measured as amplicon sequence variant richness, decreased within the rye group during the intervention, but not between groups or in the control group. Shannon index also displayed a decreasing trend in the rye

group, but no significant difference was observed within or between groups. A meta-analysis published in 2018 concluded that DF interventions do not affect microbial alpha diversity (So *et al.* 2018), but two more recent studies report decreased Shannon index after a DF intervention (Oliver *et al.* 2021; Lancaster *et al.* 2022). Decreased taxa richness or diversity following rye intake has not been reported in previous studies. It is likely that introduction of a large amount of bread into the diet of subjects made the overall diet less varied, affecting microbiota. Moreover, the large increase in DF intake may have caused a loss of microbes lacking the ability to metabolize DF. However, there was no significant change in Shannon index, which indicates that microbial taxa diversity was not affected even though the number of taxa decreased.

In summary, baseline microbiota composition had a strong impact on the microbiota results in both studies. In Paper I, the fecal microbiota composition of the donors had a strong effect on the microbiota composition of the fermentation samples. In Paper III, inter-individual variation had a stronger effect on microbiota composition than the intervention itself. *Subdoligranulum* and *Bifidobacterium* increased in rye samples in one donor in Paper I, but *Subdoligranulum* was not among the observed taxa in donors in Paper III. Butyrate producers *Anaerobutyricum hallii* and *Eubacterium ventriosum* group increased significantly in the rye group in Paper III, but were not among the taxa that were included in the analysis in Paper I as they had low relative abundance in the samples. The relative abundance of these taxa was also low in Paper III, and their clinical significance on plasma or fecal butyrate levels is unclear.

4.2 Effect of WG bread on SCFA levels

In Paper I, the levels of acetate, propionate, and butyrate at 8 h of *in vitro* fermentation were higher in the Donor II samples than the Donor I samples (Figure 12). There were indications that the fermentation process started more slowly in Donor I samples, which can at least partly explain the differences at 8 h. After 24 h, only butyrate levels differed between the donors, where higher levels were observed in the Donor II samples compared with Donor I samples. Whole grain rye in particular contributed to high butyrate levels in Donor II. In the Donor I samples, butyrate levels were similar between WG oats and WG rye at both time points (Figure 12).

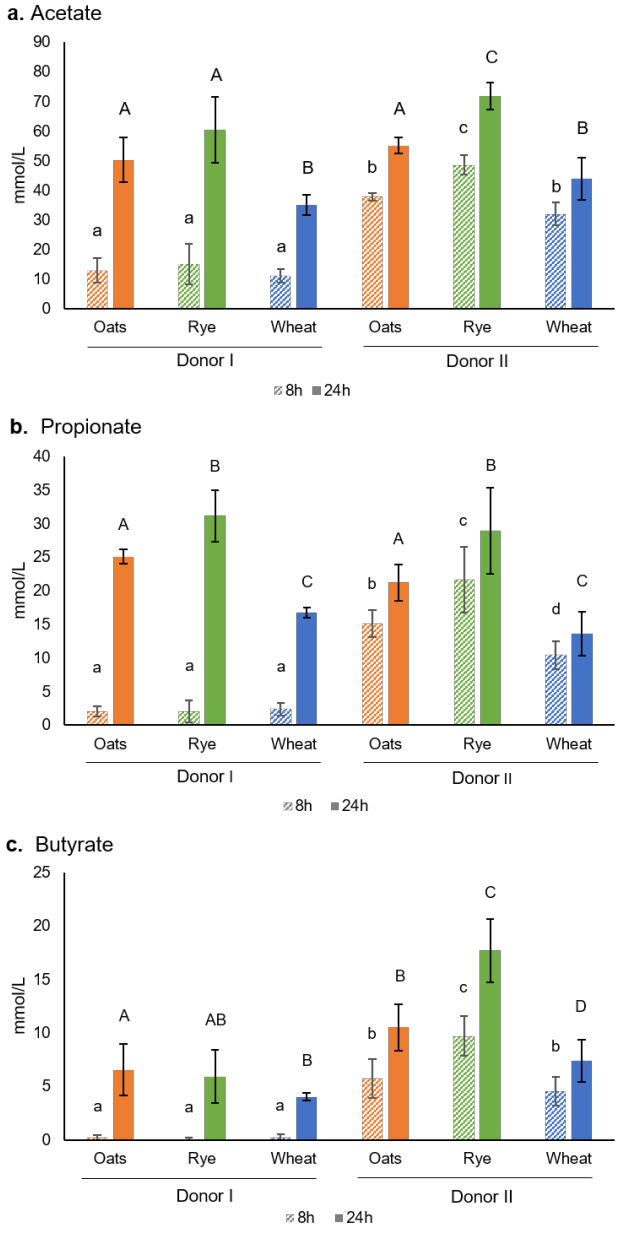

Figure 12. Levels of the short-chain fatty acids (a) acetate, (b) propionate, and (c) butyrate in the fermentation samples with different substrates (oats, rye, wheat) and fecal material from Donors I or II at 8 h and 24 h of fermentation. Different letters indicate significant differences (p<0.05) between substrate-donor combinations (lowercase 8 h, uppercase 24 h) (modified from Paper I).

As mentioned previously, metabolic ability varies between microbial taxa, and only certain bacteria can produce propionate and butyrate. The high relative abundance of *Subdoligranulum* can explain the high butyrate levels in the Donor II samples with rye substrate, as the species *Subdoligranulum variable*, which is commonly found in human feces, can form butyrate (Louis & Flint 2017). The most abundant genera in Donor I samples are not suggested butyrate producers, which likely explains the observed differences in butyrate levels between the donors. Higher propionate production has previously been linked to the *Bacteroides* enterotype (Yang *et al.* 2013; Chen *et al.* 2017), but in Paper I propionate levels did not differ between the donors after 24 h. Certain *Bacteroides* and *Prevotella* species form propionate (Louis & Flint 2017), which might explain why no differences were observed in Paper I.

In Paper III, individual or total plasma or fecal SCFAs did not change significantly between or within the intervention groups. The change closest to statistical significance was butyrate concentration in plasma (p=0.051), which showed an increasing trend in the rye group and a slightly decreasing trend in the control group (Table 5).

Table 5. Median short-chain fatty acid levels in plasma and feces before (V1) and after (V2) the three-week intervention (n=29).

	Rye		**Control**	
	V1	V2	V1	V2
Plasma (µg/mL)				
Acetate	8.9	7.4	8.4	5.6
Propionate	0.2	0.2	0.2	0.3
Butyrate	0.1	0.2	0.1	0.1
Total SCFA[1]	9.4	7.8	8.6	6.2
Feces (µg/mg)				
Acetate	74.2	103.0	77.1	203.2*
Propionate	35.2	34.4	67.0	123.5
Butyrate	43.6	48.1	34.3	77.3*
Total SCFA[1]	157.7	195.0	204.7	414.1*

[1]Sum of acetate, propionate, butyrate, valerate, and caproate. *Significant change in within-group testing ($p<0.01$)

Increased blood or fecal butyrate levels after rye intake have been reported in earlier studies. However, some of these were meal studies, in which the postprandial effects of rye on SCFA levels may be more pronounced. On the other hand, the three-week intervention in Paper III may have been too short to reveal the long-term effects of WG rye on SCFA levels reported in earlier studies of longer duration (4-12 weeks). However, in a previous six-week intervention, the WG rye diet did not alter SCFA levels (Vuholm *et al.* 2017). Unexpectedly, fecal acetate, butyrate, and total SCFA levels increased significantly within the control group, but not in the rye group in Paper III. The DF intake from six slices of the control bread was 8.2 g, which may have increased DF intake in subjects with low DF intake in their habitual diet. However, the control bread did not significantly change plasma SCFA levels, and in fact resulted in a decreasing trend.

To sum up, in Paper I, butyrate was the only SCFA that differed between the donors after 24 h of fermentation. High butyrate levels were observed specifically in rye samples from Donor II, which also had a high abundance of *Subdoligranulum* and *Bifidobacterium*. Certain *Subdoligranulum* species are known butyrate producers, so the presence of *Subdoligranulum* in Donor II samples likely contributed to the higher butyrate levels seen for rye samples. In Paper III, rye intake did not increase butyrate levels significantly, but there was an increasing trend in plasma butyrate levels after WG rye intake that was close to statistical significance. In both studies, rye increased the abundance of butyrate-producing taxa, which, together with the SCFA results, indicates that rye may have butyrate-promoting properties, which is also in agreement with previous studies.

4.3 Dietary fiber utilization during fermentation

Recovery of insoluble and soluble DF after 24 h of *in vitro* fermentation was studied in Paper I. The amount and percentage recovery of arabinose, xylose, and glucose residues, which are the main structural components of cereal DF, are presented in Figure 13. Recovery of insoluble arabinose and xylose residues in rye and oats was lower for the Donor II samples compared with the Donor I samples, especially for xylose in rye, which indicates more effective fermentation of AX in Donor II. Moreover, lower recovery of insoluble glucose residues was observed in fermentation samples with rye and wheat in Donor II compared with Donor I, which indicates more

effective fermentation of insoluble BG and possibly cellulose in Donor II. The lowest recovery of insoluble sugar residues was observed for wheat samples, as could be expected, since the total amount of DF in the wheat substrate was lower than in the rye and oat substrates, and thus bacteria could consume a larger proportion of DF.

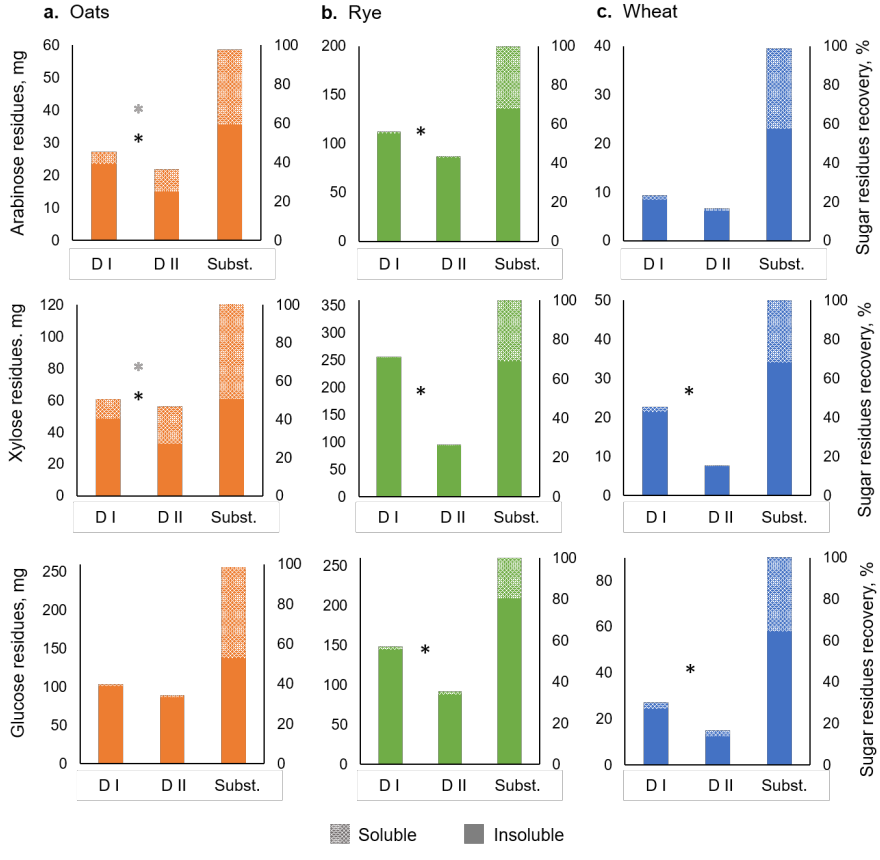

Figure 13. Amount and percentage recovery of arabinose, xylose, and glucose residues after 24 h of *in vitro* fermentation and sugar residue composition in substrates (subst.) in (a) oat, (b) rye, and (c) wheat samples with fecal material from the two donors (DI and DII). Low recovery of sugar residues indicates high utilization of dietary fiber. The amount of sugar residues (right axis) varied between the substrates due to differences in dietary fiber composition and content. Each bar presents soluble and insoluble sugar residues separately. (*p<0.05, grey for soluble and black for insoluble sugar residues) (modified from Paper I).

Prominent amounts of soluble arabinose and xylose residues were detected only in the fermentation samples with oat substrate, where sugar residue recovery was lower in the Donor I samples compared with Donor II samples (Figure 13). Part of the insoluble AX was possibly solubilized during fermentation in the oat substrate, but soluble AX residues were not present in the rye or wheat samples, indicating that AX was not solubilized or that it was consumed completely by microbes. For samples with oat substrate, recovery of soluble or insoluble glucose residues did not differ between the donors. In general, the amount of soluble sugar residues was very low in most samples after fermentation, a result that was not unexpected as soluble DF is generally readily fermentable. Altogether, the results indicate that DF utilization was more effective in Donor II samples. These findings highlight the effect of differences in microbiota composition on utilization of different DF types.

4.4 Tryptophan and phytochemical metabolites

The effects of *in vitro* fermentation on the metabolite profile and fate of precursor molecules present in the breads and substrates were investigated with untargeted and semi-targeted metabolomics in Paper II. The metabolic profile of fermentation samples with rye substrate differed from that of the other two substrates, as clear separation was observed in the PCA plot (Figure 14). This was expected, as WG rye and WG oats have different phytochemical content. The metabolic profile was separated between the donors on PC1 at the 8 h time point, but not at 24 h.

In total, 68 compounds had significantly different abundance in the different substrates between the 8 h and 24 h time points. Some previously known or suggested microbial metabolites, such as glutaric acid, hydroxyindoleacetic acid, 4-hydroxyphenyllactic acid, 3-indoleacetic acid, and leucic acid (Wishart et al. 2018), were among those displaying significant changes. In preclinical studies, some of these, and other annotated metabolites, have been associated with the GBA or immunomodulation (Schirmer et al. 2016; Ahmed et al. 2022). In addition to the change in abundance, the metabolite levels of the potential GBA metabolites were compared between the two donors.

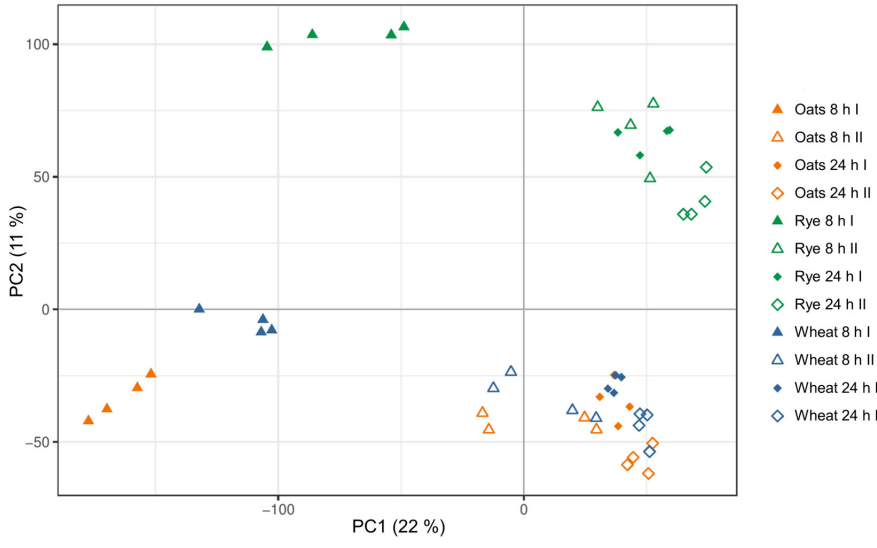

Figure 14. Principal component analysis (PCA) score plot of metabolite profiles of the different *in vitro* fermentation samples (oat, rye, wheat) (filled and hollow markers for Donor I and II, respectively). PCA was performed on normalized data for all molecules that met the quality metrics (PC1, the first principal component; PC2, the second principal component).

The levels of 3-indolepropionic acid differed between the donors at both time points, with higher levels observed in the Donor II samples after 8 h (p<0.001), and in the Donor I samples after 24 h of fermentation (p<0.001) (Figure 15). Higher levels of 3-indoleacetic acid were observed in Donor II samples than in Donor I samples at both time points (p<0.05). The levels of hippuric acid were markedly higher in the Donor I samples compared with the Donor II samples (p<0.001) at 8 h of fermentation. High ferulic acid levels were found in fermentation samples with rye substrate at 8 h in Donor II, and at 24 h in Donor I, but the difference between the donors was significant only at 24 h (p=0.001). High levels of hMPP were observed in rye and oat samples after 24 h of fermentation, and the levels were higher in the Donor I samples compared with Donor II samples after 24 h of fermentation (p<0.001).

In summary, the metabolites showing significant changes included many suggested microbiota-derived metabolites, some of which have been associated with *e.g.*, neuroprotection or regulation of inflammatory responses. Metabolite levels differed between the donors, which may indicate an effect of microbiota composition on metabolite levels.

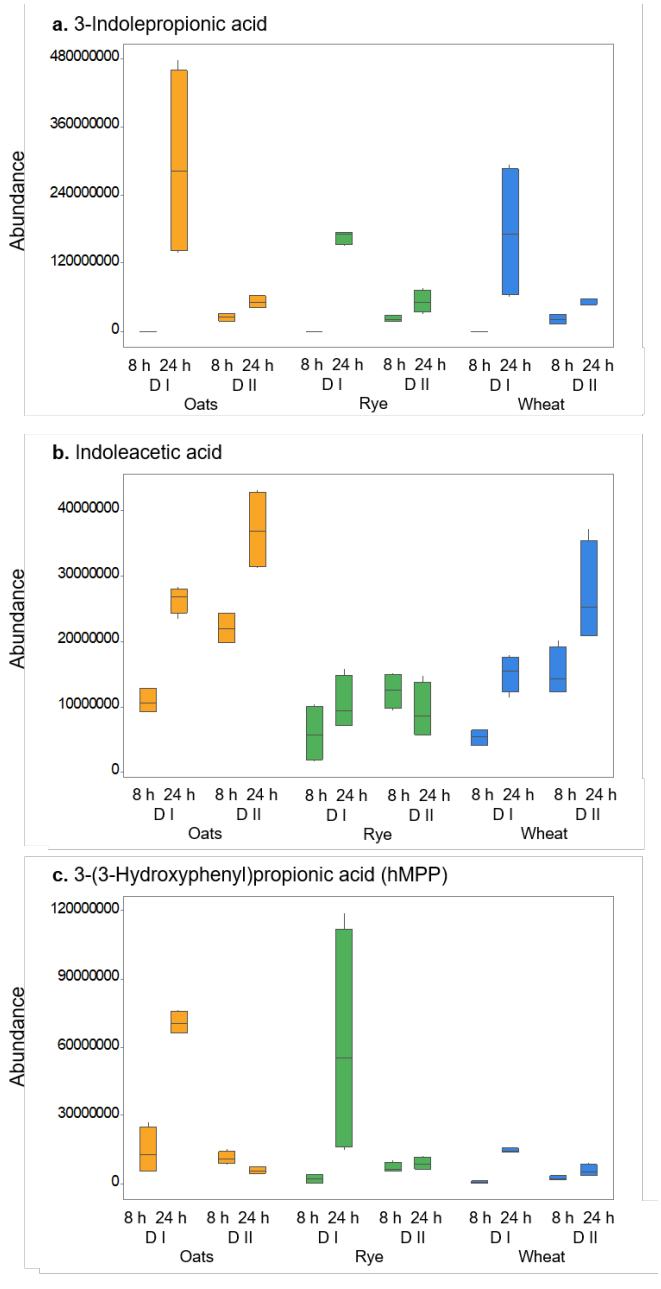

Figure 15. Abundance of the tryptophan metabolites (a) 3-indolepropionic acid and (b) indoleacetic acid, and of the ferulic acid metabolite (c) 3-(3-hydroxyphenyl)propionic acid, in the different fermentation samples (oats, rye, and wheat) with fecal material from the two donors (DI and DII)) at time points 8 h and 24 h.

4.5 Effect of WG rye bread on the gut-brain axis

The effects of the three-week daily intake of WG rye bread on GBA markers, stress responses, and cognitive performance in healthy subjects were studied in Paper III.

4.5.1 Blood markers and intestinal permeability markers

The levels of BDNF, gut hormones, and proinflammatory cytokines did not differ between or within the intervention groups (Table 6). Of the measured gut hormones, only total GLP-1 levels showed a small increasing trend in both groups. Increased PYY levels have been reported following rye intake after rye evening meals (Sandberg *et al.* 2016; Sandberg *et al.* 2017; Sandberg *et al.* 2018) but in the present study there was a decreasing trend in PYY levels in the rye group. There was a small numerical increase in BDNF levels after rye intake, but the change was not significant between or within the groups. In a previous study, BDNF levels were observed not to change after intake of a rye evening meal (Sandberg *et al.* 2018). Other previous studies have reported inflammation-suppressing effects of rye (Jonsson *et al.* 2018; Sandberg *et al.* 2018; Iversen *et al.* 2022a;), but the levels of pro-inflammatory cytokines did not change in Paper III.

Intestinal permeability, measured with blood markers (Table 6) and the multi-sugar test, did not differ between or within the groups. In an earlier study, rye intake did not affect intestinal permeability in healthy subjects (Vuholm *et al.* 2017).

Table 6. Median levels of brain-derived neurotrophic factor (BDNF), gut hormones, proinflammatory cytokines, and intestinal permeability markers in blood before (V1) and after (V2) the three-week whole grain rye bread intervention.

	Rye		Control	
	V1	V2	V1	V2
BDNF (ng/mL)	26.5	28.5	37.1	33.1
Gut hormones				
GLP-1 total (pg/mL)	27.5	32.6	21.0	24.4
GLP-1 active (pg/mL)	1.0	1.2	0.6	0.6
GLP-2 (pg/mL)	1.9	1.9	2.8	2.9
PYY (pg/mL)	58.6	50.5	40.4	39.7
Proinflammatory cytokines				
IFN-γ (pg/mL)	4.9	4.0	3.9	4.8
IL-1β (pg/mL)	0.1	0.1	0.0	0.1
IL-6 (pg/mL)	0.3	0.3	0.3	0.5
TNF-α (pg/mL)	1.3	1.4	1.5	1.4
Intestinal permeability markers				
CD14 (µg/mL)	1.9	1.9	2.2	2.0
LBP (µg/mL)	13.8	13.1	17.3	16.8

4.5.2 Stress responses and cognitive performance

Acute stress markers (salivary cortisol and alpha-amylase) were analyzed before, during, and after the stress test. Cortisol values peaked at 10-15 min after the end of the MAST (Figure 16), but cortisol and alpha-amylase levels did not differ between the groups. There was a trend for lower cortisol levels after the intervention in both groups, which indicates that the study subjects were less stressed at V2. No difference in perceived stress was observed between the groups and there were no significant differences in any of the measures of HRV or skin conductivity between the groups. Perceived long-term stress did not differ between or within the groups. Production of SCFAs has been shown to attenuate the cortisol response to psychosocial stress in men when delivered to the colon in capsules, with serum SCFA levels co-varying with the change in cortisol response (Dalile *et al.* 2020). In addition, SCFAs have been demonstrated to alleviate behavior alterations after psychosocial stress in mice (van de Wouw *et al.* 2018). On the other hand, a small increase in serum SCFA levels following extruded wheat bran intake did not affect stress responses (Dalile *et al.* 2022).

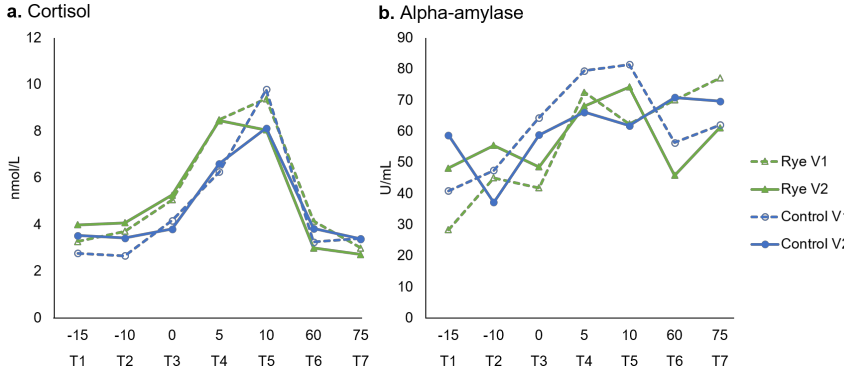

Figure 16. Median salivary level of (a) cortisol and (b) alpha-amylase levels before (T1), during (T2-T3), and after (T4-T7) the Maastricht acute stress test (MAST), before (V1) and after (V2) the three-week whole grain rye bread intervention.

No effect on cognitive control or working memory was observed in either of the groups. It might be challenging to improve cognitive performance in healthy, young adults. However, some DF interventions have been demonstrated to result in improvements in sustained attention, emotional information processing, and memory tasks in healthy adolescents or adults (Berding *et al.* 2021).

The hypothesis tested in Paper III was that intake of WG rye bread increases the relative abundance of DF-utilizing bacteria and SCFA levels, especially butyrate, which in turn affects GBA signaling, improves cognitive performance, and attenuates stress responses. However, no broad-scale changes in microbiota composition were observed. The abundance of the butyrate-producing bacteria *Anaerobutyricum hallii* and *Eubacterium ventriosum* group increased in the rye groups, but this was not reflected in the SCFA results. Thus, it is not surprising that no changes in the GBA markers, intestinal permeability, stress responses, and cognition were observed.

4.5.3 Biomarker of whole grain intake

Alkylresorcinol levels in blood were used as a compliance measure in Paper III. Alkylresorcinols are grain phytochemicals found in WG wheat, WG rye, and in small levels in WG barley, and have been suggested as a biomarker of WG wheat and WG rye intake (Landberg *et al.* 2019). The levels of total AR increased within the rye group (Figure 17), but the

difference was not significant between the groups, even though the rye group had high WG intake from the bread whereas the control bread did not contain any WG wheat or rye. Total AR levels decreased or were unchanged in four subjects in the rye group, which potentially indicates low compliance in these subjects. Moreover, total AR levels increased substantially in two subjects in the control group, but the AR C17:0/C21:0 ratio, a biomarker of WG rye intake, did not increase. The ratio of AR C17:0/C21:0 increased significantly in the rye group compared with the control group (Figure 17). Overall, the AR results suggest shortcomings in compliance in the intervention.

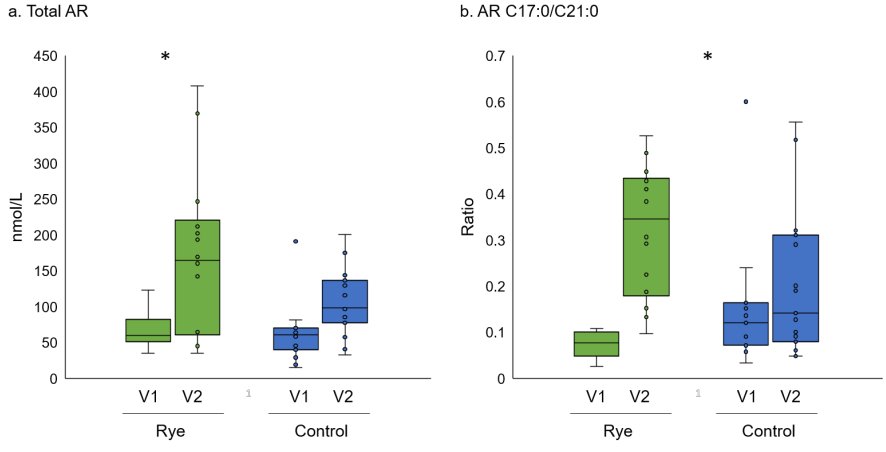

Figure 17. The levels of whole grain (WG) biomarkers (a) total alkylresorcinols (AR), and (b) the ratio of AR C17:0/C21:0, biomarker of WG rye intake, before (V1) and after (V2) the three-week intervention (*Significant change ($p<0.01$) within or between the intervention groups).

4.6 Strengths and limitations

An overview of the strengths and limitations of this thesis is presented below, while methodological limitations are discussed more in detail in Papers I-III. An overall strength of the work in this thesis is that whole grain products were studied, instead of isolated DFs or parts of the grain since whole grains contain beneficial nutrients beyond DF. Moreover, people seldom consume unprocessed bran or isolated DF, but instead eat whole grain products or other DF-rich foods that include different DF structures and other nutrients. On the other hand, using WG products instead of isolated DF can make it

challenging to interpret the observations, as several factors can contribute to the results.

4.6.1 *In vitro* fermentation study

The *in vitro* study (Papers I and II) included only two fecal donors and the microbiota composition of these individuals had a strong impact on the results. Moreover, *in vitro* conditions are very simple compared with the complex environment of the human gut. Thus, the results of the studies in Papers I and II are only indicative.

The *in vitro* study was designed to model colon fermentation of DF corresponding to a standardized amount of bread, and thus the amount of fermentation substrate depended on the DF content of the bread. Therefore, the observed differences between the substrates might be explained at least partly by differences in DF amount between the substrates. The chosen fermentation substrate processing method successfully removed starch and restored DF, but only part of the protein was removed and sugar content was not substantially decreased. Fructan is usually lost using *in vitro* digestion of food or grain material (Roye *et al.* 2020) and the method used in this study was chosen to preserve fructan, which is an important DF component in rye.

The initial conditions in *in vitro* fermentation should be carefully selected and adjusted to prevent overgrowth of fast-growing bacteria at the expense of more sensitive species, which can cause deviations in microbiota community balance from the target model community (Isenring *et al.* 2023). There was an indication of rapid growth among certain bacteria with low relative abundance in the fecal samples, especially in Donor I samples. These included *Escherichia coli*, which has a robust growth mechanism (Wang *et al.* 2010). There was some exposure to oxygen of fecal samples during sample collection and inoculate processing, which likely affected the strictly anaerobic microbes in fecal samples. However, several anaerobic taxa, such as *Subdoligranulum* and *Prevotella*, remained viable, as indicated by their increased relative abundance during fermentation.

The low SCFA levels at 8 h and low gas production observed in the first hour of fermentation in the Donor I samples indicate that the fermentation process started more slowly in those samples. This might be the result of differences in fermentation, but could also have been caused by the number of actively growing bacteria in the sample, which might have biased comparisons between the donors, especially at the 8 h time point. Moreover,

the lack of analysis of early time points (0-8 h) of fermentation may have affected the results, by overlooking potential microbial metabolism occurring at the beginning of the fermentation process.

4.6.2 Dietary intervention study

There were some limitations in the study design. For example, the three-week study duration may have been too short to cause significant effects on gut microbiota and SCFA levels. Moreover, the number of subjects who completed the study was relatively low, which limited the power of statistical testing. Limitations relating to measurements affected intestinal permeability, stress responses, and cognitive tests. Intestinal permeability markers in blood were measured only at the beginning of each visit, but it might have been more relevant to measure these markers after the MAST, to assess the effect of acute stress on permeability. There was also a potential learning effect of the MAST, as there was a decreasing trend in cortisol in the second intervention visits in both groups. Moreover, stress can affect cognitive performance, and completion of the MAST before the cognitive tests might have affected the results.

One strength, but also a limitation, of the study is that females were included (n=6/30). Studies estimating stress responses measuring salivary cortisol levels often exclude females, since the menstrual cycle affects the levels. Salivary cortisol stress responses are higher in the luteal phase and comparable to those of men, whereas in the follicular phase of the menstrual cycle, the levels are lower (Kudielka *et al.* 2009). Only females using hormonal contraceptive methods were included in the study in Paper III, to minimize the effect of menstrual cycle as the intervention duration was three weeks. However, females taking oral contraceptives have been shown to have lower salivary cortisol responses than men (Kudielka *et al.* 2009). Although this might have affected the cortisol results, it was important that the effect of the menstrual cycle was minimized and that women could be included in the study.

The challenges with compliance observed in the intervention study, in combination with relatively short study duration and small sample size, probably affected the results obtained. Compliance might have been better if different product types or supplements had been included in the intervention. Higher variation in the intervention diet might have also allowed longer study duration. On the other hand, the blinding was successful, which might

have been difficult to achieve with different product types. Moreover, the control bread could have been chosen differently. The control bread was a specially produced refined wheat bread containing oat endosperm flour and dark malt, so that it resembled WG bread. Even though this was positive from the perspective of blinding, the control bread might have increased DF intake in the subjects with low DF intake in the baseline diet and affected the study results.

4.7 General discussion

Whole grain is rich in vitamins and minerals, DF, and phytochemicals that can be metabolized by gut microbiota, and WG consumption may therefore affect the brain through the GBA or non-microbiota-related routes. Gut microbiota composition affects metabolite production, as different microbes have different metabolic abilities. This thesis explored the interplay between gut microbiota, DF, and microbiota-derived metabolites during gut fermentation of WG oat, WG rye, and refined bread, and also studied the effects of WG rye bread on gut microbiota and GBA.

The WG rye, WG oat, and refined bread differed in terms of SCFA levels, DF utilization, and metabolite profiles in Papers I and II. Whole grain rye and oats differ particularly in terms of DF and phytochemical content and composition, and refined cereals differ from WG, so the effects of the breads were expected to differ. Metabolite levels differed also between the two donors, indicating the role of microbiota composition in metabolite production, as reported in previous studies. Some interplay between microbiota composition and breads was observed in Paper I, as rye differed from the other two breads in terms of microbiota composition and butyrate levels in Donor II.

The three-week WG rye intervention did not cause broad-scale changes in microbiota composition in Paper III, which could be expected as healthy adults have a rather stable gut microbiota that is not usually affected by short-term dietary changes (Lozupone *et al.* 2012). Moreover, no significant change in SCFA levels was observed after rye intake. Thus, it is not surprising that the measured GBA markers, stress responses, and cognition were unchanged. Overall, the results indicate that it might be challenging to induce changes in inflammation, intestinal permeability, stress responses, or cognitive performance in healthy adults following a relatively mild and short

dietary intervention. Moreover, nutrition has subtle effects that might be difficult to detect due to intra-individual and inter-individual variation.

In Papers I and II, the metabolites produced from different breads and microbiota composition were studied *in vitro*, while in Paper III the effects of WG rye bread were studied in a dietary intervention study. In Paper I, the different substrates caused a strong shift in microbiota composition and affected SCFA levels, whereas in Paper III, rye intake did not have a strong impact on microbiota and SCFA levels were not significantly altered. This highlights the difference between *in vitro* modeling and human studies. *In vitro* gut fermentation models are a useful complement to human and animal studies when exploring microbiota modulation approaches and their direct effects on microbiota composition and activity (Isenring *et al.* 2023). However, it is challenging to model the complex gut environment, and thus the results of *in vitro* fermentation are only indicative and suitable for formulation of hypotheses that need to be further evaluated in human studies.

High butyrate levels were observed specifically in WG rye samples that had a high relative abundance of *Subdoligranulum* and *Bifidobacterium* in Paper I. In Paper III, WG rye intake did not increase butyrate levels significantly, but there was an increasing trend in plasma butyrate levels, and the relative abundance of butyrate-producing *Anaerobutyricum hallii and Eubacterium ventriosum* increased. These results demonstrate that rye may have butyrate-promoting effects, probably depending on gut microbiota composition.

5. Conclusions

- SCFA levels, DF utilization, and metabolite profiles differed between WG rye, WG oat, and refined bread during *in vitro* fermentation. Samples with WG rye bread differed from the other two breads.

- Gut microbiota composition affected metabolite levels and DF utilization during *in vitro* fermentation, and WG rye, WG oat, and refined bread had differing effects on microbiota composition in the samples. High butyrate levels were observed in the rye fermentation samples that had a high relative abundance of *Subdoligranulum* and *Bifidobacterium*.

- A three-week intake of WG rye bread, contributing 19 g to daily intake of DF, did not have any significant effects on SCFA levels, blood markers related to the gut-brain axis, intestinal permeability, stress responses, cognitive performance, or perceived long-term stress and well-being in healthy subjects.

- Broad-scale changes in microbiota were not observed between the groups after the three-week WG rye bread intervention, but the relative abundance of the butyrate-producing bacteria *Anaerobutyricum hallii and Eubacterium ventriosum* increased.

- The results indicate that rye may have butyrate-promoting properties, potentially depending on gut microbiota composition.

6. Future perspectives

Whole grain rye and other WG cereals contain several nutritional factors that have the potential to exert positive effects on the microbiota-gut-brain axis. However, such effects were not observed in the dietary intervention study reported in this thesis. Human intervention studies with a high number of study subjects that take into account the effect of microbiota composition and microbiota-derived metabolites are needed to study the role of WG in brain health and the GBA. Moreover, the effect of intra- and inter-individual variation should be better assessed in intervention studies. Metabolomics analysis of biological samples can also add valuable insights to human intervention studies, as it can be used to create hypotheses on mechanisms of whole grain health effects, inter-individual responses and the role of gut microbiota (Ross 2015).

The relative abundance of genus *Subdoligranulum* increased in rye samples in Donor II in Paper I. *Subdoligranulum* has previously been found to be associated with *Akkermansia muciniphila*, a bacterial species causally linked with an improvement in several metabolic parameters (Van Hul *et al.* 2020). *Subdoligranulum* has also been found to be correlated with metabolic health independently, although causality to obesity and T2D markers has not been shown. It can be hypothesized that the previously demonstrated metabolic health benefits of rye might be explained in part by increased abundance of microbes, such as *Subdoligranulum*, mediating these health benefits to the host through *e.g.*, SCFA formation, immunomodulation, and gut hormone regulation. The association between rye and potentially health-beneficial bacteria, such as *Subdoligranulum*, and butyrate production should be investigated in future studies.

It can be hypothesized that certain cereals or DFs benefit individuals with a specific microbiota composition. The increasing evidence about the role of

gut microbiota composition and its effect on microbiota-derived metabolites presents an interesting approach to personalized nutrition and product innovations. However, knowledge about the complex interactions between microbiota composition, DF utilization, and health effects is still very limited, and microbiota composition analysis is not accessible to all. Thus, it is beneficial to recommend increased intake of WG and the amount and variety of DF in the diet. Current WG and DF intake does not meet the recommendation in Western countries, which can have negative health effects directly or indirectly via impacts on gut microbiota-mediated processes. Moreover, intake of rye has decreased in Sweden and Finland, especially in younger people (Sandvik *et al.* 2017; Tammi *et al.* 2021). Further research is required to identify the barriers to WG and DF intake and to find the best means to increase consumption of WG foods.

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Popular science summary

Whole grain has been shown to decrease the risk of many diseases, such as type 2 diabetes and certain cancers, but less is known about the effects of whole grain on gut microbiota and the gut-brain axis. Gut microbiota means trillions of microbes, mainly bacteria, found in the human gastrointestinal tract. The gut-brain axis is a two-way communication route between the gut and the brain in which gut microbiota plays a central role. Whole grain is rich in dietary fiber and plant bioactive compounds that gut microbiota can use as a nutrient source. Microbes can also produce new compounds, metabolites, from these dietary compounds. For example, gut microbiota produces short-chain fatty acids (SCFA) from dietary fiber and these SCFAs can play an important role in the gut-brain axis. However, there is a large inter-individual variation in gut microbiota composition, which can affect the production of metabolites.

In this thesis, the effects of whole grain oat bread, whole grain rye bread, and low-fiber refined bread on microbiota and the levels of SCFAs and other metabolites were studied using a so-called fecal fermentation model. The effect of microbiota composition was investigated using fecal samples from two different donors in the model. In addition, the effects of whole grain rye bread on gut microbiota and the gut-brain axis were studied in a dietary intervention study, where the study subjects consumed a high amount of whole grain rye bread or a control bread daily for three weeks.

Differences in SCFA and other metabolite levels and the utilization of dietary fiber were observed during fermentation between the samples with microbiota from different donors. This indicates that microbiota composition can affect how nutrients from bread are used by gut microbiota. The levels of a specific SCFA, butyrate differed between the two donors, especially in samples with rye. Butyrate is considered a key metabolite in the gut-brain

axis. No significant changes in fecal microbiota composition or the gut-brain axis were observed in the three-week intervention study. However, a small increase in the abundance of two microbes that produce butyrate was observed in the participants consuming whole grain rye.

Altogether, the results presented in this thesis indicate that rye may have the potential to increase butyrate production, likely depending on gut microbiota composition. Rye has been shown to increase butyrate levels in previous human studies, but the effects of whole grain rye on the gut-brain axis remain unclear.

Populärvetenskaplig sammanfattning

Fullkorn har visat sig minska risken för många sjukdomar, såsom typ 2-diabetes och vissa cancerformer, men det är mindre känt om effekterna av fullkorn på tarmmikrobiotan, d.v.s. bakterierna i tarmen, och tarm-hjärna-axeln. Tarm-hjärna-axeln är en tvåvägskommunikationsväg mellan tarmen och hjärnan där tarmmikrobiota spelar en central roll. Fullkorn är rikt på kostfiber och växtbioaktiva föreningar som tarmmikrobiotan kan använda som näringskälla. Mikrober kan också producera nya föreningar, metaboliter, av dessa näringsämnen. Tarmmikrobiota kan producera kortkedjiga fettsyror (SCFA) från kostfiber och dessa SCFA kan spela en viktig roll i tarm-hjärna-axel. Det finns dock stor individuell variation i tarmmikrobiotans sammansättning, vilket kan påverka produktionen av metaboliter.

I denna avhandling studerades effekterna av fullkornshavre-, fullkornsråg- och lågfiber raffinerat bröd på mikrobiota och nivåerna av SCFAs och andra metaboliter med hjälp av en så kallad fekal fermentationsmodell. Effekten av mikrobiotasammansättningen undersöktes genom att avföring från två olika donatorer användes i modellen. Dessutom studerades effekterna av fullkornsrågbröd på tarmmikrobiotan och tarm-hjärna-axel i en studie där försökspersoner konsumerade en stor mängd fullkornsrågbröd eller ett kontrollbröd dagligen i tre veckor.

Vi kunde se skillnader i SCFA och andra metabolitnivåer och hur kostfiber förbukades mellan proverna med mikrobiota från olika donatorer. Detta indikerar att mikrobiotans sammansättning kan påverka hur näringsämnen från bröd används av bakterierna. Nivåerna av en spesific SCFA, butyrat, skilde sig mellan de två donatorerna, särskilt i prover med råg. Butyrat anses vara är en nyckelmetabolit i tarm-hjärna-axeln. Inga signifikanta förändringar i fekal mikrobiotasammansättning eller tarm-

hjärna-axel observerades i den tre veckor långa studien med försökspersoner. En liten ökning av mängden av två mikrober som producerar butyrat observerades hos deltagarna som konsumerade fullkornsråg.

Sammantaget indikerar resultaten som presenteras i denna avhandling att råg kan ha potential att öka butyratproduktionen, troligtvis beroende på tarmmikrobiotans sammansättning. Råg har visat sig öka butyratnivåerna i tidigare studier studier på människor, men effekterna av fullkornsråg på tarm-hjärna-axeln är fortfarande oklara.

Yleistajuinen tiivistelmä

Täysjyväviljan käytön on osoitettu vähentävän monien sairauksien, kuten tyypin 2 diabeteksen ja tiettyjen syöpien riskiä, mutta täysjyvän vaikutuksia suolistomikrobistoon ja suoli-aivoakseliin ei tunneta hyvin. Suolistomikrobisto tarkoittaa ruoansulatuskanavan miljardeja mikrobeja, pääasiassa bakteereita. Suoli-aivoakseli and kaksisuuntainen viestintäjärjestelmä suoliston ja aivojen välillä, jossa suolistomikrobistolla on keskeinen rooli. Täysjyvävilja sisältää runsaasti kuitua ja kasviperäisiä bioaktiivia yhdisteitä, joita suolistomikrobit voivat käyttää energianlähteenä. Mikrobit voivat myös muodostaa näistä ravintoaineista uusia yhdisteitä, metaboliitteja. Suolistomikrobisto muodstaa esimerkiksi kuidusta lyhytketjuisia rasvahappoja, joita pidetään keskeisinä yhdisteinä aivo-suoliakselin toiminnassa. Suolistomikrobiston koostumuksessa on suuria yksilöiden välisiä eroja, mikä voi vaikuttaa metaboliittien muodostukseen.

Tässä väitöskirjassa täysjyväkaura-, täysjyväruis- ja vähäkuituisen puhdistettua viljaa sisältävän leivän vaikutuksia mikrobistoon ja lyhytketjuisten rasvahappojen ja muiden metaboliittien muodostumiseen tutkittiin ulostefermentaatiomallin avulla. Suolistomikrobiston koostumuksen vaikutusta metaboliitteihin arvioitiin käyttämällä mallissa ulostenäytteitä kahdelta eri luovuttajalta. Tämän lisäksi täysjyväruisleivän vaikutuksia suolistomikrobistoon ja aivo-suoliakseliin tutkittiin ravitsemusinterventiotutkimuksessa, jossa tutkimushenkilöt söivät runsaasti täysjyväruisleipää tai vähäkuituisista kontrollileipää kolmen viikon ajan.

Lyhytketjuisten rasvahappojen ja muiden metaboliittien määrä ja kuidun hyötykäyttö erosi ulostemikrobeja eri luovuttajilta sisältävien näytteiden välillä. Tämä antaa viitteitä siitä, että mikrobiston koostumus vaikuttaa leivästä peräisin olevien ravintoaineiden hyödyntämiseen. Erään lyhytketjuisen rasvahapon, butyraatin, määrä erosi luovuttajien välillä,

erityisesti ruisnäytteiden kohdalla. Butyraattia pidetään keskeisenä yhdisteenä aivo-suoliakselin toiminnassa. Täysjyväruisleivällä ei havaittu olevan vaikutuksia suolistomikrobiston koostumkseen tai aivo-suoliakseliin kolmen viikon interventiotutkimuksessa. Kahden butyraattia tuottavan mikrobin suhteellinen määrä kuitenkin lisääntyi tutkimushenkilöillä, jotka söivät täysjyväruisleipää.

Kaiken kaikkiaan tämän väitöskirjan tulokset antavat viitteitä siitä, että rukiilla voi olla butyraatin tuotantoa lisääviä vaikutuksia, jotka todennäköisesti riippuvat suolistomikrobiston koostumuksesta. Rukiin on havaittu lisäävän butyraatin määrää myös aiemmissa tutkimuksissa, mutta täysjyvärukiin vaikutuksia aivo-suoliakseliin tulee tutkia lisää.

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I

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Fecal microbiota composition affects in vitro fermentation of rye, oat, and wheat bread

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Fermentation of dietary fiber by gut microbes produces short-chain fatty acids (SCFA), but fermentation outcomes are affected by dietary fiber source and microbiota composition. The aim of this study was to investigate the effect of two different fecal microbial compositions on in vitro fermentation of a standardized amount of oat, rye, and wheat breads. Two human fecal donors with different microbial community composition were recruited. Bread samples were digested enzymatically. An in vitro fermentation model was used to study SCFA production, dietary fiber degradation, pH, and changes in microbiota. Feces from donor I had high relative abundance of *Bacteroides* and *Escherichia/Shigella*, whereas feces from donor II were high in *Prevotella* and *Subdoligranulum*. Shifts in microbiota composition were observed during fermentation. SCFA levels were low in the samples with fecal microbiota from donor I after 8 h of fermentation, but after 24 h acetate and propionate levels were similar in the samples from the different donors. Butyrate levels were higher in the fermentation samples from donor II, especially with rye substrate, where high abundance of *Subdoligranulum* was observed. Dietary fiber degradation was also higher in the fermentation samples from donor II. In conclusion, fermentation capacity and substrate utilization differed between the two different microbiota compositions.

Consumption of whole grain cereals, rich in dietary fiber, is associated with beneficial health effects, such as lower risk of type 2 diabetes, cardiovascular disease, and colorectal cancer[1]. Arabinoxylan, β-glucan, and cellulose are the major non-starch polysaccharides (NSP) in whole grain cereals[2]. In the Nordics, wheat, rye, and oats are the most commonly consumed whole grain cereals[3,4]. Rye and oats differ in their fiber composition, as oats have a high content of water-soluble β-glucan, whereas rye is rich in arabinoxylan and fructan[2,5]. Whole grain rye and wheat have similar dietary fiber composition, but the content and water solubility of arabinoxylan is higher in rye[5]. Soluble dietary fiber is generally considered to be readily fermentable, whereas water-insoluble fiber, such as cellulose, has lower fermentability[2]. Processing method also seems to affect the fermentability of whole grain NSP[6].

The gut microbiota is estimated to consist of 10^{14} of microbes that inhabit the gastrointestinal tract, mainly the large intestine[7]. Inter-individual variation in gut microbiota composition is considered greater than changes in microbiota community within an individual. Diet is a major factor affecting gut microbiota composition and functioning, both directly and indirectly[8]. Dietary fiber is the main nutrient source for gut microbes, and fermentation of fiber produces short-chain fatty acids (SCFA), most importantly acetate, propionate, and butyrate[9]. In human physiology, SCFA act as metabolic substrate and as signaling molecules influencing energy homeostasis and the immune system[10–12].

In 2011, Arumugam et al.[13] published their findings on three different types of microbial communities in the human gut and referred to these as enterotypes, dominated by different genera (*Prevotella*, *Bacteroides*, or *Ruminococcus*) considered to be drivers of community composition. Of these, only the first two have been confirmed in later studies[14]. Enterotypes are suggested to be complex and cannot be explained by human properties, such as age or body mass index. However, the validity of enterotypes has been questioned because they may oversimplify the complexity of human gut microbiota[15]. Metabolic diversity has been observed between the enterotypes, with lower lipolytic and proteolytic fermentation potential in the *Prevotella* enterotype and with the *Bacteroides* enterotype characterized by higher saccharolytic and proteolytic capacity[16]. Recent in vitro studies

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	Bread			Substrate		
	Oats	Rye	Wheat	Oats	Rye	Wheat
Protein	14.8	10.5	11.9	28.5	19.6	35.2
Lipids	14.0	1.9	3.4	7.9	1.1	5.4
Starch	35.8	47.7	57.9	0.3	0.3	0.1
Sugars total	4.9	1.7	2.9	13.3	8.0	12.4
Glucose	0.9	0.3	0.1	5.8	4.8	3.0
Fructose	1.6	0.9	0.3	3.7	2.3	1.3
Sucrose	0.3	0.0	0.2	0.6	0.1	0.7
Maltose	2.1	0.5	2.3	3.3	0.7	7.4
Dietary fiber[a]	11.3	17.3	5.2	33.3	46.5	23.8
Insoluble	7.0	12.1	3.2	18.3	31.8	13.8
Soluble	4.3	5.2	2.0	15.0	14.7	10.0
Fructan	0.1	1.5	0.4	0.2	2.4	1.2
Arabinoxylan[b]	3.4	8.3	1.6	11.2	24.8	8.6
β-Glucan	3.2	1.9	1.0	9.8	5.8	6.0
Raffinose	0.1	0.0	0.0	0.1	0.0	0.0
Insoluble-to-soluble fiber ratio	1.6	2.3	1.6	1.2	2.2	1.4

Table 1. Nutritional composition (% of dry matter) and the ratio of insoluble-to soluble dietary fiber of breads and fermentation substrates derived from the breads, analyzed in duplicate samples. [a]Calculated as the sum of fructan (AOAC Method 999.03) and dietary fiber analyzed by AOAC Method 994.13. [b]Calculated from arabinose, xylose and galactose residue values (analyzed by AOAC Method 994.13) assuming that arabinose to xylose ratio is 0.69 in arabinogalactan.

have shown differences in fermentation between the enterotypes with respect to time, SCFA production, changes in microbiota composition, and preference for different polysaccharides[17–19]. In a study involving in vitro fermentation of fructooligosaccharides (FOS), sorghum arabinoxylan, and corn arabinoxylan, a *Prevotella*-dominated microbiota was found to produce higher levels of SCFA, and propionate production was 2- to threefold higher than for *Bacteroides*-dominated microbiota[17].

Some previous in vitro studies have shown higher fermentation rate and SCFA production for oat bran compared with rye or wheat bran[20,21], although in one in vitro fermentation study oat and rye bran were comparable in terms of SCFA production and pH[22]. In human intervention studies, fiber from wheat, rye, or oats has been shown to affect gut microbiota composition and increase the level of fermentation metabolites, but the number of studies is relatively low and the studies have methodological limitations and differences[23]. Nevertheless, current evidence supports the role of intact cereal fiber in promoting microbiota diversity and abundance.

The aim of this study was to investigate the effect of different fecal microbial community compositions from two human donors on in vitro fermentation of oat, rye, and wheat breads in terms of fiber utilization and fermentation outcomes. Bread is a complete food product containing a combination of different fibers, whereas most other in vitro fermentation studies have studied isolated polysaccharides. This study was designed to model gut fermentation of dietary fiber corresponding to a standardized amount of bread with two different microbiotas. The amount of fermentation substrate reflected the dietary fiber content of the breads, and thus differed between rye, oats, and wheat.

Results

In vitro fecal fermentation experiments were conducted to study SCFA levels, dietary fiber degradation, pH, and changes in microbiota. Fecal samples from two donors with different microbiota composition were used. Two separate experiment occasion per donor resulted into four replicates of each substrate and donor combination. Before experiments, bread samples were enzymatically digested. Study outline is presented in Supplementary Fig. S1 online.

Chemical composition of bread and fermentation substrates. The three breads differed in chemical composition and especially in the amount and type of dietary fiber (Table 1). Fermentation substrate preparation from bread increased the proportional amount of fiber in all samples, and over 80% of the fiber in bread was recovered (94.2% for oats, 87.8% for rye, 82.7% for wheat). Starch was almost completely removed from the samples (<0.5% recovered) and the amount of protein and lipids was lowered, with approximately 40% of proteins and 80% of lipids removed during the process. The ratio of insoluble and soluble fiber was only slightly affected by the substrate preparation process (Table 1). The proportion of fiber in the substrates varied from 23.8% in wheat to 46.5% in rye, and the calculated amount of fiber in the fermentation experiments was 0.55 g in oats, 1.09 g in rye, and 0.25 g in wheat, respectively.

Microbiota composition. The microbiota composition of fecal samples used in the fermentation experiments differed between the two donors (Supplementary Fig. S2 online). Analysis of the fecal samples from

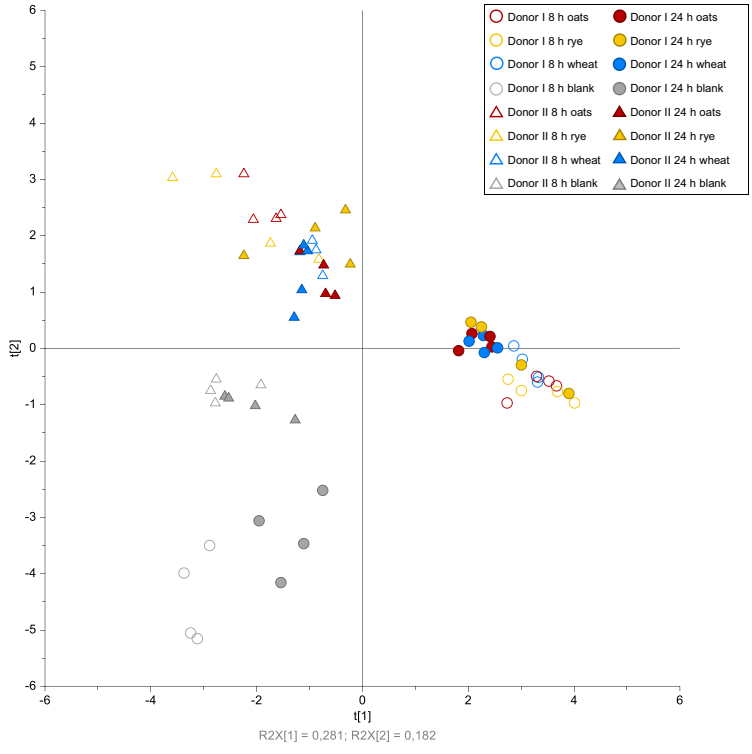

Figure 1. Principal component analysis (PCA) scores plot of the 20 most abundant genera in fermentation samples. In PCA, the first principal component (PC1, horizontal) accounts for the largest variance in the dataset. Residuals R2X (1) and R2X (2) indicate the amount of variation in the model described by PC1 and the second principal component PC2 (vertical), and t(1) and t(2) are co-ordinates of the PCA projection. Blank indicates samples without substrate, and oats, rye and wheat indicate samples with fermentation substrate.

donor I showed high relative abundance of the genera *Bacteroides*, *Christensenellaceae* R-7 group, *Blautia*, and *Akkermansia*. In contrast, the fecal samples from donor II had high relative abundance of the genera *Prevotella*, *Subdoligranulum*, and *Bacteroides*.

In principal component analysis (PCA), the microbiota composition of the fermentation samples at 8 h and 24 h was clearly separated for the two donors based on the first principal component (Fig. 1). Moreover, blank samples were separated from the samples with substrate, but the fermentation substrates were not clearly separated from each other. Shifts in relative abundance between genera were observed during the 24 h fermentation (Fig. 2). In the fermentation samples with fecal microbiota from donor I (hereafter referred to as donor I samples), all three fermentation substrates gave similar microbiota composition, with high relative abundance of *Bacteroides*, *Escherichia*/*Shigella*, and *Streptococcus* at 8 h and 24 h. The relative abundance of *Bacteroides* increased from 8 to 24 h, while the relative abundance of the other two genera decreased. In the fermentation samples with fecal microbiota from donor II (hereafter referred to as donor II samples), high relative abundance of *Subdoligranulum* (0.48 ± 0.15) was found for the rye substrate after 24 h fermentation, compared with oats (0.016 ± 0.0052) and wheat (0.049 ± 0.024). In the donor II samples, the highest relative abundance of *Bifidobacterium* was detected for the rye substrate, while the samples with oat and wheat substrates had high relative abundance of *Prevotella* (0.54 ± 0.21 for oats and 0.46 ± 0.18 for wheat) compared with rye substrate (0.022 ± 0.016) at 24 h. Similar, but less pronounced, differences between the substrates were observed at 8 h.

The analysis of similarities (ANOSIM) showed a clear difference in microbiota composition between the donors ($R = 0.918$, $p < 0.001$). In the donor I samples, experiment occasion had the highest effect on the dissimilarities in microbiota composition when samples at the 8 h and 24 h time points were analyzed separately ($R = 0.787$, $p = 0.003$ for 8 h; and $R = 0.820$, $p = 0.002$ for 24 h, respectively), followed by the effect of time point (Supplementary Table S1 online). Substrate effect on dissimilarities was not significant in donor I samples. In

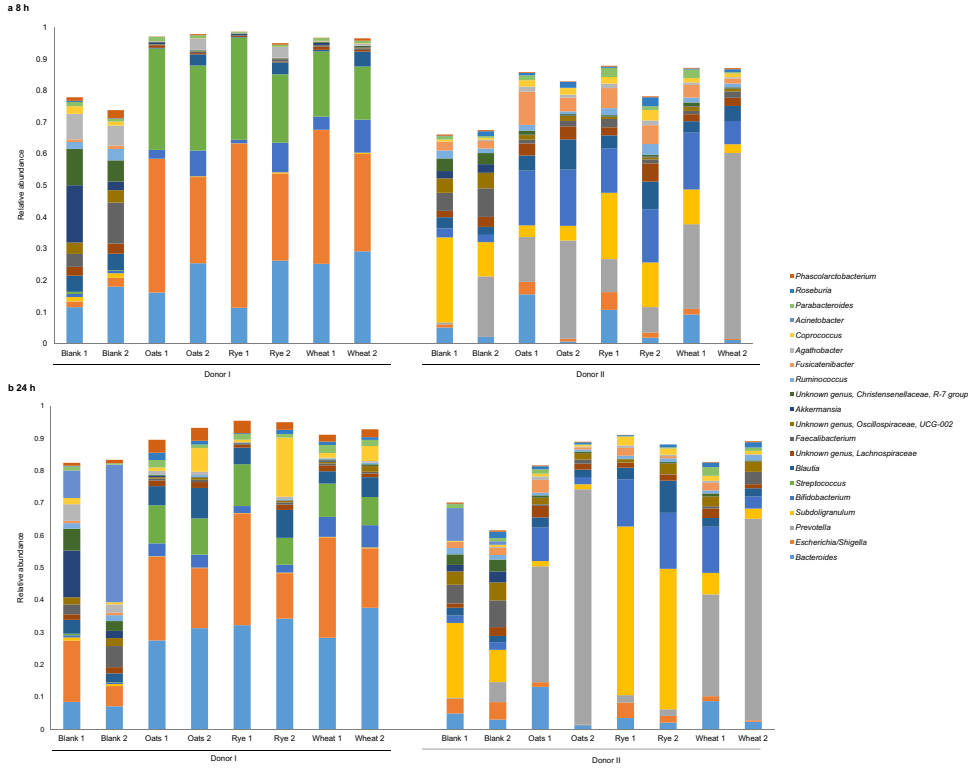

Figure 2. The 20 most abundant microbial genera in (**a**) 8 h fermentation samples, and (**b**) 24 h fermentation samples. Each bar represents mean of replicates (n = 2) from one fermentation experiment occasion (1 and 2). Blank indicates samples without substrate, and oats, rye and wheat indicate samples with fermentation substrate.

the donor II samples, substrate had the highest effect on dissimilarities in microbiota composition, especially at 24 h ($R = 0.676$, $p = 0.003$). The time point effect was small, and the experiment occasion effect was significant at 8 h but not at 24 h.

SCFA and branched-chain fatty acid (BCFA) levels. After 8 h of fermentation, the levels of acetate, propionate, butyrate, and valerate were higher in the donor II samples ($p < 0.0001$) (Fig. 3). A small interaction between donor and substrate type in the statistical model was observed for acetate, propionate, and butyrate levels. Significant differences between the substrates were observed in most pairwise comparisons of the donor II samples, with the highest SCFA levels in rye and lowest in wheat (except for valerate). No differences between the substrates were observed for the donor I samples at 8 h. The levels of total SCFA were aligned with the individual SCFA results at 8 h.

After 24 h fermentation, similar acetate and propionate levels were observed for the samples from both donors. An interaction between donor and substrate type in the statistical model was not detected for acetate or propionate level at 24 h, whereas a small interaction was detected for butyrate level, which was explained by high butyrate levels in rye substrate in donor II samples. Butyrate levels were higher in the donor II samples after 24 h of fermentation ($p < 0.0001$). Moreover, in pairwise comparison the 24-h butyrate levels in the donor II samples differed between all fermentation substrates, with rye in particular contributing to high butyrate levels. In the donor I samples, butyrate levels were similar between oats and rye. For valerate, a difference in 24-h levels was seen in comparison between the donors, but not between the substrates.

There was no difference in BCFA levels at 8 h between the samples from the different donors, or between the substrate types. The BCFA levels were higher in the donor II samples after 24 h of fermentation ($p < 0.0001$). A small interaction between donor and substrate type in the statistical model was observed for BCFA levels at 24 h. In the donor II samples, the oats and wheat substrates gave higher BCFA levels than rye. Lactate was detected in the donor I samples at 8 h (16.97 ± 1.40 mmol/L for oats, 23.07 ± 1.27 mmol/L for rye, and 9.59 ± 0.75 mmol/L for wheat) but not at 24 h.

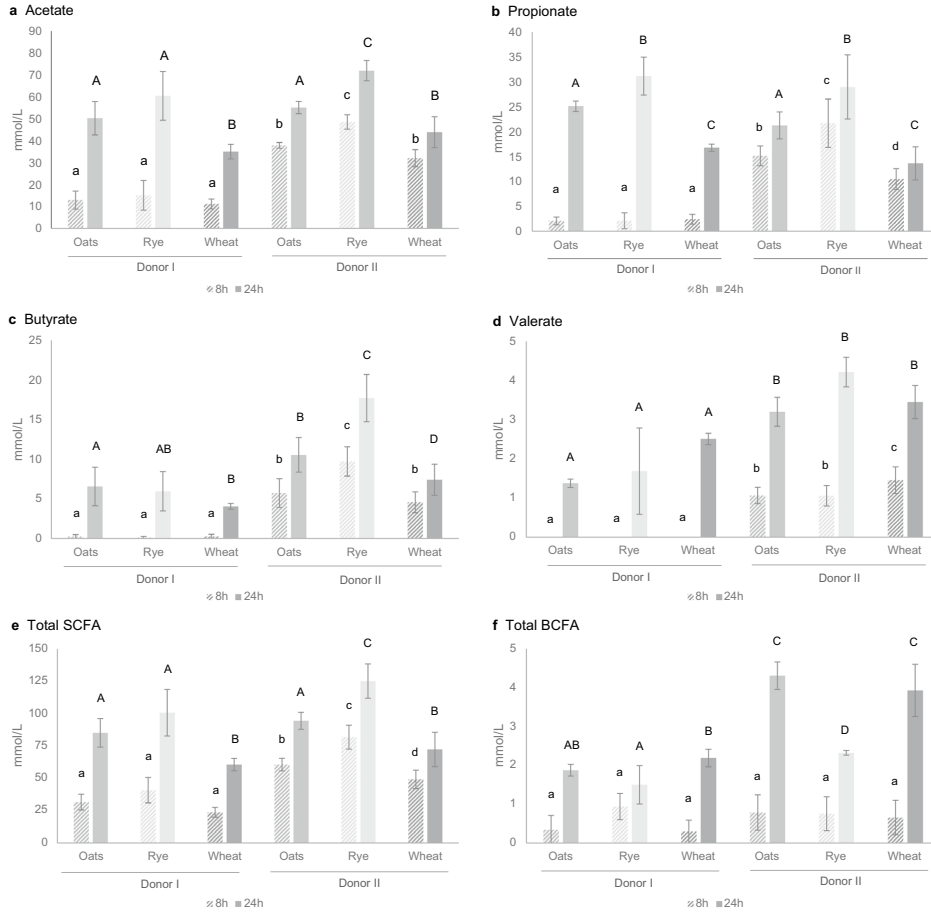

Figure 3. (**a**–**e**) Short-chain fatty acid and (**f**) branched-chain fatty acid concentrations after 8 h and 24 h of fermentation (mean of n = 4 replicates, error bars indicate s.d.). Different letters above bars indicate statistically significant differences in pairwise comparisons for each substrate-donor combination (lowercase 8 h, and uppercase 24 h, $p < 0.05$). Oats, rye and wheat indicate samples with fermentation substrates (*BCFA* branched-chain fatty acids, *SCFA* short-chain fatty acids).

In the ANOSIM, SCFA levels were dissimilar between the donors ($R = 0.210$, $p = 0.003$), and the effect of substrate on the dissimilarities in SCFA was higher in the donor II samples (Supplementary Table S1 online). Experiment occasion effect on SCFA dissimilarity was significant only in the donor I samples at 8 h. Time point (8 h vs 24 h) effect was seen in the both donor samples, and was high in the donor I samples ($R = 0.999$, $p < 0001$).

Recovery of NSP sugar residues. The lowest recovery of insoluble sugar residues was observed for wheat samples, for which the amount of sugar residues in the fermentation substrate was also lower than in rye and oat substrates (Fig. 4). Recovery of insoluble arabinose residues was lower for the donor II samples compared with donor I samples with oat and rye substrate. In addition, insoluble xylose residue recovery was lower for the donor II samples, and the difference between the two donor samples was substantial, especially for the rye substrate (25.5 vs 70.2%, $p < 0.0001$). A small interaction in the statistical model between substrate and donor was observed for insoluble xylose residues. Moreover, lower recovery of insoluble glucose residues was observed for the donor II samples ($p < 0.0001$), but the difference in oat substrate was not significant in pairwise comparison. Prominent amounts of soluble arabinose and xylose residues were detected only in the oat substrate samples after fermentation, and sugar residue recovery was lower in the donor I samples. A moderate interaction between donor and substrate type in the statistical model was observed for soluble arabinose and xylose and was

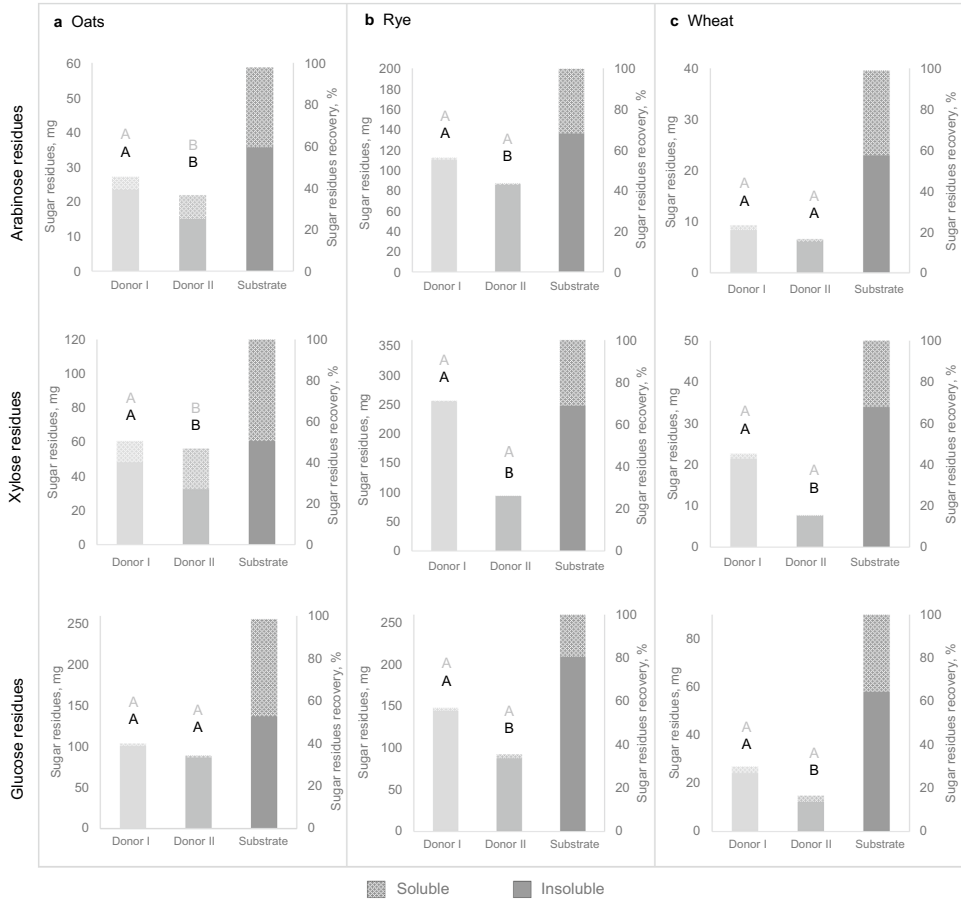

Figure 4. Amount and percentage recovery of arabinose, xylose, and glucose residues after 24 h of fermentation, and sugar residue composition in substrates in (**a**) oats, (**b**) rye and (**c**) wheat. Sugar residue amount varied between the substrates due to differences in fiber composition and substrate amount. Each bar includes soluble and insoluble sugar residues separately (mean of n = 4 replicates), and different letters (grey for soluble and black for insoluble sugar residues) above bars indicate statistically significant differences ($p < 0.05$) in pairwise comparisons between the two donor samples. Oats, rye and wheat indicate samples with fermentation substrate.

explained by higher recovery of these sugar residues in the donor II oat substrate samples. There was no difference in recovery of soluble glucose residues between the fermentation samples with fecal microbiota from the two donors or any substrate. Only a small amount of insoluble sugar residues was observed in the blank samples after fermentation (2.1 ± 1.6 mg).

Lower recovery of insoluble ($p = 0.0001$) and soluble ($p = 0.0002$) mannose residues was observed in donor II samples compared with donor I samples (Supplementary Table S2 online). The amount of insoluble galactose residues was approximately the same or higher after 24 h of fermentation when compared with the sugar residue levels in the substrate and did not differ between the fermentation samples with fecal microbiota from the two donors. Recovery of soluble galactose residues was lower in the donor II samples for oat ($p < 0.0001$) and wheat ($p < 0.001$) substrate, but higher for rye substrate ($p = 0.01$).

pH. The inoculate pH was 7.06 ± 0.02. Samples containing substrate had lower pH values than blanks at 8 h and 24 h (Table 2). A moderate interaction between donor and substrate type was detected in the statistical model. The pH was lower in the donor II samples than the donor I samples for rye substrate at 8 h ($p < 0.0001$)

	Donor I		Donor II	
	8 h	24 h	8 h	24 h
Blank	7.22 ± 0.02	7.00 ± 0.18	7.20 ± 0.01	7.12 ± 0.03
Oats	6.96 ± 0.05	6.89 ± 0.14	6.94 ± 0.06	6.84 ± 0.05
Rye	6.98 ± 0.13	6.75 ± 0.17	6.69 ± 0.07	6.45 ± 0.17
Wheat	7.05 ± 0.03	6.99 ± 0.16	7.06 ± 0.04	7.06 ± 0.04

Table 2. Changes in pH during the fermentation experiments (mean ± s.d.). Blank indicates samples without substrate, and oats, rye and wheat indicate samples with fermentation substrates.

and at 24 h ($p < 0.001$) but did not differ for the other substrates. In the donor II samples, pH differed between all substrates in pairwise comparisons at both 8 and 24 h and was lowest for the rye substrate ($p < 0.05$).

Discussion

This study was designed to model gut fermentation of dietary fiber corresponding to a standardized amount of bread, and thus the amount of fermentation substrate used depended on the fiber content of the bread. SCFA levels, and fiber degradation were higher for the samples inoculated with microbiota dominated by *Prevotella*, *Subdoligranulum* and *Bacteroides* (donor II) than for the samples inoculated with microbiota high in *Bacteroides, Christensenellaceae* R-7 group, *Blautia*, and *Akkermansia* (donor I). SCFA levels were highest for the rye substrate, probably due the higher fiber content.

The microbiota composition in the fermentation samples with fecal microbiota from donor I was similar between all three substrates, with the highest relative abundance of *Bacteroides* after 24 h of fermentation. Interestingly, the relative abundance of *Escherichia/Shigella* increased notably during fermentation in donor I samples, as the relative abundance of the genus was very low in the fecal samples. *Escherichia coli* has a very robust growth mechanism[24], and our hypothesis is that the genus had a competitive benefit in the beginning of the fermentation. Microbiota composition in the donor II samples differed between the substrates, which was also observed in the ANOSIM. The abundance of *Subdoligranulum* was high for the rye substrate, whereas the relative abundance of *Prevotella* was high for the oat and wheat substrates, especially after 24 h of fermentation. The highest relative abundance of *Bifidobacterium* was detected for the rye substrate, which is in line with findings by Eriksen et al.[25] that an 8-week rye diet can increase the relative abundance of *Bifidobacterium*. In vitro studies have demonstrated that rye bran and soluble oat fiber can enhance the growth of *Bifidobacterium*, and that rye bran can enrich *Prevotellaceae*[26]. A study by Chen et al.[17] comparing fermentation of different fiber types in the *Bacteroides* and *Prevotella* enterotypes showed a higher diversity of taxa that responded to fiber substrates in the *Bacteroides* enterotype, whereas in the *Prevotella* enterotype, *Prevotella* was the only taxa to increase on the fiber substrates. This was partly confirmed by results in the present study, since either *Prevotella* or *Subdoligranulum* had distinctly high relative abundance after 24 h in the donor II samples depending on the substrate, whereas high abundance of any single genus was not observed in the donor I samples. The connection between rye and *Subdoligranulum* observed in the present study needs to be verified in future studies.

At 8 h, SCFA levels were low in the fermentation samples with fecal material from donor I, but after 24 h of fermentation, only butyrate levels differed between the samples with fecal microbiota from the two donors. The higher butyrate production from rye substrate in the donor II samples is likely explained by differences in microbiota composition. For BCFA, no differences were observed at 8 h, but after 24 h, the levels were higher in the donor II samples. The levels of BCFA, which are metabolites of branched-chain amino acid fermentation in gut and biomarkers of protein fermentation, were higher for the oat and wheat substrates, reflecting the higher protein content of these substrates. It is also possible that higher fiber fermentation inhibited protein fermentation in the samples with rye substrate[27]. Protein fermentation causes changes in microbiota composition and metabolite production that can have negative health effects, but the evidence is still controversial, and especially the physiological role of BCFA is not well known[28]. The strong effect of time point on SCFA in the donor I samples observed in the ANOSIM can be explained by lactate production that was observed only at 8 h time point.

Rye gave the highest SCFA levels in this study, but previous in vitro studies have shown high fermentability and SCFA levels for oats. In one study, carbohydrates from oat bran fermented at a higher rate and SCFA production was slightly higher than with carbohydrates from rye and wheat bran[20]. In another in vitro fermentation study[21], oat bran increased propionate and acetate production. On the other hand, in an in vitro fermentation study conducted by Roye et al.[22], oat and rye bran were comparable in terms of SCFA production. In that study, bran was removed from residual endosperm without removal of fructan and water-extractable arabinoxylan, which, according to the authors, explained the better fermentability of rye than seen in other studies. Fructan was recovered also in the present study, which probably improved the fermentability of the rye substrate.

Acetate is produced by many bacterial groups in the human colon, but bacteria that produce propionate and butyrate are fewer and there are several pathways of SCFA metabolism that vary between bacterial groups[29]. High relative abundance of *Subdoligranulum* can explain the high butyrate production seen for the donor II samples compared with the donor I samples in the present study, as it has been shown that certain *Subdoligranulum* species form butyrate through the butyrate kinase route[30]. Chen et al.[17] found that the *Prevotella* enterotype produced higher levels of SCFA with FOS and arabinoxylan, and that propionate production was 2–3 times higher than for the *Bacteroides* enterotype. Yang et al.[31] found that *Bacteroides* was positively correlated with propionate production in in vitro fermentation. Yu et al.[19] reported higher butyrate and propionate levels in *Prevotella* than

Bacteroides enterotype in in vitro fermentation of pea cell wall polysaccharides. In the present study, donor II samples had high butyrate levels, and propionate levels were similar between the two donor samples.

In the present study, the fiber composition in post-fermentation samples is reported as recovery of insoluble and soluble sugar residues. These sugar residues originate from grain NSPs, most importantly arabinose and xylose from arabinoxylan, soluble glucose from β-glucan, insoluble glucose from cellulose, mannose from glucomannan, and galactose from arabinogalactan[2]. Low recovery of sugar residues indicates high utilization of substrate fiber by bacteria during fermentation. Recovery was calculated from total sugar residues in the substrate (i.e. the sum of insoluble and soluble sugar residues), based on the hypothesis that some of the insoluble sugar residues could have been solubilized during fermentation. Some differences in sugar residue recovery were detected between the samples with fecal microbiota from the different donors. Lower recovery of insoluble arabinose and xylose was observed in the donor II samples with oat and rye substrate which indicates more effective utilization of insoluble arabinoxylan, possibly due to higher fermentation capacity of the bacteria in donor II samples. Interestingly, only oat substrate samples contained prominent amounts of soluble arabinose and xylose sugar residues. It is possible that part of the insoluble arabinoxylan was solubilized during fermentation of oat substrate, but not rye or wheat substrate. Lower recovery of insoluble glucose residues from the rye and wheat substrates was observed in the donor II samples, indicating more effective fermentation of insoluble β-glucan and possibly cellulose. For samples with oat substrate, differences were not detected in soluble or insoluble glucose residues, which is probably explained by the high content of easily fermentable soluble β-glucan in oats. In general, the amount of soluble sugar residues was very low in most samples after fermentation, which indicates that these were more readily fermentable than the insoluble sugar residues.

The chosen method of processing bread samples to fermentation substrate had certain strengths and limitations. It was successful to remove starch and restore fiber, but only around half the protein was removed for all sample types. The process did not substantially decrease the sugar content of samples. In similar fermentation studies, in vitro digestion with mammalian enzymes is commonly used. The method used in the present study was chosen because it decreased the starch content to almost zero and enabled retention of fructan, which is usually removed during in vitro digestion of food or grain material before fermentation[22]. There were some weaknesses with the method. First, retention of fructan retained also other ethanol-soluble molecules, such as glucose, in the substrate. Second, the method is not alike to the digestion process in the human small intestine and the enzymes used were not of mammalian origin.

A limitation as regards dietary fiber recovery is that the polysaccharide levels in the fecal inoculates were not measured. However, the fiber amount in blank samples after fermentation was minimal and no gas production was detected, which indicates that the amount of fiber originating from the inoculate was negligible. The samples used for fiber analysis after fermentation experiments were autoclaved before analysis to avoid any pathogen risk, which may have affected the fiber structures present. However, the soluble and insoluble fractions were separated before autoclaving, and thus the treatment did not affect the ratio of insoluble to soluble fiber in the fermentation samples, and effects on sugar residue content are unlikely.

The aim in this study was to mimic colonic fermentation of three bread products with different fiber content, and thus the amount of fermentation substrate and the fiber amount differed between the three substrates. This difference in fiber amount limited between-substrate comparisons, since the amount of available fermentable compounds affects production of SCFA and BCFA. The difference in fiber amount also limited comparison of the results with those of studies in which the fiber or substrate amount was similar for different grains.

The low SCFA levels at 8 h, and low gas production observed during the first 8 h of the fermentation experiments in the donor I samples indicate that the fermentation process started more slowly than in the donor II samples. This might relate to differences in fermentation capacity of different bacterial taxa but can also have been caused by the number of actively growing bacteria in the sample. Viable cell counts were not conducted on fecal samples or inoculates, which is a limitation. Moreover, the handling of fecal samples and oxygen exposure could have affected the results, since donors collected a sample shortly before each experiment, but the time between sample collection and fermentation was not standardized. The fecal inoculate was not processed under strictly anaerobic conditions, although oxygen exposure was minimized. Processing of the sample to produce inoculate slightly changed the relative abundance of certain genera.

Since previous studies have reported difference in fermentation capacity between the enterotypes, we used *Bacteroides* and *Prevotella* genera as a premise to find fecal donors with contrasting microbiota composition. We did not find donors with clear *Bacteroides* or *Prevotella* dominated microbiotas, but the two donors had different microbiota composition and fermentation outcomes differed between the donor samples. There was some variation in fecal sample microbiota composition between the two experiment occasions, especially in donor II. However, the microbiota composition was similar between the experiment occasions after 24 h fermentation in the samples with substrate, and the replicates showed overall good repeatability within and between experiments. In the ANOSIM, a strong effect of the experiment occasion on dissimilarities in microbiota was observed in the donor I samples, but in the donor II samples it was significant only at 8 h but not at 24 h. In donor II samples, substrate had a pronounced effect on microbiota composition.

In most of previous in vitro fermentation studies, the fermented material was grain bran or isolated fiber, not a complete food product containing a combination of different fiber structures. A strength of this study is that the breads used were existing commercial consumer products or similar. The specially produced refined wheat bread containing oat endosperm flour was used in the present study because it was developed as a placebo bread for a clinical trial within the same project. Oat endosperm flour has high starch content and contains 4.0–5.0% of dietary fiber[32], and the amount of oat endosperm flour in the bread was only 25% of flour ingredients. As the aim of the study was not to compare rye and oat bread to whole grain wheat bread, the wheat bread was made of refined flours and had low fiber content.

Gut microbiota composition differs between individuals, which can affect gut fermentation, as shown in this and previous studies. SCFA outcomes, which depend on microbiota-fiber interactions, can lead to differences in health effects between individuals. SCFA play an important role in human physiology and energy balance, and studies with animal models have even identified a role of SCFA as mediators in the gut-brain axis, the bi-directional communication pathway between the gut and the brain[33]. Conclusive evidence from human trials that different fiber structures promote SCFA production in individuals with different gut microbiota composition can lead to more personalized dietary recommendations for prevention and treatment of different diseases and conditions.

In conclusion, in 24 h in vitro batch culture fermentation experiments, there were clear differences in SCFA production and in fiber degradation between samples with fecal microbiota from two donors with different fecal microbiota composition. Differences in butyrate, propionate, and acetate concentrations were found between oat, rye, and wheat bread substrates, especially in donor II fermentation samples. Microbiota composition changed during the fermentation experiments. The relative abundance of *Bacteroides* and *Escherichia/Shigella* increased in the donor I samples, while the relative abundance of *Prevotella*, *Subdoligranulum* and *Bifidobacterium* increased in the donor II samples. These results indicate contrasting fermentation capacity and substrate utilization potential between different microbiota profiles in the human gut. This suggests that differences in microbiota profile could in part explain intra-individual differences in diet-related health outcomes, due to differences in metabolite production.

Methods

Bread samples. Three different bread products were used: a commercial whole grain rye bread, a commercial whole grain oat bread, and a refined wheat bread containing oat endosperm flour (25% of flour). Ingredient lists and other details are presented in Supplementary Table S3 online. The breads were freeze-dried for 5 h at 30 °C and 0.01 mbar, followed by approximately 20 h at 0 °C and 1.5 mbar. Dried samples were milled in a laboratory mill to pass a 0.5 mm screen. After milling, the bread samples were stored at − 20 °C.

Removal of lipids, available starch, and savinase-degradable protein from bread. Freeze-dried and milled bread samples (35 g) were weighed into centrifuge bottles. To remove lipids, each sample was mixed with 50 mL of n-heptane, vortexed twice for 2 min, and centrifuged (10 min, 2000×*g*), after which the heptane layer was discarded. For rye and wheat bread samples, this heptane washing was repeated once, while for the oat bread samples it was repeated twice, after which the residues were air-dried.

To extract fructan, the dried residue was mixed with 250 mL of ethanol (80% v/v) and incubated at 80 °C for 45 min with magnetic stirring (500 rpm). After incubation, the sample was centrifuged (10 min, 1000×*g*) and the supernatant was collected. Thereafter, the sample was washed three times by adding 30 mL of ethanol (80% v/v), mixing, and centrifuging (10 min, 1000×*g*), with the supernatant collected after each centrifugation. Ethanol was removed from the pooled supernatants by vacuum rotor evaporation, and the unevaporated residue containing fructan was mixed with 50–100 mL of deionized water and frozen at − 20 °C.

To remove starch, an amylolytic treatment was carried out. The solid residue from ethanol washing was dried overnight at 40 °C and dispersed in 175 mL of acetate buffer (0.1 M, pH 5.0 and 5 mM $CaCl_2$) in a bottle. Then 1.75 mL thermostable α-amylase (3000 U/mL) was added and the sample was incubated at 100 °C for 60 min, with mixing three times during incubation. The solution was cooled to 40 °C, followed by addition of 10.5 mL of amyloglucosidase solution (140 U/mL), and overnight incubation at 60 °C in a shaking water bath. For oat bread samples, 25 mL of acetate buffer were added before amyloglucosidase treatment, to ensure homogeneous mixing.

To remove proteins, the dispersion was cooled to room temperature, and 1.85 mL Savinase (≥ 16 U/g, Sigma-Aldrich) was added, followed by incubation for 3 h at 50 °C in a shaking water bath. Thereafter, the sample was cooled to room temperature and ethanol (99.5% v/v) was added to make 80% (v/v) ethanol solution. The solution was shaken vigorously for 2 min, centrifuged (15 min, 1000×g), and the supernatant liquid was discarded. The pellet was washed three more times with 60 mL of ethanol (80% v/v). The solid residue was dried overnight at 40 °C, and mixed with the extract containing fructan. The mixture was frozen, freeze-dried, and milled as described above, and stored at − 20 °C.

Chemical analysis of bread and substrate samples. Chemical composition of bread samples and of substrates derived from the bread samples was analyzed in duplicate, with the results presented on a dry weight basis after drying at 105 °C for 16 h. Dietary fiber content and composition were analyzed according to the AOAC Method 994.13[34], with previously described modifications[35] to analyze the extractable and non-extractable dietary fiber separately. For the analysis of substrates, sample amount of 75 mg was used. The β-glucan content was analyzed with K-BGLU kit (Megazyme) as described previously[36]. The fructan content was determined with a K-FRUC kit (Megazyme) as previously described[37], with modifications described in Supplementary methods online. Starch content was analyzed enzymatically according to a previously published method[38]. Protein content was analyzed according to the Kjeldahl method[39] as Kjeldahl-N × 6.25. Fat content was analyzed as described previously[40]. The concentration of glucose, fructose, sucrose, maltose, and raffinose was analyzed as described previously (modified)[41].

Study subjects and fecal sample collection. Healthy study subjects (n = 10) were recruited and screened according to exclusion and inclusion criteria (Supplementary Table S4 online) to find two fecal donors with contrasting gut microbiota composition. The Swedish Ethical Review Authority approved the study protocol (application number 2019-04229) and the study was performed following the relevant guidelines and regulations. All study subjects signed an informed consent before being enrolled.

All study subjects collected a screening fecal sample using *EasySampler for stool collection* (GP Medical Devices) and a small sample tube. The screening fecal samples were stored at − 80 °C. For rapid screening of donor microbial profile, the molecular fingerprinting method terminal restriction fragment length polymorphism (T-RFLP) was used, according to a previously described protocol[42]. The T-RFLP data generated by screening samples from all study subjects were evaluated in order to identify two donors with different microbial community composition, with regard to terminal restriction fragments associated with *Bacteroides* and *Prevotella* in previous studies. Based on the T-RFLP data, two donors with contrasting microbiota composition were selected to provide fecal samples for the in vitro experiments.

Fecal samples for the fermentation experiments were collected within two hours before each experiment (including sample processing described below). The donors collected sample at home using an *EasySampler* and a plastic beaker (500 mL) with a sealed cap for collecting minimum 30 g of feces, and the samples were stored at room temperature until the experiment. Approximately 1 g of each fermentation fecal sample was frozen and stored at − 80 °C for microbiota composition analysis.

In vitro fermentation experiments. Four batch fermentation experiments were conducted with fecal samples from each donor at two separate occasions, resulting in four replicates of each substrate and donor combination The amount of fermentation substrate was energy-standardized between the breads. In addition, inulin (Merck KGaA) was used in control samples to monitor the fermentation process. Substrate (1.65 g of oats, 2.35 g of rye, 1.03 g of wheat substrate, or 1.00 g of inulin) was added to fermentation bottles. Thereafter, 50 mL of buffer (8.5 g $NaHCO_3$, 5.8 g K_2HPO_3, 0.5 g $(NH_4)_2HPO_4$, 1.0 g NaCl, 0.5 g $MgSO_4{\cdot}7\ H_20$, 0.01 g $FeSO_4{\cdot}7\ H_20$, 0.1 g $CaCl_2$ to 1 L of deionized water, pH 7.0)[43] were added to each bottle and to two bottles without substrate (blank controls). All bottles were treated with CO_2 gas until addition of inoculate. Inoculate was produced by mixing fecal sample (20 g) with buffer (1500 mL) in a bottle with CO_2 gas treatment, to obtain 1% (w/v) solution for the fermentation. The fecal slurry was filtered through a kitchen sieve and one layer of polyester filter cloth, and 50 mL were immediately added to the bottles containing buffer and substrate or blank controls. The bottles were closed and incubated at 37 °C for 24 h. Bottle contents were mixed with a motor stirrer throughout the experiment (60 s stirring, 60 s break). Gas production was measured throughout the experiment using the Gas Endeavor system (Bioprocess Control) to follow the fermentation process.

At time points 8 h and 24 h, liquid (5 mL) was collected from each bottle with a syringe and divided into three 1 mL-aliquots, and pH was measured. Aliquots were stored at − 20 °C for later analysis of microbiota composition and volatile compounds. After 24 h of fermentation and sample collection, the fermentation residue material was centrifuged (5 min, 5000×*g*), and the supernatant liquid was separated from the pellet. The supernatant and pellet were autoclaved at 125 °C for 15 min, frozen to − 20 °C, freeze-dried as described above, and stored at − 20 °C.

Analysis of fermentation samples. Fecal samples, inoculates, and fermentation samples at time points 8 h and 24 h were analyzed for microbiota composition with 16S rRNA gene sequencing as described in Supplementary methods online. Acetate, propionate, butyrate, valerate, BCFA, and lactate concentrations were analyzed as described previously[44].

Dietary fiber amount and composition after fermentation was analyzed using the fermentation residue material. Pellet composition was analyzed to estimate insoluble fiber degradation, and supernatant composition to estimate soluble fiber degradation. Dietary fiber was analyzed according to the AOAC Method 994.13[34] with published modifications[35], and additional modifications described in Supplementary Methods online. All samples were analyzed in duplicate, and results are presented on a dry weight basis, after drying at 105 °C for 16 h.

Data processing and statistical analysis. To estimate fiber degradation, the amount of each insoluble and soluble sugar residue in fermentation samples was calculated as a percentage of total sugar residues (insoluble plus soluble) in the fermentation substrate. Total SCFA content at 8 h and at 24 h was calculated as the sum of acetate, propionate, butyrate, and valerate, while total BCFA content was calculated as the sum of isobutyrate and isovalerate. The microbiota composition data were analyzed to determine relative abundance on genus level. The cut-off value for data was set at 0.9% of average relative abundance, which represented 85% of total genera abundance. These comprised the 20 most abundant genera in the dataset and were used in further data analysis.

PCA was used for exploratory data analysis of microbiota data (Simca v. 16, Umetrics). For PCA modelling, the data were scaled (univariate scaling) and log-transformed. Analysis of similarities (ANOSIM) was used to statistically test for multivariate differences in microbiota and SCFA data between categorical variables (donor, substrate, time point and experiment occasion) (PAST v. 4.11[45]). The ANOSIM was based on Bray Curtis metrics where the effect of substrate, time and experiment was evaluated for each donor separately. SCFA and BCFA levels, fiber degradation, and pH were statistically compared between the fermentation samples with fecal microbiota from the two donors and between the different fermentation substrates, using a generalized linear fixed-effects model and two-way ANOVA with interaction (RStudio v. 1.2.5019[46]). The generalized linear model included the following fixed-effects variables: donor, substrate, the interaction between donor and substrate, and experiment occasion. Homoscedasticity and normality of residuals in each linear model were checked and, if either was detected, the response variables were log-transformed. This was the case for 8 h butyrate, 8 h valerate, and soluble glucose residues. Statistically significant interactions between donor and substrate variables were examined with an interactions plot, and post hoc pairwise comparison of estimated marginal means was conducted (R package *emmeans*[47]). All analyses were adjusted for multiple comparisons (Tukey's HSD). Inulin controls and blank samples were not included in the statistical analyses. Descriptive statistical analysis was conducted in Microsoft Excel.

Data availability

16S rRNA gene sequences of fermentation samples generated and analyzed during the current study are available in the Sequence Read Archive (SRA) repository, accession number PRJNA853911. The other datasets generated in the study are available from the corresponding author on reasonable request.

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Author contributions

L.P., J.D., and R.A. planned the study, analyzed the data, and interpreted the results; L.P. and J.D. performed the statistical analysis; L.P. conducted the research, wrote the manuscript, prepared figures and was responsible for the final content; J.L. and I.M. contributed to analysis and interpretation of the data; and all authors read, commented upon, and approved the final manuscript.

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Competing interests

L.P. is an industrial PhD student employed by Fazer Sweden AB. J.L. is employed by Oy Karl Fazer AB. Authors J.D., I.M., and R.A. declare no competing interest.

Additional information

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Supplementary information

Supplementary table S1. Model variables, R-values and p-values for Analysis of similarities (ANOSIM) of microbiota and SCFA. The effect of substrate, sample time point (8 h vs 24 h) and experiment occasion was evaluated for each donor separately. Statistically significant R-values are bolded; an R-value close to 1.0 suggests dissimilarity between groups. (SCFA, short chain fatty acids).

Target data	Donor	Categorical variable	Sample time	R-value	p-value
Microbiota	Donor I	Substrate	8 h	0.014	0.382
			24 h	0.132	0.167
			8 h & 24 h	-0.018	0.549
		Experiment occasion	8 h	0.787	0.003
			24 h	0.820	0.002
			8 h & 24 h	0.239	0.005
		Time point	8 h & 24 h	0.606	<0.001
	Donor II	Substrate	8 h	0.336	0.036
			24 h	0.676	0.003
			8 h & 24 h	0.424	<0.001
		Experiment occasion	8 h	0.507	0.003
			24 h	0.172	0.095
			8 h & 24 h	0.282	0.002
		Time point	8 h & 24 h	0.136	0.039
SCFA	Donor I	Substrate	8 h	0.331	0.035
			24 h	0.574	0.004
			8 h & 24 h	0.035	0.255
		Experiment occasion	8 h	0.780	0.003
			24 h	0.098	0.196
			8 h & 24 h	0.045	0.184
		Time point	8 h & 24 h	0.999	<0.001
	Donor II	Substrate	8 h	0.604	<0.001
			24 h	0.681	0.001
			8 h & 24 h	0.269	0.005
		Experiment occasion	8 h	0.013	0.364
			24 h	-0.046	0.502
			8 h & 24 h	0.007	0.331
		Time point	8 h & 24 h	0.331	0.002

Supplementary Table S2. Mannose and galactose residue recovery of the respective combined insoluble (IS) and soluble (S) sugar residues in the fermentation substrates (mean ±SD).

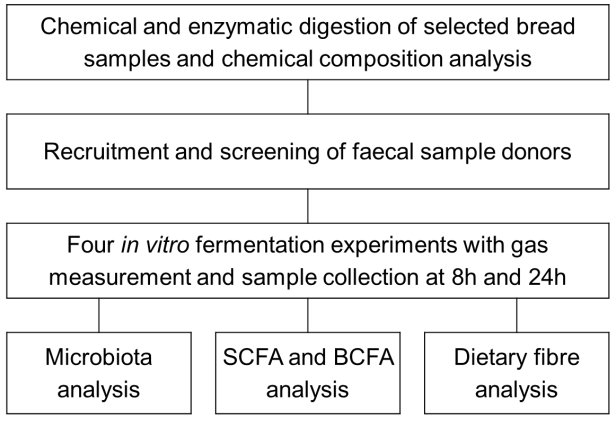
	Oats, recovery-%		Rye, recovery-%		Wheat, recovery-%	
	Donor I	**Donor II**	**Donor I**	**Donor II**	**Donor I**	**Donor II**
IS mannose	26.51 ±6.39	17.87 ±1.83	26.40 ±5.64	12.22 ±2.80	11.91 ±2.24	6.58 ±1.44
S mannose	3.08 ±0.09	2.42 ±0.35	2.72 ±0.27	2.40 ±0.12	2.65 ±0.25	1.83 ±0.42
IS galactose	56.75 ±0.63	54.65 ±5.59	70.69 ±3.35	71.91 ±9.65	43.11 ±4.55	35.80 ±10.0
S galactose	26.64 ±2.20	17.07 ±2.18	6.55 ±1.02	11.36 ±0.54	21.0 ±4.47	13.10 ±3.29

Supplementary Table S3. Bread ingredient lists and baking information.

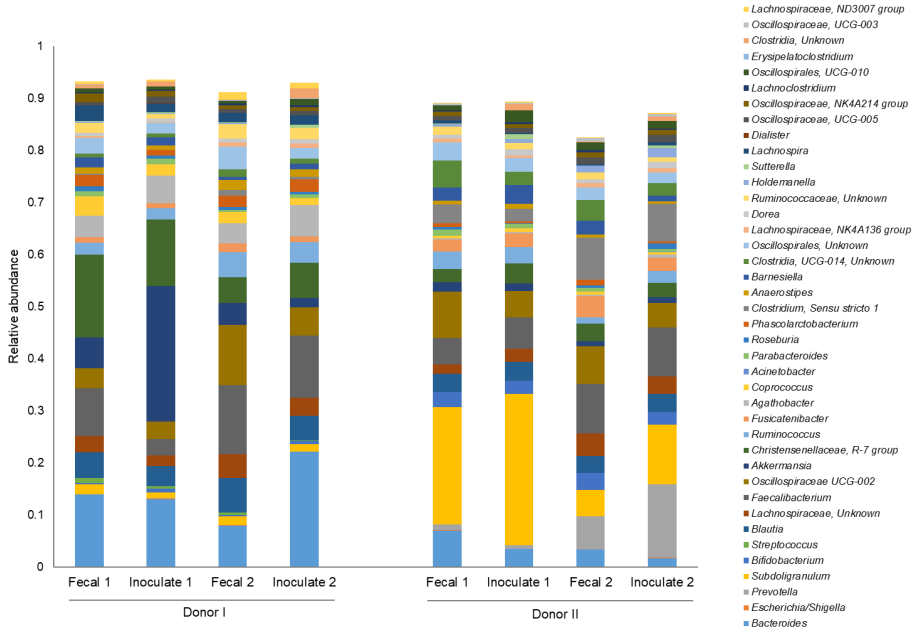
Bread type	Ingredients	Baking date	Bakery
Oats	Water, whole grain oats (flour, flakes and groats), sunflower seed, refined oat flour, pumpkin seed, rapeseed oil, psyllium, dried starter (oats), oat fiber, yeast, salt, preservative (E200), thickening agent (E412). Oats 100% of grain ingredients.	10/05/2018	Fazer Leipomot Oy, Lahti, Finland
Rye	Whole grain rye flour, whole grain wheat flour, refined wheat flour, water, sourdough from whole grain rye flour, rye fiber, yeast, salt, barley malt extract. Rye 58% of grain ingredients	10/01/2018	Fazer Bageri AB, Lidköping, Sweden
Wheat	Water, refined wheat flour, refined oat flour, malt, salt, yeast.	03/21/2019	Fazer Bageri AB, Lidköping, Sweden

Supplementary Table S4. Inclusion and exclusion criteria for study subjects.

Inclusion criteria
Age 18-65 years
Body mass index (BMI) 18,5-30 kg/m^2
Working or studying at SLU in Campus Ultuna
Available to deliver samples during the study experiment period
Exclusion criteria
Use of internal antimicrobial medication during the past 3 months before the study
Irregular bowel function
Type 2 diabetes, metabolic syndrome or an inflammatory or a functional disease of gastrointestinal tract
Suspicion or a diagnosis of a following infectious disease: HIV, hepatitis or Salmonella during the past 6 months
Planning to change diet considerably during the study time



Supplementary Figure S1. Simplified study outline.



Supplementary Figure S2. The 40 most abundant microbial genera in fecal samples and inoculates used in the fermentation experiments. In each donor, the same number in fecal and inoculate indicate same experiment occasion.

Supplementary method: Modifications in the fructan content analysis method with a K-FRUC kit

1) Pre-treatment with α-galactosidase was carried out to remove galactosyl-sucrose oligosaccharides;
2) The extraction step was scaled down to 100 mg of sample and 10 mL deionized water with incubation in a glass tube at 80 °C for 20 min; and
3) The filtration step was replaced with centrifugation of 1 mL of sample for 15 min at 10 500 × g, and the supernatant was used for analysis.

Supplementary method: Modifications in the analysis method of dietary fiber content and composition in fermentation samples

1) Sample amount for insoluble fiber samples was 20 mg and for soluble samples 100 mg;
2) Analysis started directly from the hydrolysis step, scaled down to 1/12 for insoluble fiber samples and to a volume of 3 mL for the soluble fiber samples;
3) The amount of myoinositol was 0.5 mg; and
4) Sample volume was not standardized after the hydrolysis step, and thus samples were weighed and mass was then converted to volume.

Supplementary Method: 16S rRNA gene sequencing of fermentation samples and fecal samples

DNA was extracted with a NucleoSpin® 96 Soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) with bead beating horizontally at 2700 rpm for 5 min on a Vortex-Genie 2 (Scientific Industries Inc., Bohemia, NY, USA). A minimum of one positive control (ZymoBIOMICS™ Microbial Community Standard, Zymo Research Co., Irvine, CA, USA) and one negative control was included with each batch of samples. Polymerase chain reaction (PCR) was conducted using universal bacterial 16S rRNA gene primers targeting the V3-V4 region; the forward primer S-D-Bact-0341-b-S-17 and the reverse primer S-D-Bact-0785-a-A-21 (Klindworth *et al.* 2013), with Illumina adapters attached. The cycling conditions used in PCR were as follows: Initial denaturation at 98 °C for 30 s, followed by 25 cycles at 98° C for 10 s, at 55 °C for 20 s, and at 72 °C for 20 s, with a final elongation step at 72 °C for 5 min. Amplification was verified by gel electrophoresis. Index tags were added in a subsequent PCR using the Nextera Index Kit V2 (Illumina Inc., San Diego, CA, USA) with the PCR cycling conditions described above, but with only eight cycles instead of 25. Products from the second PCR were pooled based on band intensity and the resulting library was cleaned with AMPure XP magnetic beads (Beckman Coulter Ltd, Bread, CA, USA). The DNA concentration in pooled libraries was measured using an AccuLite 470 fluorometer (Biotium Inc., San Francisco, CA, USA). Sequencing was performed on an Illumina MiSeq desktop sequencer using the MiSeq Reagent Kit V3 (Illumina Inc., San Diego, CA, USA) for 2 × 300 bp paired-end sequencing. An adjusted DADA2 pipeline was used for bioinformatics processing of the sequence data into the amplicon sequence variant (ASV) abundance table, performed as described earlier (Callahan *et al.* 2016). Taxonomic assignment of the different ASVs detected was carried out using a naive Bayesian classifier algorithm comparing the ASV sequences to the SILVA reference database (version 138) (Quast *et al.* 2013).

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Callahan B.J., et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 13, 581-583, doi:10.1038/nmeth.3869 (2016).

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Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2023:81

This thesis aimed to study the effects of whole grain bread on gut microbiota and the gut-brain axis. Differences in microbiota-derived metabolites, changes in microbiota composition, and dietary fiber utilization were observed both between the three different breads and two different fecal microbiota compositions during *in vitro* fermentation. A three-week intervention with whole grain rye bread did not demonstrate any significant effects on the microbiota-gut-brain axis. Overall, the results indicated that whole grain rye may have butyrate-promoting effects.

Laura Pirkola received her graduate education at the Department of Molecular Sciences, SLU. She obtained her MSc degree in human nutrition at the University of Helsinki.

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