Modulating the human intestinal microbiome

in healthy adults and elderly through dietary supplements

Ran An

Propositions

1. Ageing related changes in intestinal microbiota composition relate to alterations in health status, instead of chronological ageing.
   (this thesis)

2. The existence of a universal microbiome modulation strategy is unlikely.
   (this thesis)

3. The COVID-19 pandemic clearly demonstrates that microbes control our planet.

4. Microbial ecology mirrors human social behaviour.

5. It's important to hear others' voices, but one should not forget to listen to one's own voice.

6. Embracing what we don't know is crucial for directing research.

Propositions belonging to the thesis entitled:

"Modulating the human intestinal microbiome in healthy adults and elderly through dietary supplements"

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Wageningen, 10th of December 2020

Modulating the human intestinal microbiome in healthy adults and elderly through dietary supplements

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Modulating the human intestinal microbiome in healthy adults and elderly through dietary supplements

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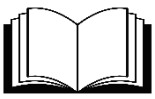
Table of contents

1

General introduction and thesis outline



1. General Introduction and Outline of the Thesis

1.1. Introduction

Over billions of years, microorganisms have been on earth and comprise a major portion of global biomass [1]. Microorganisms have been involved in the formation of the biosphere and play a key role in the cycling of nutrients, acting as our functional partners. Without microbes there is no life on earth. There is evidence that microbes were used to make beverages as early as 7000BC. Nowadays, single microbial strains or microbial communities (*i.e.* combination of different microbes) are used in food preservation and production, like the starter cultures used for cheese and wine fermentation [2]. In turn, overgrowth of some microbes in food can cause food spoilage and foodborne disease [3]. Besides their application in food, microbes are also widely used in biotechnological production of enzymes, chemicals and antibiotics [4, 5]. Microorganisms can be found basically everywhere, including air, surfaces, the deep sea, and even in and on the human body, especially in the gastrointestinal (GI) tract. Nevertheless, the environmental conditions, for example, temperature, pH, presence of oxygen and pressure, are selective forces for the microbial residents of a specific niche. The metabolic activity of microbes, in return, constantly affects their living environment.

The GI tract of humans and other animals encompasses different niches, which are colonized by distinct microbial communities that interact with the host [6]. The earliest report about human GI microbiota can be traced back to the 1670-1680s, when Antonie van Leeuwenhoek, a Dutch businessman and scientist who built the first microscope, revealed the differences in microbial composition between body sites when he studied material from mouth and faeces [7]. Moving forward to 1885, the first GI microbe was isolated by Theodor Escherich [8]. Later, in 1944, the development of techniques to culture microbes under anoxic conditions boosted the field of anaerobic microbiology, allowing the isolation, cultivation and characterization of strictly anaerobic microorganisms, which also represent the major portion of microbes living in the human GI tract [9]. Only in 1996, 16S ribosomal RNA (rRNA) gene sequencing was for the first time compared to cultivation-based profiling and revealed a good agreement between the two approaches [10], although this against the common assumption nowadays. Thereafter, the rapid introduction of a variety of molecular, cultivation-independent methods in GI tract microbial community research boosted this field, and it has been estimated early on that at the time of publication, approximately 80% of GI microbes were still uncultured [11]. More recently, breath-taking advancements in next generation sequencing technologies [12] and analysis platforms, like QIIME and NG-Tax [13, 14], now allow gaining increasingly refined and comprehensive insights in GI microbiota composition and activity, as well as implications of these insights for health. Concurrently, a number of studies investigated the factors contributing to the observed microbiota variation and revealed possible contributions from a range of factors, including, for example, host health status [15], medication (*e.g.* antibiotics) [16], diet [17] and age [18]. With respect to the latter factor, studies have been mainly conducted in infants and adults, whereas less attention has been paid to the increasing elderly population.

Worldwide, humans' life expectancy has increased, especially in developed counties, fuelling interest to delay the onset of ageing related diseases. Impairment of human health, such as through chronic disease and obesity, is highly prevalent in elderly, and it has been in many cases associated with GI microbiota composition and/or functionality. On top of that, with increased age and changes in living conditions (*e.g.* placement in nursing homes or even hospitalization), elderly themselves tend to have decreased capacity to cope with health deterioration, *i.e.* increased frailty, and thus rely on medications, which could also contribute to the microbiota variation [19]. A number of studies investigated the effect of ageing on the GI microbiota via comparing adults and elderly, but observations vary drastically between studies [20, 21]. This inconsistency between studies may in part be attributed to differences in methodologies and recruitment strategies. The recruited subjects often underwent a large variation in lifestyle, health status and dietary habits. Dietary interventions are known to affect the GI microbiota in infants and adults. In contrary, the impact of dietary interventions on the GI microbiota of elderly, especially frail elderly, and how that differs from effects observed in adults, received limited attention.

Before we move to the next section discussing methodologies to study microbial communities, I would like to introduce some terminologies which are often used ambiguously in this field. The most comprehensive and recent set of definitions was proposed by Marchesi and Ravel in 2015 [22], comprising, among others, the following terms: *Microbiota* is the assemblage of microorganisms present in a defined environment; *Microbiome* involves the entire habitat, including the microorganisms (bacteria, archaea, eukaryote and virus), their genomes, activities and abiotic and biotic conditions; *Metagenomics* describes the approach to study the collection of genomes and genes from the members of a microbiota; *Metatranscriptomics* refers to the analysis of the RNA expressed by the microbiota in a sample via sequencing of the corresponding cDNA; *Metaproteomics* refers to the characterization of all proteins produced by the microbiota in a given sample; *Metabonomics* refers to the analytical approaches used to determine the metabolite profile(s) from complex systems.

1.2. Studying microbial communities using different methodologies

Methods used to study the GI microbiota are often divided into cultivation dependent and independent approaches, targeting different aspects of the microbial community (Fig. 1). Generically, culture dependent approaches are employed to isolate and culture microbes using predefined media in different cultivation systems (batch or continuous, reviewed elsewhere [23]). Culture dependent methods, however, substantially limit the discovery of new microorganisms or unknown species, due to the uncertainty of required nutrients and/or living conditions, like oxygen concentration, pH and availability of carbon sources. Historically, one of the hallmark cultivation independent approaches, *i.e.* random 16S rRNA gene sequencing, was used for the first time to characterize human associated GI microbiota in 1996 [10]. Shortly after, comparative analysis revealed nearly 80% of the microorganisms in the human GI tract were still unknown or uncultured at that time [11]. Simultaneously, scientists continue to develop new approaches [9, 24, 25] to culture the yet uncultured microbes. In this context, culturomic approaches, *i.e.* high-throughput culturing, came to the spotlight [26] and is becoming increasingly popular nowadays [27, 28], resulting in a rapid increase in the number of cultured species [26]. Nevertheless, studies investigating the microbial composition, diversity and functionality in large (pre-clinical) populations still largely depends on culture independent approaches.

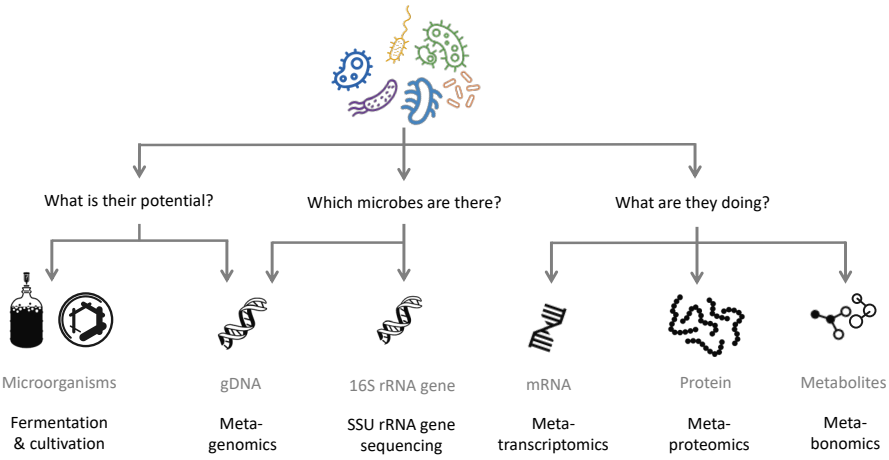


Figure 1 Microbiome analysis methodologies. Modified from Zoetendal *et al.* 2013 [29]; gDNA: genomic DNA; SSU rRNA: small subunit ribosomal RNA; mRNA: messenger RNA; Terminologies adopted from Marchesi and Ravel [22].

Rapid developments and further improvements in sequencing platforms and corresponding sequencing chemistries brought wide opportunities for microbiome research [12]. 16S rRNA gene amplicon sequencing is now routinely used to study microbial community composition, although it is criticized for low taxonomic resolution and primer bias (*i.e.* coverage and identification of microbes depending on the targeted variable region) [30]. Deep shot gun metagenome sequencing can provide information on microbial community composition at higher taxonomic resolution up to the strain level, and it also allows identifying the genetic potential of the members of a microbial community, intrinsically providing information on microbial transmission and functionality [29]. Moreover, metatranscriptomics allows

moving from genetic potential to genes that are actively expressed in the microbial community [25]. Nevertheless, metagenomics and metatranscriptomics are also not free of potential methodological biases, including DNA/RNA isolation/extraction, sequencing platforms, read length, and analysis pipeline, like HUMAnN2, PanPhlAn and MG-RAST [30-32]. To this end, studying consequential protein expression and metabolite profiles can be determined with metaproteomics [33] and metabonomics, using mass spectrometry and nuclear magnetic resonance spectrometry [34]. A primary limitation of metaproteomics and metabonomics is to distinguish between molecules derived from microbiota or the environment. To this end, it is important to compare and integrate data using different approaches, although methods to integrate different data are still at their early stage [35]. For instance, cultivation independent approaches have the potential to inspire the development of new strategies to culture yet uncultured microbes [36, 37]. Cultivation dependent approaches, in return, can provide urgently needed experimental support for predictions of microbial metabolic potential and activity, making taxonomic and functional predictions more reliable.

1.3. Microbes in the human intestinal tract

The intestinal tract includes the major sites for food digestion, fermentation and nutrient absorption [38, 39]. Specifically, after food intake, the ingested food passes through pharynx, oesophagus, stomach, small intestine and colon, after which it exits from the anus. Collectively, these different sections comprise the GI tract (Fig.2). During mastication, host digestive enzymes are secreted, and macromolecules are further digested in the stomach that is characterized by a low pH. Digestible food components are broken down to smaller molecules, *e.g.* simple sugars and amino acids, which are absorbed in the small intestine. Comparing to the stomach, the small intestine has lower oxygen levels and is less acidic, and up to 10^8 microbial cells per gram of intestinal content are found in the ileum. Thereafter, the intestinal content, including remaining food components, enter the large intestine that is characterized by further decreased oxygen concentrations and a nearly neutral pH. Here, part of the indigestible food components, including *e.g.* resistance starch and plant derived fibre, are broken down by inhabiting microbes, *i.e.* a mix of facultative anaerobes and obligate anaerobes, the number of which reaches $10^{11\text{-}12}$ cells/g or higher [40].

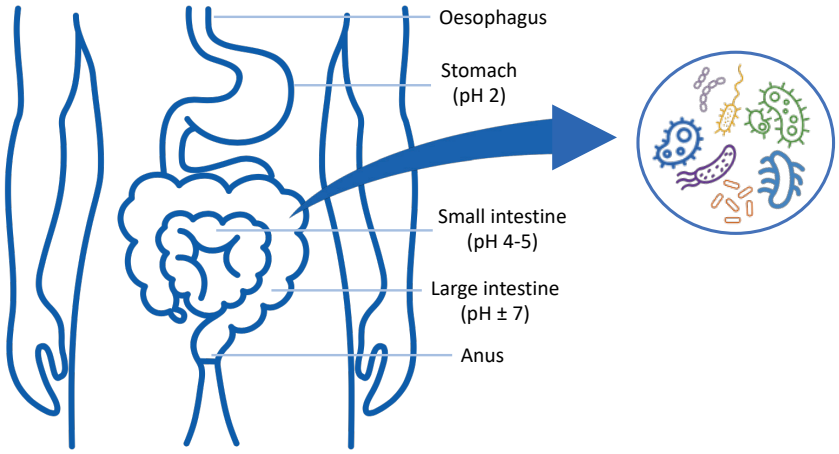


Figure 2. Schematic representation of human gastrointestinal tract, in addition to mouth and pharynx.

Specifically, the adult GI microbiota is dominated by prokaryotic cells, although other microorganisms, like fungi (the mycobiome), viruses (the virome) and bacterial viruses (the phageome) exist and have been suggested to play a role in human health [37]. At finer resolution, the major portion of prokaryotic cells is bacteria, with a small fraction of methanogenic archaea. Notably, members of the bacterial phyla Bacteroidetes and Firmicutes predominate the GI microbiota. Together with other microorganisms, the GI microbiota exhibits a strong host specificity [41], although it can be perturbed after disturbances, such as drastic dietary changes (*e.g.* changing from omnivore to vegan) and broad spectrum antibiotic intake [42]. To what extent disturbances affect microbial community composition and functionality depends on the resilience of the microbial community and the strength of the perturbation [43]. Nevertheless, considering the nutrient availability, oxygen level and physical changes from small intestine, proximal colon, distal colon to faeces, studies investigating the spatial differences along the GI tract are warranted.

Remarkably, the majority of currently available GI microbiota studies are based on faeces, as stool collection is non-invasive and easily implemented for large cohorts [44]. In contrast, studies investigating the small intestinal microbial community are rather limited due to the difficulties in sampling and downstream analysis. Specifically, the small intestine is located in the middle of our body (Fig.2), and thus collecting samples from the small intestine unavoidably will be invasive and challenging in healthy volunteers. Next to that, after food passing through the stomach, small intestinal fluid contains complex structural and biochemical background noise (*e.g.* residual food particles blocking sampling tubes, digestive enzymes and bile), which render reproducible and representative microbial analyses (*e.g.* DNA/RNA isolation) difficult. In one of the first studies of small intestinal microbiota, samples were collected from ileostomy patients and studied in terms of small intestinal microbiota composition and activity. It was shown that the microorganisms living in the small intestine are mostly rapid nutrient utilizers that can respond quickly to changes in nutrient availability, such as enterococci and streptococci [45]. Although differences in microbial composition have often been observed between different studies, a general

consensus is that the small intestinal microbiota is less diverse, but more dynamic, compared to that of the colon [46]. Furthermore, the small intestine is composed of duodenum, jejunum and ileum, that differ in terms of microbial load and composition [45, 47-49], in addition to the differences between individuals [48, 50]. For instance, the number of microbes is higher in the ileum, compared to duodenum and jejunum [48]. Similar to studies of small intestinal microbiota, studies investigating spatial differences in colonic microbiota also face methodological challenges, including, for example, bowel preparation effects [51, 52]. A recent study using a two-colonoscope approach without bowel preparation procedure, revealed distinct microbial composition between proximal and distal colon samples in 20 healthy subjects [53]. Nevertheless, the spatio-temporal dynamics of the small intestinal and colonic microbiota composition and activity is still largely unknown.

1.4. Microbiota during life

For over a century, it has been believed that the foetal environment is sterile. Microbes colonize the human GI tract after birth, vertically from the mother and horizontally from the environment [54]. In recent years, however, a few studies challenged the *sterile womb paradigm* in healthy pregnancies and described the microbial community in foetus, placenta and amniotic fluid [55-57]. Nevertheless, these studies are criticized for lacking relevant negative controls and for being prone to contaminant DNA in studies using culture independent approaches [58].

After the initial colonization, the human microbiome undergoes constant compositional and functional changes, until reaching a relative stable adult like community [59]. The process of colonization and further maturation is highly impacted by delivery mode, feeding mode (*i.e.* breast or formula feeding) and medication (*e.g.* antibiotics) [60]. It is now generally believed that these events have a possible contribution to quality of life at later ages [61]. Disruption of the normally balanced microbial community has been associated with adverse effects, such as obesity and inflammatory diseases (*e.g.* inflammatory bowel diseases) [43]. A number of studies showed differences in microbiota of young adults and elderly, suggesting alterations during ageing, with a pronounced decrease in microbial diversity [62] and alteration in microbial composition (*e.g.* decrease in *Bifidobacterium* abundance) [63-65], whereas contradictory results have also been reported [66]. This inconsistency could in part be attribute to the differences in methodologies [30] and recruitment strategy (in/exclusion criteria), including dietary habit, smoking, living situation and health status [67, 68] (details in Chapter 2). Notably, the GI microbial community of elderly in different health states has received limited attention.

Although the role of microbiota in the ageing process is still under debate (Chapter 2), modulating microbiota composition has been the aim of many studies. In most cases, studies employed dietary changes, specifically changing the whole diet (*e.g.* Mediterranean diet intervention) or adding specific food components. Comparing to comprehensive diet changes, dietary supplementation with additional food components is more favourable to consumers in their daily routines. One of the most commonly supplemented/advised food components is dietary fibre.

1.5. Dietary fibre and GI microbiota

Of all dietary components that enter our intestine, dietary fibre is most well-known for its health promoting effect, especially for GI physiology via structuring the digesta (*e.g.* bulking), modulating the digestive process (*i.e.* transit time) or acting as energy source for the GI microbiota [69]. Microbes living in the GI tract, especially bacteria, break down non-digestible food components, such as non-digestible carbohydrates (NDCs), which can escape endogenous enzyme digestion and absorption from the small intestine. In the meantime, a vast variety of microbial metabolites, including gas (*e.g.* CH_4, H_2 and CO_2), vitamins (*e.g.* B_{12} and K) and organic acids (*e.g.* acetic, propionic and butyric acids) are generated (Fig. 4). Although the effect of some specific compounds on human health is still unclear, products like vitamins and organic acids are known to contribute to human health [70]. Remarkably, supplemented NDCs, in return, also could contribute to GI microbiota modulation, with some of the NDCs conveying a prebiotic potential [39].

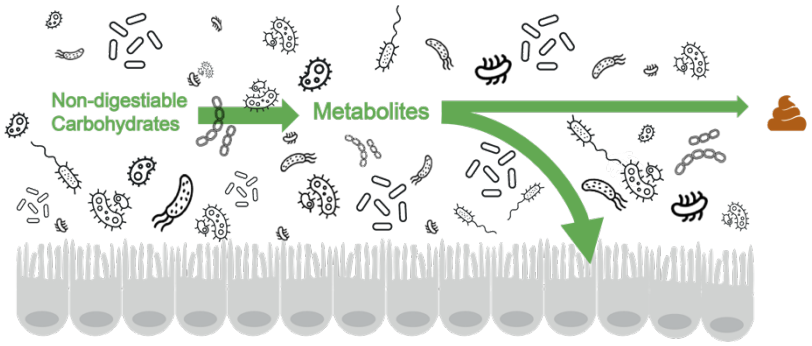


Figure 3. Interaction of non-digestible carbohydrates and gastrointestinal microbiota

The first definition of a prebiotic was introduced in 1995 by Glenn Gibson and Marcel Roberfroid [71] as “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon”. Although the probiotic concept has remained the same, its exact definition has been adjusted many times [72-74]. In 2017, Gibson *et al.* provided the most recent definition as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [73]. Although the definition of prebiotics kept changing, numerous research groups have dived into this field in the quest for novel potential prebiotics [75]. Among all candidates, inulin type fructans and galactans have been most frequently documented for their health promoting effects, as well as promoting the (relative) abundance of *Bifidobacterium* and/or *Lactobacillus* [75, 76], and decreasing the amount of potentially pathogenic microbes [75]. Remarkably, the changes in the GI microbial communities is associated with many factors, including but not limited to, properties of supplemented food components (*i.e.* NDCs for this thesis) and the individual-specific resident microbiota. On one hand, supplemented NDCs can affect microbiota composition (*e.g.* promoting the growth of certain bacteria, like *Bifidobacterium*) and diversity. The extent of this effect will, in part, be depending on the property of supplemented NDCs (*e.g.* type of linkage, degree of polymerization and side chain

properties). On the other hand, the resident microbial community determines whether, how and to what extent supplemented NDCs get utilized as well as corresponding gene/protein expression and metabolites yield, *i.e.* the functionality of the GI microbial community [70]. Investigations of the changes in microbiota composition and metabolic activities in response to dietary interventions, ranging from major dietary changes to addition of specific dietary supplementations, have been to date often based on adults [77, 78].

Collectively, we are still at the beginning of understanding the microbial communities residing in different sections along the GI tract, as well as their response to different dietary supplementations in elderly, especially elderly of different health status, and if (and how) those differ from adults.

2. Research aims and thesis outline

The work described in this thesis aims to decipher the composition and function of the GI tract microbiota in adults, especially elderly, as well as the response of the microbiota to different dietary fibre supplementations and how this is related to health.

This thesis comprises seven chapters, including one review (**Chapter 2**) and four research chapters. Three of the research chapters focus on different dietary interventions (**Chapter 3-5**), whereas one research chapter focusses on the metabolic capacity of intestinal microbiota (**Chapter 6**).

Chapter 1 briefly introduces the microbes in the human GI tract and gives an outline of this thesis, highlighting aims and motivation for carrying out studies included in this thesis. **Chapter 2** provides a literature **review**, summarizing the current understanding of age-related changes in microbiota composition, and how microbiota-targeted interventions could be used to counteract these changes.

The **three intervention studies** are presented in Chapter 3-5. Here we investigate the effect of different fibre supplementations on microbiota composition in adults and elderly, as well as the differences in their microbial function along the GI tract. **Chapter 3** compares the faecal microbial composition between healthy adults and healthy elderly, as well as their response to the supplementation of sugar beet pectin, using a randomized, double-blind, placebo-controlled, parallel study. **Chapter 4** focuses on pre-frail elderly, investigating differences in microbial composition of adults and pre-frail elderly, and their response to galacto-oligosaccharide (GOS) supplementation using a randomized, double-blind, placebo-controlled, cross-over study. This study provides insights into the impact of health status (*i.e.* frailty level) on faecal microbiota composition, as well as its impact on the response of the microbiota to GOS supplementation. **Chapter 5** describes a synbiotic intervention using a combination of chicory fructo-oligosaccharide (FOS) and the multispecies probiotic Ecologic® 825 on faecal microbiota composition, as well as the microbiota composition of duodenum, jejunum and ileum in healthy adults over time. This study highlights the temporal dynamics in small intestinal microbiota composition, as well as its response to the supplemented synbiotic.

A sub-group of subjects included in the GOS intervention (Chapter 4), has been recruited as donor of faecal inoculum for two *in vitro* fermentation studies. **Chapter 6** compares the faecal microbiota metabolic capacity of pre-fail elderly and healthy adults using non-food grade components which differ in molecular structure.

In **Chapter 7**, I summarize and discuss critically the research findings and give recommendations for future studies.

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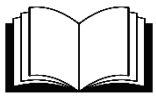
Age dependent changes in GI physiology and microbiota: time to reconsider?



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Abstract

Our life expectancy is increasing, leading to a rise in the ageing population. Ageing is associated with a decline in physiological function and adaptive capacity. Altered gastrointestinal (GI) physiology can affect the amount and types of nutrients digested and absorbed as well as impact the intestinal microbiota. The intestinal microbiota is considered a key player in our health, and a variety of studies have reported that microbiota composition is changing during ageing. Since ageing is associated with a decline in GI function and adaptive capacity, it is crucial to obtain insights into this decline and how this is related to the intestinal microbiota in the elderly. Hence, in this review we focus on age-related changes in GI physiology and function, changes of the intestinal microbiota with ageing and frailty, how these are associated, and how intestinal microbiota-targeted interventions may counteract these changes.

Highlights

What is already known on this subject?

- Ageing is associated with alterations in gastrointestinal function, on both the organ and cellular level, including an impaired immune function.
- The composition of the intestinal microbiota changes during ageing.

What are the new findings?

- The direct impact of age-related alterations in gastrointestinal function on nutrient digestion and absorption, as well as on the intestinal microbiota composition and functionality, is largely unknown.
- Intestinal microbiota perturbations in the elderly are more likely to be associated with health status, medication use and lifestyle factors, rather than with chronological ageing per se.

How might it impact on clinical practice in the foreseeable future?

- The intestinal microbiota could be a promising target to improve health outcome in relevant subgroups of elderly.
- More studies are needed on the impact of manipulating the intestinal microbiota, using a targeted approach and clear focus on well characterized and relevant subgroups of elderly (such as frail elderly, specific comorbid conditions and/or medication use).

1. Introduction

Although geographical differences exist, the overall life expectancy is increasing worldwide, leading to a steady rise in the ageing population. In Europe, the proportion of individuals aged 65 years and over has been estimated to increase from 17% in 2010 to 30% in 2060 [1]. Ageing is associated with an inevitable time-dependent decline in physiological function and adaptive capacity, as a result of lifelong accumulating molecular and cellular damages [2-4]. Several studies have shown that this decline is host-specific and can be influenced by various factors such as host genetics, lifestyle (*e.g.* diet and smoking), sociodemographics (*e.g.* age, socioeconomic status and ethnic background), living situation (*e.g.* community-dwelling or institutionalized) [5] and co-morbidities (including medication use), contributing to large heterogeneity in the elderly population. Whereas conventionally elderly were defined as being >65 years of age, nowadays the overall rate of biological ageing is decreasing, and cut-offs as well as age definitions vary widely. In scientific research as well as in clinical settings, it is therefore relevant to assess physiological functionality rather than focusing on chronological age. Some studies include subjects with specific co-morbidities or (pre-)frail elderly to address differences in age-related physiology. Others focus on centenarians to get further insight in genetic and lifestyle factors associated with longevity and resilience to disease [6].

Frailty is "a syndrome of decreased reserve and resistance to stressors, resulting from cumulative declines across multiple physiological systems, causing vulnerability to adverse outcomes" as defined by Fried *et al*. [7] Applying this definition in a meta-analysis of 2009, the pooled prevalence of frail elderly in community-dwelling adults aged 65 years and older in Europe was 17.0% [8]. The frailty phenotype of Fried *et al*. [7] has been operationalised to assess physical frailty by evaluating unintentional weight loss, self-reported exhaustion, weakness (*e.g.* by handgrip strength), slow walking speed, and low physical activity. Besides these widely adopted Fried criteria, several multidisciplinary scores exist that include measures of medical, psychological, cognitive, functional, and/or social loss [9]. In an extensive systematic review, 79 different frailty assessment tools have been identified, though a 'gold' standard is lacking [9].

Since frailty is associated with an increased risk of negative health outcomes leading to disability and impaired quality of life [10], the increasing group of frail elderly leads to more direct (*e.g.* consultations, diagnostic procedures, hospitalisations, medication use) and indirect healthcare costs (*e.g.* social and daily support). Therefore, further insights into modifiable factors and preventive strategies are highly relevant.

The intestinal microbiota, *i.e.* the collection of microbes found in the intestine, may be a promising target as it clearly links to a myriad of host functions, is affected by environmental factors, and perturbations have been reported in the ageing population. Nutritional interventions, aiming to modulate intestinal microbiota composition and functionality, may contribute to intestinal health and general wellbeing (of the ageing population). Therefore, our aim was to review 1) the current knowledge on the effect of ageing on gastrointestinal (GI) physiology and on intestinal microbiota, 2) whether other factors besides ageing, such as

frailty, affect GI physiology and microbiota, and 3) potential targets to counteract the changes in GI physiology and microbiota observed in elderly and/or frailty. Hence, current knowledge on GI physiology and function, intestinal microbiota composition and activity, and manipulation of the intestinal microbiota in relation to ageing and frailty will be summarised, paying specific attention to age-definitions and associated health status.

2. Physiology and function of the ageing GI tract

The GI tract has a core function for the human body as it ensures adequate digestion and absorption of nutrients, involving a sequence of events from the mouth until the anus. These are facilitated by GI motility and digestive secretions and regulated by neural and hormonal control. Age-related functional declines have been reported for some of the organs involved (Figure 1).

In the mouth, masticatory function and taste are often found to be impaired in elderly in general [11]. Moreover, Watanabe *et al*. [12] found that frail elderly had significantly fewer teeth, lower occlusal force and muscle thickness when compared to healthy and pre-frail elderly, indicating that impaired masticatory function becomes more pronounced with frailty [12]. Swallowing problems were reported by 10%-30%, in a heterogeneous group of individuals aged 65 years and over [13]. Swallowing depends amongst others on the presence of saliva. Results from a meta-analysis, including 47 original controlled studies, showed that salivary flow rate decreases with healthy ageing, resulting from degenerative changes of cellular structures of the salivary glands [14]. While reduced salivary secretion has been associated with medication use such as anticholinergic drugs, psychotropic drugs, antihistamines and diuretics, sub analyses showed that medication use does not impact salivary flow rate [14]. Frailty criteria were, however, not taken into account. A recent study by Rogus-Pulia *et al*. [15] showed that perception of mouth dryness was also associated with increasing age, although potential contributing factors like comorbidities and medication use were not addressed [15]. The observed dryness is probably due to compositional changes in saliva, which may negatively impact thickness and adherence of the oral salivary film [15,16]. Increased impairment in mouth functions as reported for elderly in general, can contribute to food avoidance, reduced or altered intake (*e.g.* more easily digestible food), and eventually poor nutritional status [11]. This can also impact intestinal microbiota composition and activity. Deteriorated oesophageal functions repeatedly reported in elderly in general, include reduced peristalsis [17,18], increased number of non-propulsive contractions [18], and decreased compliance of the oesophagus [18]. These alterations can be found already from the age of 40 years [19]. Healthy ageing was not or only to a modest extent found to be associated with slowing of gastric emptying of both solids and liquids [20,21]. In frail compared to non-frail elderly, gastric emptying of liquids was even found to be enhanced [21]. Furthermore, the gastric compliance was reduced, while gallbladder emptying and oro-caecal transit time were not different between frail and non-frail elderly [21]. On the other hand, in a recent review it was stated that healthy elderly have a longer oro-caecal and colonic transit time than healthy young adults [22]. The decreased rectal compliance and decreased rectal sensation associates with healthy ageing and may contribute to complaints such as constipation [18]. Several studies have provided evidence for alterations in motility associated with healthy ageing, being most pronounced for the oesophagus and large intestine. It should be acknowledged that increased intestinal transit time affects composition and activity of intestinal microbiota, both *in vitro* [23] and *in vivo* [24], but data in elderly or frailty are lacking.

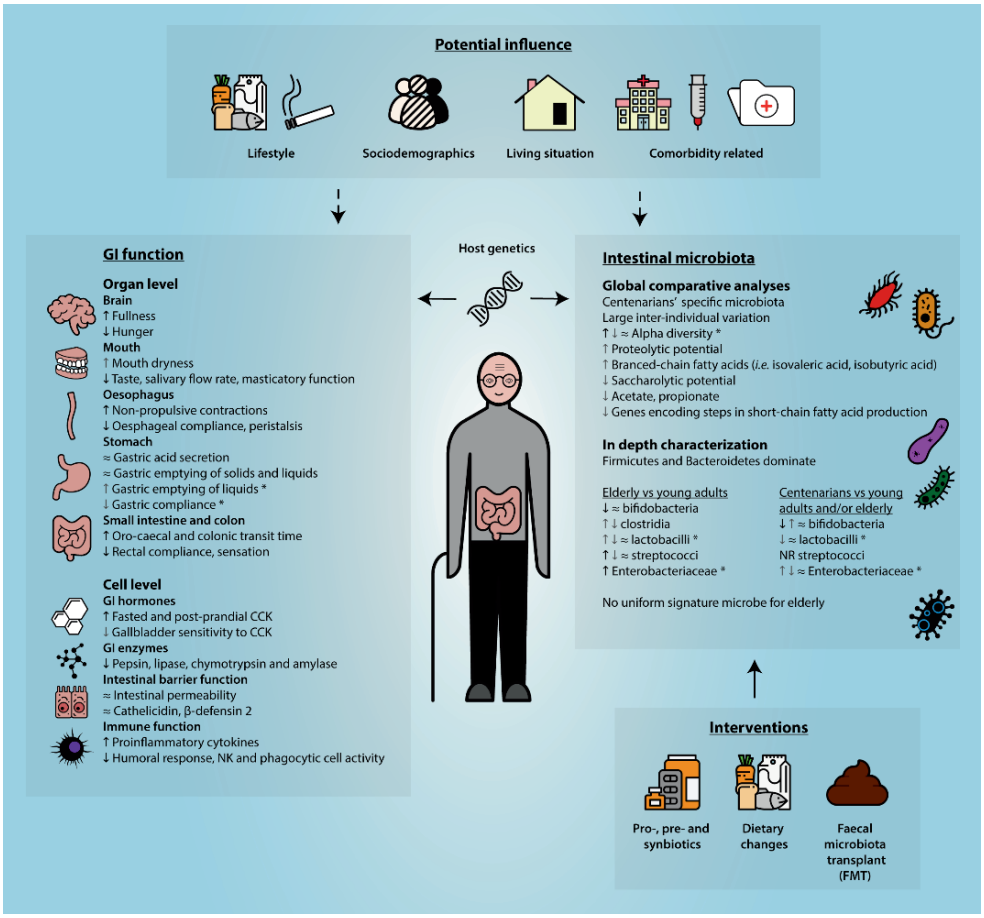

Figure 1. Non-exclusive listing of key changes in gastrointestinal function and the intestinal microbiota during ageing, including potential influencing factors as well as interventions to beneficially manipulate the intestinal microbiota. Legend: * Frailty related; ≈ No significant difference; NR Not Reported; ↑ A few or the minority of studies showing a significant increase; ↑ Several or the majority of studies showing a significant increase; ↓ A few or the minority of studies showing a significant decrease; ↓ Several or the majority of studies showing a significant decrease.

Most GI functions, including the secretion of gastric juice, bile and digestive enzymes, as well as GI motility, are regulated by a complex interplay of hormonal and neuronal factors, involving the central and the enteric nervous system. In the stomach, marked alterations in gastric acid secretion could not be demonstrated in healthy elderly [25]. Though, the higher prevalence of atrophic gastritis and proton pump inhibitor use in elderly [20] will lead to decreased acid production and subsequently bacterial overgrowth in subgroups. Rémond *et al.* [26] reviewed studies on the effects of ageing on excretion of digestive secretions and found that bicarbonate secretion, as well as enzyme concentrations of pepsin in the stomach, and lipase, chymotrypsin and amylase in duodenal fluids were lower in healthy elderly compared to young adults. However, bile acid secretion, was not affected by ageing [26]. In an extensive review on (an)orexigenic GI hormones, strongest evidence was found for elevated fasted and post-prandial concentrations of cholecystokinin (CCK) in elderly in

general compared to younger adults [27]. Although gallbladder emptying was similar, gallbladder sensitivity to endogenous CCK was significantly reduced in healthy elderly compared to young adults [28]. Evidence on the effects of ageing on peptide YY, glucagon-like peptide-1 and ghrelin levels were inconclusive, partly as a consequence of different methodologies used, whereas pancreatic polypeptide and oxyntomodulin are hardly studied [27]. Data on other relevant GI hormones and peptides (*e.g.* secretin, gastrin, motilin, somatostatin, chromogranin A) as well as specifically addressing subgroups of elderly are scarce. More studies using standardised methods and clear definitions of the target population are needed. The enteric nervous system also plays an important role in the regulation of *e.g.* motility, secretions and local blood flood, by bidirectional communication between the brain and the gut, and by local reflexes. Several rodent studies found the ageing process to induce neuronal loss as determined by lower neuronal density in oesophageal, small intestinal and colonic tissue [20]. This effect was more pronounced in cholinergic when compared to nitrergic myenteric neurons [29]. Human data are, however, lacking.

Although several of the above processes will impact GI function, studies on intestinal absorption of nutrients per se are largely lacking. In rodent studies, ageing was associated with a decrease in surface area because of villus degeneration [20] and malabsorption of carbohydrates, lipids, proteins, minerals and vitamins [30], but these findings need confirmation in humans. Food intake was, however, found to be altered in healthy elderly, which was associated with decreased hunger and appetite scores [27]. A meta-analysis including 59 studies confirmed that hunger scores were 25% and 39% lower after overnight fasting and in postprandial state, respectively, and fullness 37% higher in healthy elderly compared to young adults [31]. Furthermore, reduced nutritional status was found to be related to frailty in elderly [32].

Apart from the segment-specific functions with regard to digestion and absorption, an adequate GI barrier function is pivotal for protection against the external environment, including epithelial integrity, mucus and defensin secretion, as well as the gut associated lymphoid tissue. Although studies in primates suggest an impaired epithelial barrier function in older animals [33], Wilms and colleagues [34] did not observe differences between healthy elderly versus adults based on results from a combined *in vivo* (using the multi-sugar permeability test) and ex vivo approach (evaluating colonic biopsies in Ussing chambers) [34]. Although not specifically studied in elderly, factors associated with ageing such as nonsteroidal anti-inflammatory drugs (NSAID) use, alcohol intake, obesity and diabetes have, however, been shown to increase intestinal permeability. The 'chemical' barrier is hardly studied in elderly. One study showed that serum levels of the host defensive peptides cathelicidin and β-defensin 2, were similar between healthy elderly and young adults [35]. Data on mucosal and/or faecal samples as well as on the role of other antimicrobial peptides or mucus and analyses in frail elderly specifically are lacking. Many studies explored the impact of ageing on the intestinal immune response. It has repeatedly been shown that the human intestinal mucosal immune system is compromised with ageing, as nicely reviewed by Mabbott *et al*. [36] Alterations in dendritic cell subsets have been reported in elderly in general [36]. Further, healthy ageing is characterized amongst others by a pro-inflammatory cytokine profile ('inflammageing', *i.e.* increased levels of amongst others TNF-α and IL-6), a decreased humoral response such as reduced secretory IgA levels, and decreased natural

killing and phagocytic cell activity [37]. This will be further affected in immune-related comorbid conditions and/or frailty [37]. The observed immunosenescence can contribute to the increased risk of recurrent and persistent infections reported in the elderly [37].

3. Intestinal microbiota in elderly

Ageing is associated with several alterations in GI physiology and function, which can impact the amount and types of nutrients delivered to the small intestine and colon, thereby affecting intestinal microbiota composition and functionality in these segments. Additionally, the altered immune function will impact host-microbe interactions, which can also contribute to alterations in intestinal microbiota composition and functionality in this population.

The GI tract encompasses different biotic environments. Although different types of microorganisms, such as archaea, fungi, viruses and bacteriophages may play a role in intestinal health [38], most studies focus on bacteria. Furthermore, it is evident that different locations of the GI tract harbour distinct microbial communities [39], but determination of the bacterial composition is mostly performed on faecal samples as their collection is non-invasive and feasible for large ((pre-)clinical) populations. Nevertheless, other intestinal sites can be sampled using luminal brushes, rectal swabs, colonic lavage, and mucosal biopsies as reviewed earlier [38]. In elderly, studies have mainly focused on analyses of the microbiota composition in faecal samples, which is generally considered to be representative for the distal large intestinal content.

In this section, we summarize the current knowledge on microbiota composition of elderly and discuss whether the faecal microbiota of young adults is different from elderly, paying special attention to age-related health status (*i.e.* (pre)frail and centenarians) and confounding factors.

3.1. Global faecal microbiota comparative analyses

A wide variety of studies have compared the faecal microbiota composition in elderly versus young adults and centenarians. In supplementary table S1, a non-exclusive overview is given of studies investigating the faecal microbiota profile of elderly. Their overall microbiota composition was generally visualized and verified by ordination and multivariate analysis, *e.g.* significant age-group-based separation in diagrams. At phylum level, the faecal microbiota of young adults and elderly was found to be rather similar in some studies [40-43], whereas others reported significant differences [40,44,45]. Remarkably, the faecal microbiota of centenarians, who are reported to have a lower incidence [46] of chronic illness than 80-99 years old elderly and considered a “successful” ageing model [47], was reported to be different from that of 70-years-old non-institutionalized elderly [42]. Although these studies were conducted in different countries, a clear link with the geographic origin cannot be observed. Part of the contradictory findings between young adults and elderly could be due to differences in recruitment strategy (in/exclusion criteria), age definitions and confounding factors, such as comorbidity, medication use, lifestyle and socioeconomic factors, hampering an adequate comparison. For instance, the microbiota composition of smokers was different from that of non-smokers, aged 20-59 yrs [48]. The effect of smoking in combination with

ageing has not been reported to date. Overall, the microbiota of elderly is highly variable [45]. Therefore, it is hard to define typical microbiota of elderly and that of centenarians.

Observations with regard to the effect of ageing on the alpha diversity, *i.e.* compositional complexity based on richness and evenness of the microbial ecosystem, vary, especially due to frailty and within the group of centenarians (Table 1). Initially, alpha diversity was found to decline during ageing [49,50], which was mainly based on cultivation and classical 16S ribosomal RNA (rRNA) gene approaches. However, this observation could not be confirmed by high throughput 16S rRNA gene sequencing and phylogenetic microarrays. Several studies even reported higher alpha diversity [51-54] in the microbiota of community dwelling elderly versus young adults, while others reported no significant differences [42,55,56]. A high alpha diversity has often been suggested to be associated with better homeostasis and resilience to disturbance [57]. In terms of centenarians, their alpha diversity was reported to be higher than that of elderly [43,58,59], but not exclusively [42,51,59]. Moreover, a broad range of confounding factors can affect the varying microbiota alpha diversity observed, including host and/or lifestyle factors. For instance, a decreased alpha diversity was reported for smokers comparing to non-smokers [48]. In addition, although not exclusively [60,61], lower alpha diversity has been reported to be associated with increased [53,55,56] frailty (Table 2), which suggests that the health status of elderly rather than ageing itself is associated with a lower alpha diversity of the faecal microbiota.

Table 1. Ageing associated changes in alpha diversity of faecal microbiota in elderly and centenarians

Ref. (Country of study cohort)	Young		Elderly		Group being compared	Microbiota profiling[a]	Elderly[b]		Centenarian[c]	
	Nr.	Age range	Nr.	Age range			Diversity	Richness	Diversity	Richness
Zwielehner 2009 (Austria)[49]	17	18-31y	17 institutional	78-94y	Young adults vs elderly	PCR-DGGE, qPCR, clone libraries	↓	n.r.	n.a.	n.a.
Biagi 2010 (Italy)[42]	20	25-40y	43	59-78y	Young adults vs elderly vs centenarians	Phylogenetic microarray & qPCR	≈	n.r.	↓	n.r.
			21	99-104y						
Kong 2016 (China)[58]	47	24-64y	54	65-83y	Nonagenarians and centenarians vs young adults and elderly	Illumina MiSeq	n.r.	n.r.	↑	↑
			67	90-102y						
Biagi 2016 (Italy)[43]	15	22-48y	15	65-75y	Young adults vs elderly vs centenarians	Illumina MiSeq	n.r.	n.r.	↑	↑
			39	99-109y						
Wang 2015 (China)[59]			16	80-99y	Elderly vs centenarians	Illumina MiSeq	n.a.	n.a.	≈	≈ or ↑[d]
			8	100-108y						
O'Toole 2015 (Ireland)[56]			282	64-102y	Age association	Pyrosequencing	≈	n.r.	n.r.	n.r.
Falony 2016 (Belgium)[52]	1106 (19-85y)[e]				Adults <40y vs middle-aged 40-59y vs elderly >60y	Illumina MiSeq	n.r.	↑	n.a.	n.a.
Odamaki 2016 (Japan)[51]	367 (0-104y)[e]				Age association	Illumina MiSeq	↑	↑	↓	↓
Jackson 2016 (UK)[53]	728 (42-86y)[e]				Age association	Illumina MiSeq	↑	↑	n.a.	n.a.
Bian 2017 (China)[54]	1095 (3-100y+)[e]				Age association	Illumina MiSeq	↑	n.a.	n.r.	n.r.
Maffei 2017 (US)[55]	85 (43-79y)[e]				Age association	Illumina MiSeq	≈	≈	n.a.	n.a.

Nr.: number of subjects; y: years of age; Centenarian: people aged >100y; Nonagenarian: people aged 90-100y; PCR-DGGE: polymerase chain reaction denaturing gradient gel electrophoresis; qPCR: quantitative polymerase chain reaction; ↓ significant decrease; ↑ significant increase; ≈ not significantly different; n.a. not available; n.r. not reported; [a] 16S rRNA (gene)-based; [b] microbiota comparison between elderly and young adults; [c] microbiota comparison between centenarians and elderly/young adults; [d] depending on which subgroup of elderly they compared; [e] studies did not report on the definition of young adult, elderly and centenarian.

Table 2. Summary of studies associating faecal microbiota profiles with frailty

Ref. (Country of study cohort)	Young		Elderly		Determination of frailty	16S rRNA approach	Main findings with increased frailty
	Nr.	Age range	Nr.	Age range			
van Tongeren 2005 (The Netherlands)[72]			23	70-100y	Groningen Frailty Indicator	FISH	↓lactobacilli/ *Enterococcus* group ↓*Bacteroides/Prevotella* group ↓*Faecalibacterium prausnitzii* ↑Enterobacteriaceae
Claesson 2012 (Ireland)[77]	13	28-46y	178	64-102y	Barthel Index & Functional Independence Measure	Pyrosequencing	↓*Ruminococcus* CAG, ↓*Prevotella* ↓*Oscillibacter* CAG ↑*Bacteroides* CAG
O'Toole 2015 (Ireland)[56]			282	64-102y	Barthel Index & Functional Independence Measure	Pyrosequencing	↓alpha diversity
Jeffery 2016 (Ireland)[60]	13	28-46y	371	64-102y	Barthel Index & Functional Independence Measure	Pyrosequencing	↓alpha diversity ↑alpha diversity
Ticinesi 2017 (Italy)[61]			76 multimorbid	71-97y	Rockwood Frailty Index	Illumina MiSeq	≈ microbial richness ↑unclassified member of Clostridiaceae 1 family ↑unclassified member of Lachnospiraceae family ↑*Oscillospira*, ↑*Peptococcus*, ↑*Porphyromonas* ↑*Fonticella*, ↑*Prevotella*
			25	65-87y			
Jackson 2016 (UK)[53]	728 (42-86y)[a]				Rockwood Frailty Index	Illumina MiSeq	↓microbial diversity and richness ↓a sub-set of Lachnospiraceae OTUs ↓*Faecalibacterium*, ↓*Faecalibacterium prausnitzii* ↑*Eubacterium*,↑*Eggerthella*, ↑*Eubacterium dolichum* ↑*Eggerthella lenta*, ↑13 Enterobacteriaceae OTUs
Maffei 2017 (US)[55]	85 (43-79y)[a]				34-item frailty index	Illumina MiSeq	↓microbial richness ↓*Paraprevotella*,↓*Sutterella* ↓Rikenellaceae family OTU ↑*Coprobacillu*, ↑*Dialister* ↑TM7 candidate-phylum OTU

Nr.: number of subjects; y: years of age; CAG: co-abundance groups; FISH: fluorescence in situ hybridisation; OTU: operational taxonomic unit; Increased Barthel index indicates decreased frailty; Increased functional independence measure indicates decreased frailty; ↓ significant decrease; ↑ significant increase; ≈ not significantly different; [a] studies did not report on the definition of young adult, elderly and centenarian.

3.2. In depth characterization of faecal microbiota composition

The first paper that associated the dynamics of several bacterial genera during life was published by Mitsuoka [62] in 1990. This culture-based hallmark paper described that in comparison to young adults, the faecal microbiota of elderly comprised a lower abundance of bifidobacteria, whereas clostridia, lactobacilli, streptococci, Enterobacteriaceae were increased. In this review, we compared and contrasted subsequent culture-based as well as culture-independent studies with respect to these identified ageing-associated bacterial groups.

The reduction in the abundance of bifidobacteria in the faecal microbiota of elderly, has been confirmed in many studies [40,43,49,63-65], irrespective of elderly or frailty definitions, though not exclusively [42,43,66]. Surprisingly, a decreased abundance of bifidobacteria has also been reported in centenarians (*i.e.* 99-104yrs), compared to young adults [65] or elderly (>65yrs) [43], whereas an increased abundance (of bifidobacteria) was reported in (super)centenarians (>105yrs) [43]. In an Italian cohort, no significant differences in bifidobacteria abundance were observed between elderly and centenarians (*i.e.* 99-104yrs) [42]. The abundance (of bifidobacteria) was reported to be lower in the subgroup of institutionalized geriatric elderly [49], in the subgroup of hospitalized elderly [64] and in the subgroup of *Clostridium difficile* associated disease (CDAD) elderly [63], compared to healthy elderly and young adults. Furthermore, the abundance of bifidobacteria was higher in another Italian cohort than in Swedish, German and French cohorts, irrespective of age (*i.e.* both young adults and elderly), which was concluded to be due to differences in dietary habits [40]. These observations indicate that comorbidity and habitual diet may affect the alterations in the abundance of bifidobacteria during ageing, but to what extent and how they could contribute to the observed changes is still not clear.

Clostridia, lactobacilli and streptococci belong to the Firmicutes phylum (often referred to as Gram-positive bacteria with low guanine-cytosine content in their DNA). In line with observations in young adults, the phyla Firmicutes and Bacteroidetes together form the most dominant fraction of the microbiota in elderly. However, whether Firmicutes [51,67,68] or Bacteroidetes [44,45,59] is the most dominant phylum, differs between studies.

The number of studies that has reported an effect of ageing on the abundance of clostridia is rather small when compared to other bacterial groups. This can in part be attributed to the continuous reclassification and renaming of anaerobic spore-forming bacterial isolates after the introduction of 16S rRNA gene-based taxonomy that were traditionally named Clostridium. Although increases and reductions in the abundance of clostridia have been described in the faecal microbiota of elderly versus young adults [64,69], comparative analyses between studies is hampered by this continuous reclassification.

The reported increase in abundance of lactobacilli during ageing by Mitsuoka *et al*. could not be confirmed in other culture-based studies [63,64,70], whereas it was confirmed in several culture-independent studies [40,69,71]. The abundance of lactobacilli in centenarians did not differ from that of young adults in the above-mentioned Italian cohort

[65]. However, in a Chinese cohort, the abundance of lactobacilli in rural centenarians (consuming a high fibre diet) was not different from that in healthy urban elderly (consuming a low carbohydrate and low fibre diet), but was lower in healthy rural elderly (consuming a high fibre diet) [59]. Moreover, compared to healthy elderly the abundance of lactobacilli was found to be higher in the subgroup of elderly with CDAD [63,70] and in hospitalized elderly [64]. However, the CDAD and hospitalized elderly were under metronidazole and undefined antibiotic treatment, respectively, which could have impacted the comparative analyses. Van Tongeren *et al*. found that decreased abundance of lactobacilli was associated with deteriorated health status (increased frailty) [72]. These contrasting observations again highlight the complexity of the impact of chronological age and/or additional host or environmental factors on microbiota composition.

The abundance of streptococci was reported to be mainly higher in healthy elderly and elderly with altered bowel habits compared to young adults [40,64,66,69], though not exclusively [40]. None of the studies reported on difference in the abundance of streptococci in centenarians compared to young adults or elderly so far. In addition, the abundance of streptococci was reported to be lower in the subgroup of hospitalized elderly, compared to healthy elderly [64], whereas NSAID use had no effect [69].

The family Enterobacteriaceae has diverse ecological (*i.e.* being able to survive in diverse environments) and metabolic characteristics and includes many potentially pathogenic microorganisms (*e.g.* members of the genera Escherichia, Salmonella, Klebsiella, Proteus). The abundance of enterobacteria, was reported to be higher in faecal samples of healthy elderly than that of young adults from different countries. [40,64] Remarkably, a decreased abundance was reported in centenarians compared to young adults in an Italian cohort [65]. In a Chinese cohort, however, the abundance of Enterobacteriaceae, as well as the abundance of the genus Escherichia, was higher in rural centenarians than in healthy urban elderly, but was not different from that in healthy rural elderly [59]. This indicates that, in addition to health status, the living situation (*e.g.* rural versus urban with differences in for example dietary intake and hygiene or antigen exposure) might also contribute to the alterations of intestinal microbiota composition [59]. In addition, although the abundance of Enterobacteriaceae was reported to be not significantly different between healthy elderly and hospitalized elderly [73], a higher abundance of Enterobacteriaceae was found in the subgroup of elderly carrying *Clostridium difficile* compared to *Clostridium difficile* negative elderly [74]. Moreover, increased Enterobacteriaceae abundance was associated with increased frailty [53,72]. This supports the observation that alterations in the intestinal microbiota composition are more pronounced in frail or comorbid elderly.

The observation of our comparative analyses based on the groups identified as ageing-associated by Mitsuoka [62], indicates that in comparison to young adults, the intestinal microbiota of elderly comprised lower levels of bifidobacteria and higher levels of streptococci and enterobacteria, which seem to be more pronounced in frail or comorbid elderly. However, it should be noted that contradicting findings have also been reported, such as bifidobacteria levels in centenarians. In line with the above observations, a detailed comparative analysis between different studies for other potential microbial taxa associated to elderly or relevant subgroups did not reveal a single microbial group that was

consistently positively or negatively associated with ageing (Table S1). Moreover, studies that included centenarians or different frailty phenotypes (Table 2) hint towards the observation that a decline in health status rather than ageing itself is associated to changes in intestinal microbiota composition. Comparative analyses are further hampered by the myriad of methods [38,75,76] used to study the faecal microbiota composition as well as the lack of consistent definitions of frailty [53,55,61,72,77] and age for elderly [40,43-45,59,64,72], which could be 80 yrs+ [59], 70yrs+ [44,72], 65 yrs+ [43,45,64] or 60yrs+ [40]. This stresses the need for well-designed longitudinal studies. Such studies monitoring intestinal microbiota changes over time and also taking into account the large inter-individual variation [45], provide the ideal setting to study intestinal microbiota dynamics during ageing. These studies are obviously long-lasting, adding to complexity, solid scientific infrastructure and costs. Nevertheless, several large-scale population cohorts have already been initiated [67,68]. Follow up of such cohorts may ultimately provide insight into long-term intestinal microbiota dynamics and their relation to ageing, frailty and comorbid conditions.

Overall, we clearly observed that intestinal microbiota compositional changes during ageing are more likely to be associated with health status of the elderly and confounding factors than with ageing itself. This is summarized in Figure 1 and Table 2 (See also Table S1 for details per study). It has to be taken into account that most studies focusing on the intestinal microbiota in elderly describe the faecal microbial composition based on 16S rRNA genes. Given the high level of functional redundancy within and across microbial groups as well as the fact that bacteria are very versatile and can quickly adapt and respond to changes in their environment, indicates that only considering composition of the faecal microbiota has its limitations [78]. So far, studies focusing on the metabolic capacity or activity of the intestinal microbiota in elderly via metagenomics, metabolomics or other activity-based profiling approaches, are very limited. One study reported that in comparison to young adults, the faecal microbiota of non-institutionalized elderly showed an increase in proteolytic potential, but decreased saccharolytic potential with a low abundance of genes encoding steps in short-chain fatty acids production pathways [41]. In line with this observation, decreased concentrations of acetate and propionate, but increased faecal dry matter content and concentrations of branched-chain fatty acids (*i.e.* isovaleric acid, iso-butyric acid) have been reported in institutionalized and non-institutionalized elderly compared to young adults [79,80]. Furthermore, the living situation of elderly was shown to correlate with the faecal metabolites profile, with higher concentrations of acetate, propionate and valerate in community-dwellers [77]. A recent study investigated the functional capacity and activity of the faecal microbiota in a large cohort of healthy elderly (308 men, aged 65-81 years old) using metagenomics and metatranscriptomics at four time points over 6 months. However, the study did not report on comparisons between specific subgroups of elderly or dietary habits [67,68]. It has been reported that short-term dietary changes can have a drastic impact on microbial metabolite production and host physiology without drastically changing faecal microbiota composition [81]. Hence, we argue that approaches addressing functional capacity and activity of the faecal microbiota are crucial to further unravel the role of the microbiota in host physiology of the ageing and/or frail population.

4. Manipulating the intestinal microbiota of elderly

Although a typical microbiota profile of elderly is hard to define, manipulating the intestinal microbiota of elderly and host outcome has been subject of several studies. Supplementation of functional foods like pro-, pre- or synbiotics are nutritional approaches to beneficially alter the microbiota (Figure 1). Several studies have been performed on the effect of probiotics in elderly, of which many focuses on the risk of infections and immunosenescence. In a systematic review, including 15 randomised clinical trials in 5916 patients with a mean age of 75 years, Wachholz *et al*. did not find significant effects of probiotics on the occurrence and durations of infections, nor on mortality rate, when compared to placebo [82]. Also with regard to inflammatory and immunosenescence markers, Calder *et al*. concluded in a recent review that the evidence for the efficacy of probiotics in elderly is limited and/or inconsistent [83]. Several elderly studies did, however, show changes in faecal microbiota composition, being most pronounced for increased abundances of bifidobacteria, after use of for example *Bifidobacterium* lactis HN019, *Bifidobacterium longum* 46 and *Bifidobacterium longum* 2C or multi-species probiotics [84]. Although probiotic use is often considered to be safe, specific safety studies in elderly are still limited and extra caution is warranted, especially in subjects with impaired host defence mechanisms.

Well known prebiotics, including galacto-oligosaccharides (GOS), inulin and fructo-oligosaccharides [85], have often been evaluated for their effect on bowel habits in constipated elderly, and showed an increase in defecation frequency [86]. With regard to immune function, a limited number of prebiotic intervention studies have been performed in elderly. Although beneficial effects on specific parameters have been reported [87], no effect was shown on vaccination efficacy [88]. In a synbiotic study, Costabile *et al*. observed a significant and more pronounced effect on NK cell activity, microbiota composition and blood lipids in elderly treated with the combination of *Lactobacillus rhamnosis* GG and soluble corn fibre, when compared to soluble corn fibre alone [89]. Furthermore, different pre- and synbiotic food supplements studies have been shown to lead to an increased abundance of faecal bifidobacteria and/or lactobacilli in elderly [84]. The clinical relevance hereof, without additional effects on health outcome parameters, is still a matter of debate.

It should be noted that effects of probiotic strains, prebiotic compounds and/or combinations thereof, differ and largely depend on the duration of the intervention period, subject population and mechanism [90] to be targeted. We are only at the beginning of understanding the association between specific microbes and our health, especially in elderly and their associated comorbidity or frailty. Although several pro-, pre- and synbiotic studies have been performed in adults in general, caution is needed when extrapolating these findings to elderly and subgroups thereof. Therefore, more insights in the exact microbial composition and underlying mechanistic effects are needed to enable more targeted interventions in relevant subgroups.

Several studies have evaluated dietary intake in general, showing *e.g.* changes in macronutrient intake as well as deficiencies in micronutrients [84], but studies targeting the microbiota composition by changing habitual dietary intake in the elderly are still scarce.

Current studies are mainly performed in young adults, showing changes in microbiota composition and especially metabolic activity, relatively quickly after major changes in dietary habits [81,91,92]. It should be noted, however, that the observed compositional changes did not exceed the inter-individual variation. At present, analyses of the Nu-Age dietary intervention study [93] are ongoing, investigating the effect of major diet changes (Mediterranean diet, 1 year randomized, single-blind controlled trial) in 1250 elderly subjects.

Other strategies to manipulate the intestinal microbiota include faecal microbiota transplantation (FMT). The success rate is variable, and largely depends on the disease or disorder to be treated [94]. FMT efficacy is most convincing for treating Clostridium difficile infection [94] which has a rather high incidence in the elderly, also because of the high antibiotic use [95]. In a specific review focusing on elderly, Cheng *et al*. confirms the efficacy of FMT for *C. difficile* in this group but recommends to use this treatment strategy early in disease course to prevent complications [96]. Clear criteria for stool donor selection and screening are still warranted, especially in susceptible (frail)- elderly given the risk of transplanting concomitant pathogens and or antibiotic resistance genes.

5. Summary and conclusions

Several small, age-related declines in the physiology of the GI tract have been reported for ageing in general, whereas frailty and impaired health status seem to play a role in the (further) decline (Figure 1). Studies on GI physiology and function in subjects with age-related health decline or being resilient to diseases while ageing, *e.g.* centenarians, as well as studies directly linking host function and outcome in elderly to microbiota composition and activity are, however, still scarce. Regarding the intestinal microbiota composition, alterations are more pronounced in frail or comorbid elderly, although age-related changes in the abundance of *Bifidobacterium* and Enterobacteriaceae have been reported in general. However, a "typical" intestinal microbiota of elderly is hard to define, given the large inter-individual differences in the intestinal microbiota of elderly. Moreover, intestinal microbiota of elderly is more likely to be affected by a broad range of potentially confounding factors, such as lifestyle (including *e.g.* diet and smoking), health status, medical treatment (including medication), and living situation rather than by ageing per se. Although, we acknowledge that unravelling causes and consequences will be challenging since possible confounders such as diet will influence both the microbiota and GI physiology. Based on our current knowledge, future longitudinal studies should shift towards investigating the role of GI physiology and intestinal microbiota as well as their dynamics over time in specific well-characterised subgroups of elderly, such as frailty and elderly with a specific health decline, and how these can be modulated by targeted interventions or improvements in lifestyle and living situation.

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Sugar beet pectin supplementation did not alter profiles of faecal microbiota and exhaled breath in healthy young adults and healthy elderly

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Abstract

Ageing is accompanied with increased frailty and comorbidities, which is potentially associated with microbiome perturbations. Dietary fibres could contribute to healthy ageing by beneficially impacting gut microbiota and metabolite profiles. We aimed to compare young adults with elderly, and to investigate the effect of pectin supplementation on faecal microbiota composition, short chain fatty acids (SCFAs), and exhaled volatile organic compounds (VOCs) using a randomized, double-blind, placebo-controlled parallel design. Fifty-two young adults and 48 elderly consumed 15g/day sugar beet pectin or maltodextrin for four weeks. Before and after the intervention period, faecal and exhaled breath samples were collected. Faecal samples were used for microbiota profiling by 16S rRNA gene amplicon sequencing, and for analysis of SCFAs by gas chromatography (GC). Breath was used for VOC analysis by GC-tof-MS. Young adults and elderly showed similar faecal SCFA and exhaled VOC profiles. Additionally, faecal microbiota profiles were similar, with five genera significantly different in relative abundance. Pectin supplementation did not significantly alter faecal microbiota, SCFA, nor exhaled VOC profiles, in elderly nor young adults. In conclusion, aside from some minor differences in microbial composition, healthy elderly and young adults showed comparable faecal microbiota composition and activity, which were not altered by pectin supplementation.

Keywords: microbiota; exhaled air; dietary fire; pectin; ageing; elderly; young adults

1. Introduction

In line with the rising life expectancy, the ageing population is increasing globally, leading to an increase in direct and indirect healthcare costs [1,2]. General health status may decline with ageing and has been associated with changes in gastrointestinal (GI) tract microbiome characteristics; *e.g.* changes in microbial diversity, microbiota composition as well as microbiota function [3]. On the other hand a substantial group of elderly is capable to maintain the functional ability that supports wellbeing, which is defined as "healthy ageing" [4]. Various studies investigated the effect of age on microbiota composition by comparing the microbiota of healthy elderly and healthy young adults. Nevertheless, the definition of "healthy" and of age cut offs used for elderly varies between studies. Mueller *et al.* demonstrated a lower relative abundance of *Bifidobacterium* and a higher relative abundance of enterobacteria in the elderly in four European study populations (France, Germany, Italy and Sweden) [5]. In contrast, increased levels of *Bifidobacterium* in the microbiota of higher-aged individuals (*i.e.* centenarians) has also been reported, as compared to that of young adults [6]. Furthermore, compared with that of young adults, the microbiota of non-institutionalized elderly had lower abundance of genes coding for carbohydrate metabolism, but increased proteolytic potential (increased abundance of genes coding for the degradation of branched-chain amino acids) [7]. Reported alterations in microbiota composition and/or activities, could in part be attributed to changes in alterations with respect to nutritional factors [8]. Intake of dietary fibres, such as the non-digestible carbohydrates fructo-oligosaccharides (FOS) [9], galacto-oligosaccharides (GOS) [10-12], and resistant starch, has been shown to beneficially impact intestinal microbiota composition. Supplemented non-digestible carbohydrates that reach the colon are fermented by microbes and thereby contribute to the production of metabolites including SCFAs, which are known for their health promoting effects [13].

Pectin is an important member of dietary fibre that is present in many fruits, vegetables and legumes. Pectin supplementation has been shown to affect microbiota composition both *in vitro* [14,15] and *in vivo* in rats [16-18], mice [19], piglets [20], but also in humans [21,22], specifically in patients with active ulcerative colitis [21] and adults with slow-transit constipation [22], although specific effects depend on the solubility and chemical fine structure of supplemented pectin. For instance, pectin supplementation increased the relative abundance of *Bifidobacterium,* compared with controls in an *in vitro* study [23] as well as in adults with slow-transit constipation [22], but not *in vivo* in piglets [20] or rat [17], nor in patients with active ulcerative colitis [21]. Considering the impact of pectin supplementation on microbial activity, most studies have focused on faecal SCFA levels, although it should be noted that the majority of the metabolites are absorbed in the intestine. Currently published studies have been reported *in vitro* and *in vivo* in animals [16-20] on the effects of pectin on faecal metabolite profiles. Some of these metabolites, so called volatile organic compounds (VOCs) [24], are also present in exhaled breath and have shown distinct profiles in health and disease states [25], *e.g.* in patients with gastrointestinal diseases, like irritable bowel syndrome (IBS) [26]. The exhaled VOC profiles have been associated with the intestinal microbial composition [27] and can be affected by major dietary changes [28]. Data on the impact of pectin supplementation on VOC profiles are, however, currently lacking. The varying effects on microbiota composition and/or faecal

SCFA levels are likely due to different methodologies [29], and differences in dosage [19], chemical structure [30] and/or source (*e.g.* from lemon, apple or sugar beet) [17,20] of pectin used.

Sugar beet pectin, which can be produced from sugar beet pulp as a by-product in sugar beet industry, received much attention as a potential health promoting food and feed ingredient in the recent years. Sugar beet pectin as compared to citrus and apple pectin for example, comprises acetylation of homogalacturonan. A rat model assessed health effects of sugar beet pectin supplementation in comparison with low- and high-methyl esterified citrus pectin and soy pectin, respectively [17]. Low-methyl esterified citrus pectin and soy pectin significantly increased concentrations of total SCFA, and of propionate and butyrate in the cecum, whereas sugar beet pectin supplementation led to a stronger increase in the relative abundance of *Lactobacillus* and Lachnospiraceae [17]. Furthermore, in the TIM-2 *in vitro* colon fermentation model, the propionate production was higher when sugar beet pectin was added in comparison to citrus fruits derived pectin [31]. In addition, it has been reported that sugar beet pectin derived galacturonide oligosaccharides demonstrated prebiotic potential through promoting anti-inflammatory commensal bacteria in the human colon based on an *in vitro* model using bacterial and host cell cultures [32]. Therefore, the next step would be to investigate whether sugar beet pectin consumption also beneficially impacts the microbiota *in vivo* in humans. Beneficial modulation of the intestinal microbiota is especially important in people who are prone to develop intestinal problems, such as the elderly. The intestinal microbiota of this group was previously shown to have a lower saccharolytic capacity [7]. A decrease in saccharolytic fermentation and consequently an increased proteolytic fermentation is considered to be less desired for optimal gut homeostasis, as this is associated with the production of potentially toxic metabolites such as phenolic and sulfide-containing compounds [33]. Therefore, with this study we compared faecal microbiota composition, faecal SCFA profiles and VOCs in exhaled breath of young adults versus elderly, and to investigate the impact of four weeks sugar beet pectin supplementation on these parameters. We hypothesised that the intestinal microbiota and metabolite profiles in faeces and breath differ between young adults and elderly, with a greater response to four weeks pectin supplementation in elderly versus young adults.

2. Materials and Methods

2.1. Study overview

This study was part of a larger project on the effect of pectin on GI function [34]. This study was designed as a randomized, double-blind, placebo-controlled, parallel study (Figure S1), which has been approved by the Medical Ethics Committee of the University Hospital Maastricht and Maastricht University (azM/UM, The Netherlands) and has been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT02376270). It was performed according to the Declaration of Helsinki (latest amendment in Fortalesa, Brasil, 2013) and Dutch Regulations on Medical Research involving Human Subjects (1998) at the Maastricht University Medical Center+ (MUMC+) between March 2015 and April 2016. All participants gave written informed consent prior to participation.

2.2. Participants

Healthy young adults (18-40 years) and healthy elderly (65-75 years) with a body mass index between 20-30 kg/m^2 were recruited by public advertisements. Key exclusion criteria included GI diseases, abdominal surgery interfering with GI function, use of nonsteroidal anti-inflammatory drugs and/or vitamin supplementation within 14 days prior to testing, administration of pro-, pre-, or antibiotics in the 90 days prior to the study, pregnancy, lactation, smoking and history of side effects towards intake of prebiotic supplements. Other medications use was checked by medical doctor. Sample size calculation was based on a previous study in which the effects of five weeks dietary fibre-enriched pasta intake was investigated [35]. For the sample size calculation, data of the primary study outcome parameter of the original research protocol, intestinal permeability (not included in this manuscript), were used. The sample size calculation showed that each age group should contain at least 48 completers (*i.e.* 24 per intervention group).

2.3. Dietary intervention

Each subject was randomly assigned to the pectin or placebo group (Figure S1). A person not involved in the study generated the list of random allocations using a computerized procedure. Subjects in the intervention (pectin) group received 15g/day of pectin (GENU® BETA pectin, CP Kelco, Grossenbrode, Germany). GENU® BETA pectin is a high ester pectin extracted from sugar beet pulp, with a degree of acetation of the homogalacturonan backbone of the pectin of the pectin of 18-26%, and molecular weight > 60,000 Da. Subjects in the placebo group received 15g/day maltodextrin (GLUCIDEX® IT 12, Roquette Freres, Lestrem, France). Both maltodextrin and pectin were supplemented as dry powders free from off-flavours and odours, and packed in closed sachets of a single dose of 7.5 grams. Subjects were asked to ingest the supplements twice daily for four weeks, before breakfast and before diner, respectively. Prior to consumption, the content of a sachet was transferred into a glass, mixed with flavoured syrup (Karvan Cévitam®, Koninklijke De Ruijter B.V., Zeist, the Netherlands) and approximately 200 ml of tap water. Time of consumption was recorded in a diary, and empty and remaining sachets were returned to the investigator

to check for product intake compliance. During the intervention periods, all subjects were asked to maintain their habitual diet.

2.4. Faecal samples and microbiota profiling

Faecal samples were collected before and after the intervention period and immediately stored at -20 °C in home freezers before being transported frozen to the study site. Microbiota composition was determined by sequencing of barcoded 16S ribosomal RNA (rRNA) gene amplicons using Illumina Hiseq2500 (2 x 150 bp).

DNA was isolated using Repeated-Bead-Beating [36] and purified using the Maxwell® 16Tissue LEV Total RNA purification Kit Cartridge (XAS1220). The V5-V6 region of 16S rRNA gene was amplified in triplicate using primers BSF784/R1064 and faecal DNA as template [37] . Each 35 µl reaction contained 0.7 µl 20 ng/µl DNA template, 7 µl 5×HF buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 0.7 µl of 10mM dNTPs (Thermo Fisher Scientific), 0.35 µl DNA polymerase (2 U/µl) (Thermo Fisher Scientific), 25.5 µl nuclease free water (Promega, Madison, WI USA) and 0.7 µl 10 µM of sample-specific barcode-tagged primers [37]. Cycling conditions were as follows: 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 42 °C for 10 s, 72 °C for 10 s, with a final extension of 7 min at 72 °C. Subsequently, the triplicate PCR products were pooled for each sample, purified with the CleanPCR kit (CleanNA, the Netherlands) and quantified using the QubitTM dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Eugene, Oregon USA). In total, we obtained 16S rRNA gene amplicons from 196 faecal samples, eight biological replicates plus six synthetic microbial communities, which served as a positive control to control for replicability and reflection of the actual composition by the sequencing approach, respectively [37]. An equimolar mix of purified PCR products was prepared and sent for sequencing (GATC-Biotech, Konstanz, Germany, now part of Eurofins Genomics Germany GmbH). Raw sequence reads were subsequently processed using NG-Tax [37]. The sequencing data is available at the European Nucleotide Archive with accession number PRJEB31775.

2.5. Faecal metabolite profiling

SCFAs were measured in the faeces due to their correlation with a healthy (distal) colon. In addition, we also measured BCFAs (branched chain fatty acids) since their formation indicates protein fermentation instead of only glycosidic fermentation. Concentrations of SCFAs and BCFAs were determined in duplicate. Between 200-300 mg faeces were dissolved in 1.0 ml distilled water, mixed and centrifuged (30 000 × g for 5 min). Standard solutions of acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid and isobutyric acid were prepared in concentrations of 0.01-0.45 mg/ml. Two hundred fifty microliters of internal standard solution (0.45 mg/ml 2-ethylbutyric acid in 0.3M HCl and 0.9M oxalic acid) was added to 500 µl of the standard solutions and centrifuged samples. After mixing and centrifugation, 150 µl supernatant was used for analysis. SCFAs were quantified using gas chromatography (Focus GC, Thermo Scientific, Waltham, MA, USA) coupled with a flame ionization detector (FID) (Interscience, Breda, The Netherlands). One µl was injected into a CP-FFAP CB column (25 m × 0.53 mm × 1.00 µm, Agilent, Santa Clara, CA, USA). The initial oven temperature was 100 °C, increased to 180°C (8 °C/min), held at this temperature for 1

min, increased to 200 °C at 20 °C/min and held at this temperature for 5 min, respectively. Injection was done at 200 °C with flow rate of 40 ml/min at a constant pressure of 20kPa. Data was processed using Xcalibur® (Thermo Scientific, Waltham, MA, USA). To correct for the potential impact of stool consistency (potentially altering with ageing and by prebiotic intake), SCFA concentrations were expressed per gram dry matter. Dry matter content was determined by vacuum drying of 500 mg faeces for five hours at 60 °C using a concentrator plus (Eppendorf, Hamburg, Germany).

2.6. Volatile organic compounds profiling

Exhaled air samples were collected by breathing into a 3 L Tedlar bag (SKC Limited, Dorset, UK), being transferred within one hour to carbon-filled stainless-steel absorption tubes (Markes International, Llantrisant, UK) using a vacuum pomp (VWR international, Radnor, PA, USA). Volatile organic compounds (VOCs) were measured using thermal desorption gas chromatography time-of-flight mass spectrometry (GC-tof-MS, (Markes International, Llantrisant, UK) as described previously [38]. Briefly, samples containing VOCs were injected in the system with split ratio 1:2.7. Approximately 40 % of the sample was trapped into the cold trap at 5 °C in order to concentrate the sample. The remaining amount of the sample was stored to the sorption tube. The VOCs in the cold trap were released into a capillary GC column (RTX-5ms, 30 m×0.25 mm 5 % diphenyl, 95 % dimethylsiloxane, film thickness 1 m, Thermo Electron TraceGC Ultra, Thermo Electron Corporation, Waltham, USA). The temperature of the GC was programmed as follows: 40 °C for the first 5 min, and then increased to 270 °C at 10 °C/min. Compounds in the samples were detected by *tof*-MS Thermo Electron Tempus Plus time-of-flight mass spectrometer, Thermo. Electron Corporation, Waltham, USA). Electron ionization mode was set at 70 eV and the mass range m/z 35-350 was measured. The resulting breath-o-grams were denoised, baseline corrected, aligned, normalized by probabilistic quotient normalization and scaled for further analyses [39].

2.7. Statistical analyses

Statistical analysis of baseline characteristics of study participants was performed using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY, USA: IBM Corp.). Differences in age and BMI between all young adults and elderly, or between placebo group and pectin group, were shown as means ± standard deviation (SD) and tested using T-tests. Differences in categorical variables were shown as percentages and tested with Chi-square tests or Fisher's exact tests when appropriate. Baseline samples were used to compare microbiota composition and metabolite profiles (*i.e.* faecal SCFAs and exhaled VOCs) of young adults to those of elderly individuals, while the impact of pectin supplementation was studied by comparative analysis of pre- and post-intervention samples based on intention-to-treat analysis. *P*-values ≤ 0.05 (two-sided) were considered to indicate statistical significance.

Complex data including microbiota and VOCs were analysed using multivariate statistics. Sequence read counts were normalized to microbial relative abundance, and microbiota diversity indices (Faith's phylogenetic diversity (PD) and inverse Simpson) were calculated at amplicon sequence variant (ASV) level as implemented in the *Picante* [40] and *Phyloseq*

[41] packages, respectively. Since the data was non-parametric, Wilcoxon test was applied to determine whether diversity as well as relative abundance of specific bacterial taxa were significantly different between groups. False discovery rate (FDR) was used to correct for multiple testing according to the Benjamini-Hochberg procedure. Unpaired tests were used to determine the differences between age groups at baseline. Paired tests were used to compare pre- vs post-intervention effects. Pairwise weighted UniFrac (WU) [42] and unweighted UniFrac (UU) [43] distance based principal coordinate analysis (PCoA) was used to visualize microbial community variation at ASV level [44]. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences between groups as implemented in the *Vegan* [45] package. Random Forest (RF) analysis (500 trees with four-fold cross validation) was performed to validate the findings of PCoA coupled with PERMANOVA (data not shown), *i.e.* testing if microbiota profiles could predict the age group differences and intervention effect. All microbiota based statistical analysis was performed in R (R-3.5.0) [46]. R code for the analysis is available at GitHub (https://github.com/mibwurrepo/Pectin-elderly-intervention).

Exhaled breath data was analysed with Principal Component Analysis (PCA) and RF. Data were log transformed to account for data skewed distribution and pareto-scaled to ensure equal contribution of each volatile metabolite in breath in the consequent analysis. RF analysis (with 1000 trees) was performed to discover whether VOCs in exhaled breath could predict the intervention in elderly and young adults as well as to investigate whether exhaled breath metabolites were different between young and elderly adults. In order to represent the unbiased prediction error, the data was randomly divided into a training- and a validation set. The training set was used to find discriminatory VOCs and to build the classification model. The performance of the RF classification model was demonstrated by the area under the curve of receiver operating characteristic (AUROC) for the validation set. The final results were visualized in a PCA score plot using the most discriminatory VOCs which were selected in at least 80 % of RF iterations in the training set. Statistical analyses of VOCs were performed using Matlab 2018a (The MathWorks, Natick, 2018).

SCFAs were single parameters and analysed with univariate statistics. To compare SCFA levels of young adults versus elderly independent-samples T Tests were performed. To compare SCFA levels within age groups and between intervention groups, unstructured linear mixed model analyses were performed. Individual was included as random factor. Intervention group, time and 'intervention group x time' were included as fixed factors, and corrections for baseline values were made. Statistical analyses of SCFAs were performed using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY, USA: IBM Corp.)

3. Results

3.1. Characterization of subjects

For the current study 52 healthy young adults and 48 elderly were included, of whom the baseline characteristics are provided in Table 1. Elderly had a significantly higher age, body mass index (BMI) and medication use when compared with young adults. Placebo and pectin groups did not differ for any of the baseline characteristics in either of the two age groups. Three young adults (*i.e.* two in the pectin group, one in the placebo group) dropped out during the study due to overt non-compliance or prescription of antibiotic therapy. From these drop-outs, samples were used for baseline characteristics and faecal- and exhaled breath analyses but were not included in the post intervention measurements. DNA isolation failed for one faecal sample from a young adult (placebo group, post intervention), and hence was excluded for microbiota profiling.

Table 1. Baseline characteristics of the young adults (n=52) and elderly (n=48) study populations.

	Young adult (n=52)			Elderly (n=48)			All young adults vs. all elderly
	Placebo (n=27)	Pectin (n=25)	*P*-value	Placebo (n=24)	Pectin (n=24)	*P*-value	*P*-value
Age (years)	22.8±4.1	23.4±4.5	0.614	69.8±2.4	69.5±3.2	0.723	<0.001
Female (%)	48.2	68.0	0.148	50.0	37.5	0.383	0.164
BMI (kg/m^2)	22.6±2.7	23.2±2.7	0.444	26.2±2.8	25.5±2.6	0.334	<0.001
Medication (%)	0	0	1.000	33.3	45.8	0.376	<0.001
PPI (%)	0	0	1.000	12.5	12.5	1.000	<0.001
Statins (%)	0	0	1.000	4.2	4.2	1.000	<0.001
Antihypertensives (%)	0	0	1.000	8.3	12.5	0.637	<0.001
Other medication (%)	0	0	1.000	12.5	16.7	0.683	<0.001

Differences in age and BMI between all young adults and elderly, or between placebo group and pectin group, were tested using T-tests. Differences in sex (*i.e.* female or male) were tested with chi-square tests. Differences in medication use were tested by Fisher's exact tests. Values are presented as mean ± SD or percentage (%). BMI, body mass index. PPI, proton-pump inhibitors.

3.2. Young adults and elderly showed similar faecal microbiota composition, SCFA- and exhaled VOC profiles.

PCoA based on weighted UniFrac (taking relative abundance of bacterial ASVs into account) revealed no significant differences between the microbiota of young adults and elderly (Figure 1A). However, PCoA based on unweighted UniFrac distances (only taking into account presence/absence of bacterial ASVs, placing emphasis on less abundant species), did show a small though significant difference between the microbiota of young adults and that of elderly (*P*=0.001), with 2.4 % of microbiota variation being explained by age groups (Figure 1B). The RF analysis to determine differences in microbiota profiles between young adults and elderly, showed an out-of-bag error rate of 29.29 %, indicating relatively small differences in microbiota profiles. The relative abundances of five genus-level taxa (*Enterorhabdus, Ruminiclostridium* 6*, Mogibacterium, Lachnospiraceae* UCG008 and uncultured genus within the Coriobacteriaceae) out of 224 genera were significantly different between young adults and elderly before the intervention (Figure 2). In addition, no significant differences were found in their faecal microbiota alpha diversity at baseline (Figure S2). Furthermore, in both age groups PERMANOVA analysis of microbiota profiles based on weighted UniFrac and unweighted UniFrac distance matrices showed no significant difference between placebo and pectin supplementation groups at baseline.

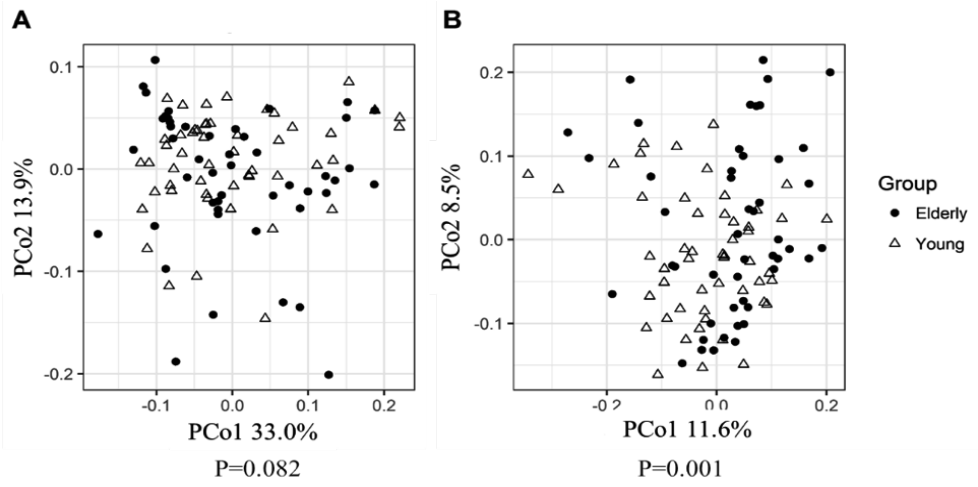

Figure 1. Baseline PCoA plots based on weighted UniFrac (A) and unweighted UniFrac (B) pairwise distance matrices using amplicon sequence variant-level data, show overlapping microbiota profiles of young adults and elderly. Significance of observed differences between groups was evaluated by PERMANOVA.

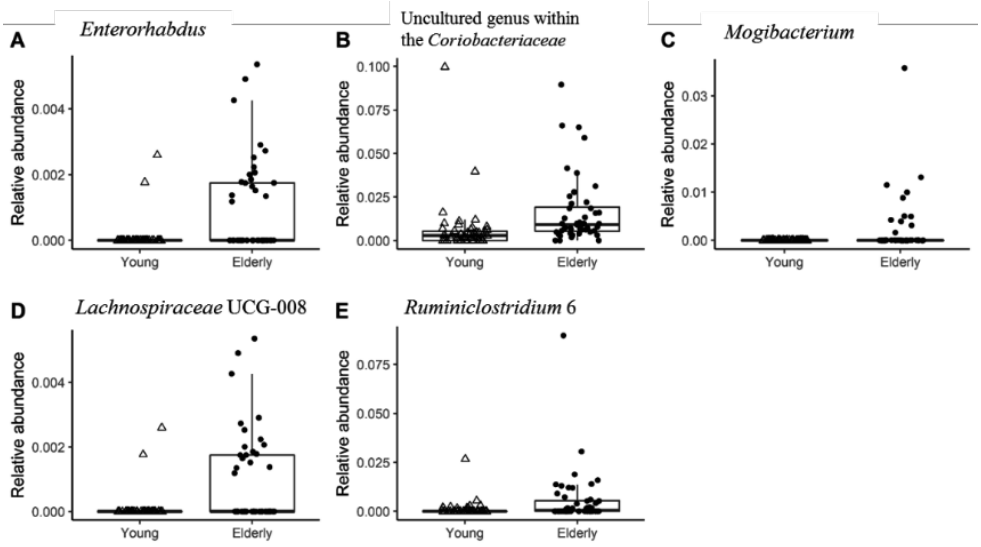

Figure 2. Genus level taxa that significantly differed (FDR<0.05) in relative abundance between young adults and elderly at baseline.

Baseline faecal SCFA and BCFA concentrations revealed no significant differences between young adults and elderly (Table 2). Independent of age group, large individual differences were found for all SCFAs as indicated by the relatively high SD.

Table 2. Faecal short-chain fatty acid concentrations (μmol/g dry content) of young adults (n=52) and elderly (n=48) at baseline.

	Young adults (n=52)		Elderly (n=48)		*P*-value*
	Mean	SD	Mean	SD	
Acetic acid	225.9	187.6	201.6	145.2	0.469
Propionic acid	71.1	66.4	58.1	53.2	0.281
Butyric acid	59.2	45.0	56.6	49.8	0.785
Valeric acid	8.4	6.4	9.3	6.7	0.473
Isobutyric acid	6.8	3.7	7.2	6.0	0.715
Isovaleric acid	10.6	5.6	11.1	9.0	0.729

*Differences between age groups were tested by independent-sample T Tests. SD, standard deviation.

The VOC-based RF analysis using a set of 15 VOCs, to determine differences in exhaled VOCs between young adults and elderly at baseline, showed an AUROC of 0.70 with sensitivity and specificity of 0.6 and 0.58 in the validation set (Figure 3A), indicating relatively small differences in exhaled breath profiles, which is in line with faecal microbiota and SCFA data. PCA analysis performed on the VOCs that were important for classification in the resulting RF model showed no clear differences between young adults and elderly (Figure 3B). This is in accordance with PCA analysis performed on the complete breath profiles (Figure S3).

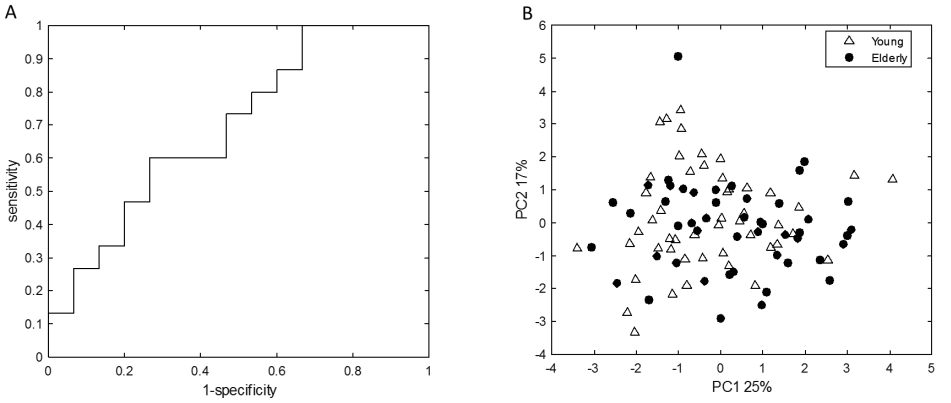

Figure 3. (A) Receiver operating characteristic curve performed on the validation set, with area under the curve = 0.70. (B) PCA score plot, performed on a set of 15 VOCs that were found important (set of the most discriminatory VOCs selected in at least 80 % of RF iterations) for classification in the RF model, showing no clear groupings in exhaled breath profiles between young adults and elderly. Percentages given at both axes indicate percentage of variation explained by either principal component.

3.3. Four weeks of sugar beet pectin supplementation did neither alter faecal microbiota composition, nor SCFA- or exhaled VOC profiles

Comparative analysis between pre- and post-intervention samples did not reveal any significant effects of pectin supplementation on global microbiota profiles at ASV level (Figure 4A-B) and in-depth microbial composition (*i.e.* detailed taxa comparison), nor impact on microbial phylogenetic diversity (Figure 4D) and InvSimpson diversity indices (Figure 4C). In addition, we did observe significantly smaller intra-individual variation over the treatment period, comparing to inter-individual variation, based on weighted and unweighted UniFrac (Table S2). Interestingly, the young pectin group showed a significantly decreased inter-individual variation in phylogenetic diversity post pectin treatment, while the other groups displayed a more heterogeneous response. Four (except for *Ruminiclostridium* 6) out of five genera which were different before the intervention, remained significantly different between age groups after the intervention (Figure S4), suggesting that these differences are consistent between elderly and young adults. In terms of subjects who were shown to have a higher relative abundance of corresponding taxa after the intervention, 72.0 % (*Enterorhabdus*), 91.5 % (*Coriobacteriaceae* uncultured), 46.2 % (*Lachnospiraceae* UCG-008) and 90.9 % (*Mogibacterium*) were the same subjects as before the intervention. These differences in bacterial relative abundance could not be explained by medication use nor other characteristics noted at baseline (Table S1).

Four weeks of sugar beet pectin intake did also not significantly change faecal SCFA or BCFA concentrations in young adults, nor in elderly (Table 3). In addition, within exhaled breath several SCFAs were detected, namely acetic acid, pentanoic acid, propionic acid and 2-methyl-propanoic acid, which did not change after the intervention.

In order to investigate the effect of pectin on the VOC profiles of exhaled breath, RF analysis was performed between pre- and post- pectin intervention data for young adults and elderly separately. Performance of the model, based on the most discriminatory VOCs in breath, resulted in an AUROC of 0.57 and 0.50 for the validation set for young adults and elderly, respectively, indicating that samples taken before and after the-intervention did not differ. The corresponding PCA score plots were performed on sets of 11 and 12 VOCs for young adults and elderly, respectively, as these were the most discriminatory compounds selected in at least 80% of RF iterations (Figure 5A-B). No clear groupings were found between post and pre-intervention indicating similarity in breath profiles.

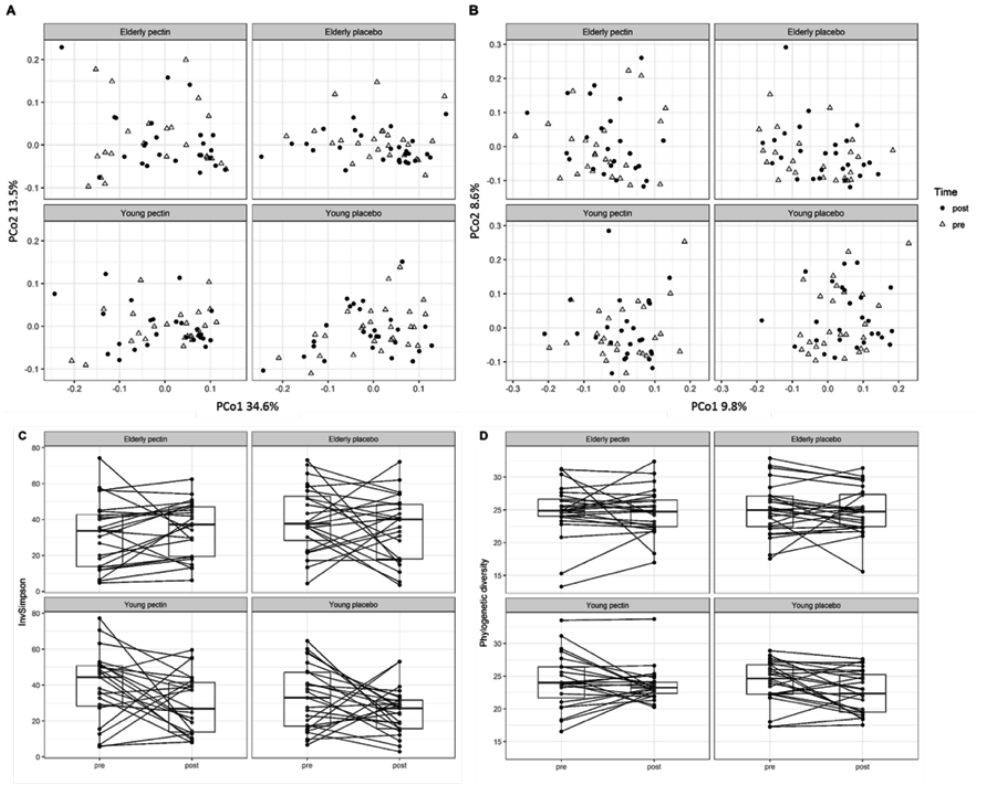

Figure 4. Intervention effects on microbiota composition and alpha diversity in young adults and elderly. PCoA plots at baseline and after four weeks sugar beet pectin or placebo supplementation based on weighted UniFrac (A) and unweighted UniFrac (B), showed no clear groupings in microbiota profiles between pre- and post-intervention. (C) Comparison of InvSimpson and (D) Phylogenetic diversity indices pre- vs post-intervention at individual level, showing no significant changes in microbial diversity pre- vs post-intervention. Significance of differences between groups was evaluated by PERMANOVA.

Table 3. Fecal short-chain fatty acid concentrations (µmol/g dry content) of placebo- and pectin intervention groups at baseline and after four weeks supplementation, in young adults and elderly.

		Young adults					Elderly				
	Intervention	Pre-intervention		Post-Intervention		P-value* (placebo vs pectin)	Pre-intervention		Post-Intervention		P-value* (placebo vs pectin)
		mean	SD	mean	SD		mean	SD	mean	SD	
Acetic acid	Placebo	210.2	182.7	263.7	233.3	0.202	167.9	95.0	230.5	188.1	0.548
	Pectin	242.8	195.1	237.8	222.4		235.3	178.0	268.4	155.2	
Butyric acid	Placebo	56.1	41.5	77.5	55.8	0.066	44.1	25.8	56.3	46.8	0.280
	Pectin	62.6	49.1	61.1	47.2		69.2	63.9	67.3	37.5	
Isobutyric acid	Placebo	6.2	2.4	7.9	4.9	0.495	6.1	3.7	7.2	4.3	0.290
	Pectin	7.5	4.8	8.2	5.3		8.3	7.5	7.8	4.0	
Isovaleric acid	Placebo	10.1	3.5	12.4	8.0	0.654	9.6	6.0	10.9	6.2	0.364
	Pectin	11.2	7.2	12.5	8.2		12.7	11.2	11.8	6.4	
Propionic acid	Placebo	71.0	69.6	99.4	131.0	0.074	40.7	18.0	52.6	32.5	0.752
	Pectin	71.2	64.3	66.8	49.0		75.6	69.4	81.8	43.8	
Valeric acid	Placebo	7.1	5.1	10.7	13.5	0.113	7.9	4.3	9.4	7.2	0.391
	Pectin	9.8	7.4	9.6	5.5		10.8	8.3	10.3	4.1	

* Corrected for baseline values. Within age groups, differences between interventions were tested by an unstructured linear mixed model and correction for baseline values. SD, standard deviation.

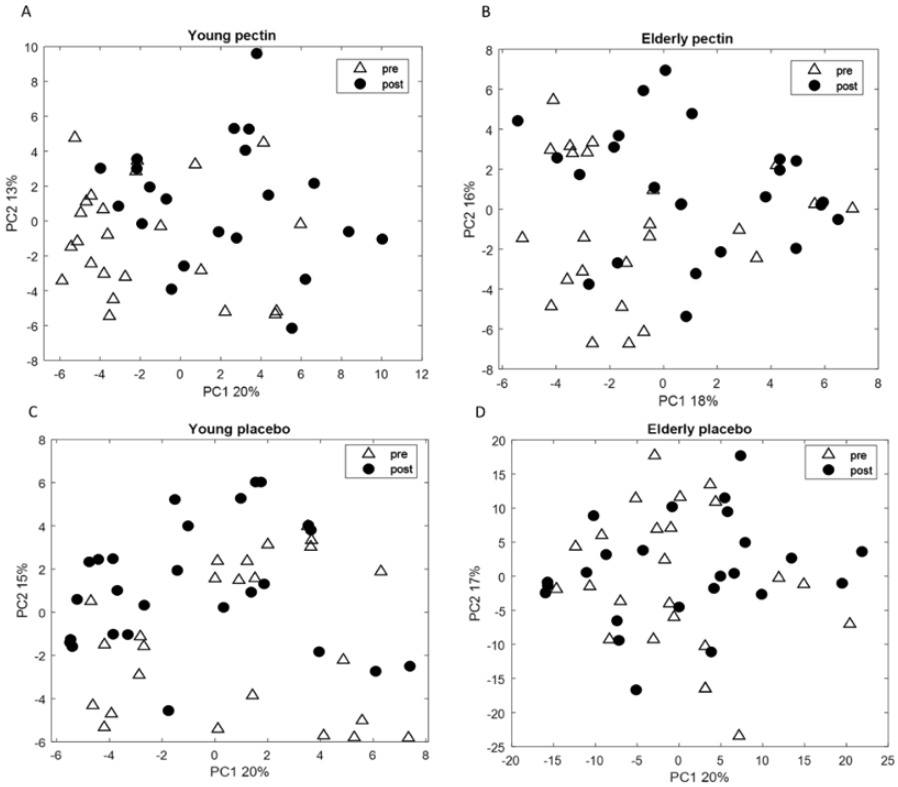

Figure 5. PCA score plot based on the set of (A) 11 volatile metabolites in the exhaled breath of young adults to discriminate between pre and post-pectin intervention; (B) 12 volatile metabolites in the exhaled breath of elderly to discriminate between pre and post-pectin intervention. PCA score plots performed on the set of 14 VOCs measured in exhaled breath of (C) young adults; and on 16 VOCs measured in exhaled breath of D) elderly; for pre- and post-placebo intervention. No groupings of the samples are observed. Discriminatory VOCs were selected in at least 80 % of RF iterations.

RF models for the placebo intervention showed AUROCs of 0.32 and 0.40 for young adults and elderly, respectively. PCA score plots shown in Figures 5C-D indicate that similarly to pectin, placebo did not alter the VOC profiles in exhaled breath in neither of the age groups.

In addition, VOCs of young adults and elderly were compared between pectin and placebo supplementation at baseline and post- intervention. The implemented RF models, separated between young adults and elderly, revealed no predictive power, indicating similar breath profiles of placebo and pectin supplementation at baseline (AUROC of 0.35 for both models). The post-intervention RF classification model led to AUROCs of 0.34 and 0.58 for the validation set for young adults and elderly, respectively, demonstrating no differences in breath profiles between placebo and pectin post-intervention.

Together with the observations on microbiota composition, SCFAs and VOCs, this suggests that in this study, pectin had no significant impact on the faecal microbiome, nor on breath metabolite profiles, neither in elderly nor in young adults.

4. Discussion

In the present study we compared healthy young adults versus healthy elderly and studied the effect of sugar beet pectin supplementation on faecal microbiota composition, faecal SCFA and exhaled breath VOC profiles. We hypothesised that intestinal microbiota and metabolite profiles in faeces and breath differ between elderly and young adults. We did observe limited and very subtle differences between age groups with respect to microbiota composition, with only 5 out of 224 genera being significantly higher in relative abundance in elderly compared with young adults. No significant differences were found in faecal SCFA and exhaled VOC profiles between age groups. In addition, in neither of the two age groups, any effects of pectin supplementation on faecal microbiota, SCFA, and exhaled VOC profiles were observed.

Aside from the small differences in the composition of the intestinal microbiota between the age groups, microbiota composition and its activity in the healthy elderly was comparable with profiles in the healthy young adults. This suggests that health status rather than chronological age, may affect microbiota composition and activity, an observation in line with findings in previous studies [3]. Biagi *et al.* [47] compared the microbiota of young adults with that of non-institutionalized elderly with good physical and cognitive health status and also demonstrated high similarity between young and elderly. Jackson *et al.* [48], specified the health status (*i.e.* frailty level) of recruited community dwellers according to the Rockwood frailty index, and revealed an association between microbiota profile (*e.g.* decrease in microbial diversity) and increased frailty. Claesson *et al.* [49] classified elderly into four different groups (*i.e.* community dwellers, outpatients, short-term hospitalized and long-term hospitalized) and demonstrated that changes in residency (*e.g.* changing from community dwellers to long-stay), which suggests differences in health status, correlated with dietary intake patterns. This difference in food intake could contribute to perturbations in the microbiota composition and/or microbial activity [49]. Specifically, the long stay subjects showed decreased acetate, propionate, valerate and butyrate levels compared to community dwellers [49]. This was further confirmed by functional analysis, showing that institutionalized elderly [50] and elderly using medication [7] had a decreased number of genes coding for SCFAs production in their microbiota when compared with young adults.

Five genera (*Enterorhabdus, Ruminiclostridium* 6*, Coriobacteriaceae* uncultured*, Mogibacterium, Lachnospiraceae* UCG-008) were significantly higher in relative abundance in the faecal microbiota of elderly, compared to that of young adults. *Mogibacterium* spp. have previously been isolated from oral cavities [51] and the prevalence of dental caries is higher in the elderly [52,53]. Moreover, one recent study employing metagenomic sequencing showed the translocation of oral microbes to the intestine [53]. Nevertheless, the role of *Mogibacterium* in the intestine remains unclear. The aerotolerant genus *Enterorhabdus* was previously shown to have a higher relative abundance in prediabetic subjects, compared to healthy controls [54]. Moreover, the increased prevalence of prediabetes was associated with higher BMI [55]. This is confirmed in the present study, as the BMI of elderly was significantly higher than that of young adults while the relative abundance of *Enterorhabdus* was also increased in the elderly. *Ruminiclostridium* 6,

Coriobacteriaceae uncultured and *Lachnospiraceae* UCG-008 are not well classified genus-level groups, up to now. In addition, subjects maintained their habitual diet during the study. It cannot be ruled out that possible confounders, such as differences in habitual diet or other lifestyle factors have contributed to the minor differences between the microbiota of young adults and elderly in the current study.

Pectin supplementation did not affect faecal microbiota, SCFA and exhaled VOC profiles in elderly, nor in young adults, respectively. Interventions designed to study the effects of non-digestible carbohydrates on microbiota composition and/or activity in elderly, so far mainly focused on inulin [56], FOS [9], GOS [12], trans-galacto-oligosaccharide mixture (BGOS) [10,11] and a non-digestible carbohydrate mixture (of resistant starch, GOS, corn fibre, polydextrose and wheat dextrin) [57]. In all studies bifidogenic effects were demonstrated, but only two studies reported changes in microbial activity, *i.e.* increase in lactic acid [11] and butyrate [12] levels, when BGOS or GOS was provided, respectively. Studies investigating the effects of pectin on the intestinal microbiota have been based on both *in vitro* systems [14,15,23], *in vivo* models [16-20] and in humans [21,22], demonstrating increases in SCFA levels and/or alteration in microbial composition. One human intervention study with 24 g/day pectin (unspecified origin) in constipated adults showed significant increases in faecal *Bifidobacterium* and *Lactobacillus* levels, as well as a significant decrease in *Clostridium* [22]. In the present study, however, pectin supplementation did not affect faecal microbiota composition. Differences with the present study could in part be explained by differences in the source, chemical structure and/or amount of pectin supplemented (15 g/day present study vs 24 g/day), as well as differences in health status (*e.g.* constipated adults have relatively long residence time in colon).

In line with the present study, no bifidogenic effect was observed, when the same sugar beet pectin was supplemented to rats for seven consecutive weeks continuously [17]. The duration of the present study was even shorter (*i.e.* four weeks) compared to the above rat study, which may have also impacted on potential intervention effects. It has previously been shown in *in vitro* fermentation studies with human faecal microbiota that an increased degree of esterification decreased pectin fermentation rate [58], and the production of SCFAs was found to be decreased in the cecum of conventional rats (rats colonized with rat faecal material) [30]. Consistent with our current study, the rat model demonstrated that the sugar beet pectin did neither affect SCFA profiles in cecum nor in colon, except for a significantly decreased propionate level in the colon [17]. To this end, it should also be noted that metabolites that are produced in the gut lumen are known to be readily absorbed and transported to different compartments of our body, after which a proportion of the metabolites will be exhaled by the lungs and thereby detected in breath.

Recent studies have shown that VOC profiles in exhaled air have diagnostic potential [26,59-61]. It has been demonstrated previously that exhaled VOCs showed a very strong correlation with intestinal microbiota composition as studied in patients with Crohn's disease [27], but also in IBS [26]. Therefore, exhaled VOCs can also be used as an indicator of intestinal microbiota activity, either by their direct metabolic activity or by conversion of metabolites derived from host processes. In the studies of Blanchet *et al.* [62] and Dragonieri *et al.* [63], the effect of age on exhaled metabolic breath profiles was

investigated using two different analytical methodologies, *i.e.* mass spectrometry and the electronic nose, respectively. In both studies the effect of age on VOCs profiles was very limited. In the study by Blanchet *at al.* the VOCs profiles have been found to be statistically significant between age ranges divided in segments of ten years. Although, the VOCs profile was statistically significant between those age segments, the overall effect was not strong enough to lead to discriminatory model. In the similar study by Dragonieri *et al.* exhaled breath profile of young (below 50 years old) and older individuals showed no differences using canonical discriminant analysis. This is in accordance with the present study, where healthy young adults and elderly showed high similarity in exhaled VOC profiles in line with the microbiota profiles. Several investigators have pointed to effects of dietary nutrients on VOC profiles of the exhaled breath both in clinical and animal studies [26,64-66]. The changes in exhaled breath composition due to dietary nutrients have been related to their direct impact on metabolism and/or because they modify the intestinal microbiota (composition and/or activity). In a recent study by Smolinska *et al* significant differences in exhaled VOC profiles of adults were observed 240 min after consuming two infant formula diets that only differed with respect to lipid structures, showing that differences in dietary nutrients can lead to short term changes in exhaled breath composition [67]. Although pectin is a dietary fibre which potentially could alter VOC profiles by increasing the intestinal metabolite production, in the current study, no intervention effect was shown on exhaled VOC profiles of young adults and elderly. This is in contrast to a study by Raninen *et al.* [68] which investigated the level of 15 VOCs in exhaled breath of subjects that consumed either a high fibre diet (44g/day of whole grain rye) or a low fibre diet (17g/day of whole grain rye) and demonstrated significant differences in VOC profiles. In addition, a single test meal (mixture of different carbohydrates) also affected exhaled VOC profiles. Observed differences between studies may be explained by different types (cereal vs. fruit or vegetable source) and/or dosages of fibres used.

5. Conclusion

In this study, aside from the subtle differences in microbiota composition, healthy young adults and healthy elderly showed similar profiles in microbiota composition and microbial activity, as well as breath metabolite profiles at baseline. These findings are in line with our recent understanding that the microbiota composition and activity are preserved in healthy ageing and changes are primarily due to alterations in health status and lifestyle factors [3]. In addition, no effects of pectin supplementation on microbiota composition, faecal SCFA- or breath metabolite profiles were observed, indicating resilience towards pectin exposure. It would be interesting to investigate the effects of pectin in more susceptible subgroups of elderly (*i.e.* frail, or with specific comorbidities). For future research, studies investigating the dynamics of intestinal microbial composition, activity and exhaled VOC profiles under different health conditions, as well as how they response to different dietary fibre supplementations, are warranted.

Author Contributions: The authors contributions were as follows: Conceptualization, Ellen Wilms, Ad A.M. Masclee, Paul de Vos and Freddy J. Troost; Formal analysis, Ran An, Ellen Wilms, Agnieszka Smolinska and Gerben D.A. Hermes; Funding acquisition, Ad A.M. Masclee and Freddy J. Troost; Investigation, Ran An, Ellen Wilms and Agnieszka Smolinska; Methodology, Ellen Wilms, Ad A.M. Masclee and Freddy J. Troost; Resources, Ad A.M. Masclee, Henk A. Schols, Frederik J. van Schooten and Hauke Smidt; Supervision, Ad A.M. Masclee, Hauke Smidt, Daisy M.A.E. Jonkers, Erwin G. Zoetendal and Freddy J. Troost; Writing – original draft, Ran An, Ellen Wilms, Agnieszka Smolinska, Gerben D.A. Hermes and Erwin G. Zoetendal.

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Supplementary Material:

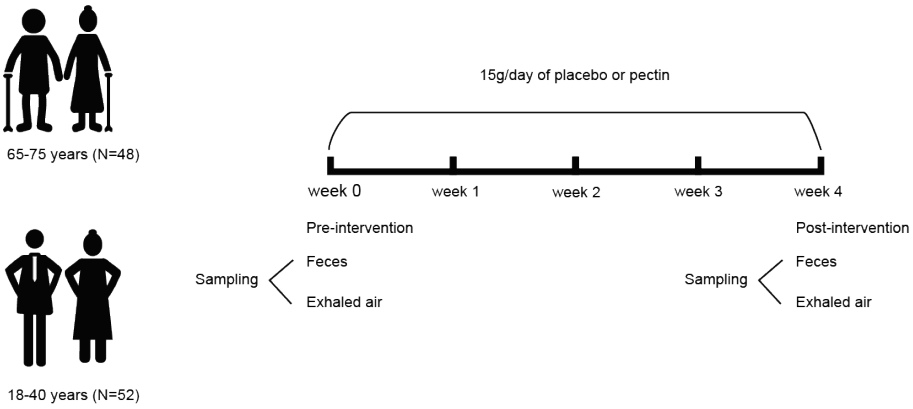

Figure S1: Schematic overview of the study design. Forty-eight healthy elderly and 52 young adults started this study. Participants consumed either 7.5g pectin or 7.5g maltodextrin (placebo) twice daily for four weeks. Feces and exhaled air were sampled before and after the intervention for analyses.

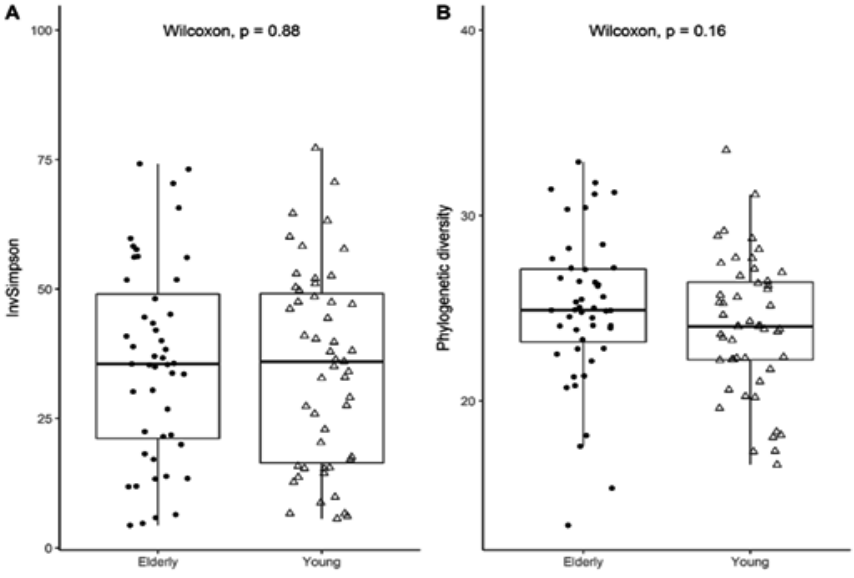

Figure S2: Faecal microbiota of young adults and elderly did not show significant differences in alpha diversity at baseline. The diversity of the microbiota was evaluated by inverse Simpson's index and phylogenetic diversity.

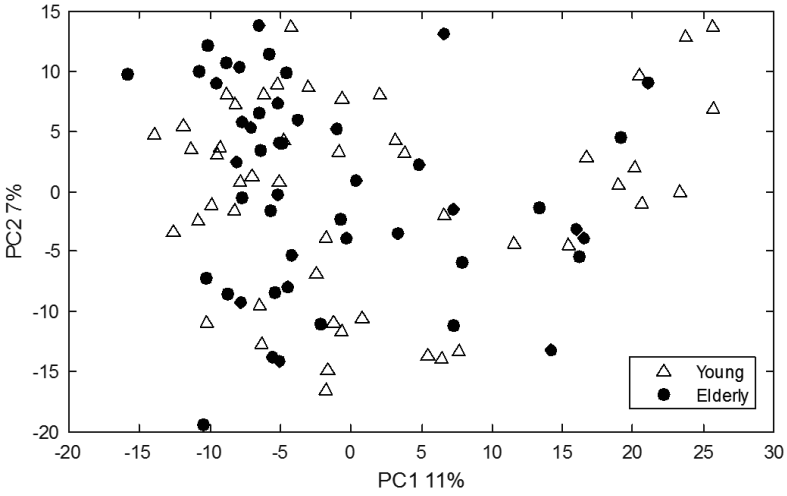

Figure S3: PCA score plot performed on the complete breath profiles of young adults and elderly. No clear groupings in exhaled breath profiles could be observed between young adults and elderly.

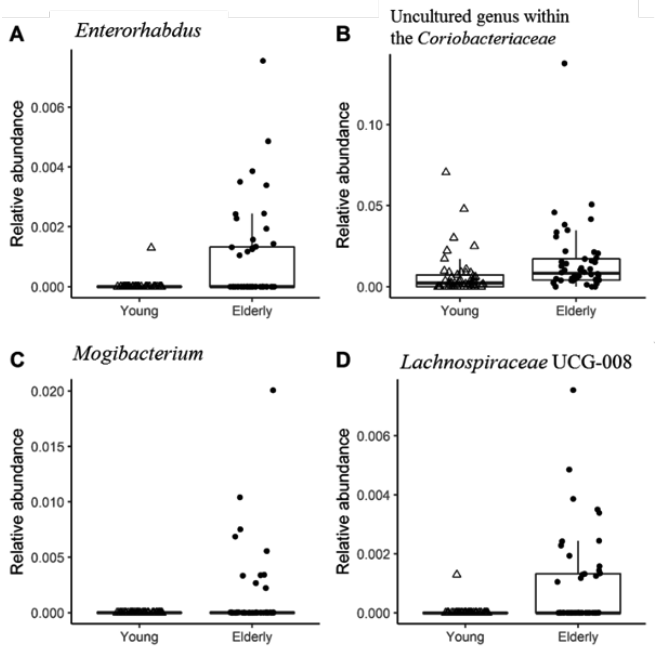

Figure S4: Significantly different genus level taxa (FDR<0.05), comparing the microbiota of young adults and elderly after intervention.

Table S1. Contribution of participants' baseline characteristics to baseline microbiota variation.

	weighted UniFrac		unweighted UniFrac	
	R-square	*P*-value	R-square	*P*-value
Age	0.0141	0.5039	0.0049	0.7895
BMI (kg/m2)	0.0044	0.8098	0.0009	0.9590
Alcohol (units/week)	0.0264	0.2752	0.0271	0.2810
Sport (hours/week)	0.0023	0.8904	0.0003	0.9867
Sex (male/female)	0.0047	0.6284	0.0094	0.4016
Medication (yes/no)	0.0006	0.9349	0.0029	0.7593
Allergy (yes/no)	0.0035	0.6957	0.0024	0.7910
Vegetarian (yes/no)	0.0007	0.9299	0.0240	0.0921
Food supplements (yes/no)	0.0094	0.3805	0.0029	0.7524
Disease history (yes/no)	0.0116	0.3201	0.0197	0.1478

Contribution of baseline characteristics of participants was tested by fitting available variables to the ordination object. The variation of baseline microbiota profiles could not be explained by any of the included baseline variables.

Table S2. Inter- and intra-individual distance over the intervention period.

	Inter-individual distance	Intra-individual distance	*P*-value
Weighted UniFrac	0.21±0.07	0.14±0.07	<0.001
Unweighted UniFrac	0.43±0.07	0.24±0.09	<0.001

Differences between inter-individual distance and intra-individual distance, were tested using T-tests. Values are presented as mean ± SD. Higher value in distance indicates smaller similarity.

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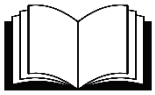
Galacto-oligosaccharides supplementation in prefrail elderly and healthy adults increased faecal bifidobacteria, but did not impact immune function and oxidative stress



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Abstract

Ageing is associated with an increased risk of frailty, intestinal microbiota perturbations, immunosenescence and oxidative stress. Prebiotics such as galacto-oligosaccharides (GOS) may ameliorate these ageing-related alterations. We aimd to compare the faecal microbiota composition, metabolite production, immune and oxidative stress markers in prefrail elderly and younger adults, and investigate the effects of GOS supplementation in both groups. In a randomised controlled cross-over study, 20 prefrail elderly and 24 healthy adults received 21.6 g/day Biotis^{TM} GOS (containing 15.0 g/day GOS) or placebo. Faecal 16S rRNA gene-based microbiota and short-chain fatty acids were analysed at 0, 1 and 4 weeks of intervention. Volatile organic compounds were analysed in breath, and stimulated cytokine production, CRP, malondialdehyde, trolox equivalent antioxidant capacity (TEAC) and uric acid (UA) in blood at 0 and 4 weeks. Principal coordinate analysis showed differences in microbial composition between elderly and adults ($P \leq 0.05$), with elderly having lower bifidobacteria ($P \leq 0.033$) at baseline. In both groups, GOS affected microbiota composition ($P \leq 0.05$), accompanied by increases in bifidobacteria ($P < 0.001$) and decreased microbial diversity ($P \leq 0.023$). Faecal and breath metabolites, immune and oxidative stress markers neither differed between groups ($P \geq 0.125$) nor were affected by GOS ($P \geq 0.236$). TEAC values corrected for UA were higher in elderly versus adults ($P < 0.001$), but not different between interventions ($P \geq 0.455$). Elderly showed lower faecal bifidobacterial (relative) abundance than adults, which increased after GOS intake in both groups. Faecal and breath metabolites, parameters of immune function and oxidative stress were not different at baseline, and not impacted by GOS supplementation.

Key words: microbiome, nutrition, probiotics/prebiotics, immunology

1. **Introduction**

In line with a continuously rising life expectancy, the ageing population is increasing worldwide. Ageing is associated with a decline in general physiological functioning, including immunosenescence, contributing to frailty.[1]. By applying the Fried criteria to determine physical frailty, a recent study showed prevalence rates of 50.5% and 16.0% for prefrail and frail status, respectively, in a European population of community-dwelling elderly (aged 75-84 years) [2]. Frailty has been associated with impaired quality of life, increased risk of comorbidity and increased healthcare costs [3].

The intestinal microbiota comprises a complex community of microbes dominated by anaerobic bacteria, and plays a key role in intestinal immunity and defense capacity [4], either directly by microbe-cell interactions or indirectly via bacterial metabolites. Lower microbial diversity, richness and alterations in relative abundance of several bacterial groups have been found in elderly, being most pronounced in frail elderly and in association with lifestyle risk factors [5]. The mechanisms through which these changes in the intestinal microbiota contribute to age-related immunosenescence remain unclear up to now [6]. Immunosenescence refers to age-related alterations in immune function, and is characterised by *e.g.* dysfunctional immune cells such as monocytes and T-cells, and a pro-inflammatory cytokine profile [7]. Moreover, immunosenescence is associated with oxidative stress [8], a condition in which the balance between pro- and antioxidants is disrupted leading to an oxidizing state [8, 9]. Both immunosenscence and higher oxidative stress levels are considered risk factors for age-related morbidities such as infections [8], as well as for cardiovascular [10] and neurodegenerative [11] disorders.

Since frailty is not a unidirectional process, early identification (*i.e.* prefrail status) and targeted interventions may help to improve health status and thereby decrease comorbidity and healthcare costs in elderly. One such approach could be via the intake of prebiotics such as galacto-oligosaccharides (GOS). GOS are non-digestible oligosaccharides, which have been classified as prebiotic because of their impact on the intestinal microbiota and host health. Increased production of short-chain fatty acids (SCFAs) after GOS intake may contribute to anti-inflammatory and antioxidant effects. Vulevic *et al.* [12] showed that administration of 5.5 g/day GOS for five weeks beneficially altered the faecal microbiota and some markers of immune function in healthy elderly. However, data on the effects of GOS in prefrail elderly are lacking.

The aims of the current study were twofold: 1) to compare prefrail elderly and healthy younger adults with respect to faecal microbiota composition, metabolites in faeces and exhaled breath, immune markers and oxidative stress markers, and 2) to study the impact of four weeks GOS supplementation in both groups. We hypothesised that faecal microbiota composition, metabolites in faeces and exhaled breath, and parameters of immune function and systemic oxidative stress show perturbations in prefrail elderly when compared with healthy adults, and that these markers can be beneficially altered by four weeks of GOS supplementation.

2. Materials and methods

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center+ and registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT03077529). The study was performed at the Maastricht University Medical Center+ between March 2017 and September 2018, according to the Guidelines of the Declaration of Helsinki (latest amendment of 2013, Fortaleza, Brazil), Dutch Regulations on Medical Research involving Human Subjects and Good Clinical Practice (GCP) guidelines. All participants gave written informed consent before participation.

2.1. Subjects

Men and women without gastrointestinal (GI) complaints, Body Mass Index (BMI) 20 - 30 kg/m2 and being weight stable were recruited in two age groups: elderly 70 – 85 years of age and adults 25-50 years of age. The Fried criteria to determine physical frailty [13] were used to classify and include prefrail elderly (score 1 or 2) and robust adults (score 0). Other inclusion criteria were hemoglobin, C-reactive protein (CRP), creatinine, alanine transaminase (ALT) and gamma-glutamyl transpeptidase (GGT) within the normal range of reference values, or being slightly outside the normal range but not at risk of severe comorbidities (as determined by a medical doctor). Key exclusion criteria were history of any chronic disorder or major surgery, which potentially limited participation, completion of the study or interfered with the study outcomes; self-reported human immunodeficiency virus or lactose intolerance; use of antibiotics 90 days before the start of the study, average alcohol consumption of >14 alcoholic units per week, pregnancy, lactation, institutionalization (e.g. hospital or nursing home), use of probiotics, prebiotics or laxatives 14 days before the start of the study, and history of side effects towards prebiotic supplements. As medication use and comorbidities are more frequent in elderly, they were not general exclusion criteria, but considered to reflect (prefrail) ageing. Both were checked by a medical doctor, and subjects were only allowed to participate if medication and comorbidities were not expected to interfere with the outcome parameters. Furthermore, medication use had to be on stable dosing at least 14 days before and during the study.

2.2. Study design

This study was designed as a randomised, double-blind, placebo-controlled, cross-over study with a 4-6 weeks wash-out period between the intervention periods (Supplementary Figure 1). Per age group (*i.e.* elderly and adults), randomisation was performed with concealed block sizes of four, to assign participants to the placebo or the GOS intervention period. An independent person generated two randomization lists of random allocations using a computerised program (http://www.randomization.com), which was stratified for gender. Both study participants and investigators were blinded to the interventions until analyses were completed. During the GOS intervention period of four weeks, participants received 21.6 g/day of BiotisTM GOS Powder (FrieslandCampina Ingredients, Amersfoort, the Netherlands), containing 15.0 g/day of pure GOS. In the placebo intervention period, 21.6 g/day of maltodextrin (GLUCIDEX® IT 12, Roquette Frères, Lestrem, France), which is

completely absorbed in the small intestine, was supplemented for four weeks. Both GOS and placebo were provided as white powdered supplements with similar appearance (*i.e.* colour, taste and odour), and packed in closed sachets of a single dose of 7.2 g. Participants were asked to ingest the content of three sachets daily (before breakfast, lunch and dinner), by transferring the powder in a glass, mixing with approximately 200 mL tap water and consuming the complete drink. Time of consumption had to be recorded, and empty sachets were returned to assess compliance. At baseline, after one week and after four weeks GOS and placebo intake, faecal samples were collected, and three-day dietary records and stool characteristics scores were completed at home (Supplementary Figure 1). Faecal samples were stored at -20 °C until arrival at the study site, and subsequently stored at -80 °C for later analyses. At baseline and after four weeks GOS and placebo supplementation, venous blood and exhaled air samples were collected after an overnight fast, and stored at -80 °C and room temperature, respectively, until further use. The GI symptom rating scale (GSRS) was completed at baseline and at weekly intervals. Supplement intake continued until all samples were collected and questionnaires completed.

2.3. Microbiota composition

Total DNA was isolated from faeces and subsequently purified as described previously [14]. Microbiota composition was determined by sequencing of barcoded 16S ribosome RNA (rRNA) gene amplicons using Illumina Hiseq2500 (2 x 150 bp). The V4 region of prokaryotic 16S rRNA genes was amplified in triplicate using 515F [15]-806R [16] primers and purified DNA as template. Polymerase chain reaction (PCR) was performed as described previously [14], with annealing temperature of 50 °C. An equimolar mix of purified PCR products was sent for sequencing (GATC-Biotech, Konstanz, Germany). Raw sequence data was processed using NG-Tax default settings [17, 18].

2.4. qPCR analysis

Total DNA was also used for quantitative PCR (qPCR) using a CFX384 Touch TM Real-Time PCR Detection System (Bio-Rad, California, USA). The reaction mixture was composed of 6.25 µl iQ TM SYBR ® Green Supermix, 0.25 µl forward primer (10 µM), 0.25 µl reverse primer (10 µM), 3.25ul nuclease free water and 2.5 µl DNA template. Primers were used that targeted either total bacteria [19] or *Bifidobacterium spp*. [20]. The program for amplification of total bacteria was initiated at 95 °C for 3 min, followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 52 °C for 30 s and elongation at 72 °C for 30 s. The program for total bifidobacteria was: 94°C for 5 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 50 s, 72 °C for 50 s. Both programs were ended with a melt-curve analysis from 60 °C to 95 °C with 0.5 °C per step. Reactions for the quantification of total bacteria and total bifidobacteria were performed in triplicates. Data was analysed using the CFX manager TM (Bio-Rad).

2.5. Faecal metabolites

Faecal organic acids resulting from microbial fermentation were measured using high performance liquid chromatography (HPLC), equipped with a SUGAR SH1011column (Shodex, Japan). The column was operated at 45 °C, with a flow rate of 0.8ml/min, using 0.1N H_2SO_4 as eluent. The compounds were detected by a RID-20A (Shimadzu, Kyoto, Japan) refractive index detector at a temperature of 40 °C. One gram faeces was suspended in 4.0 ml Milli-Q water, mixed and centrifuged at 4 °C 2000 × g for 20 min. Four hundred µl of supernatant was mixed with 600 µl of 10mM DMSO in 0.1N H_2SO_4. Ten µl of samples was injected and subjected to analysis. The chromatograms were depicted and analysed with Chromeleon™ Chromatography Data System (CDS) Software (ThermoFisher Scientific, Massachusetts, USA). Organic acids were expressed per gram dry matter to correct for stool consistency. Dry matter content was determined by vacuum drying of 500 mg faeces for five hours at 60 °C (Concentrator plus, Eppendorf, Hamburg, Germany).

2.6. Volatile organic compounds

Metabolites were also analysed in exhaled breath. Therefore, each participant delivered exhaled air samples by breathing into a 3L Tedlar bag (SKC Limited, Dorset, UK). The content of the bag was transferred within one hour to carbon-filled stainless steel absorption tubes (Markes International, Llantrisant, UK) using a vacuum pomp (VWR international, Radnor, PA, USA). Volatile organic compounds (VOCs) captured into stainless tubes were measured using thermal desorption gas chromatography coupeled to time-of-flight mass spectrometry (GC-tof-MS, (Markes International, Llantrisant, UK) as described previously [14, 21]. The resulting breath-o-grams were denoised, baseline corrected, aligned, normalised by probabilistic quotient normalisation and scaled for further analyses [22].

2.7. Immune parameters

Blood was collected in BD Vacutainer® sodium heparin and serum tubes (BD Biosciences, San Jose, CA, USA). Sodium heparin blood was used within three hours for whole blood stimulations to determine ex vivo cytokine production. RPMI 1640 medium with HEPES and glutamax (Thermo Fischer Scientific, Waltham, MA, USA), supplemented with 10% heat inactivated Fetal Bovine Serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin (Gibco™, Thermo Fischer Scientific, Waltham, MA, USA) was used as culture medium. Whole blood was mixed with medium in 1:5 ratio, and incubated with 10 µg/ml Phytohemagglutinin-M (PHA) (L8902, Sigma-Aldrich, St. Louis, MO, USA) to stimulate T-cells, and 10 µg/ml E. coli 055:B5 Lipopolysaccharides (LPS) (L4524, Sigma-Aldrich, St. Louis, MO, USA) to stimulate monocytes. After 24 hours incubation at 37°C and 5% CO2, samples were centrifuged (8 min, 283 × g) and plasma aliquots were stored at -80 ºC for further analyses. Plasma samples were thawed and kept at 4 ºC until incubation with Interleukin (IL)-1β, IL-6, IL-8, IL-10, Interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α antibodies (Catalogue numbers 558279, 558276, 558277, 558274, 558269 and 560112, respectively, BD Biosciences, San Jose, CA, USA). The Cytometric Bead Array (BD Biosciences, San Jose, CA, USA) was performed according to manufacturer's instructions and samples were measured using a FACSCanto II flow cytometer (BD Biosciences, San Jose,

CA, USA) and analysed with FACSDiva TM Software (BD Biosciences, San Jose, CA, USA). Serum CRP concentrations were determined by immunoturbidimetric assay using Cobas 6000 analyser (Roche, Mannheim, Germany).

2.8. Parameters of systemic oxidative stress

Lithium heparin blood (BD Vacutainer®, BD Biosciences, San Jose, CA, USA) was collected for malondialdehyde (MDA), trolox equivalent antioxidant capacity (TEAC) and uric acid (UA) analyses. MDA plasma concentrations were quantified after derivatisation with thiobarbituric acid into a fluorescent chromogen.[23] The chromogen was extracted from the samples with butanol after centrifugation at 30,000 x g for 5 min. Fluorescence of the butanol phase was measured in black bottom 96-wells plates by using an excitation wavelength of λ_{ex} = 530 nm and an emission wavelength of λ_{em} = 560 nm. Plasma antioxidant capacity was quantified as TEAC values, according to Fischer *et al.* [24] and corrected for inter-individual differences in plasma UA concentrations [25] quantified by HPLC [26].

2.9. Dietary intake

Participants were asked to complete dietary records on three consecutive days before each test day. Before the start of the first intervention period, participants were instructed how to record their food, beverage and dietary supplement intake based on standard household units. Energy and nutrient intake were analysed using the online dietary assessment tool of The Netherlands Nutrition Centre (www.voedingcentrum.nl). As polyphenols are a major class of dietary antioxidants in addition to some vitamins and minerals, their intake was also calculated using PhenolExplorer 3.6, a comprehensive database containing polyphenol content values of over 400 food items [27, 28].

2.10. Gastrointestinal tolerance and stool characteristics

The GSRS was used to monitor GI tolerance. It consists of 15 items using a seven-point Likert-type scale (where 1 represents absence of troublesome symptoms and 7 represents very troublesome symptoms) and calculated into five subscales including reflux, abdominal pain, indigestion, diarrhoea, and constipation [29]. Stool characteristics (*i.e.* stool frequency and stool consistency) were scored on three consecutive days using the Bristol Stool Scale. Stool consistency is an ordinal scale score ranging from hard (type 1) to soft (type 7). Frequencies of hard stools and loose stools were calculated and analysed as described previously [30].

2.11. Statistical analyses

In a study investigating the effects of GOS on different bacterial groups using fluorescent in situ hybridisation (FISH) in healthy elderly volunteers, significant changes in bifidobacteria and Bacteroides spp. were observed [12]. The estimated effect after five weeks GOS intervention was an increase of 0.59 ± 0.44 log10 cells/g faeces for bifidobacteria and a decrease of 0.018 ± 0.022 log10 cells/g faeces for Bacteroides spp. The power (1-β) was set at 0.8, and the significance level (α) at 0.025 as we were interested in the effects of GOS as well as the differences between age groups. Using these numbers, the sample size per age group equalled 8 and 17 subjects, respectively. O'Keefe *et al*. [31] investigated microbiota composition in healthy individuals by comparing two weeks high-fibre and low-fibre diets in a cross-over setting, it was shown that 20 subjects was sufficient to robustly identify differences in microbiota composition between treatment groups. Taking all above into account, 20 subjects were included per age group, which should be sufficient to pick up changes in microbiota composition, as well as in specific genera.

Per parameter, we compared elderly and adults, and subsequently identified effects of GOS versus placebo interventions in both age groups. For this, normality of the data was tested by histograms and summarised accordingly using the median and interquartile range (IQR; 25–75th IQR) or means ± standard deviation (SD) for numerical variables, and by percentages for categorical variables. Subsequently, independent-sample t-tests and Mann-Whitney U tests were performed for numerical variables, and Chi-square tests for categorical variables to test for differences between elderly and adults.

The 16S rRNA gene sequence read counts were normalised to relative abundance and the microbial alpha-diversity indices (Faith's phylogenetic diversity (PD) and Inverse Simpson) were calculated based on amplicon sequence variants (ASVs) as implemented in Phyloseq and vegan packages [32, 33]. Homogenicity of the dataset was evaluated by detrended correspondence analysis (DCA) and subsequently using linear or unimodal constrained ordination for multivariate analysis. Redundancy analysis (RDA) was used to assess the contribution of environmental variables (*e.g.* BMI, age, alcohol *etc.*) to the observed microbiota variation. Principal coordinate analysis based on weighted UniFrac, unweighted UniFrac, Bray-Curtis and Jaccard distances was used to evaluate variation in microbiota composition at ASVs level. Significance of observed differences between groups was tested by permutational multivariate analysis of variance (PERMANOVA). Intervention effects on single bacterial taxa and microbiota diversity indices were evaluated using a linear mixed model as implemented in the *nlme* package [34], with variance components (random intercept) and correction for baseline values. All statistical analyses of the microbiota data were conducted in R (R-3.5.0).

Exhaled breath data were analysed with ANOVA-simultaneous component analysis (ASCA) [35] and unsupervised Random Forest (uRF) [36]. The ASCA model was used to investigate the effect of the treatment and uRF for visualisation of the data. Data were log transformed to account for data skewed distribution and pareto-scaled to ensure equal contribution of each volatile metabolite in breath in the ASCA analysis. In case of uRF analysis data were only log transformed. The statistical analysis was carried out separately for each age group.

The differences in VOC profiles at baseline between elderly and adults were explored using a supervised version of RF analysis. The training set was used to find the discriminatory VOCs and to build the classification model. The performance of the RF classification model was expressed by the area under the curve of receiver operating characteristic (AUROC) for the validation set. Statistical analyses of VOCs were performed using Matlab 2019a (The MathWorks, Natick, 2018).

Both intention to treat and per protocol analyses were performed. Within age groups, differences between intervention periods were assessed by variance components (random intercept) linear mixed model analyses with intervention group, time, intervention period, 'intervention group × time', 'intervention group × intervention period', and 'time × intervention period' as fixed factors, and correction for baseline values. For significant intervention effects, differences between age groups were assessed by addition of the fixed factor 'intervention × time × age group'. All statistical analyses were performed for adults and elderly separately using IBM SPSS Statistics for Windows (version 25.0, IBM Corporation, Armonk, NY, USA). *P*-values ≤ 0.05 (two-sided) were considered to indicate statistical significance. P-values were corrected for multiple testing by the false discovery rate (FDR) of Benjamini–Hochberg per cluster of parameters (*i.e.* bacterial taxa, metabolites, immune, oxidative stress and dietary intake) per age group. GI symptoms were corrected by FDR for multiple time points.

3. Results

3.1. Study subjects, GI tolerance and dietary intake

After evaluating 66 elderly and 33 adults for eligibility, 20 elderly (all with Fried frailty score 1) and 24 adults (Fried frailty score 0) were randomised in the study. Three adults dropped out and one adult was non-compliant to the intervention (Supplementary Figure 2). Elderly and adults returned on average 97.6% and 95.0% of the empty sachets, respectively.

As per protocol analyses led to the same conclusions, only the results of the intention to treat analyses are shown. Concerning baseline characteristics, besides age, also medication use and BMI were significantly different between age groups (Table 1).

Table 1. Baseline characteristics of the elderly (n=20) and adults (n=24)

	Elderly (n=20)	Adults (n=24)	*P*-value
Age (yrs, mean ± SD)	74.3 ± 3.7	38.2 ± 7.8	<0.001
Sex (% female)	45.0	66.7 †	0.149
BMI (kg/m^2, mean ± SD)	26.4 ± 3.0	23.1 ± 2.6	<0.001
Smoking (%)	10.0	12.5	0.795
Habitual alcohol consumption (%)			
<1 unit/wk	25.0	33.3	
1 – 7 units/wk	55.0	62.5	0.251
8 – 14 units/wk	20.0	4.2	
Medication use (%) ‡			
No	60.0	95.8	0.003
Anticoagulation	25.0	4.2	0.045
Antihypertensives	25.0	0	0.009
Statins	15.0	4.2	0.213
Blood parameters (mean ±SD) §			
Hemoglobin (mmol/L)	8.8 ± 0.8	8.8 ± 0.7	0.984
CRP (mg/L)	1.1 ± 1.3	1.5 ± 3.1	0.592
Creatinin (µmol/L)	83.3 ± 17.6	74.8 ± 12.4	0.068
ALT(U/L)	22.5 ± 10.1	23.3 ± 9.2	0.787
GGT (U/L)	22.5 ± 11.1	20.8 ± 11.9	0.629

ALT: alanine transaminase, BMI: body mass index, CRP: C-reactive protein, GGT: gamma-glutamyl transpeptidase. † Drop-outs and non-compliant subject were all female, and replaced by females. ‡ Most commonly used medication. § Taking into account gender specific reference values, five adults and seven elderly showed a limited increase or decrease in one of the blood parameters not associated with clear comorbidity. Age, BMI and blood parameters were compared between intervention groups with the use of an independent samples t-test. Sex, smoking, habitual alcohol consumption and medication use were compared between intervention groups with the use of a Pearson's Chi-square test.

GI tolerance towards the intervention products was assessed weekly by use of the GSRS questionnaire. After FDR correction for multiple testing, GI symptom scores were not significantly different between GOS and placebo intervention in elderly nor in adults (all $P \geq 0.058$) (Supplementary Figure 3). In addition, average stool frequency as well as average frequencies of hard stools and loose stools were not significantly different between GOS and placebo supplementation in elderly and adults after FDR correction for multiple testing (all $P \geq 0.170$).

Energy, macronutrient, micronutrient as well as polyphenol intake levels did not differ significantly between elderly and adults at baseline nor after GOS as compared to placebo supplementation (Supplementary Table 1).

3.2. Faecal microbiota composition

Principal coordinate analysis (PCoA) based on Bray-Curtis (considering relative abundance of ASVs, Supplementary Figure 4A) and weighted UniFrac (considering relative abundance of ASVs and their position in the phylogenetic tree) revealed significant differences between the microbiota of adults and that of elderly (Figure 1A), with 3.49% and 4.53% of variation explained by age groups at baseline, *i.e.* at the start of the first intervention period. However, PCoA based on Jaccard (only based on presence or absence) and unweighted UniFrac (based on presence or absence, and their position in the phylogenetic tree) distances showed no significant differences (Supplementary Figure 4B and C), indicating the differences in microbiota profile are mainly driven by differences in relative abundance rather than presence/absence of bacterial taxa. We investigated the differences between elderly and adults, and the effects of GOS on genus level (Table 2). Only the relative abundance of *Bifidobacterium* was significantly lower in elderly compared with adults *(P* = 0.033, Table 2). This was confirmed by qPCR *(P* = 0.025, Figure 1C). The relative abundance of all the other genera as well as microbial richness and diversity (both *P* = 0.942, Supplementary Figure 4) were not significantly different between elderly and adults.

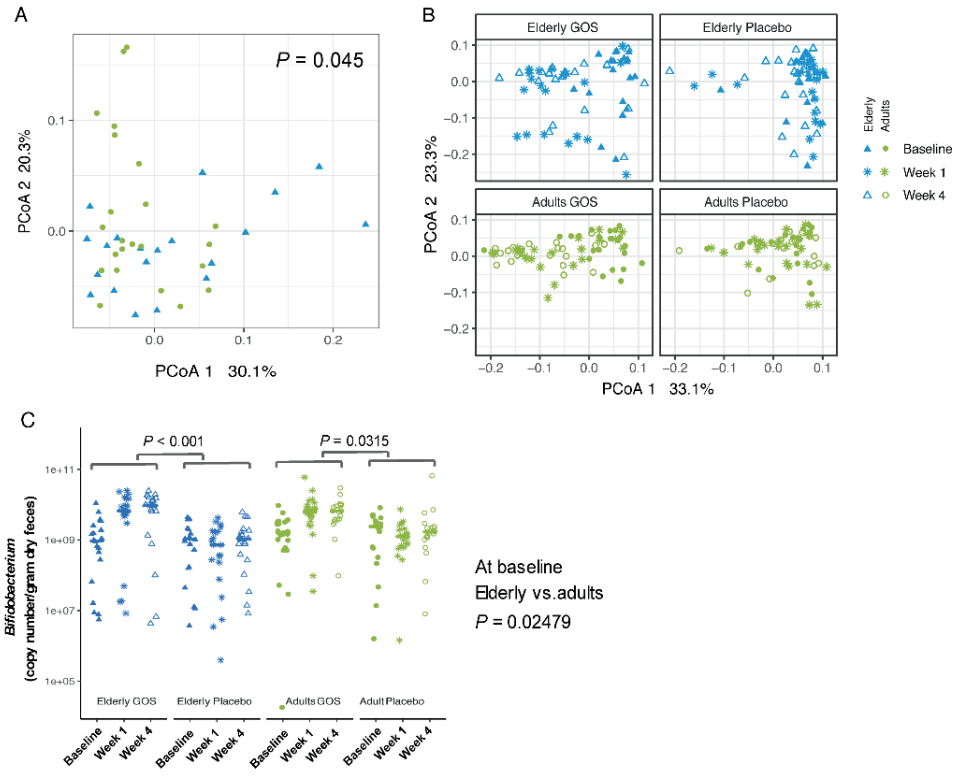

Figure 1. Faecal microbiota at baseline (A), and comparing baseline microbiota to that after 1 week and 4 weeks GOS and placebo intervention (B) in 20 elderly and 23 adults based on 16S rRNA gene sequences. PCoA plots are based on weighted UniFrac distance matrices. PERMANOVA was used to compare the microbiota of elderly and adults in the PCoA plots at baseline, as well as for intervention effects between time points per group of subjects (*e.g.* Elderly baseline GOS vs Elderly Week 1 GOS). (C) *Bifidobacterium* (copy number/g dry faeces) based on qPCR, pre and post GOS and placebo intervention periods in 20 elderly and 23 adults. Sample sizes vary due to drop-outs and technical reasons. Values are presented in scatter plots with median line. Elderly vs. adults at baseline of the first intervention period were compared by a Mann-Whitney U test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values. PCoA; Principal coordinate analysis.

Table 2. Most abundant genera based on sequencing of the 16S rRNA gene, pre and post GOS and placebo intervention periods of the elderly (n=20) and adults (n=23)

	Elderly (n=20)							Adults (n=23)							P-value (elderly vs adults at baseline)
	GOS			Placebo			P-value	GOS			Placebo			P-value	
	Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		
Blautia	0.091 [0.061; 0.105]	0.066 [0.036; 0.087]	0.079 [0.042; 0.101]	0.100 [0.073; 0.127]	0.073 [0.040; 0.109]	0.079 [0.054; 0.116]	1.000	0.092 [0.068; 0.138]	0.067 [0.046; 0.086]	0.056 [0.038; 0.077]	0.098 [0.075; 0.132]	0.082 [0.069; 0.101]	0.088 [0.065; 0.099]	0.415	0.797
Bifidobacterium	0.024 [0.011; 0.071]	0.294 [0.208; 0.400]	0.260 [0.080; 0.339]	0.027 [0.016; 0.046]	0.020 [0.007; 0.038]	0.042 [0.008; 0.071]	<0.001	0.070 [0.049; 0.144]	0.348 [0.208; 0.484]	0.304 [0.240; 0.517]	0.083 [0.027; 0.150]	0.083 [0.035; 0.149]	0.113 [0.045; 0.171]	<0.001	0.033
Faecalibacterium	0.065 [0.038; 0.077]	0.049 [0.035; 0.071]	0.058 [0.038; 0.08]	0.073 [0.033; 0.096]	0.066 [0.032; 0.111]	0.061 [0.024; 0.090]	0.979	0.096 [0.070; 0.117]	0.074 [0.047; 0.102]	0.070 [0.051; 0.089]	0.077 [0.050; 0.102]	0.092 [0.050; 0.115]	0.075 [0.057; 0.107]	0.214*	0.610
Unclassified genus within Lachnospiraeceae family	0.030 [0.016; 0.081]	0.030 [0.018; 0.051]	0.046 [0.023; 0.067]	0.043 [0.022; 0.083]	0.037 [0.016; 0.072]	0.044 [0.018; 0.051]	0.850	0.039 [0.029; 0.064]	0.041 [0.014; 0.05]	0.034 [0.012; 0.058]	0.038 [0.019; 0.058]	0.041 [0.023; 0.061]	0.062 [0.022; 0.082]	0.270	0.715
Subdoligranulum	0.037 [0.020; 0.075]	0.019 [0.010; 0.031]	0.022 [0.014; 0.037]	0.038 [0.019; 0.059]	0.032 [0.013; 0.053]	0.022 [0.012; 0.047]	0.213	0.036 [0.028; 0.046]	0.025 [0.013; 0.031]	0.016 [0.013; 0.025]	0.040 [0.022; 0.062]	0.034 [0.019; 0.041]	0.032 [0.018; 0.056]	0.476	0.857
Prevotella 9	0.001 [0.000; 0.073]	0.023 [0; 0.211]	0.002 [0; 0.077]	0.002 [0; 0.063]	0.001 [0; 0.111]	0.000 [0.000; 0.086]	0.873	0.000 [0.000; 0.008]	0.000 [0.000; 0.007]	0.000 [0.000; 0.005]	0.000 [0.000; 0.001]	0.000 [0.000; 0.017]	0.000 [0.000; 0.003]	0.407	0.486
Ruminococcus 2	0.024 [0.002; 0.081]	0.016 [0; 0.025]	0.012 [0; 0.06]	0.043 [0.003; 0.059]	0.024 [0.003; 0.058]	0.027 [0.004; 0.05]	0.298	0.004 [0.000; 0.060]	0.007 [0.000; 0.049]	0.012 [0.000; 0.028]	0.022 [0.000; 0.046]	0.007 [0.000; 0.037]	0.026 [0.000; 0.049]	0.469	0.606
Christensenellaceae R-7 group	0.010 [0.004; 0.036]	0.004 [0.000; 0.023]	0.011 [0.002; 0.022]	0.019 [0.005; 0.033]	0.017 [0.002; 0.039]	0.015 [0.003; 0.033]	0.083*	0.017 [0.006; 0.032]	0.008 [0.006; 0.014]	0.013 [0.003; 0.026]	0.022 [0.007; 0.040]	0.017 [0.010; 0.041]	0.021 [0.008; 0.028]	0.481	0.827
Bacteroides	0.014 [0.003;	0.004 [0.001;	0.007 [0.003;	0.025 [0.006;	0.028 [0.011;	0.015 [0.002;	1.000	0.023 [0.004;	0.016 [0.006;	0.012 [0.005;	0.027 [0.009;	0.017 [0.007;	0.033 [0.022;	0.523	0.771

GOS: galacto-oligosaccharides. Selection made based on average relative abundance (in baseline samples) above 2.5%. Data are expressed as median [IQR; *i.e.* Q1; Q3]. Sample sizes vary due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention period were compared by Mann-Whitney U tests. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and corrected for baseline values. Analysis has been performed in all genera. For visibility, here only genera with average relative abundance >2.5% have been shown. P-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. * Significant before correction for multiple testing.

PCoA based on weighted UniFrac (Figure 1B) indicated that microbiota composition was significantly different after one and four weeks of GOS supplementation, both in adults (both $P = 0.003$) as well as elderly *(*$P = 0.003$ and $P = 0.010$, respectively), when compared to the corresponding data from subjects consuming the placebo supplementation. Similarly, for Bray-Curtis distance-based analysis (Supplementary Figure 4D), microbiota profiles differed significantly after one and four weeks of GOS supplementation both in adults (both $P = 0.003$) and elderly *(*$P = 0.003$ and $P = 0.005$, respectively). However, no significant differences were observed based on unweighted UniFrac and Jaccard distance (Supplementary Figure 4E and F; *(*$P > 0.05$)). The relative abundance of *Bifidobacterium* increased significantly after one and four weeks GOS supplementation in elderly and adults (both $P < 0.001$, Table 2), which was confirmed by qPCR for elderly ($P < 0.001$) and adults ($P = 0.0315$) (Figure 1C). Furthermore, the relative abundance of bifidobacteria (illustrating intervention effects) in the group of subjects receiving GOS in the first intervention period, dropped back to baseline levels after four weeks of wash-out, indicating the absence of carry-over effects after GOS supplementation (Supplementary Figure 5). The relative abundance of all the other genera was not significantly different between GOS versus placebo neither in elderly, nor in adults ($P > 0.05$). Microbial diversity (InvSimpson) decreased significantly after four weeks GOS supplementation in both adults *(*$P = 0.011$) and elderly ($P = 0.023$) (Supplementary Figure 6A). Microbial richness (Faith's PD) dropped significantly in elderly ($P = 0.009$), but it was not significantly affected by GOS supplementation in adults ($P > 0.05$) (Supplementary Figure 6B). Furthermore, the significant effects of GOS on relative abundance of *Bifidobacterium* and on microbial diversity were not significantly different between elderly and adults ($P \geq 0.337$).

3.3. Faecal metabolites

Acetate, propionate, butyrate, isobutyrate and succinate concentrations did not significantly differ between elderly and adults at baseline ($P \geq 0.125$) (Table 3). Furthermore, metabolite concentrations did not change after four weeks GOS versus placebo supplementation neither in elderly, nor in adults ($P \geq 0.520$).

Table 3. Faecal metabolite concentrations (µmol/g dry content) before, during and after GOS and placebo interventions in elderly (n=20) and adults (n=23)

	GOS			Placebo			*P*-value	GOS			Placebo			*P*-value	*P* - value (elderly vs adults at baseline)
	Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		
Acetate	64.83 [40.74; 89.41]	80.01 [57.90; 120.72]	69.53 [45.84; 107.39]	81.71 [54.42; 139.90]	90.71 [55.56; 113.99]	73.8 [49.62; 140.69]	0.976	94.81 [72.89; 135.44]	76.98 [53.10; 154.52]	111.00 [58.20; 162.04]	77.90 [59.34; 112.65]	69.74 [36.03; 100.90]	85.90 [44.87; 112.30]	0.972	0.188
Propionate	20.79 [15.00; 33.61]	27.78 [16.73; 34.41]	20.91 [16.11; 31.01]	28.71 [17.03; 39.18]	26.23 [19.15; 32.73]	28.21 [17.20; 38.63]	0.737	31.66 [19.21; 42.52]	26.29 [18.38; 36.98]	27.64 [19.37; 38.75]	28.28 [19.13; 38.58]	25.50 [17.03; 42.88]	25.86 [15.75; 40.36]	0.654	0.125
Butyrate	14.74 [9.14; 20.45]	17.94 [9.13; 23.43]	15.28 [11.29; 19.13]	16.55 [10.72; 23.20]	16.17 [9.78; 23.59]	24.40 [8.05; 32.13]	0.270	24.93 [19.20; 41.89]	23.23 [16.79; 30.75]	22.14 [13.85; 36.07]	21.71 [14.05; 36.11]	22.55 [5.45; 37.06]	17.78 [11.71; 40.37]	0.951	0.125*
Isobutyrate	2.32 [1.31; 3.41]	2.315 [1.23 ;3.69]	3.30 [1.93; 3.64]	3.25 [1.88; 5.46]	3.15 [1.41; 4.48]	2.88 [2.01; 4.75]	0.560	2.64 [0.00; 4.26]	1.73 [0.00; 3.24]	2.82 [0.59; 4.48]	3.48 [1.59; 3.77]	2.24 [0.00; 3.63]	3.38 [0.68; 6.42]	0.288	0.487
Succinate	0.45 [0.10; 1.76]	0.18 [0.06; 0.36]	0.35 [0.13; 1.04]	0.285 [1.13; 1.92]	0.355 [0.06; 1.15]	0.18 [0.10; 0.61]	0.537	0.34 [0.00; 1.32]	0.59 [0.19; 3.11]	0.345 [0.06; 0.79]	0.18 [0.02; 0.83]	0.28 [0.00; 0.79]	0.23 [0.02; 1.40]	0.253	0.487

GOS: galacto-oligosaccharides. Data are expressed as median [IQR; *i.e.* Q1; Q3]. Sample sizes vary due to drop-outs and technical reasons. Lactate and formate concentrations were under the detection limit. Elderly vs. adults at baseline of the first intervention period were compared by Mann-Whitney U tests. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and corrected for baseline values. P-values were corrected for multiple testing by false-discovery-rate (FDR) according to Benjamini-Hochberg. * Significant before correction for multiple testing (P = 0.034).

3.4. Volatile organic compounds

The VOC-based statistical analysis revealed no major differences between elderly and adults at baseline with an AUROC of 0.62 in the validation set (Figure 2A), indicating relatively small differences in exhaled breath profiles, which is in line with microbiota composition and faecal metabolite data. The visualisation of the data using the complete VOC-profile as shown in Figure 2B showed no clear differences between adults and elderly.

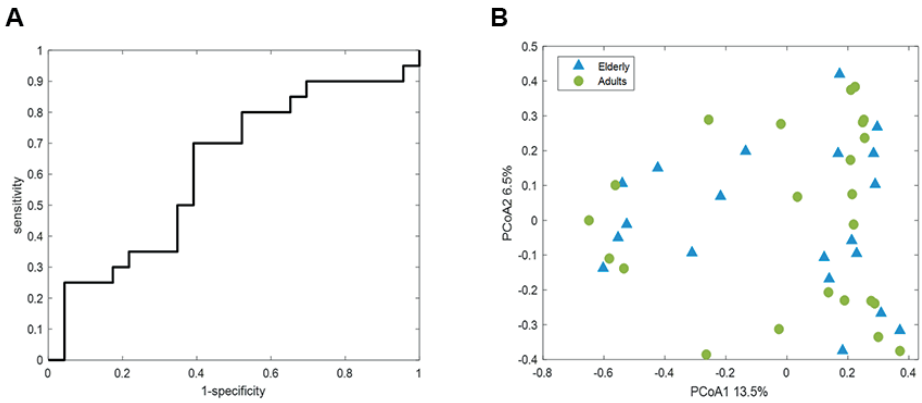

Figure 2. (A) Receiver operating characteristic curve performed on the validation set, with area under the curve = 0.62. (B) unsupervised random forest score plot performed on the VOC profiles obtained from participants at baseline, showing no clear groupings in exhaled breath profiles between 20 elderly (blue triangles) and 23 adults (green dots).

With respect to the effect of treatment on the VOC profiles of exhaled breath, ASCA analysis did not show any significant differences between baseline and treatment for either of the two age groups. The uRF score plots show no clear groupings between samples taken before and after the GOS and placebo four weeks intervention, indicating similarity in breath profiles (Figure 3A-D).

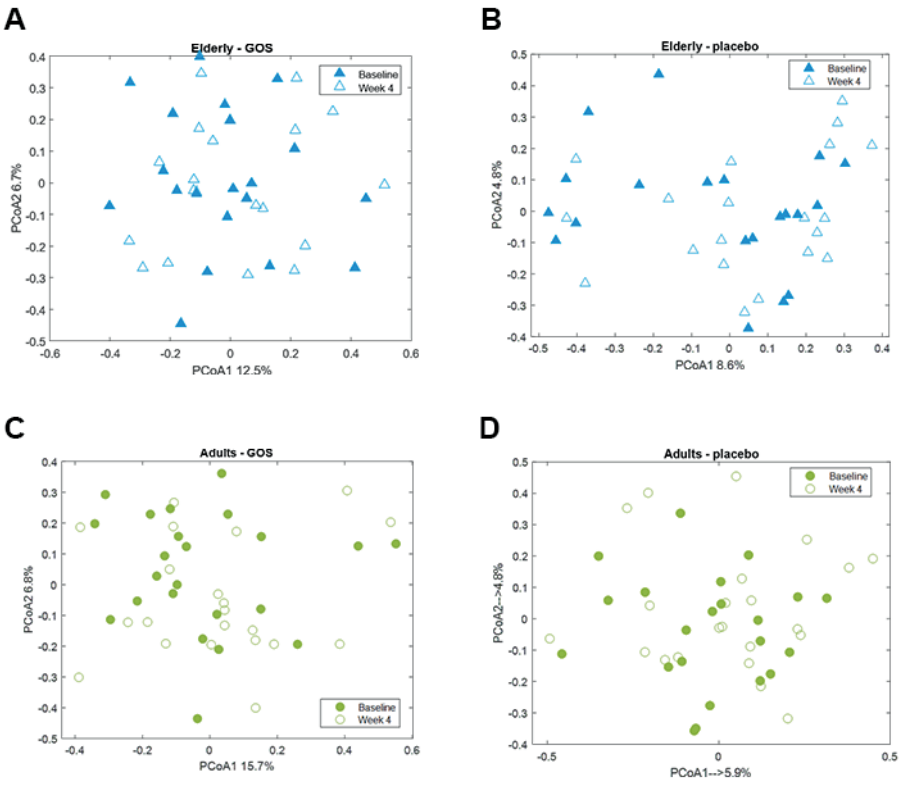

Figure 3. Unsupervised random forest score plots based on the complete VOC profiles (A) found in the exhaled breath of 20 elderly at baseline and 4 weeks post-GOS intervention; (B) found in the exhaled breath of 20 elderly at baseline and post-placebo intervention; (C) found in the exhaled breath of 23 adults at baseline and 4 weeks post-GOS intervention; (D) found in the exhaled breath of 23 adults at baseline and 4 weeks post-placebo intervention. Sample sizes vary due to drop-outs and technical reasons.

3.5. Immune parameters

Cytokine production (IL-1β, IL-6, IL-8, IL-10, TNFα and IFN-γ) in plasma after 24 hours PHA or LPS whole blood stimulations and serum CRP concentrations were not significantly different between elderly and adults at baseline (Figure 4, all $P \geq 0.803$). Moreover, these parameters did not differ significantly between four weeks GOS and placebo supplementation neither in elderly nor in adults (Figure 4, all $P \geq 0.964$). Overall, these results suggest that GOS did not significantly impact parameters of immune response in elderly or adults.

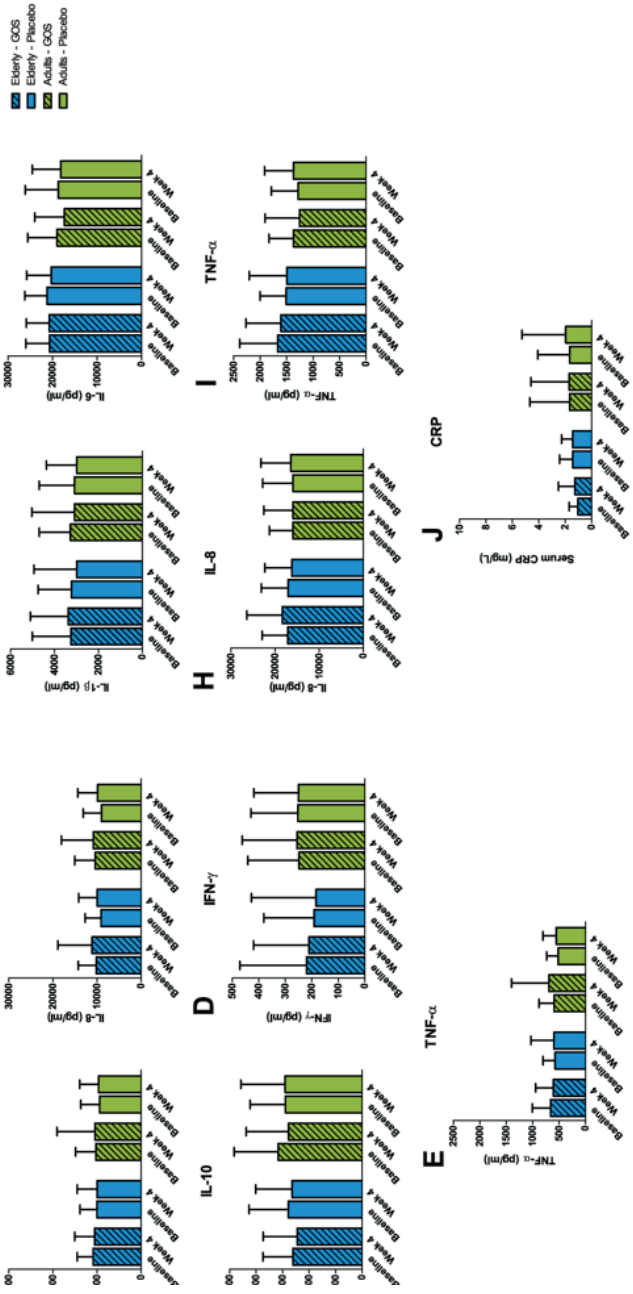

Figure 4. Whole blood cytokine production after 24 hours 10 µg/ml PHA (A-E) or 10 µg/ml LPS (F-I) stimulation and serum CRP concentrations (J), at baseline and after 4 weeks of GOS (striped) and placebo (unstriped) intervention in 20 elderly (blue bars) and 24 adults (green bars). Values are presented in bars with mean and SD. Sample sizes vary slightly due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention period were compared by an independent samples t-test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values. P-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. CRP, C-reactive protein; LPS, lipopolysaccharide; PHA, phytohemagglutinin-M

3.6. Parameters of systemic oxidative stress

Plasma MDA and UA concentrations as well as TEAC values did not differ significantly between elderly and adults at baseline (Figure 5A-C) (all $P \geq 0.128$). However, baseline TEAC values corrected for UA plasma concentrations (Figure 5D) were significantly higher in elderly compared with adults ($P < 0.001$). None of the markers significantly differed between subjects after four weeks GOS or placebo supplementation, neither in elderly, nor in adults (Figure 5A-D, all $P \geq 0.236$). These observations indicate that GOS did not significantly alter parameters of systemic oxidative stress in elderly or in adults.

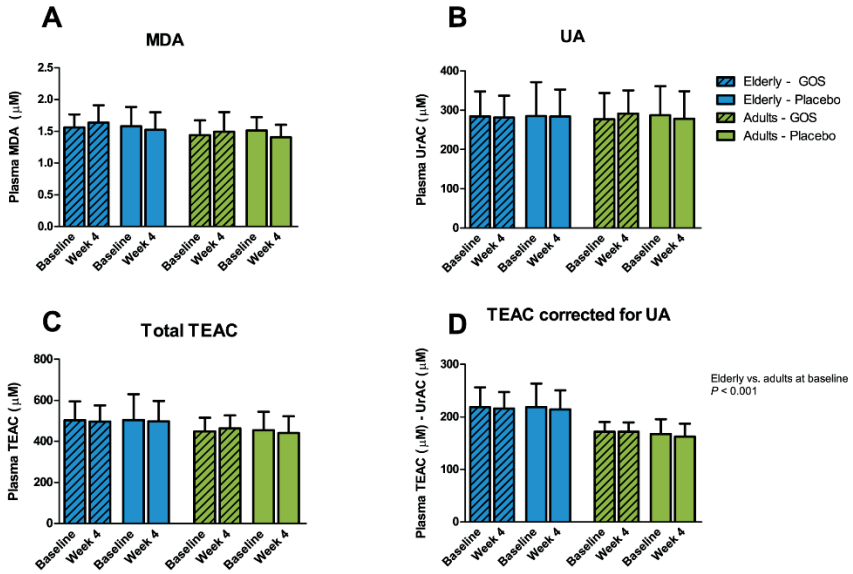

Figure 5. Plasma concentrations of MDA (A) and UA (B), total TEAC values (C), and TEAC values corrected for UA plasma concentrations (D), at baseline and after 4 weeks GOS (striped) or placebo (unstriped) intervention in 20 elderly (blue bars) and 24 adults (green bars). Values are presented in bars with mean and SD. Sample sizes vary due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention period were compared by an independent samples t-test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values. P-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. MDA, malondialdehyde; TEAC, trolox equivalent antioxidant capacity; UA, uric acid.

4. Discussion

We have shown that faecal microbiota composition differed significantly between age groups, with significantly lower relative abundance of *Bifidobacterium* in the elderly when compared with adults. Faecal and breath metabolites, as well as parameters of immune function and systemic oxidative stress did not show significant differences between elderly and adults. After four weeks of GOS supplementation, microbiota composition of both age groups changed significantly, accompanied by significant increase in *Bifidobacterium*, significant decrease in microbial diversity (in adults and in elderly) and microbial richness (in elderly). In the elderly and adults, faecal and breath metabolites, and parameters of immune function and systemic oxidative stress were not significantly different when comparing subjects that either received GOS or placebo intervention.

Microbiota perturbations in elderly have been shown to be more pronounced in elderly with impaired health status (*e.g.* frailty) [5]. In the current study, in addition to significantly lower levels of bifidobacteria in prefrail elderly versus healthy adults, the overall microbiota composition differed significantly, with around 4% of the total microbiota variation explained by subject age group. Consistently, Claesson *et al.* [37] found that elderly subjects (aged 64-102 yrs) did cluster based on residence location, with microbial diversity being lowest in people staying long-term in residential care, being indicative for increased comorbidity and frailty. In the current study, we included community dwelling elderly without major comorbidities and applied the widely used Fried criteria [13] to identify physically 'prefrail' elderly. Others using the Rockwood Frailty index (including a broader range of deficits) or the Barthel Index (assessing performance in activities of daily living), did find a negative association between microbial richness and frailty level [38, 39]. In the current study, although no negative correlation between frailty score and microbial diversity has been observed, the significantly lower faecal bifidobacterial abundance in prefrail elderly compared with healthy adults is in line with previous studies [40-42]. In addition, as found previously [12, 43-45], in the current study, four weeks GOS supplementation (15 g/day) resulted in a significant increase in faecal bifidobacteria abundance and differences in overall faecal microbiota composition of adults and elderly. Despite differences in bifidobacterial abundance, faecal metabolites did not show significant differences neither between elderly and adults, nor between subjects consuming either GOS or placebo supplements. It should be noted that these findings should be interpreted with care, because SCFAs are readily absorded and faecal metabolites do not reflect metabolite production of the proximal colon (*i.e.* assumed site of GOS fermentation).

Previous studies showed VOCs to be linked to both the microbial activity [46, 47] as well as host pathophysiological processes such as inflammation [48, 49]. In the current study, however, the analyses of exhaled breath revealed no significant differences in VOCs between elderly and adults. This is in line with a previous study of our group [14].

In addition, no significant changes in VOC profiles were found after four weeks of GOS supplementation both in elderly as well as in young adults. It has been suggested that diet may impact the generation of compounds found in exhaled breath [50-52]. GOS is a dietary fibre, of which we assumed that due to changes of the intestinal microbiota activity, VOC

profiles could potentially be altered. However, no significant effects of GOS on faecal metabolites or VOCs in exhaled breath were found. A recent study by Drabińska *et al.* [53] demonstrated moderate changes in urine VOC patterns after 12 weeks supplementation of oligofructose-enriched inulin (*i.e.* Synergy 1). It should be noted, however, that that study was performed in children suffering from celiac disease.

As several studies have observed that immunosenescence is common in elderly, and the intestinal microbiome plays a role in intestinal immune functioning, we have evaluated cytokine responses after ex vivo PHA and LPS stimulation of whole blood for 24 hours. We found that LPS-stimulated whole blood IL-1β, IL-6, IL-8 and TNF-α concentrations were not significantly different between elderly and adults. Our findings contrast with those of Bruunsgaard *et al*. [54] who found that LPS-stimulated IL-1β and TNF-α, but not IL-6 levels, were lower in a mixed group of healthy and comorbid elderly (80-81 yrs, n=168) compared with healthy young adults (19-31 yrs, n=91). Our results that PHA-stimulated IL-6, IL-8, IL-10, IFN-γ and TNF-α concentrations did also not differ significantly between elderly and adults, are in line with previous findings on IL-6 production after PHA stimulation in isolated peripheral blood mononuclear cells (PBMC) [55, 56]. We also evaluated serum CRP as frailty has been associated with inflammation [57]. CRP levels were not significantly different between elderly and adults in our study. Taken together, our findings point towards a relatively healthy elderly population with a preserved immune response, despite being defined as physically prefrail by the Fried criteria [13].

Four weeks of GOS supplementation (*i.e.* 21.6 g/day of BiotisTM GOS Powder) did not significantly impact cytokine production by LPS or PHA whole blood stimulations, neither in elderly nor in adults. Interestingly, in the study of Vulevic *et al*. [12] 5.5 g/day of GOS intake for five weeks in healthy elderly (64-79 yrs) resulted in significantly decreased LPS-stimulated production of IL-6 and TNF-α in PBMC, pointing towards anti-inflammatory properties of this relatively low dose of GOS. On the other hand, IL-1β and IL-8 production were not affected in that study [12]. Data on habitual dietary intake and other lifestyle factors were not reported, and can therefore not be compared. Further, apart from some small methodological differences, a clear biological explanation for the contrasting findings cannot be given. We speculate that these differences might be cohort-specific based on a recent study that showed that differences between cohorts with similar selection criteria can already have a huge impact on observations [58].

As immune function is associated with oxidative stress [8], and elevated oxidative stress levels and lower antioxidant capacity have been reported in elderly [59-63], we also determined the concentrations of the lipid peroxidation marker MDA and the antioxidant capacity of plasma (TEAC) in elderly and adults. Both were not significantly different between elderly and adults.

In addition, we showed that four weeks GOS intervention did not significantly alter markers of oxidative stress and antioxidant capacity in either of the age groups. Interestingly, when we corrected TEAC values (*i.e.* total antioxidant capacity) for the most abundant antioxidant in plasma, UA, the antioxidant capacity was found to be even higher in elderly compared with adults. However, dietary intake (*i.e.* polyphenol intake) did not differ significantly between age groups.

Our data indicate that the included prefrail elderly turned out to be relatively healthy, with no significant baseline differences between elderly and adults, and lack of a GOS effect on faecal metabolites as well as parameters of immune function and systemic oxidative stress. The Fried frailty criteria refer to physical (pre)frailty, and do not necessarily select for subjects with an altered intestinal microbiota or immune-related deficits. Further, we have shown that GI symptom scores and stool frequency (including frequencies of hard stools and loose stools) were not significant different between GOS and placebo intervention neither in elderly, nor in adults. Therefore, the addition of a relatively high dose of GOS (15.0 g/day) was well-tolerated, and these findings are in line with other dietary fibre intervention studies in comparable populations [64, 65]. Moreover, compliance as determined by returned empty sachets was high (95.0% in adults and 97.6% in elderly), although we acknowledge this is a subjective measure.

5. Conclusion

In conclusion, we showed that bifidobacteria were lower in prefrail elderly compared with adults, but faecal and breath metabolites, as well as parameters of immune function and oxidative stress were not significantly different. It remains to be determined what the potential consequences of low bifidobacterial counts in *e.g.* prefrail elderly are for the longer term. By performing longitudinal studies, it can be investigated whether low bifidobacterial abundance will be a risk factor for impaired health functions, such as frailty. Further, in this well-controlled study, four weeks GOS supplementation increased bifidobacteria and decreased microbial diversity, but did not affect faecal and breath metabolite concentrations, immune function and oxidative stress, based on the parameters measured. Future intervention studies aiming to improve immune-related health status should select more vulnerable subgroups of (frail) elderly, preferably by using biomarkers and/or based on the outcome parameters.

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Author contributions: Ellen Wilms: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original draft, Visualization, Project administration. Ran An: Conceptualization, Methodology, Formal analysis, Data curation, Writing – Original draft, Visualization. Agnieszka Smolinska: Formal analysis, Resources, Data curation, Writing – Original draft, Visualization. Yala Stevens: Investigation, Writing – Review & Editing. Antje R. Weseler: Resources, Writing – Review & Editing. Montserrat Elizalde: Formal analysis: Marie-José Drittij: Formal analysis. Athanasia Ioannou: Formal analysis, Writing – Review & Editing. Frederik J. van Schooten: Resources, Writing – Review & Editing. Hauke Smidt: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision: Ad A.M. Masclee: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision. Erwin G. Zoetendal: Conceptualization, Methodology, Resources, Writing – Orginal draft, Supervision, Funding acquisition. Daisy M.A.E. Jonkers: Conceptualization, Methodology, Resources, Writing – Original draft, Supervision, Funding acquisition.

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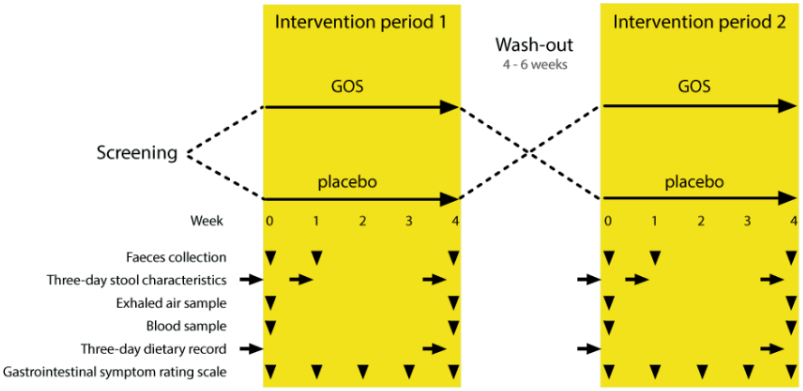
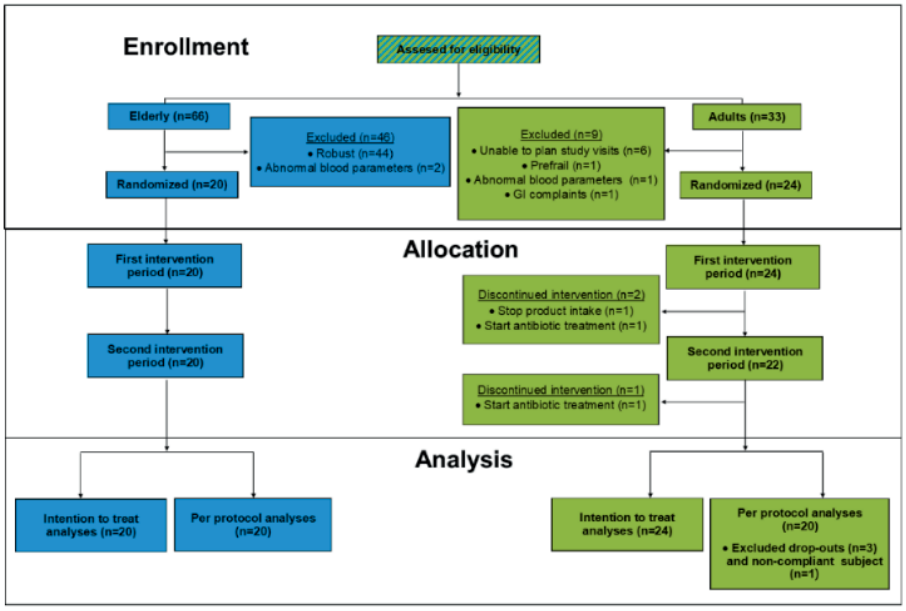
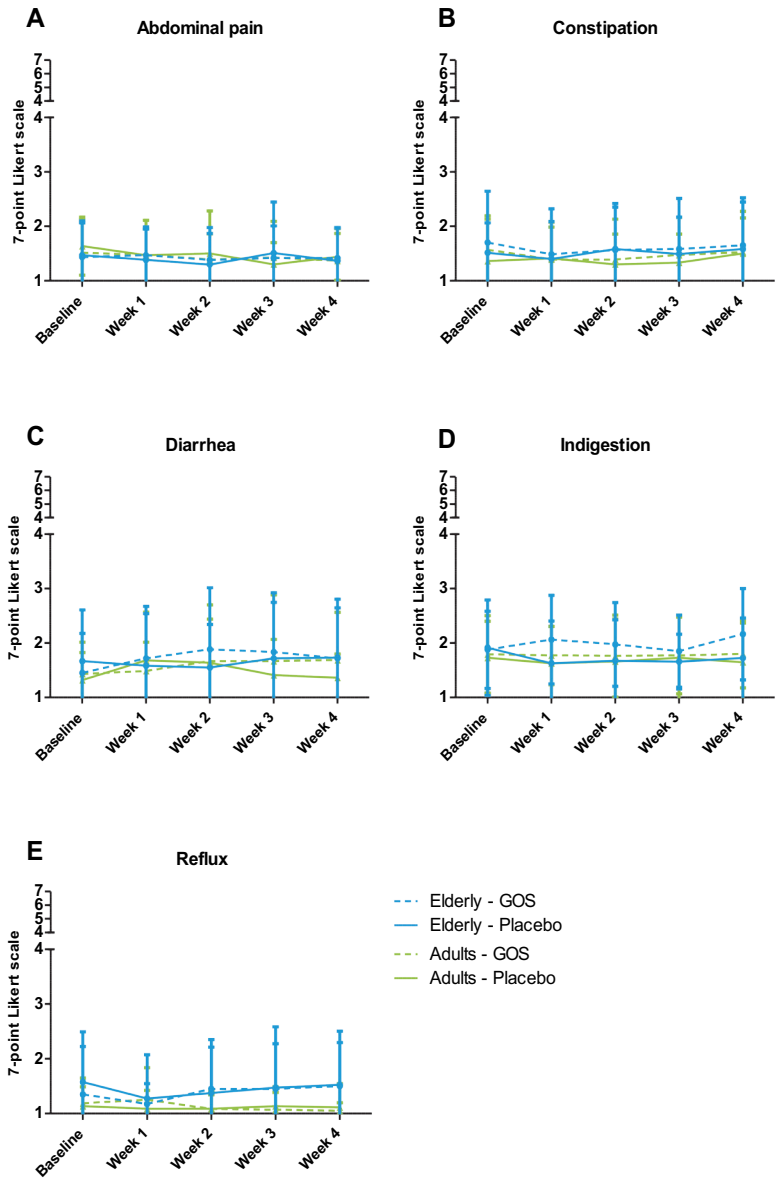
Supplementary Material:



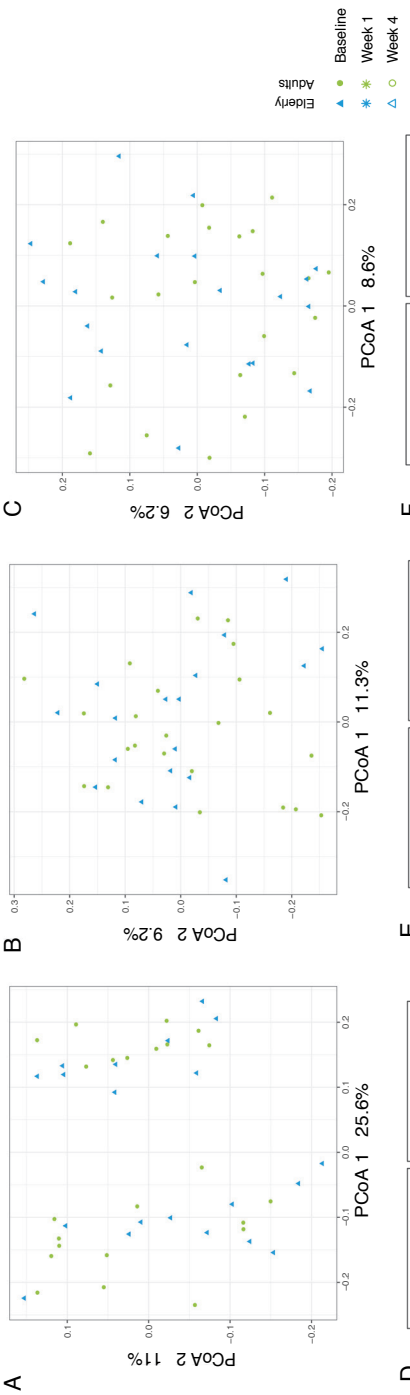
Supplementary Figure 1. Study timeline. Screening, GOS and placebo supplementation, faeces collection, three-day dietary records and stool characteristics, blood and exhaled air sampling, gastrointestinal symptom rating scales were completed on the weeks as indicated by arrows. The wash-out period was four to six weeks. Intake of GOS and placebo continued until all measurements of the specific intervention period were finished.



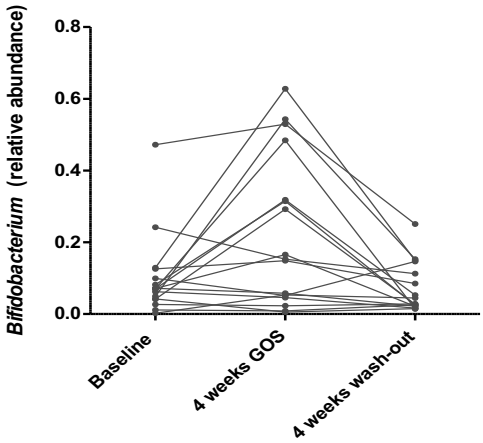
Supplementary Figure 2. Flow diagram of the study



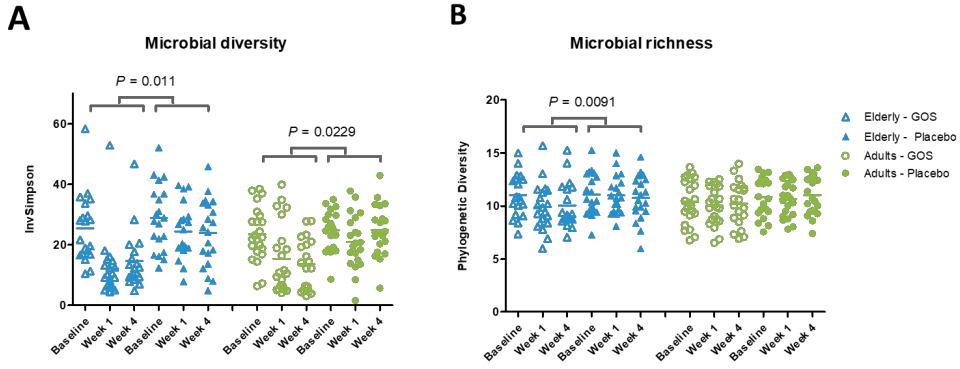
Supplementary Figure 3. Gastrointestinal symptoms at baseline and every week of GOS (dashed lines) and placebo (fixed lines) intervention periods in elderly (blue) and adults (green grey). A: Abdominal pain scores. B: Constipation scores. C: Diarrhea scores. D: Indigestion scores. E: Reflux scores. Means and standard deviations are shown. Missing values at specific weeks were due to drop-outs. Gastrointestinal symptom scores were compared between intervention groups with variance components (random intercept) linear mixed models and correction for baseline values. P-values per time point were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg.



Supplementary Figure 4. Faecal microbiota at baseline (A, B, C), and comparing baseline microbiota to that after 1 week and 4 weeks GOS and placebo intervention (D, E, F) in 20 elderly and 23 adults based on 16S rRNA gene sequences. PCoA plots are based on unweighted UniFrac (A,D), Bray-Curtis (B,E) and Jaccard (C,F) distance matrices. PERMANOVA was used to compare the microbiota of elderly and adults in the PCoA plots at baseline, as well as for intervention effects between time points per group of subjects (e.g. elderly baseline GOS vs elderly Week 1 GOS).



Supplementary Figure 5. *Bifidobacterium* based on sequencing of the 16S rRNA gene in the group of subjects (n=17) receiving GOS in the first intervention period, dropped back to baseline after four weeks of wash-out period, indicating no carry-over effects after GOS supplementation.



Supplementary Figure 6. Faecal microbial diversity (A) and richness (B) in 20 elderly and 23 adults. Values are presented in scatter plots with median line. Sample sizes vary due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention period were compared by a Mann-Whitney U test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values.

Supplementary Table 1. Dietay intake at baseline and after 4 weeks GOS and placebo intervention periods of the elderly (n=20) and adults (n=24)

	Elderly (n=20)					Adults (n=24)					*P*-value
	GOS		Placebo		*P*-value	GOS		Placebo		*P-value*	elderly vs adults
	Baseline	Week 4	Baseline	Week 4		Baseline	Week 4	Baseline	Week 4		at baseline
Energy (kcal)	1905 ± 518	1836 ± 543	2070 ± 498	1913 ± 519	0.859	2099 ± 514	1978 ± 762	2065 ± 594	1920 ± 484	0.906	0.808
Carbohydrate (g)	208 ± 51	201 ± 57	207 ± 44	203 ± 42	0.859	236 ± 69	212 ± 70	223 ± 53	221 ± 64	0.825	0.808
Carbohydrate, sugars (g)	89.2 ± 29.6	83.7 ± 31.2	89.3 ± 28.3	90.4 ± 28.5	0.859	100.1 ± 37.8	91.7 ± 38.9	91.6 ± 32.1	92.2 ± 37.9	0.825	0.808
Fibre (without GOS) (g)	21.4 ± 6.6	20.9 ± 8.4	22.4 ± 7.4	21.0 ± 6.6	0.905	22.5 ± 7.2	19.5 ± 6.6	20.4 ± 7.2	19.4 ± 7.2	0.825	0.948
Fat (g)	75.3 ± 29.9	71.9 ± 28.7	88.2 ± 29.4	77.2 ± 35.4	0.859	84.2 ± 26.8	85.0 ± 45.9	85.8 ± 36.9	75.8 ± 25.5	0.825	0.808
Fat, saturated (g)	28.8 ± 11.9	29.0 ± 14.1	34.0 ± 15.1	29.8 ± 14.5	0.825	32.5 ± 9.4	31.4 ± 14.1	33.4 ± 16.0	28.7 ± 10.3	0.825	0.808
Protein (g)	70.7 ± 17.5	69.9 ± 21.6	82.6 ± 26.5	72.0 ± 21.9	0.825	76.6 ± 17.6	73.4 ± 33.1	77.9 ± 25.5	66.4 ± 14.3	0.825	0.808
Calcium (mg)	806 ± 297	782 ± 438	848 ± 435	754 ± 372	0.859	839 ± 333	992 ± 1243	875 ± 331	719 ± 236	0.825	0.808
Iodine (µg)	176 ± 61	161 ± 63	181 ± 69	174 ± 63	0.859	155 ± 43	155 ± 105	156 ± 55	146 ± 34	0.825	0.808
Iron (mg)	9.9 ± 2.2	10.3 ± 2.9	11.2 ± 3.3	10.5 ± 2.8	0.825	10.7 ± 3.3	9.6 ± 3.6	10.1 ± 3.2	9.7 ± 2.6	0.825	0.950
Magnesium (mg)	288 ± 66	278 ± 89	326 ± 84	292 ± 64	0.859	305 ± 89	317 ± 257	278 ± 85	268 ± 89	0.825	0.949
Phosphorus (mg)	1245 ± 284	1212 ± 399	1381 ± 454	1252 ± 330	0.859	1267 ± 286	1141 ± 401	1287 ± 358	1130 ± 303	0.825	0.912
Polyphenol (mg)	1798 ± 900	1676 ± 865	1900 ± 900	1832 ± 912	0.859	1366 ± 722	1386 ± 551	1430 ± 889	1179 ± 834	0.825	0.808
Potassium (mg)	2945 ± 723	2793 ± 845	3167 ± 523	2979 ± 651	0.905	3050 ± 1081	2663 ± 885	2789 ± 1036	2612 ± 890	0.825	0.912
Sodium (mg)	2360 ± 916	2389 ± 774	2590 ± 1042	2588 ± 993	0.825	2230 ± 601	2656 ± 1524	2648 ± 888	2273 ± 685	0.783*	0.948
Selenium (µg)	37.7 ± 12.3	38.7 ± 13.1	48.9 ± 17.9	43.0 ± 14.8	0.825	44.8 ± 18.0	51.5 ± 42.1	40.3 ± 13.7	43.8 ± 20.9	0.825	0.950
Vitamin A (µg)	837 ± 516	807 ± 506	724 ± 500	874 ± 616	0.825	886 ± 1003	811 ± 1090	737 ± 472	590 ± 347	0.825	0.950
Vitamin B1 (µg)	1.01 ± 0.40	0.86 ± 0.38	1.08 ± 0.51	0.96 ± 0.45	0.859	0.94 ± 0.28	0.97 ± 0.41	0.93 ± 0.38	0.82 ± 0.34	0.825	0.808
Vitamin B2 (mg)	1.44 ± 0.89	1.38 ± 0.91	1.48 ± 0.93	1.38 ± 0.89	0.975	1.19 ± 0.41	1.07 ± 0.39	1.20 ± 0.44	0.97 ± 0.37	0.825	0.808
Vitamin B3 (mg)	14.7 ± 3.9	14.2 ± 4.7	16.7 ± 5.6	15.9 ± 6.4	0.905	15.6 ± 6.2	15.3 ± 8.3	15.4 ± 5.5	13.6 ± 5.6	0.825	0.808
Vitamin B6 (mg)	1.43 ± 0.53	1.26 ± 0.51	1.46 ± 0.54	1.35 ± 0.49	0.859	1.42 ± 0.58	1.23 ± 0.55	1.22 ± 0.53	1.16 ± 0.39	0.825	0.950
Vitamin B11 (µg)	275 ± 116	247 ± 116	262 ± 94	242 ± 104	0.859	254 ± 92	205 ± 85	226 ± 108	195 ± 63	0.825	0.950
Vitamin B12 (µg)	3.55 ± 1.93	3.55 ± 2.15	4.14 ± 2.89	3.87 ± 2.33	0.905	6.70 ± 16.00	3.02 ± 1.41	3.77 ± 1.79	2.58 ± 1.09	0.825	0.808
Vitamin C (mg)	115.0 ± 76.5	90.5 ± 57.8	94.2 ± 38.8	89.2 ± 53.3	0.825	194 ± 275	187 ± 300	174 ± 305	163 ± 295	0.980	0.808
Vitamin D (µg)	4.50 ± 1.93	3.99 ± 3.47	4.43 ± 3.79	4.71 ± 3.54	0.825	2.92 ± 2.66	2.69 ± 2.54	2.60 ± 3.44	2.37 ± 2.93	0.825	0.808
Vitamin E (mg)	10.2 ± 5.2	10.6 ± 4.4	12.3 ± 5.2	11.8 ± 5.8	0.860	10.9 ± 5.1	10.2 ± 5.0	9.7 ± 4.1	9.7 ± 4.0	0.825	0.808
Zinc (mg)	8.8 ± 2.8	8.7 ± 3.2	10.3 ± 3.1	8.7 ± 3.0	0.825	9.2 ± 2.3	8.6 ± 3.0	9.7 ± 3.3	8.1 ± 1.8	0.825	0.950

GOS: galacto-oligosaccharides. Values are presented in mean and SD. Sample sizes vary slightly due to drop-outs. Elderly vs. adults at baseline of the first intervention period were compared by an independent samples t-test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values. P-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. * Significant before correction for multiple testing (*P* = 0.029).

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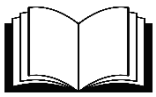
5

Tracing the effects of ingested probiotics in combination with short chain fructo-oligosaccharides through longitudinal sampling from different regions of the small intestine



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Abstract

The microbial communities inhabiting the different sections of the human intestine are associated with our health and respond to dietary changes. Nevertheless, most studies investigating intestinal microbiota employ faecal samples, whereas studies assessing small intestinal microbiota are scarce. Here we compared microbial communities residing in duodenum, jejunum, proximal ileum and faeces. Additionally, we studied the response of these communities to the multispecies probiotic Ecologic® 825 in combination with short-chain fructo-oligosaccharides (scFOS) using a randomized double-blind placebo-controlled parallel design. Twenty healthy volunteers (18-65 yrs) ingested either the synbiotic (daily 1.5 * 10^{10} CFU Ecologic® 825 + 10 g FOS P6) or the placebo twice daily for 14 days. Before (*i.e.* empty stomach) and after (*i.e.* empty stomach, *ca.* 2 h after final product ingestion and *ca.* 3 h after lunch) the supplementation period, luminal samples were collected from duodenum, jejunum and proximal ileum using a multi-lumen catheter in addition to faeces for the analysis of microbiota composition, targeting the variable region V4 of the 16S rRNA gene and followed by HiSeq Illumina sequencing. At baseline, we observed significant differences between the microbiota of duodenum, jejunum, proximal ileum and faeces, with nine out of 189 observed genus-level taxa having higher relative abundance in luminal duodenal microbiota compared to that of proximal ileum (all $P < 0.05$). Of note, the microbial diversity gradually decreased from duodenum to jejunum and subsequently proximal ileum, and was highest in faeces. Two weeks synbiotic supplementation did not significantly change overall microbiota composition of duodenum, jejunum, proximal ileum or faeces, although the duodenal microbial diversity significantly decreased *(P*= 0.0498). In addition to the strong individuality, the duodenal, jejunal and ileal microbiota demonstrated considerable dynamics within a day. Ingestion of the synbiotic led to a short-term spike in the relative abundance of corresponding genera in the small intestine within approximately two hours after ingestion, after which relative abundances quickly returned to baseline levels. Collectively, the current study is the first study investigating the spatio-temporal kinetics in small intestinal microbiota in healthy subjects. Findings of the current study reinforce the instability of small intestinal microbiota, challenge the distribution of microbial diversity along the small intestine and represent the first effort to study implications of synbiotic supplementation on small intestinal microbiota.

Keywords: synbiotic, microbiota, duodenum, jejunum, ileum, probiotic, prebiotic.

1. Introduction

The human intestinal tract plays an important role in food digestion, nutrient absorption, and fermentation of unabsorbed components. Along its entire length the intestinal tract is inhabited by a large number of microbes, collectively called intestinal microbiota, which plays an important role in human health, including (but not limit to) immune modulation and metabolite production (*e.g.* short chain fatty acids and vitamins) [1]. In most scientific studies in humans, faecal material is used to study the intestinal microbiota due to ease of access to faecal samples in large pre-clinical cohort studies. Although the faecal microbiota composition is considered to preserve relevant and representative information for distal colonic content, it is evident that it does not represent microbial communities inhabiting the small intestine [2].

The small intestine can be divided into upper, mid and lower sections, namely duodenum, jejunum and ileum. The small intestinal environment is characterized by short transit time and rapid luminal flux, as well as high concentrations of digestive enzymes, antimicrobial peptides and bile acids, which together act as selective forces for its inhabiting microbes [3, 4]. For instance, the bile acids secreted from the bile duct, near the duodenum, are known to broadly inhibit the growth of intestinal microorganisms, especially those living in the small intestine [4, 5]. Together with the duodenum, the jejunum also plays an important role in nutrient assimilation and absorption [6]. Towards the ileum, the transit rate and the concentration of oxygen decrease, whereas the number of microbes, especially anaerobes, increases [7]. Thereafter, decreased concentrations of simple sugars, oxygen and antimicrobial compounds, and a further decreased transit rate facilitate the growth of complex polysaccharide-utilizing anaerobes in the colon [2]. Nevertheless, available studies investigating the regional differences in small intestinal microbiota are often based on only one or a few subjects, or comparing samples from different locations that are derived from different subjects, which can be attributed to the difficulties in sample collection [8-11]. As an alternative, collection of ileostoma effluent from ileostomists, *i.e.* subjects without a colon, enables repeated sampling in a non-invasive way [12], however, ileostoma effluent cannot be used to study the spatial differences in small intestinal microbial composition and functionality.

The small intestinal microbiota interacts with its host through many ways, including immune modulation, production of antimicrobial compounds and metabolites (*e.g.* short-chain fatty acids (SCFAs) and vitamins) [1, 13]. Moreover, the expression of SCFAs receptors, *i.e.* G-coupled protein receptors (GPR41, GPR43 and GPR109a), was found higher in human ileum than distal colon [14]. Therefore, investigating and modulating composition and functionality of the small intestinal microbiota can have important implications for host health. In addition to faecal microbiota transplantation, dietary interventions, including supplementation with prebiotics, probiotics and synbiotics (combination of pre-and probiotics) are considered as a promising tool to modulate the intestinal microbiota. Notably, van Baarlen *et al*. demonstrated remarkable differences in NF-κB pathways' modulation in duodenum after ingestion of a *Lactobacillus plantarum* strain harvested at different growth phases [15]. Later this research group also revealed changes in duodenal

mucosal gene-regulatory networks in the human small intestine after ingesting different lactic acid bacteria [16]. Furthermore, small intestinal microbiota was suggested to be more amenable to dietary changes [13]. Considering remarkably lower numbers of microbes residing in the small intestine (*ca.* 10^4-10^8 CFU/ml intestinal fluid) compared to those inhabiting the colon, one dosage of probiotics (*ca.* 10^{10} CFU/day) with 10% survival rate, would result in 10^9 live cells that would be expected to confer greater impact on the microbiota of the small intestine than on colonic microbiota [17], although microbes not necessarily need to be alive to confer a heath effect [18]. However, studies investigating the *in vivo* response of small intestinal microbiota to dietary changes, especially synbiotics or probiotics, are still lacking.

In the current study, we aimed to compare the microbial communities inhabiting different parts of the small intestine (*i.e.* duodenum, jejunum and proximal ileum) and faeces in healthy individuals, as well as the response of these microbial communities to 14 days of synbiotic supplementation (*i.e.* the multispecies probiotic Ecologic® 825 in combination with short-chain fructo-oligosaccharides (FOS P6)). This synbiotic product was selected since the probiotic mixture conveyed positive effects *in vivo*, ex vivo and in animal studies on intestinal barrier function [19, 20], and FOS P6 has been shown to stimulate growth and activity of the probiotic strains included in Ecologic® 825 [21]. We hypothesised that microbiota differs between intestinal locations and that these location-specific microbial communities respond differently to the synbiotic supplementation.

2. Materials and Methods

Details of the study design were reported earlier [21]. Briefly, this study was approved by the Medical Ethics Committee of Maastricht University Medical Centre+ and performed following the Declaration of Helsinki (latest amendment by the World Medical Association in 2013) and Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study has been registered at the US National Library of Medicine (http://www.clinicaltrials.gov) under registration number NCT02018900.

2.1. Study overview

This study followed a double-blind, randomized, controlled, parallel design. Twenty healthy adults (aged 18-65yrs, BMI 20-30 kg/ m2) completed the study. None of the participants took any medication 14 days prior to the study, nor antibiotics 90 days prior to the study. Detailed description of ex/inclusion criteria can be found elsewhere [21].

Recruited subjects were randomly assigned into either the synbiotic group or the control group. Ecologic®825, FOS P6 and maltodextrin were provided by Winclove Probiotic BV (Amsterdam, the Netherlands). Subjects in the synbiotic group took 6 g/day of the probiotic mixture Ecologic®825 (1.5 $*10^{10}$ CFU/day) combined with 10 g/day FOS P6 (degree of polymerization (DP) 3-5) in two equal dosages each provided in a duo-sachet containing 3 g of the probiotic mixture and 5 g scFOS. Ecologic®825 was composed of nine probiotic strains, namely *Lactococcus lactis* (W19), *Lactobacillus paracasei* (W20), *Lactobacillus acidophilus* (W22), *Lactobacillus salivarius* (W24), *Lactobacillus casei* (W56), *Lactobacillus plantarum* (W62), *Bifidobacterium bifidum* (W23), *Bifidobacterium lactis* (W51) and *Bifidobacterium lactis* (W52). The control group received 10 g/day maltodextrin and 6 g/day of the probiotic carrier material in two equal dosages. The carrier material was composed of maize starch, maltodextrin, mineral mix, inulin and FOS (P6; inulin and FOS comprised a maximum of 15% of the total carrier material, *i.e.* less than 0.9 g/day). The synbiotic and control mixtures had an identical appearance. Subjects ingested the supplements after dissolving the contents of the duo sachets in 200 ml water every morning and evening at the same time for two weeks. The intervention lasted for 14 days.

2.2. Sample collection and storage

Before and after the intervention period, luminal content was sampled using a multi-lumen customized sampling catheter (Mui Scientific, Mississauga, Ontario, Canada). Specifically, it was composed of nine smaller lumina for simultaneous sampling of luminal content from different sites of the small intestine. Six days prior to the starting of the intervention (day -6) the luminal content of the duodenum, jejunum and proximal ileum was sampled in the morning, after an overnight fast. At day 14, on the last day of the intervention, the luminal content was sampled from the same locations at three time points within that day, *i.e.* in the morning after overnight fast, around noon after taking the supplement before lunch (*i.e.* approximately 2 h after consumption of the supplements), and around 16:00 h, *i.e.* three hours after lunch. Immediately after collection, all small intestinal samples were snap-frozen in liquid nitrogen and subsequently stored at -80 °C until analysis. Similarly, before

and after the intervention (*i.e.* the same day as luminal content collection) faecal material was immediately frozen after defecation and stored at -80 °C until analysis.

2.3. Microbiota composition analysis

Microbiota composition was determined with barcoded 16S ribosomal RNA (rRNA) gene amplicon sequencing (Illumina Hiseq2500 (2 × 150 bp)). The intestinal content (≤ 1 ml) was immediately transferred to a screw cap tube containing 0.25 g of 0.1 mm zirconia beads and 3 glass beads (diameter 2.5 mm) to which 300 µl Stool Transport and Recovery (STAR) buffer (Roche Diagnostics, United States) was added. The mix was subjected to repeated bead beating (5.5 ms 3 × 60 s), followed by 15 min heating at 95 °C at 1000 rpm. The lysate was then centrifuged for 5 min at 4 °C (14000 g). The supernatant was collected and stored in a separate tube. The pellet was subjected to another cycle of cell disruption with 200 µl of STAR buffer. Supernatants from both cycles were pooled. Two times 250 µl of the supernatant was purified using a Maxwell extraction instrument (Promega, United States) with a Maxwell® 16Tissue LEV Total RNA purification Kit Cartridge customized for DNA extraction (XAS1220) and eluted in 40 µl of nuclease free water. For faecal material, 0.25 g faeces were transferred to a screw cap tube containing 0.5 g of 0.1 mm zirconia beads and 5 glass beads (diameter 2.5 mm) to which 700 µl STAR buffer was added. Subsequent procedures were the same as described above for small intestinal samples, except for the second-round of cell disruption that used 300 µl STAR buffer and only one time 250 µl supernatant being used for purification. Obtained total DNA was then diluted to 20 ng/µl before amplification.

The V4 region of the 16S rRNA gene was amplified in triplicate using primers 515F [22] and 806R [23] and extracted DNA as template. The amplification program was as described previously [24], but with an annealing temperature of 50 °C. Purified PCR products were mixed in equimolar amounts and sent for sequencing (Eurofins Genomics, Konstanz, Germany). Throughout the process, mock communities (*i.e.* synthesised microbial community), biological replicates of random samples were included for quality control. Raw sequencing reads were processed using the NG-Tax 1.0 pipeline using default settings [25]. Taxonomy assignment was based on the SILVA database (version 128) [26, 27].

2.4. Statistical analysis

The 16S rRNA gene sequence read counts were normalised to relative abundance. The microbial diversity indices (Faith's phylogenetic diversity (PD) and Inverse Simpson) were calculated based on amplicon sequence variants (ASVs). Normality of the data was checked by Shapiro-Wilk test. As data was not normally distributed in the current study, the Friedman test and pairwise Wilcoxon signed-rank tests were used for paired measurements. At baseline, the number of samples per sample type, *i.e.* faeces, jejunum, duodenum and proximal ileum, was, however, unequal, due to failure in sample collection and/or DNA isolation. Therefore, the pairwise Mann-Whitney U test was used as an alternative. Significant differences between groups were tested by permutational multivariate analysis of variance (PERMANOVA) based on pairwise weighted and unweighted UniFrac distances at ASV level. Principal coordinate analysis (PCoA) was used to visualize the microbiota variation. Intervention effects on individual genus-level taxa were tested using a linear mixed effect model, with subject as random factor, and time (*i.e.* days after intervention) and treatment (*i.e.* intervention) as fixed factors, while the analysis was performed per sample type. P values were corrected for multiple testing by the false discovery rate (FDR) using the Benjamini-Hochberg procedure. Corrected p values < 0.05 were considered to indicate significance. All statistical analyses were conducted in R (R-3.5.0).

3. Results

3.1. Characterization of subjects

Twenty participants completed the entire study protocol and were randomly assigned into control or synbiotic group, with no significant differences in sex, age and BMI (Table 1).

Table 1 Baseline characteristics of the control (n=10) and synbiotic (n=10) group

	Control (n=10)	Synbiotic (n=10)	*P*-value
Sex (% female)	70.0	20.0	0.070
Age (yrs)	21.7 [20.0, 24.0]	19.7 [19.1, 21.8]	0.082
BMI (kg/m^2)	24.1 [22.9, 24.9]	22.9 [21.7, 24.1]	0.226

Percentage of sex was compared using Fisher exact test between treatment groups. Age and BMI are expressed as median [Q1, Q3] (IQR). Between groups, parameters, *i.e.* age and BMI, were tested with Mann-Whitney U test. BMI: body mass index; yrs: years of age. IQR: interquartile range.

3.2. Microbial composition differed between duodenum, jejunum, proximal ileum and faeces

Spatial differences in microbial composition along the small intestine (*i.e.* duodenum, jejunum and proximal ileum), and in faecal samples taken prior to the intervention were characterised using 16S rRNA gene amplicon sequencing. This revealed a clear difference between the composition of small intestinal and faecal microbiota, in addition to a large inter-individual variation (Fig. 1). At family level, the faecal microbiota was in general predominated by members of Lachnospiraceae and Ruminococcaceae, whereas several subjects also had high relative abundance of Bifidobacteriaceae, and few subjects showed high relative abundance of Prevotellaceae. Concurrently, Steptococcaceae predominated the duodenal and jejunal microbiota, with some subjects having high relative abundance of Prevotellaceae in duodenum, as well as Veillonellaceae, Pasteurellaceae or Enterobacteriaceae in jejunum and/or ileum.

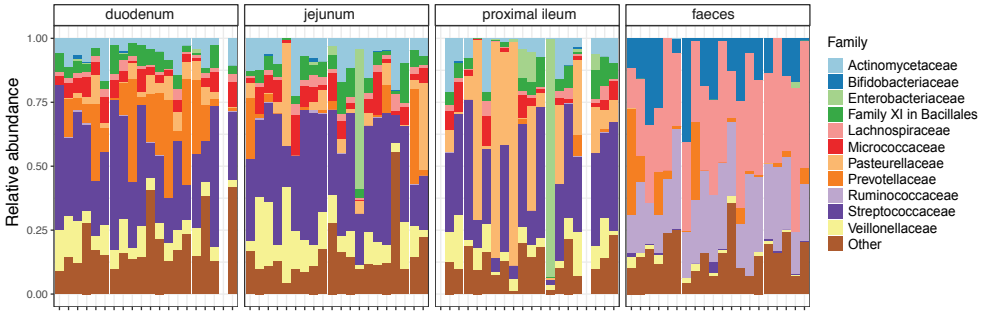

Figure 1. Relative abundance of most predominant bacterial families (top 12, ranked base on the average relative abundance across the entire dataset) in different types of samples derived from 20 healthy subjects. Top 12 microbial families are listed in the legend. Other families are summarized as "Other". Each column represents a given type of sample from one subject. Missing columns are due to technical reasons (*i.e.* failure in sample collection and/or DNA isolation).

The relative abundance of nine out of 189 observed genus-level taxa was significantly (all $P < 0.05$) higher in luminal duodenal microbiota compared to proximal ileum (Fig. 2). The relative abundance of three out of these nine genera, namely *Prevotella* 7, *Porphyromonas* and *Fusobacterium*, was also significantly (all $P < 0.05$) higher in the duodenal microbiota comparing to that of jejunum (Fig. 2). In contrast, no significant (all $P > 0.05$) differences were observed in the relative abundance of individual taxa between jejunum and proximal ileum. Collectively these data indicate a gradual change in microbial composition along the small intestine from upper, mid to lower sections.

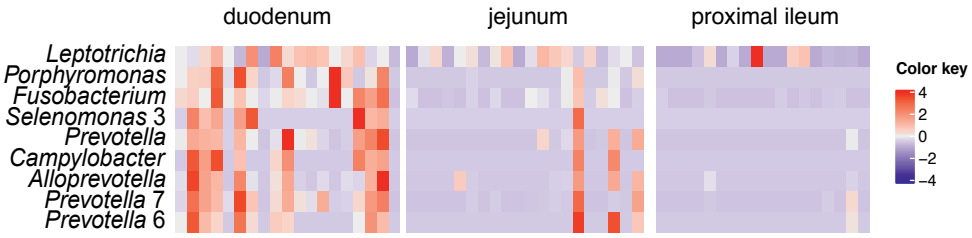

Figure 2. Heatmap of microbial genus-level taxa that significantly differed between samples taken at different locations along the small intestine. Colour in the heatmap reflects the relative abundance normalized per taxon, with blue colour indicating lower than average relative abundance and red colour indicating higher than average relative abundance. Scaling of colours in the heatmap (*i.e.* colour key) was done by subtracting the overall mean of a given genus across the entire dataset from its value for a specific sample (*i.e.* specific location and individual) and divided by the standard deviation of the given genus. Unequal sample size is due to technical reasons (*i.e.* failure in sample collection and/or DNA isolation).

PERMANOVA based on weighted UniFrac (considering relative abundance of ASVs and their position in the phylogenetic tree) and unweighted UniFrac (considering only presence or absence of ASVs and their phylogenetic position) distance matrices, revealed significant (all $P < 0.05$) differences between the microbiota of faeces and that of small intestinal content. Within the small intestine, PCoA based on weighted and unweighted UniFrac distance matrices exhibited significant differences between duodenum and jejunum ($P = 0.0096$ and $P = 0.0070$, respectively), between jejunum and proximal ileum ($P = 0.0160$ and $P = 0.0060$, respectively), as well as between duodenum and proximal ileum (both $P = 0.0015$) (Fig. 3A&B).

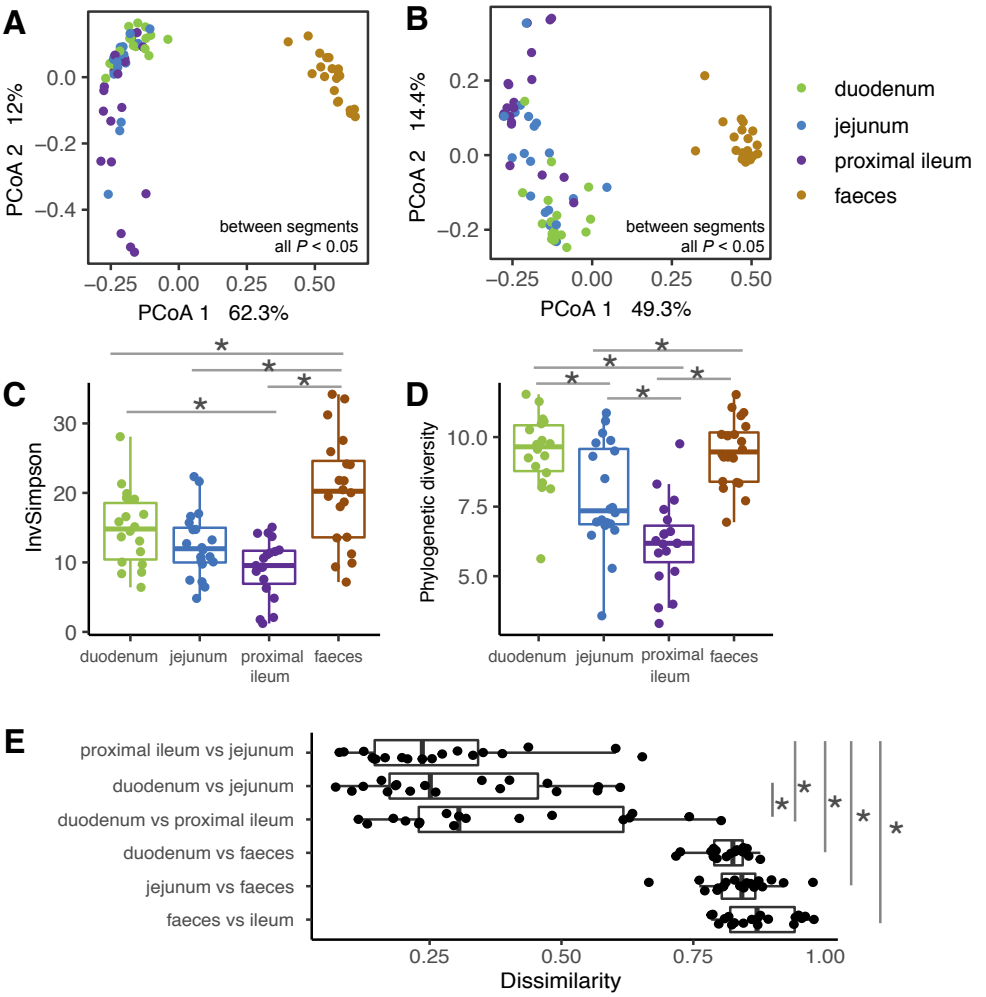

Figure 3. PCoA of microbiota composition based on (A) weighted or (B) unweighted UniFrac distance matrices. Significant differences between samples based on pairwise weighted and unweighted UniFrac distances at ASV level were tested by PERMANOVA. (C) microbial diversity and (D) richness at baseline. (E) Dissimilarity between different sample types within each individual. Microbial diversity and richness as well as pairwise dissimilarity comparisons are presented in box-whisker plots with median line as well as the 1st and 3rd quartiles. Pairwise comparisons between different sample types were evaluated by Wilcoxon signed-rank test. When the sample size was unequal, pairwise Mann-Whitney U test was used as an alternative. Dissimilarity was calculated based on weighted UniFrac distance. Scale ranges from 0 to 1, with lower values indicating higher similarity. Comparison of dissimilarities between sample types was done using a linear mixed effect model with subject as random intercept. Sample sizes vary due to technical reasons (*i.e.* failure in sample collection and/or DNA isolation). PCoA; Principal coordinate analysis. InvSimpson: Inverse Simpson. PERMANOVA: permutational multivariate analysis of variance. * indicates significant differences ($P < 0.05$) between groups.

Concerning microbial alpha-diversity as measured using the Inverse Simpson diversity index (Fig. 3C), the faecal microbial diversity was significantly higher than that of duodenum (P = 0.0391), jejunum (P = 0.0047) and proximal ileum (P = 0.0001). The faecal microbial richness (Faith's PD), however, was only higher than that of jejunum (P = 0.0101) and proximal ileum (P < 0.0001), but not significantly different from that of duodenum (Fig. 3D). When comparing the different locations within the small intestine, no significant differences were found in microbial diversity between duodenum and jejunum, nor between jejunum and proximal ileum, but the microbial diversity of duodenum was significantly higher than that of ileum (P = 0.0047) (Fig. 3C), which indicated a gradual change in microbial diversity along the small intestine. Differences between small intestinal locations were more pronounced with respect to microbial richness (Fig. 3D), with significant differences between duodenum and jejunum (P = 0.0135), jejunum and proximal ileum (P = 0.0042), as well as between duodenum and proximal ileum (P < 0.0001).

In line with the differences in alpha diversity described above, dissimilarity in composition between the microbiota of faeces and that of small intestinal locations was significantly (all P < 0.05) larger than that among small intestinal samples (Fig. 3E). Moreover, the dissimilarity between duodenal and ileal microbiota was significantly larger than that between the microbiota residing in duodenum and jejunum (P = 0.0348), as well as the dissimilarity between the microbiota in jejunum and proximal ileum (P = 0.0055). Hence, observed differences are in line with the biogeographical and physiological distances along the intestinal tract [6].

Overall, sample site (duodenum, jejunum, proximal ileum or faeces) explained 66.7% (based on weighted UniFrac distance) or 55.0% (unweighted UniFrac distance) of total microbiota variation. Microbiota variation within each sample site, was not significantly (all P > 0.05) explained by baseline characteristics, *i.e.* age, sex and BMI, of recruited subjects.

3.3. Impact of synbiotic supplementation on microbiota composition

Comparative analysis indicated that 14 days of synbiotic versus placebo supplementation did not significantly (all $P > 0.05$) change the overall microbial composition in duodenum, jejunum, proximal ileum or faeces (Fig. 4A-D and Supplementary Figure 1A-D). Furthermore, univariate analyses did not show significant (all $P > 0.05$) differences in the relative abundance of individual taxa before and after 14 days of synbiotic supplementation in any of the sample types (Supplementary Table 1).

Microbial diversity (InvSimpson) in duodenum decreased significantly ($P = 0.0498$) after synbiotic versus placebo supplementation (Fig. 4E). The microbial diversity in jejunum, proximal ileum and faeces (Fig. 4F-H), as well as phylogenetically weighted richness (Faith's PD) in duodenum, jejunum, proximal ileum and faeces did not change significantly (all $P > 0.05$) after synbiotic versus placebo supplementation (Supplementary Figure 1E-H). As such, the drop of microbial diversity in duodenum can most likely be attributed to a decrease of microbial evenness in the duodenum.

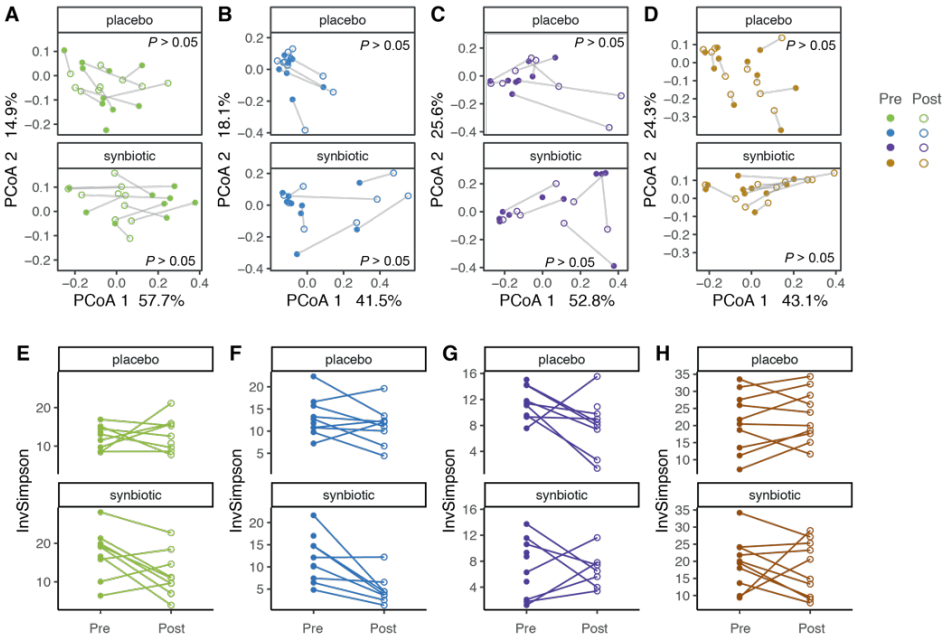

Figure 4. PCoA of microbiota composition pre vs post intervention in duodenum (A), jejunum (B), proximal ileum (C) and faeces (D), as well as corresponding changes in microbial diversity in duodenum (E), jejunum (F), proximal ileum (G) and faeces (H). PCoA plots are based on weighted UniFrac distance. Values are presented in scatter plots and linked per individual. Small intestinal samples were collected after an overnight fasting. Pre: before Intervention; Post: 14 days after start of the intervention. Comparative analysis between timepoints within each sample type and supplementation group (*e.g.* duodenum pre synbiotic vs duodenum post synbiotic), was performed with PERMANOVA. Within each sample type, intervention effects on the microbial diversity were compared with variance components (random intercept) linear mixed models and correction for baseline values. Sample sizes vary due to technical reasons (*i.e.* failure in sample collection and/or DNA isolation). PCoA; Principal coordinate analysis. InvSimpson: Inverse Simpson. PERMANOVA: permutational multivariate analysis of variance.

3.4. Kinetic analysis of the probiotic intervention

Bacterial strains included in the probiotic mixture Ecologic®825 belong to three genera, including *Lactococcus, Lactobacillus* and *Bifidobacterium*. Therefore, we assessed differences in relative abundance of these genera in samples taken before and after the two weeks' supplementation period (Fig. 5 beige background) and their dynamics on day 14 (Fig. 5 white background).

Fourteen days of supplementation did not affect the relative abundance of *Lactococcus, Lactobacillus* or *Bifidobacterium* in duodenum, jejunum, proximal ileum or faeces when considering all subjects (Fig. 5 beige background). Two subjects had increased levels of *Lactobacillus* and one subject had increased levels of *Bifidobacterium* in the jejunum after 14 days of synbiotic supplementation, however, the relative abundance of these two genera decreased again during the day (Fig. 5). This may be attributed to the temporal dynamics of small intestinal microbiota, as we observed in the placebo group that the small intestinal microbial composition fluctuated within a day (Supplementary Figure 2). At day 14 (*i.e.* post, post 2 and post 3 in Fig. 5), we observed that especially four out of ten subjects in the synbiotic group had a strong increase in the relative abundance of the three genera used in the intervention in duodenum (all P values corrected and calculated for all participants, $P > 0.2$), jejunum (*Lactococcus P* = 0.08, *Lactobacillus P* = 0.15, *Bifidobacterium P* = 0.15) and proximal ileum (*Lactococcus P* = 0.01, *Lactobacillus P* = 0.18, *Bifidobacterium P* = 0.04) *ca.* 2 h after ingestion of the synbiotic, whereas levels had returned to baseline *ca.* 3 h after lunch (Fig. 5). Subjects in the placebo group did not show any significant alteration in the relative abundance of these three genera, neither after two weeks of supplementation, nor during day 14.

At ASV level, sequences corresponding to the 16S rRNA gene sequences of all ingested probiotic species were observed in the 16S rRNA gene sequence data (Supplementary Figure 3). Specifically, the changes in the relative abundance of ASVs classified as *Lactococcus lactis* were in line with the observations at genus level, *i.e.* increased relative abundance *ca.* two hours after ingestion and rapid decrease afterwards (Fig. 5A). Moreover, we observed ASVs corresponding to all *Lactobacillus* species in the probiotic mixture, including *L. plantarum, L. acidophilus, L. salivarius*, *L. paracasei* and *L. casei.* The relative abundance of these *Lactobacillus* species was highest *ca.* two hours after the ingestion at day 14, coinciding with the observation at genus level (Fig. 5B). It should be noted, however, that the inherent limitations with respect to species-level resolution in V4 sequence data did not in all cases allow for unequivocal assignment of ASVs to specific species (Supplementary Figure 3). Similarly, for *Bifidobacterium*, observed sequences corresponding to *B. bifidum*, while *B. lactis* was classified as *B. longum*/*B. lactis*, with an increase in relative abundance after synbiotic ingestion in all sample types.

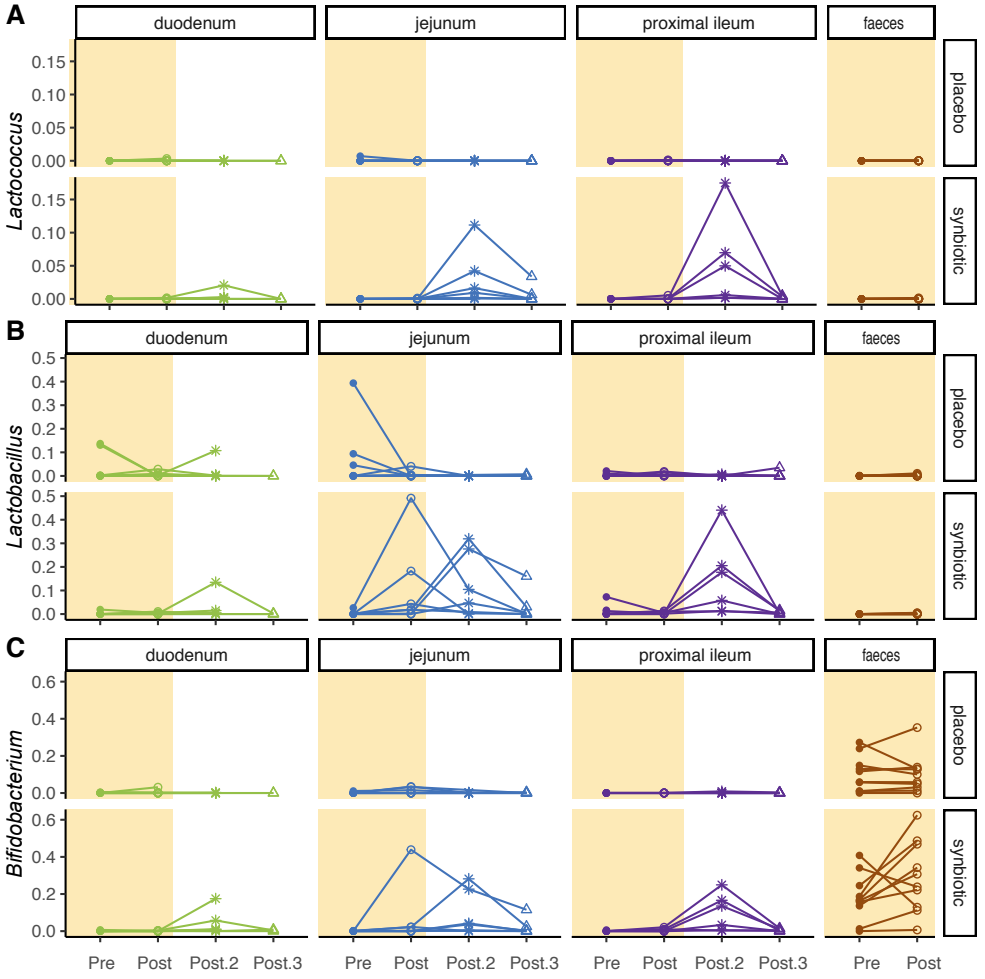

Figure 5. Kinetics of ingested bacteria, (A) *Lactococcus*, (B) *Lactobacillus* and (C) *Bifidobacterium* in duodenum, jejunum, proximal ileum and faeces after supplementation of placebo or synbiotic. Samples are linked per individual. Pre: sample collected in the morning after an overnight fasting before the intervention. At day 14 (14 days after start of the intervention), small intestinal samples were collected at three time points, *i.e.* in the morning after overnight fasting (Post), *ca.* 2 h after consumption of supplements but before lunch (Post.2) and *ca.* 3 h after lunch (Post.3). Faeces was collected at the same day as small intestinal samples.

4. Discussion

In the current study, we compared the composition of the microbial communities residing in the duodenum, jejunum, proximal ileum and faeces as well as their response to synbiotic supplementation, in healthy human volunteers. We hypothesised that microbiota composition differs per sample site and that microbial communities respond differently to the synbiotic supplementation. We indeed found significant differences between the microbiota of duodenum, jejunum, proximal ileum and faeces in microbiota composition and diversity. Furthermore, two weeks of synbiotic supplementation significantly decreased the microbial diversity in the duodenum, whereas it did not significantly affect the microbiota composition in duodenum, jejunum, proximal ileum or faeces. Moreover, small intestinal microbiota was characterised by high fluctuations within a day, and the ingested probiotic mixture induced a short-term spike in the relative abundance of corresponding genera and ASVs within approximately two hours after ingestion.

In the current study, although some subjects had high relative abundance of Veilonellaceae, Enterobacteriaceae or Pasteurellaceae, the small intestinal microbiota was in general predominated by Streptococcaceae, as found previously in ileostoma effluent from ileostomists and small intestinal fluid from healthy subjects [9, 10, 28]. In addition, spatial and physiological differences between upper, mid and lower sections of the small intestine and faeces, were reflected by significant differences in microbiota composition at baseline, in line with the conclusion of a previous review [2]. The analysis of small intestinal microbiota similarity within each individual in this study revealed that dissimilarities between communities were in line with the physical distance between sampling sites. This can most probably be attributed to the gradually change in physiological conditions along the small intestine, including pH, concentrations of oxygen and nutrients, that act as selective force, resulting in location-specific microbial communities adapted to their respective living environment [29]. This can also explain, in part, the higher relative abundance of several microbial taxa in duodenum compared to proximal ileum, as these taxa, *i.e. Leptotrichia, Prevotella sp., Selenomonas, Fusobacterium, Porphyromonas* [30, 31], *Alloprevotella* [32] and *Campylobacter* [33], are known members of the oral microbiota.

Strikingly, in contrast to what is believed in general, *i.e.* increasing microbial diversity from the stomach to the colon [34], comparative analyses in the current study showed that the microbial diversity and richness decreased from upper to lower sections of the small intestine. Early on, researchers investigated the small intestinal microbiota based on small intestinal specimen of sudden death subjects, which are often hard to obtain and small in sample size, and doubts have been raised with respect to these samples being representative of the *in vivo* situation [35]. Later on, Wang *et al.,* compared the microbial diversity of jejunum (using a biopsy capsule) and distal ileum (using colonoscopy) in a single subject, and revealed lower microbial diversity in jejunum, comparing to distal ileum [8]. As an alternative, collection of ileostoma effluent from ileostomists, which enables repeated sampling in a non-invasive way, was used in a number of small intestinal studies [9-11]. For instance, Booijink *et al*. compared the microbial composition of ileostoma effluent from seven ileostomists, and demonstrated extensive individuality in small intestinal microbiota [9]. Nevertheless, it is obvious that ileostoma effluent cannot be used to study spatial

differences in small intestinal microbiota. Recently Seekatz *et al*. used a customized multichannel catheter and collected luminal content of duodenum and the proximal, mid- and distal jejunum from eight healthy subjects, demonstrating increased microbial diversity in duodenum compared to proximal and mid jejunum [28], being in line with findings of the current study. Collectively, comparing to available studies, to the best of our knowledge the current study is the largest study to date investigating spatio-temporal variation in small intestinal microbiota based on 20 subjects. In addition, in the current study we used a multichannel aspiration catheter which enables comparative analysis among different sites of the small intestine within the same subject, therefore allowing to account for subject specificity, including subject- and location-specific responsiveness to a given intervention such as, in this study, a synbiotic supplement.

In the current study, strains included in the synbiotic (Ecologic®825 and scFOS) belong to the three genera *Lactobacillus, Bifidobacterium* and *Lactococcus*, many members of which are known for their tolerance/resistance to low pH and bile [36], as well as adhesion to mucus, epithelial cells or enterocyte-like Caco-2 cells [37-39]. Moreover, compared to single strain probiotics, multispecies probiotic mixtures are suggested to have potential advantages to convey additive or synergistic effects and exhibit better efficacy in health-related outcomes [40]. Two weeks of synbiotic supplementation in the current study did not alter overall microbiota composition in duodenum, jejunum, proximal ileum or faeces. Furthermore, we observed decreased microbial diversity, but not richness in the duodenum, suggesting reduced evenness due to the influx of a large number of probiotic cells (1.5×10^{10} CFU). Nevertheless, the cause and implication of the decreased microbial evenness on health-related parameters in the duodenum remains to be uncovered.

Considering pouch microbiota being comparable to jejunal and ileal microbiota in healthy subjects [10], findings of the current study are in line with those described earlier in which eight weeks of Ecologic®825 intervention did not affect the diversity of mucosal pouch microbiota in patients with active pouchitis [41]. In another recent study, Moser *et al*. showed that supplementation with the synbiotic mixture OMNi-BiOTiC® Stress Repair, (Institut Allergosan, Graz, Austria), containing the the same strains as Ecologic®825, increased the microbial diversity in the upper intestinal tract (*i.e.* gastric corpus and duodenum), but not in the lower intestinal tract (ascending colon or faeces), of IBS-D patients [42]. Moser and colleagues found a significant increase in the relative abundance of unclassified Lactobacillaceae, and a decrease in relative abundance of *Moraxella* and *Moryella* in faeces. In the current study, no significant effects of two weeks intervention with respect to the relative abundance of bacterial groups were observed, including the three genera included in the supplement, *i.e. Lactococcus, Lactobacillus* and *Bifidobacterium*. It should be noted, however, that comparability of both studies is limited, and differences between the current study and the study of Moser and colleagues with respect to target group (healthy subjects vs. IBD patients), dosage of the probiotic (1.5×10^{10} CFU vs. 5.6×10^{10} CFU) and prebiotic (10 g/day scFOS vs. inclusion in the carrier material only) and duration (two vs. four weeks) might at least in part have contributed to the observed differences in microbial data between studies [43, 44]. To this end, it is interesting to note that a number of studies reported reduced mucosal and/or faecal microbial diversity and richness in IBS patient vs healthy controls [45-47], and thus, the more diverse

communities in healthy individuals might be more resilient, and thus less amenable to change. In part, this might also contribute to explaining the previously observed lack of effects on intestinal permeability both under basal and under indomethacin-induced stressed conditions, immune function or intestinal symptoms in subjects receiving this synbiotic supplement [21].

Consistent with earlier observations that the small intestinal microbiota is highly dynamic [2], we also observed fluctuations in small intestinal microbial composition (*i.e.* duodenum, jejunum and proximal ileum) within a day (Supplementary Figure 2). Booijink *et al.* [9] studied the ileal microbiota of over time using ileostoma effluents from one ileostomy patient and demonstrated considerable dynamics over a period of 9-28 days. Furthermore, Seekatz *et al.,* monitored the composition of duodenum composition in six individuals and revealed considerable changes over time [28]. Adding to this, the current study not only demonstrated the temporal dynamics of duodenal microbiota in ten subjects (the control group), but also the dynamics of jejunal and proximal ileal microbiota composition in the same subjects within a day, in addition to the strong subject specificity, although food intake have a big temporal effect within a day on the community. In the synbiotic group, the relative abundance of probiotic genera fluctuated within a day (empty stomach vs *ca.* two h after final probiotic ingestion vs *ca.* three h after lunch), albeit not in all subjects, which could be attributed to individual differences in transit time [48] and differences in baseline microbiota composition. These fluctuations indicated that passage of probiotic strains through the small intestine was completed within six hours. Up to now, although studies exist investigating the probiotic effect on mucosal transcriptomic responses, the short-term effect of synbiotic supplementation on small intestinal microbiota has not been addressed before. The implications of the observed elevated levels of probiotic strains approximately two hours after the ingestion on immune and metabolic markers remain to be addressed in follow-up studies.

5. Conclusion

Overall, this is the first study investigating the spatio-temporal dynamics in small intestinal microbiota in healthy subjects, as well as their response to synbiotic supplementation, with a dedicated control panel of healthy individuals receiving placebo intervention. We demonstrated significant differences in microbial composition at baseline between duodenum, jejunum, proximal ileum and faeces. Two weeks synbiotic (Ecologic®825 + scFOS) supplementation did not affect the overall microbiota composition in the small intestine and faeces differently from placebo but it did alter the microbial diversity in the duodenum. Moreover, small intestinal microbiota demonstrated high instability, and ingested probiotic bacteria were shown to lead to a short-term spike in the relative abundance of corresponding genera and ASVs in four out of ten subjects within two hours after ingestion, after which relative abundances quickly returned back to baseline levels.

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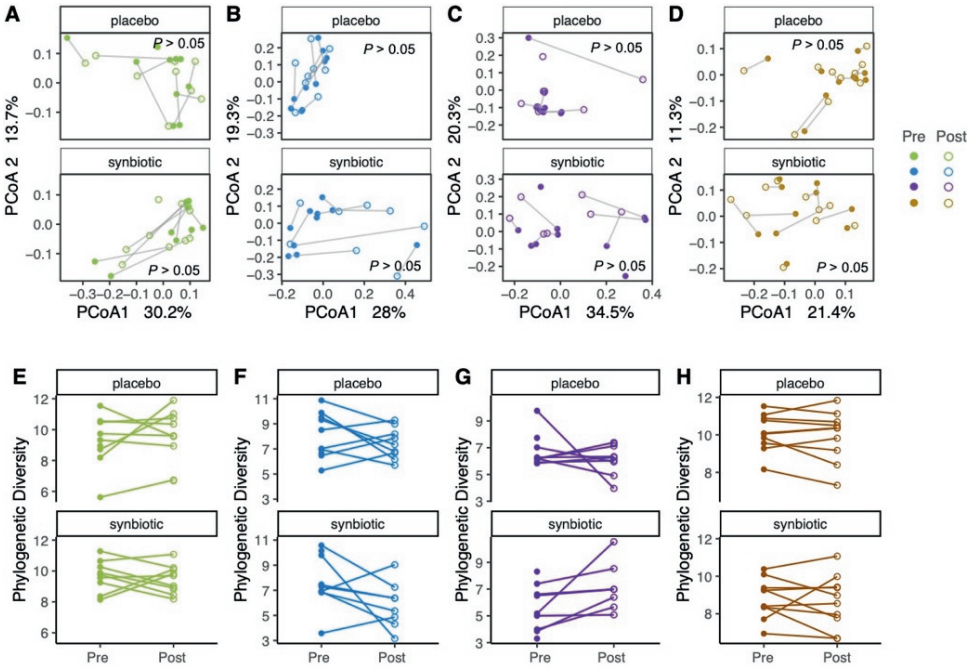
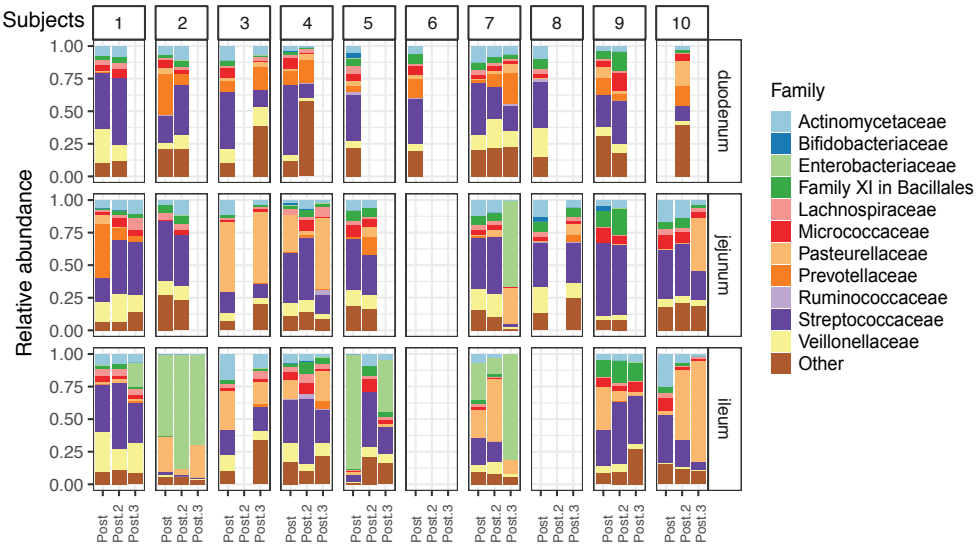
Conflicts of interest: J.G. and I.B.V. are employees of Winclove Probiotics. They have no direct or additional financial interests, and moreover this does not alter their adherence to policies on open and transparent sharing of data and materials. All other authors disclose no conflicts of interest.

Author contributions: The authors contributions were as follows: Conceptualization, H.S., F.J.T., J.G., I.B.V., A.A.M, W.M.D.V. and G.T.R.; Formal analysis, R.A.; Funding acquisition, H.S., W.M.D.V., I.B.V., A.A.M, G.T.R. and F.J.T.; Investigation, R.A., J.G., E.W. and C.S.P.; Methodology, J.G., E.W. and F.J.T.; Resources, F.J.T. and H.S.; Supervision, H.S., D.M.A.E.J., E.G.Z., G.T.R., W.M.D.V. and F.J.T.; Writing original draft, R.A.

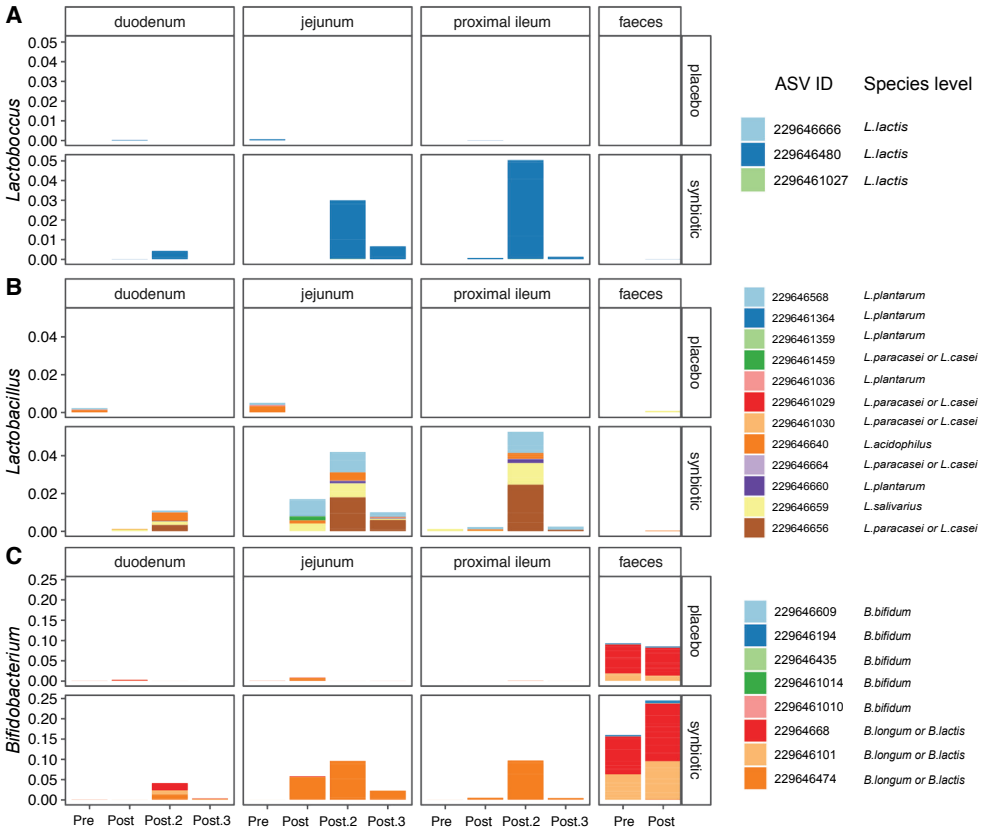
Supplementary Material:



Supplementary Figure 1. PCoA of microbiota composition pre vs post intervention in duodenum (A), jejunum (B), proximal ileum (C) and faeces (D), as well as changes in microbial richness (Faith PD) in duodenum (E), jejunum (F), proximal ileum (G) and faeces (H). PCoA plots are based on unweighted UniFrac distance. Values are presented in scatter plots and linked per individual. Small intestinal samples were collected after an overnight fasting. Pre: before Intervention; Post: 14 days after start of the intervention. Comparative analysis between timepoints within each sample type and supplementation group (*e.g.* duodenum pre synbiotic vs duodenum post synbiotic), was performed with PERMANOVA. Within each sample type, intervention effects on the microbial diversity were compared with variance components (random intercept) linear mixed models and correction for baseline values. Sample sizes vary due to technical reasons (*i.e.* failure in sample collection and/or DNA isolation). PCoA; Principal coordinate analysis.



Supplementary Figure 2. Relative abundance of different bacterial families (top 12, 12, ranked base on the average relative abundance across the entire dataset) in different sample types derived from 10 healthy subjects who were included in the placebo group. Top 12 microbial families (ranked based on the average relative abundance across the entire dataset) are listed in the legend. Each column represents corresponding type of sample from one subject at a specific time point. Missing columns are due to technical reasons (*i.e.* failure in sample collection and/or DNA isolation). Samples were collected at day 14 (after the intervention), specifically in the morning after an overnight fasting (Post), after maltodextrin before lunch (Post.2) and c.a. 3h after lunch (Post.3). Subject ID was re-coded to adhere to relevant privacy regulations.



Supplementary Figure 3. Kinetics of ingested bacteria at ASV level, (A) *Lactococcus*, (B) *Lactobacillus* and (C) *Bifidobacterium* in duodenum, jejunum, proximal ileum and faeces after supplementation of placebo or synbiotic. Data expressed as averaged relative abundance per sample type. Pre: sample collected in the morning after an overnight fasting prior to the intervention. At day 14 (14 days after start of the intervention), small intestinal samples were collected at three time points, *i.e.* in the morning after an overnight fasting (Post), *ca.* 2 h after consumption of supplements but before lunch (Post.2) and *ca.* 3 h after lunch (Post.3). Faeces were collected at the same day as small intestinal samples.

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6

The faecal microbiota of pre-frail elderly is less efficient in metabolizing typical bifidogenic carbohydrates compared to that of adults

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Abstract

Globally increased life expectancy triggered great interest to delay the onset of frailty, which has been associated with alterations in compositional and functional characteristics of intestinal microbiota. In the current study, we used an *in vitro* batch fermentation model to compare the metabolic capacity of the faecal microbiota of adults (n=6) versus pre-frail elderly (n=6), to degrade various glycosidic carbohydrates, namely galacto-oligosaccharides, 2'-fucosyllactose, chicory fructo-oligosaccharides and inulin, and isomalto/malto-polysaccharides. Analysis of 16S rRNA gene sequences and metabolites revealed a distinct variation in overall microbiota and metabolite composition during incubation was explained by individuality of the subjects and carbon source. Nevertheless, age group of the subjects also had significant impact on carbohydrate degradation and metabolite production. Moreover, the increase in the relative abundance of *Bifidobacterium* was more pronounced in the microbiota of adults compared to that in pre-frail elderly, in line with decreased effectiveness to degrade typical bifidogenic carbohydrates in the latter group. Altogether, compared to healthy adults, the microbiota of pre-frail elderly was less efficient in carbohydrate degradation which might potentially be attributed to the decreased abundance of *Bifidobacterium*, a genus that is frequently observed in lower abundance in elderly.

Keywords: frailty, elderly, adults, microbiota, capacity, carbohydrates, metabolites

1. Introduction

The human intestinal tract contains different anatomical regions that are inhabited by a large number of microorganisms, collectively called the intestinal microbiota. The intestinal microbiota plays an important role in human health as it converts indigestible carbohydrates into metabolites that we absorb, produces essential vitamins, and affects immune system development and functioning [1].

Ageing is associated with alterations in intestinal physiology and functionality, such as deteriorated oesophageal functions and rectal compliance, thereby affecting the inhabiting microorganisms [2]. A number of studies has investigated the changes in faecal microbial composition during ageing by comparing the microbiota of adults and elderly. Although contradictory findings have been reported [3], comparing with adults, the intestinal microbiota of elderly has frequently been reported to contain higher levels of streptococci and Enterobacteriaceae as well as lower levels of *Bifidobacterium*, with differences being more pronounced in frail or comorbid elderly [4]. Consistently, comparative microbiota composition analysis revealed a lower level of *Bifidobacterium* in pre-frail elderly compared to that in healthy adults [5]. In addition, Rampelli *et al.* employed a metagenomic approach, indicating that the microbiota of elderly had decreased saccharolytic potential, e.g. loss of genes involved in carbohydrate metabolism and decreased number of genes coding for short chain fatty acid production, in comparison to the microbiota of young adults [6]. Nevertheless, as it has been shown that both within and across microbial groups, microorganisms demonstrate high levels of functional redundancy [7], the impact of these reduced gene numbers on intestinal function has yet to be demonstrated.

While DNA based approaches to study the microbiota can be used to predict its functional potential, the actual activity of microorganisms depends on different biotic and abiotic conditions [8]. Moreover, microbes are very versatile and can quickly adapt to changes in their living environment [9]. Hence, DNA-based approaches do not provide information about the metabolic activity of the microbiota to degrade specific carbohydrates. Therefore, *in vitro* incubations under conditions simulating the intestinal tract can be used to verify functional prediction and provide refined information regarding functional capacity.

In the current study, we aimed to compare the metabolic capacity of microbiota in adults and elderly in response to carbohydrates of different molecular structure, including galacto-oligosaccharide (GOS) [10], 2'-fucosyllactose (2'-FL) [11, 12], chicory fructo-oligosaccharides (FOS; synonym oligofructose), inulin [13] and isomalto/malto-polysaccharides (IMMP) [14], which are known to be utilized by members of the intestinal microbiota. We hypothesised that the metabolic capacity of pre-frail elderly microbiota is lower compared to that of adults, in terms of carbohydrate degradation and metabolite production.

2. Materials and Methods

2.1. Study setup

Six adults and six elderly, which were included in a previously conducted *in vivo* GOS intervention study [5] donated their faecal material for the current *in vitro* incubation study (Fig. S1), at least four weeks after the intervention period (guaranteeing sufficient washing out). All participants defecated into a stool collector (Excretas Medical BV, Enschede, the Netherlands). Directly after defecation, donated faecal material was divided into two portions. A small portion (> 0.5 g) was frozen immediately. The remaining faeces was anoxically cryo-conserved and used as inoculum for the *in vitro* incubations. The viability of different microbial groups in the anoxically cryo-conserved faecal material was determined with propidium monoazide (PMA) dye. The incubations with faeces as inoculum lasted for 24 h with samples collected in duplicate to compare microbiota composition, carbohydrate degradation and metabolite production between age groups (adults vs elderly). The degrading capacity for two typical bifidogenic carbohydrates with different molecular structure, GOS and 2'-FL, was determined for the microbiota of all six adults and six elderly and compared to a non-carbohydrate control. To further extend these experiments, we also studied the degradation of other typical bifidogenic carbohydrates, FOS inulin, and IMMP, using the faecal inocula of three adults and three elderly for which sufficient material was still available.

2.2. Participants *in vitro* study

A subgroup (n=12) of subjects from a GOS intervention study [5], including six adults (three men, three women, aged 25-50 yrs) and six elderly (three men, three women, aged 70-79 yrs) who were randomly contacted, participated in the current study. All subjects were from Maastricht and surroundings in the Netherlands. None of the participants had taken proton pump inhibitors or acid inhibitors, nor antibiotics 90 days prior to the study, nor did any of the participants have a chronic disorder or major surgery, which potentially could have limited participation, completion of the study or interfered with the study outcomes. Detailed description of the inclusion and exclusion criteria has been given elsewhere [5]. Subject codes as shown in the results were randomly assigned in the data analysis phase and could not be traced back to individual subjects without the specific randomization key. The *in vivo* study [5] has been approved by the medical Ethics Committee of the Maastricht University Medical Center+ and registered in the US National Library of Medicine (http://www.clinicaltrials.gov) with the registration number NCT03077529.

2.3. Dietary intake

Participants in the current study completed the dietary records on three consecutive days, after instructed to record their food, beverage and dietary supplement intake based on standard household units. Their nutrient intake was analysed using the online dietary assessment tool of The Netherlands Nutrition Centre (www.voedingcentrum.nl).

2.4. Carbohydrates

In the current study, we investigated the composition and metabolic capacity of faecal microbiota in presence of five different carbohydrates, *i.e.* GOS, 2'-FL, FOS, inulin and IMMP, separately. Specifically, GOS and 2'-FL were kindly provided by Friesland Campina (Ingredients, Amersfoort, The Netherlands). In the current study, in order to mimic the actual portion of GOS utilized by intestinal microbiota, purified GOS with <3% monomers and lactose was used. Size distribution of oligomers in this preparation was as follows: 2.4% degree of polymerization (DP)1, 11.3% DP2, 41.8% DP3, 25.6% DP4, 12.1% DP5, 4.6% DP6, 1.4% DP7, 0.34% DP8, 0.11% DP9. 2'-FL (Fucα1-2Galβ1-4Glc) as one of the key human milk oligosaccharides, was also included in the current study. FOS and inulin were kindly provided by Sensus (Roosendaal, the Netherlands). FOS or oligofructose (Frutalose® OFP) is derived from partial enzymatic hydrolysis of inulin from chicory and consisted for 92 +/- 2 % of fructo-oligosaccharides (DP2-10) and 8 ± 2 % mixture of fructose, glucose and sucrose. Long chain inulin (Frutalfit® TEX!), termed as inulin in the current study, is also derived from chicory, comprising ≥ 99.5% inulin (DP 2 to 60, average chain length ≥22 monomers), and ≤ 0.5% mixture of fructose, glucose and sucrose. The IMMP used is IMMP-92 (AVEBE, the Netherlands) which is a novel indigestible α-glycan derived from starch, with 92% of α-(1→6) glycosidic linkages [15].

2.5. Faecal sample collection and storage

To store and transport freshly defecated faeces under anoxic conditions, Anaerocult® A mini (Merck KGaA, Darmstadt, Germany) was activated with 10 ml nuclease-free water (Promega, Madison, WI, USA), and placed next to the faeces in the stool collector before the lit was closed to create an anoxic atmosphere. Afterwards, the stool collector and two open bags of AnaeroGen (AnaeroGenTM 3.5 L, Thermo Scientific, USA) were put into an anaerobic box (AnaeroPack™ 7.0 L Rectangular Jar, Thermo Scientific™, USA) and stored at 4 °C until transportation. Samples were transported on ice from Maastricht to Wageningen University & Research within nine hours. After arrival, the anaerobic box was transported immediately into the anaerobic chamber (MK3 Workstation, Don Whitley, United Kingdom), filled with 4 % H_2 and 96 % N_2. For each sample, 17.5 g faecal material was then mixed using a spatula with 7.5 g dialysate (Tritium Microbiologie, Eindhoven, the Netherlands), 35.7 g nuclease free water (Promega, Madison, WI USA) and 9.8 ml sterile glycerol. Subsequently, the mixed faecal slurry was transferred into a serum bottle and sealed with a butyl rubber stopper and metal crimp cap inside the anaerobic chamber, and stored at -80 °C.

2.6. *In vitro* incubations

Anoxically cryo-conserved faecal inoculum was defrosted and transferred to a 96% N_2 and 4% H_2 filled anaerobic chamber (BACTRONEZ 300, Shel Lab, USA). Incubation of each carbohydrate (10 mg/ml) was done with 10% (v/v) faecal inoculum in duplicate, while incubations without faecal inoculum and without carbohydrates served as controls using the standard ileal efflux medium (SIEM) as described earlier [14]. Briefly this medium was composed of 40% (v/v) BCO, 10% (v/v) 2-(N-morpholino)ethanesulfonic acid buffer (1 M, pH 6.0), 1.6% (v/v) salt solution, 0.8% (v/v) $MgSO_4$ (50 g/L), 0.4% (v/v) cysteine hydrochloride (40 g/L) and 0.08% (v/v) vitamin mix. The BCO contained 60 g/L bacto peptone, 60 g/L casein and 1 g/L ox-bile. The salt solution contained (g/L): 156.3 K_2HPO_4, 281.3 NaCl, 28.13 $CaCl_2.2H_2O$, 0.31 $FeSO_4.7H_2O$ and 0.63 porcine hemin. The vitamin mix contained (mg/L): 1 menadione, 2 D(+)biotin, 0.5 vitamin B_{12}, 10 D(+)pantothenate, 5 aminobenzoic acid, 4 thiamine-HCl, 5 nicotinamide adenine dinucleotide free acid. The medium components were produced by Tritium Microbiologie BV (Eindhoven, The Netherlands). Cultures were incubated for 24 h at 37 °C on a rotary shaker at 200 rpm.

2.7. Sample collection

Samples were collected 0 h, 4 h, 10 h and 24 h after inoculation (Fig. S2). The headspace gas was sampled first to determine H_2 and CH_4 production. Three aliquots of 1 ml from each culture bottle were then distributed into 1.5 ml Eppendorf tubes. One of these aliquots was heated at 100 °C for 5 min to determine carbohydrates in the supernatant. Afterwards, all samples were centrifuged at 4 °C at 18600 rcf for 10 min. The supernatants from the other two tubes were pooled and stored at -20 °C for metabolite measurement, while the remaining pellets were stored at -80 °C for microbiota composition analysis.

2.8. Carbohydrate, gas, and metabolite measurements

Degradation of GOS, 2'-FL, FOS, inulin and IMMP was determined using High Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD). Specifically, samples taken during the incubation were diluted to 50 µg/ml or 10 µg/ml of initial substrate concentration, and then centrifuged for 15 min at 15000 g. Subsequently, 10 µl of supernatant was injected to an ISC5000 HPLC system (Dionex, Sunnyvale, CA, USA), which was composed of a CarboPac PA-1 column (250 mm × 2 mm ID), a CarboPac PA guard column (25 mm × 2 mm ID) and an ISC5000 ED detector (Dionex) in the PAD mode. The flow rate was set at 0.3 ml/min. The running solvents contained 0.1 M NaOH (A) and 1 M NaOAc in 0.1 M NaOH (B). Specifically: Gradient for GOS: 0-25 min 0-25% B; 25-30 min, 25-100% B; 30-35 min, washing step with 100% B; 35-35.1 min, 100-0% B; 35.1-50 min, equilibration with 100% A; GOS peaks were annotated according to Ladirat et el. 2014 [16]. Gradient for 2'-FL: 0-15 min 0-15% B; 15-20 min, 15-100% B; 20-25 min, washing step with 100% B; 25-25.1 min, 100-0% B; 25.1-40 min, equilibration with 100% A; Gradient for FOS: 0-35 min 0-35% B; 35-40 min, 35-100% B; 40-45 min, washing step with 100% B; 45-45.1 min, 100-0% B; 45.1-60 min, equilibration with 100% A; Gradient for inulin and IMMP: 0-40 min 0-40% B; 40-45 min, 40-100% B; 45-50 min, washing step with 100% B; 50-50.1 min, 100-0% B; 50.1-65 min, equilibration with 100% A. Additionally, the degradation and size of the large IMMP

molecules was also determined using High Performance Size Exclusion Chromatography (HPSEC) as described previously [14].

Headspace gas composition was measured using a CompactGC gas chromatograph (Global Analyser Solutions, Breda, The Netherlands), equipped with a Carboxen PDD precolumn (pressure: 200 kPa, split flow: 20 ml/min, column oven: 90 °C, valve oven: 80 °C) with a carrier gas flow of 20 ml/min and a TCD column (pressure: 200 kPa, split flow: 10 ml/min, column oven: 80 °C, valve oven: 80 °C).

Concentration of short chain fatty acids and other organic acids was determined using High Performance Liquid Chromatograph (HPLC), equipped with a SUGAR SH1821 column (SHODEX, Japan). The column was operated at 54 °C with a flow rate of 0.8 ml/min, using 0.01N H_2SO_4 as eluent. The compounds were detected by a RID-20A (Shimadzu, Kyoto, Japan) refractive index detector with a temperature of 40 °C. Four hundred µl of collected supernatant was mixed with 600 µl of 10 mM DMSO in 0.01N H_2SO_4, and 10 µl of this mixture was injected for analysis. All analytical measurement data were processed using Chromeleon ™ Chromatography Data System (CDS) Software (Thermo Fisher Scientific, Massachusetts, USA).

2.9. Microbiota composition analysis

The microbiota composition was determined by sequencing of barcoded 16S ribosomal RNA (rRNA) gene amplicons using Illumina Hiseq2500 (2 x 150 bp). Collected pellets were mixed with 350 µl Stool Transport and Recovery (STAR) buffer (Roche Diagnostics, United States) and subsequently transferred into a screw cap tube containing 0.25 g of 0.1 mm zirconia beads and 3 glass beads (diameter 2.5 mm). Samples were then subjected to repeated bead beating (3 times 5.5ms × 60 s) using the FastPrep-24™ 5G bead beating grinder and lysis system (MP Biomedicals, the Netherlands) and followed by 15 min centrifugation at 4 °C. Supernatant was collected, and the pellet was subjected to another cycle of isolation with 300 µl STAR buffer. Two-hundred-fifty µl of combined supernatants was transferred into the Maxwell® 16Tissue LEV Total RNA purification Kit Cartridge (XAS1220) and processed using the Maxwell® 16 Instrument (Promega, Leiden, The Netherlands). Subsequently, DNA was eluted in 35 µl of nuclease free water. The V4 region of the 16S rRNA gene was amplified in triplicate using barcoded 515F [17] - 806R [18] primers and diluted DNA (in nuclease free water, 20 ng/µl) as template with an annealing temperature of 50 °C. The PCR was performed as described previously [19]. An equimolar mix of purified PCR products was sent for sequencing (Eurofins Genomics, Konstanz, Germany). Raw sequencing data was processed using NG-Tax 1.0 with default settings [20]. Taxonomy was assigned based on SILVA database version 128 [21, 22].

2.10. Viability measurements

The fraction of viable microbes in the anoxically cryo-conserved faeces was determined with PMA dye, a photoreactive dsDNA-binding dye that only penetrates the envelop of dead cells [23]. Briefly, 1 ml of anoxically cryo-conserved faecal inoculum from each donor was mixed with 2.5 µl of 20 mM PMA dye (Biotium, Inc., United States). Afterwards they were incubated at room temperature for 5 min in the dark (covered with aluminium foil) and then treated with a PMA-Lite TM LED photolysis device (Biotium, Inc., United States) for 15 min. Subsequently, samples were centrifuged at 4 °C at 1500 rcf for 10 min. The pellet was collected and used for DNA isolation and subsequent barcoded amplification, using the methods described in section 2.8.

2.11. Statistical analysis

Relative abundance of microbial taxa was calculated based on 16S rRNA gene sequence read counts. The microbial diversity and richness were calculated based on amplicon sequence variants (ASVs), which were also used to calculate distance matrices. Permutational multivariate analysis of variance (PERMANOVA) was performed based on weighted and unweighted UniFrac distance matrices. Principal coordinate analysis (PCoA) was used to visualize the microbiota composition variation between samples [24]. Microbiota variation partitioning was conducted by fitting environmental variables (*i.e.* age group, sampling timepoint and type of carbohydrate) to weighted and unweighted UniFrac distance matrices, using the adonis function in the vegan package [25]. To compare and contrast alterations in microbiota composition with different carbohydrates versus no-carbohydrate control during the incubation, we used principal response curve (PRC) analysis to identify genera which fit the best (weights > 0.05) to explain the observed difference between no-carbohydrate and carbohydrate-based incubation, using the prc function in the vegan package [25]. As for the metabolite data, redundancy analysis (RDA) in combination with Monte Carlo permutation was performed to assess to what extent explanatory variables, *i.e.* incubation time, subject- and carbohydrate-specificity, could explain the overall variation in metabolite data, using the rda function in the vegan package [25]. To uncover the effect of age group (adult vs elderly) on the degradation of carbohydrates/concentration of metabolites during incubation, we analysed the metabolite data using two-way mixed ANOVA, with one between-subjects' factor (age group) and one within subject factor (incubation time), using the anove_test function in the rstatix package [26]. False discovery rate (FDR) correction according to the Benjamini-Hochberg procedure was applied for multiple testing when applicable. A corrected P value < 0.05 (q-value) was considered to indicate significant difference. All statistical analyses were conducted in R (R-3.6.3).

3. Results

3.1. Characterization of subjects

Six adults and six elderly who donated faecal samples for this study differed significantly in age, but not in sex, BMI, alcohol consumption, smoking, medication use or dietary fibre intake (Table 1).

Table 1 Characteristics of the elderly (n = 6) and adults (n = 6)

	Adults (n = 6)	Elderly (n = 6)	*P* - value
Age (yrs, mean ± SD)	34.0 ± 4.2	72.8 ± 3.0	< 0.001
Sex (% female)	50.0	50.0	1.000
BMI (kg/m^2, mean ± SD)	24.2 ± 3.7	26.5 ± 3.0	0.277
Alcohol (%)	83.3	83.3	1.000
Smoking (%)	0.0	16.7	1.000
Anticoagulation use (%)	0.0	16.7	1.000
Antispasmodics (%)	0.0	16.7	1.000
Habitual dietary fibre intake (g/day)	22.4 ± 6.5	26.6 ± 2.5	0.208

Between groups, parameters, i.e. age, BMI, were tested with independent student t-test. Percentage of sex, alcohol consumption, smoking, medication use and dietary fibre intake were compared using Fisher exact test between groups. BMI: body mass index; SD: standard deviation; yrs: years of age.

3.2. Effect of transport and storage on the viability of the microbiota

PERMANOVA based on weighted and unweighted UniFrac distance matrices did not reveal any significant differences between the microbiota composition of directly frozen faeces and that of cryo-conserved faecal inocula with or without PMA treatment (Fig. S3A&B). Although the relative abundance of some bacterial groups, such as Bacteroidaceae and Prevotellaceae, differed in some subjects, visually reflected in larger weighted as compared to unweighted distances, comparative analysis demonstrated no significant difference in these microbial groups. Overall, our results demonstrated a good recovery of all microbes with the anoxic cryo-conservation protocol used to transport and store the samples for the incubation studies (Fig. S3C).

3.3. Changes in microbiota composition in presence of different carbohydrates

PERMANOVA using weighted UniFrac distances revealed significant contribution of age group (4.87%), subject identity (32.35%), type of carbohydrate (10.43%) and sampling timepoint (18.45%) to the overall microbiota variation (Fig. 1A), while their contributions were 8.06%, 64.50%, 1.86% and 8.44%, respectively, based on unweighted UniFrac distances (Fig. 1B).

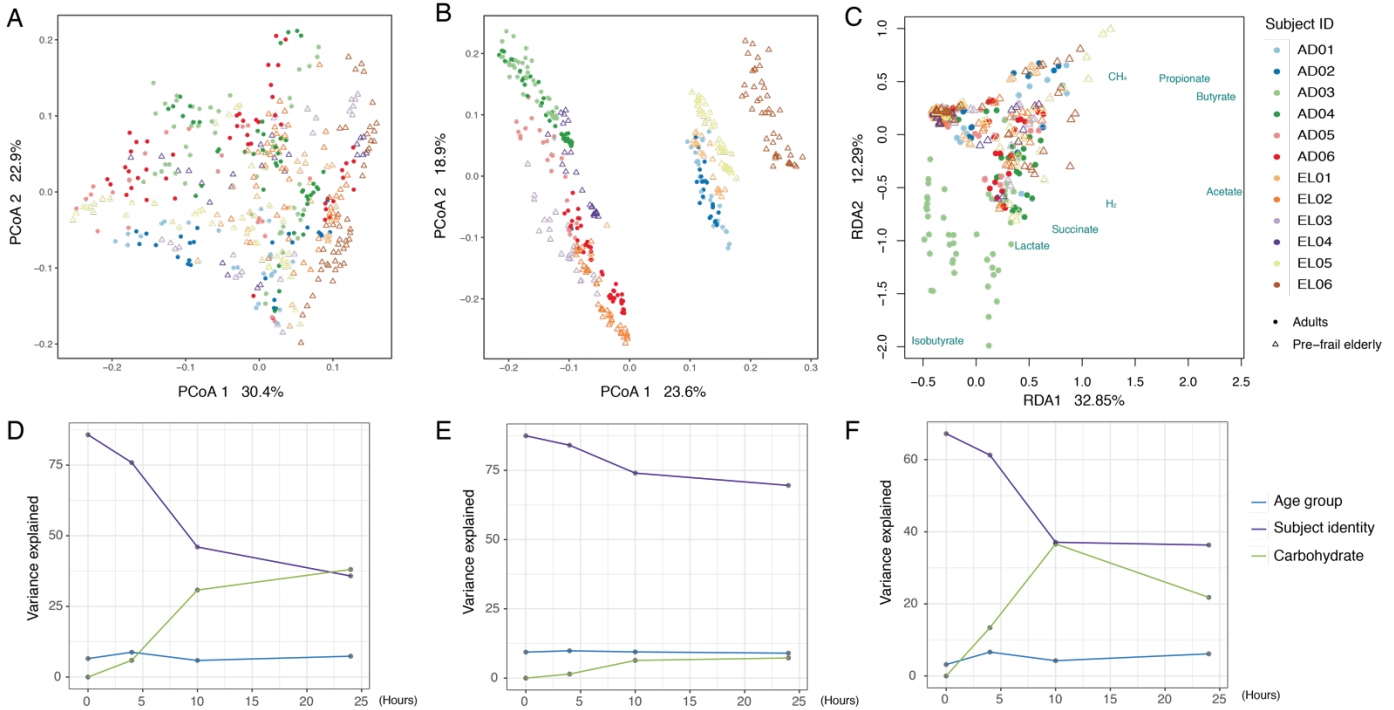

Figure 1. Microbiota and metabolite variation in the dataset. PCoA based on (A) weighted UniFrac and (B) unweighted UniFrac distance matrices of all incubation samples. (C) RDA based on total metabolite data. (D-E) Variation in microbiota composition that can be explained by age group and subject- or carbohydrate-specificity, based on (D) weighted and (E) unweighted UniFrac distance matrices or (F) variation in metabolites at 0 h, 4 h, 10 h and 24 h. AD: adult; EL: elderly. PCoA: principal coordinate analysis. RDA: redundancy analysis

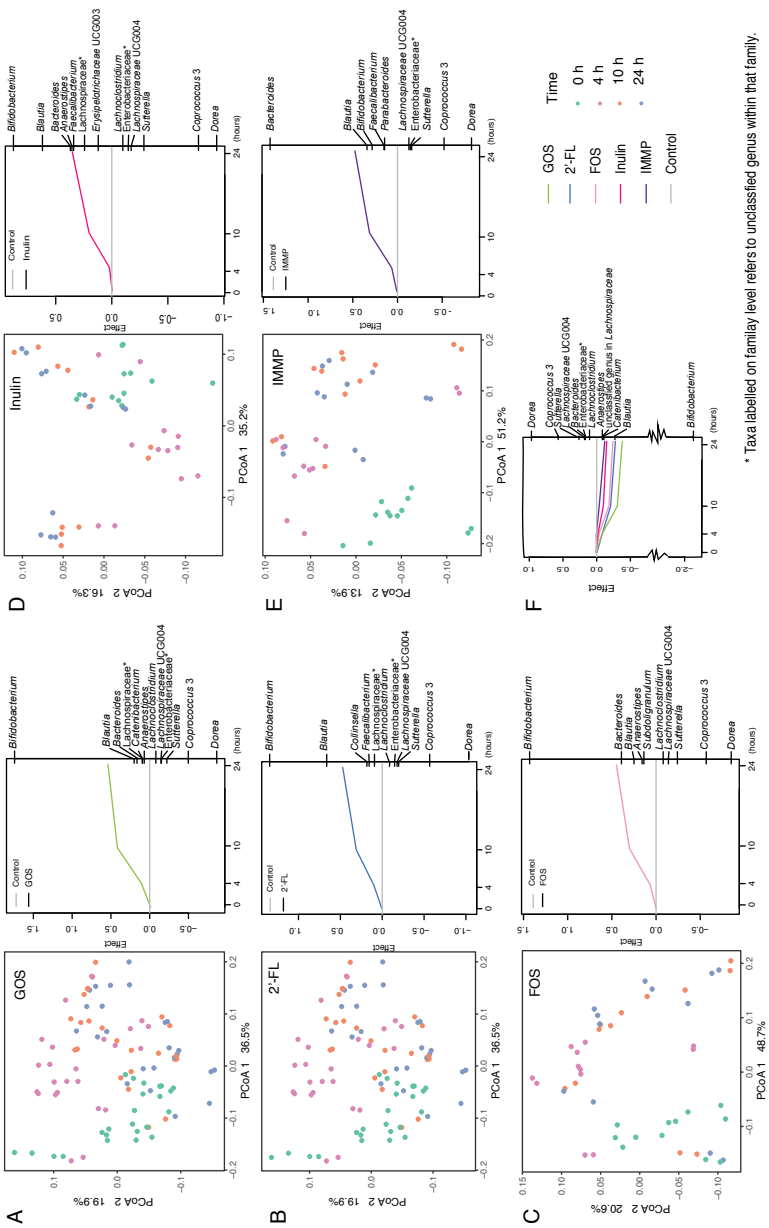

Figure 2. Alterations in microbiota composition, in response to (A) GOS, (B) 2'-FL, (C) FOS, (D) inulin, (E) IMMP and (F) all carbohydrates over time. Each section of the figure (except for F) is composed of two parts: (1) PCoA analysis based on weighted UniFrac distance (each dot represents one sample and samples are coloured per sampling time point). (2) Principal response curve, i.e. alterations in microbial composition compared to no-carbohydrate control. Genera (weights > 0.05) for which the model best explains the observed variation between no-carbohydrate and carbohydrate-based incubation are shown on the right side of each figure. Hereby data of adults and elderly were combined. PCoA: principal coordinate analysis. GOS: galacto-oligosaccharides. FOS: fructo-oligosaccharides. 2'-FL: 2'-fucosyllactose. IMMP: isomalto/malto-polysaccharide.

Variation partitioning per sampling timepoint demonstrated significant contribution of age group ($P < 0.01$) throughout the incubation period, although its contribution remained low (5.87% - 9.86%) compared to that of subject identity and carbohydrate (Fig. 1D&E). Based on weighted or unweighted UniFrac distance matrices, variation explained by subject identity was highest at 0 h (85.76% and 87.47%, respectively) and decreased gradually towards 35.78% or 69.56%, respectively, over time. In contrast, the type of carbohydrate did not contribute to the microbiota variation at the start of the incubation as expected, whereas with the progress of incubation, its contribution increased to 38.07% or 7.26%, respectively, at the end of the incubation. Moreover, during the incubation, the microbial diversity and richness decreased significantly both in the microbiota of adults and that of elderly (Fig. S4) indicative for a selective stimulation exerted by the different carbohydrates during the incubation.

During the incubation, in comparison to no-carbohydrate controls, a large number of genera changed in their relative abundance in response to different carbohydrates, while altered genera differed between carbohydrates (Fig. 2). Among other genera, *Bifidobacterium* increased the most in response to FOS, 2'-FL, GOS and inulin. Notably, compared to other carbohydrates, the increase in the relative abundance of *Bifidobacterium* was largest in response to GOS (Fig. 2F). This alteration was in general more pronounced in the microbiota of adults than that of elderly, with few exceptions (Fig. 3A). In the presence of IMMP, the relative abundance of *Bacteroides* increased over time both in adults and elderly (Fig. 2E and Fig. 3B). Finally, the relative abundance of *Dorea* and *Coprococcus* 3 only increased in the no-carbohydrate controls, except for the microbiota of EL06, which also showed an increased level of *Dorea* in the presence of carbohydrates (Fig. 2 and Fig. 3C).

To assess how well the incubation of faecal microbiota in the presence of carbohydrates *in vitro* reflects the *in vivo* observations we compared microbiota composition and its dynamics over time between the *in vitro* GOS incubations and the *in vivo* GOS effects [5] on the microbiota of the same subjects (Fig. 4). In two subjects, *i.e.* EL02 and EL06, the relative abundance of *Bifidobacterium* was lower or even below the detection threshold at the start of the incubation, with incongruent alterations in *Bifidobacterium* levels in response to GOS (Fig. 4B). The relative abundance of *Bifidobacterium* in EL02 increased after one week of GOS supplementation, whereas *in vitro* only a subtle increase was observed. In contrast, for EL06, the microbiota did not have detectable levels of *Bifidobacterium* at baseline both *in vitro* and *in vivo*. After one week of GOS intervention, the relative abundance of *Bifidobacterium* increased but in contrast to other elderly it completely disappeared after four weeks GOS supplementation. Remarkably, in line with this observation the relative abundance of *Bifidobacterium* stayed under the detection limit during the *in vitro* incubations with faecal inoculum from this subject. After four weeks of GOS intervention, the microbiota was more similar to that after 10 h of GOS incubation, compared to that after 10 h of 2'-FL or protein incubation (Fig. 4). Although we realize that the timelines between *in vivo* and *in vitro* are completely different, this comparison suggests that incubation of carbohydrates *in vitro* can to some level mimic the *in vivo* observations with respect to alterations in microbiota composition, especially its impact on the relative abundance of *Bifidobacterium* (Fig. 4).

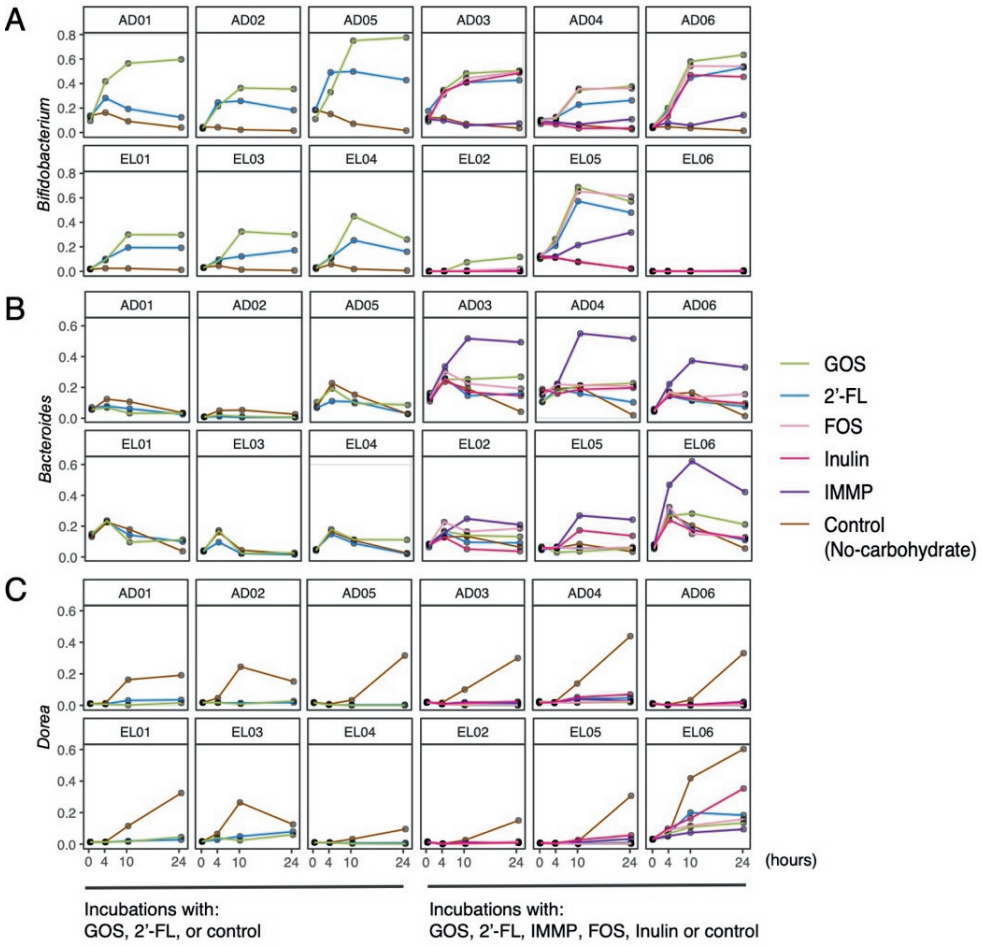

Figure 3. Changes in the relative abundance of (A) *Bifidobacterium*, (B), *Bacteroides* (C) and *Dorea* in the presence of different carbohydrates and no-carbohydrate control during the incubation. GOS: galacto-oligosaccharides. FOS: fructo-oligosaccharides. 2'-FL: 2'-fucosyllactose. AD: adult; EL: elderly.

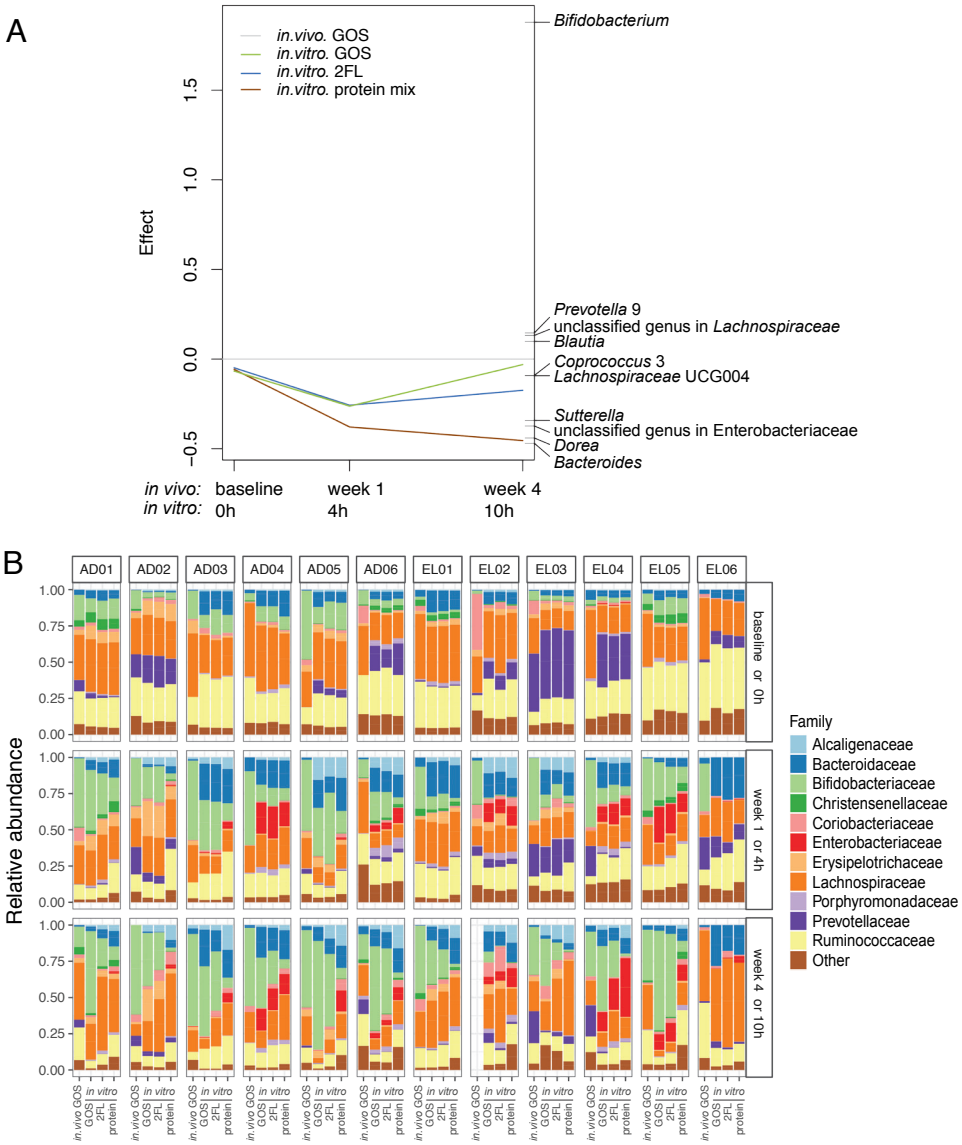

Figure 4. Comparative analysis between *in vitro* and *in vivo* [5] including both adults and elderly. (A) First axis of the principal response curve showing alterations in microbial composition over time in response to GOS *in vivo* and in response to non-carbohydrate control (protein mix) and carbohydrates (GOS or 2'-FL) *in vitro*, while taking *in vivo* GOS intervention as reference. Genera (weights > 0.05) for which the model best explains the observed variation between reference and treatments are shown on the right side of the figure. (B) Relative abundance of different bacterial families (top 12, ranked base on the average relative abundance across the entire dataset) in the microbiota of 6 adults and 6 elderly. Top 12 microbial families are listed in the legend. Other families are summarized as "Other". Each column represents the corresponding type of sample from one subject. Samples collected at 24 h were excluded from this comparative analysis as some carbohydrates were completely depleted within 10 h (details section 3.4). AD: adult; EL: elderly. GOS: galacto-oligosaccharides. 2'-FL: 2'-fucosyllactose.

3.4. Degradation kinetics of GOS, FOS, 2'-FL, inulin and IMMP during incubation

Considering all DPs of GOS as a whole, the microbiota of elderly was significantly slower in GOS degradation compared to the microbiota of adults (P = 0.041, Fig. 5A, DP distribution in Fig. S5). Zooming in on specific DPs, however, the microbiota of elderly was especially slower in the degradation of DP2 (P = 0.047) and DP3 (P = 0.068), compared to the microbiota of adults (Fig. 5A).

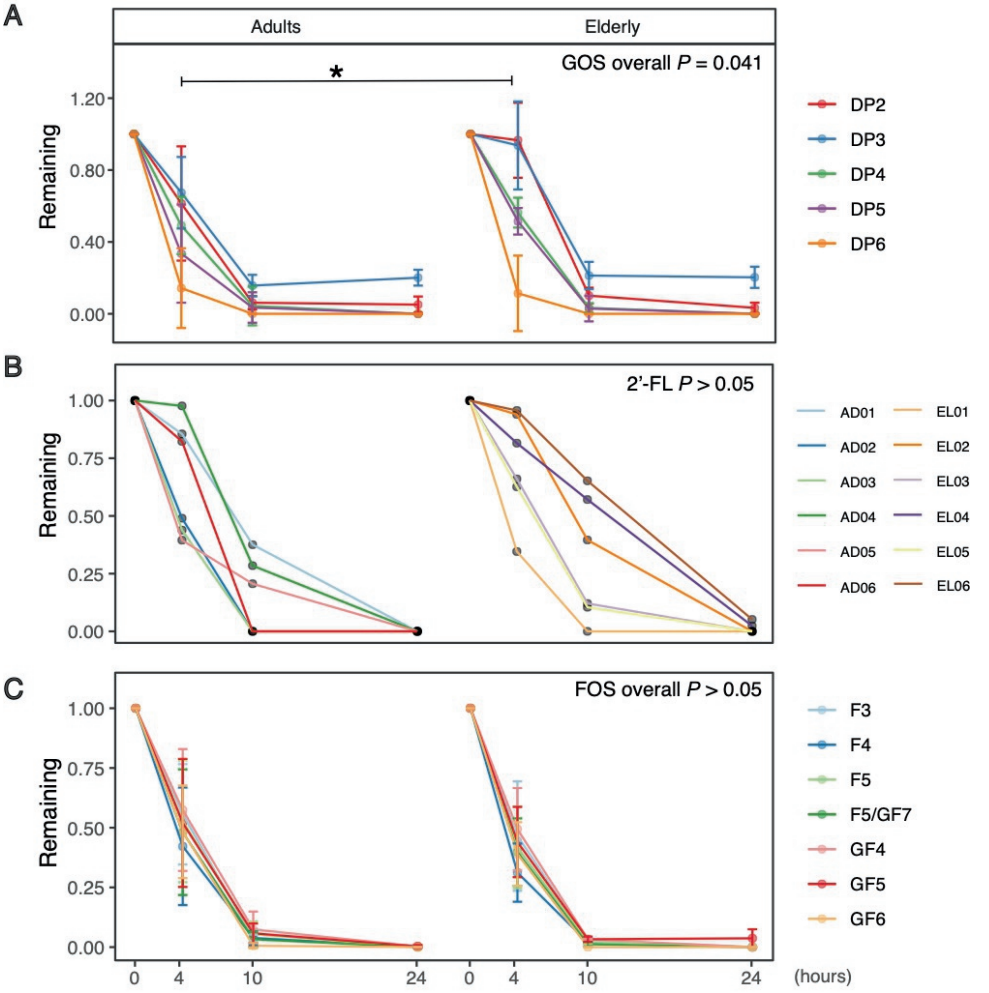

Figure 5. Degradation kinetics of (A) GOS, (B) 2'-FL and (C) FOS. Data is expressed as fraction of residual substrate as compared to the initial concentration of oligosaccharides or 2'-FL. Concentrations per DP in initial GOS and FOS were set to 1.0. Mean +/- SD are shown (Figure A and C). AD: adult; EL: elderly. DP: degree of polymerization; GOS: galacto-oligosaccharides. FOS: fructo-oligosaccharides. 2'-FL: 2'-fucosyllactose F: fructose, G: glucose. SD: standard deviation.

As for 2'-FL, the microbiota of adults and elderly did not differ significantly in its degradation (Fig. 5B), although subjects with lower abundance/no faecal *Bifidobacterium* at the start and during the incubation (*i.e.* EL02 and EL06, Fig. 3B) were remarkably slower in 2'-FL degradation, as compared to subjects with higher relative abundance of *Bifidobacterium*, like AD03 and AD05 (Fig. 3A and 5B).

FOS is composed of fructose (F) oligosaccharides with or without a terminal glucose (G) residue. Specifically, in the current study, FOS contained 2.1% F, 1.8% F_2, 3.4% GF, 2.8% GF_2, 6.0% GF_3, 27.5% F_3, 10.0% GF_4, 21.4% F_4, 7.6% GF_5, 8.9% F_5, 2.9% GF_6 (Fig. S6). Contrast to GOS, the microbiota of adults and elderly did not differ significantly in FOS degradation when considering all oligosaccharides as a whole ($P > 0.05$), nor after zooming in on specific oligosaccharides (Fig. 5C). In addition, degradation of FOS coincided with formation of fructose mono (F) and dimer (F_2) after 4 h of incubation, which were used quickly after 10 h of incubation, and completely depleted by 24 h (Table S1).

As compared to GOS, FOS and 2'-FL, inulin (DP2-60, distribution of DPs in Fig. S7) and IMMP are composed of also longer chains or larger molecules which cannot be shown as remaining fractions, but rather are shown as elution chromatograms (Fig. 6). Specifically, the inulin degradation kinetics differed between subjects (Fig. 6A). For instances, the degradation of inulin was fastest by the microbiota of subject AD-03 and slowest by that of AD06. In addition, inulin was nearly completely after 24 h of incubation, whereas FOS only took approximately 10 h (Fig. 5B and Fig. 6A).

The molecular weight of IMMP used in the current study ranged from 4.84 kDa to 36.60 kDa (Fig. 6B). IMMP was not degraded after 4 h of incubation by the microbiota of any of the subjects (Figure 6B). After 10 h, a clear shift in molecular weight of IMMP was observed, being most pronounced in AD03, AD04 and EL06 (Fig. 6B). The degradation of IMMP coincided with the formation of oligosaccharides after 10 h of incubation for all subjects (Fig. S8). The concentration of these oligosaccharides was considerably higher in case of EL05 and EL06, compared to the incubations with faeces obtained from other subjects. After 24 h, the big shoulder containing large IMMP molecules (6.10kDa-36.60kDa, Fig. 6B) and newly formed IMMP derived oligosaccharides had nearly disappeared for all subjects (Fig. S8).

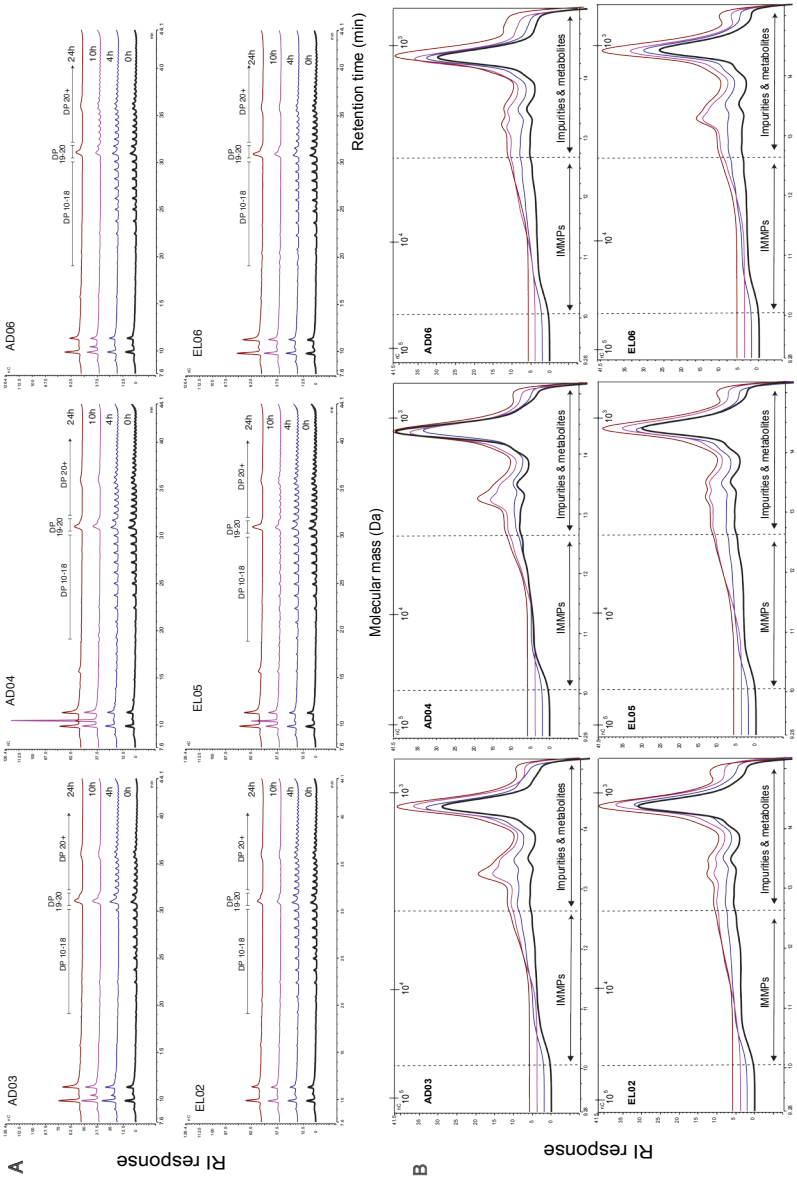

Figure 6. (A) HPAEC elution chromatograms of Inulin. (B) HPSEC elution chromatograms of IMMP. Incubation with faecal microbiota of three adults and three elderly. AD: adult; EL: elderly; DP: degree of polymerization; IMMP: isomalto/malto-polysaccharides. Incubation lasted for 24 h. Samples were taken at 0 h (Black line), 4 h (Blue line), 10 h (Purple line) and 24 h (Brown line).

3.5. Metabolite production during incubation

RDA revealed significant contribution of age group (2.16%), incubation time (34.66%), subject identity (16.31%) and type of carbohydrate (8.65%) to the overall variation in metabolite data (Fig. 1C), as shown for microbiota composition (Fig. 1A&B). RDA performed separately for the different timepoints showed that age group (3.17%-6.63%) contributed to the variation in metabolite data throughout the incubation period (Fig. 1F). Over time the relative contribution of subject identity to explaining metabolite variation decreased, while contribution of carbohydrates increased (Fig. 1E), in line with changes in microbiota composition (Fig. 1D&E). Nevertheless, from 10 h to 24 h, variation explained by carbohydrate decreased (Fig. 1E), which may be attributed to the depletion of some carbohydrates such as 2'-FL and FOS after 10 h (Fig. 5&6).

Zooming in on the effect of age group on the concentration of the different metabolites (Table 2), for the GOS incubation, the concentration of propionate and butyrate differed significantly ($P < 0.001$ and $P = 0.048$, respectively) between age groups, with significantly higher concentrations of propionate and butyrate in elderly compared to those in adults 24 h after inoculation. Simultaneously, the concentration of acetate did not differ significantly between adults and elderly.

In response to 2'-FL, the concentration of butyrate differed significantly between age groups ($P = 0.048$), with significantly higher concentration of butyrate at 24 h in elderly, compared to that in adults (Table 2). Concurrently, the concentration of acetate was significantly lower in elderly at 4 h ($P = 0.049$) and 10 h ($P = 0.003$), compared to that in adults.

Table 2 Concentration of metabolites (mM) during the incubation of GOS and 2'-FL by faecal inocula from elderly and adults.

		Elderly (n=6)				Adults (n=6)				Age group × incubation time (P value)
Incubation time (h)		0	4	10	24	0	4	10	24	
GOS	Acetate	1.36[0.42,1.79]	7.44[6.10,10.82]	45.48[36.70,61.66]	43.83[37.61,69.16]	1.34[1.21,1.47]	13.83[7.19,25.19]	55.26[52.84,60.22]	53.45[40.78,60.54]	0.519
	Propionate	**2.52[0.00,2.77]**	**4.03[2.868,5.16]**	**9.48[6.05,11.71]**	**16.84[15.37,20.41]***	**2.24[2.20,2.50]**	**3.99[2.601,5.41]**	**7.134[5.14,10.18]**	**10.74[9.55,13.11]**	**0.000**
	Butyrate	**0.00[0.00,0.11]**	**1.62[0.445,2.97]**	**17.28[7.53,24.25]**	**47.77[43.31,52.53]***	**0.00[0.00,0.00]**	**2.01[0.75,3.68]**	**8.114[4.67,18.10]**	**28.45[13.94,47.14]**	**0.048**
	Lactate	0.63[0.43,0.75]	2.81[2.48,3.23]	14.19[4.74,25.73]	0.43[0.00,7.12]	0.62[0.431,0.743]	5.80[3.47,11.11]	23.205[19.00,26.22]	9.56[0.795,20.16]	0.519
	Succinate	0.56[0.461,0.661]	1.67[1.39,2.02]	5.77[3.90,8.40]	0.00[0.00,1.75]	0.55[0.00,1.189]	1.83[0.77,3.09]	1.507[0.46,6.98]	0.51[0.00,7.80]	0.173
	Isobutyrate	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.259
	CH4	0.00[0.00,0.00]	0.20[0.00,0.47]	0.58[0.00,1.52]	1.02[0.00,4.01]	0.00[0.00,0.00]	0.00[0.00,0.32]	0.00[0.00,0.91]	0.00[0.00,2.66]	0.630
	H2	0.00[0.00,0.00]	1.07[0.95,1.23]	9.45[8.63,12.26]	8.99[0.02,12.58]	0.00[0.00,0.00]	2.06[0.72,2.73]	8.67[3.84,12.87]	6.75[0.04,9.90]	0.630
2'-FL	**Acetate**	1.54[0.87,1.73]	**7.24[5.66,14.87]***	**40.28[31.99,47.24]***	62.57[43.58,68.36]	1.61[1.12,1.77]	**14.28[7.82,22.97]**	**55.46[46.64,59.74]**	57.14[52.16,60.48]	0.148
	Propionate	2.80[0.00,2.99]	4.19[2.09,5.78]	11.89[10.58,12.83]	25.08[18.72,30.00]	2.15[0.80,2.51]	4.76[3.46,5.43]	11.71[10.29,15.15]	23.84[19.68,26.57]	0.519
	Butyrate	**0.00 [0.00,0.00]**	**1.66[0.52,3.84]**	**12.02[9.32,16.52]**	**44.58[41.30,51.62]***	**0.00[0.00,0.07]**	**2.19[0.85,4.36]**	**12.63[7.93,21.33]**	**31.10[27.12,48.71]**	**0.048**
	Lactate	0.67[0.59,0.75]	3.05[2.08,3.86]	2.97[0.71,7.31]	0.00[0.00,0.75]	0.35[0.23,0.67]	4.45 [3.37,7.136]	3.00[0.75,7.25]	0.00[0.00,0.74]	0.519
	Succinate	0.29[0.00,0.73]	1.69[1.529,2.711]	2.52[1.08,4.33]	0.50[0.00,1.70]	0.65[0.50,0.98]	2.69[0.77,3.00]	1.33[0.00,6.47]	0.00[0.00,5.31]	0.884
	Isobutyrate	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.277
	CH4	0.00[0.00,0.00]	0.18[0.00,0.53]	0.65[0.00,1.92]	0.81[0.00,2.80]	0.00[0.00,0.00]	0.00[0.00,0.43]	0.00[0.00,0.931]	0.00[0.00,3.22]	0.630
	H2	0.00[0.00,0.00]	0.88[0.68,1.40]	6.16[3.54,8.71]	5.59[0.05,8.39]	0.00[0.00,0.00]	1.90[0.72,2.73]	8.07[5.33,9.70]	9.10[4.95,15.67]	0.519

Data are expressed as median [Q1, Q3] (IQR). Changes in the concentration of each metabolite over time were analysed using two-way mixed ANOVA, with one between-subjects' factor (age group) and one within subject factor (incubation time), to uncover the effect of age group (adult vs elderly) over the incubation time period. P-values were corrected for multiple testing by false-discovery-rate (FDR) according to Benjamini-Hochberg procedure. * significant between age groups at corresponding time point. QIR: interquartile range.

Table 3 Concentration of metabolites (mM) during the incubation of FOS, inulin and IMMP by faecal inocula from elderly and adults.

		Elderly (n=3)				Adults (n=3)				Age group × incubation time (*P* value)
Incubation time (h)		0	4	10	24	0	4	10	24	
FOS	Acetate	0.77[0.16,1.24]	8.34[6.21,8.86]	29.38[23.85,64.55]	33.74[22.24,51.08]	1.05[0.86,1.33]	8.31[7.53,19.11]	54.17[52.44,57.27]	49.62[36.91,59.28]	0.469
	Propionate	2.65[0.61,3.20]	3.83[2.32,4.47]	6.03[5.62,7.14]	**11.01[10.03,15.75]***	2.51[2.31,2.53]	3.93[3.15,4.70]	6.77[4.38,7.91]	**7.63[5.06,10.53]**	0.171
	Butyrate	0.00[0.00,0.00]	0.50 [0.12,2.53]	14.29[5.57,27.58]	**41.358[27.00,43.71]***	0.20[0.00,0.43]	1.36[1.00,2.84]	8.96[5.09,14.09]	**8.78[4.93,26.66]**	0.136
	Lactate	0.69[0.54,0.91]	2.58[1.72,2.72]	27.40[7.98,37.34]	0.00[0.00,13.66]	0.50 [0.27,0.61]	3.54[3.05,8.60]	32.38[21.82,34.21]	30.20[7.12,36.30]	0.255
	Succinate	0.99[0.71,1.27]	2.01[1.78,2.59]	6.06[5.55,8.94]	2.65 [0.99,5.57]	0.60[0.14,1.30]	3.30 [1.14,4.82]	7.82[2.19,10.64]	8.33[2.92,10.13]	0.293
	Isobutyrate	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.45]	0.00[0.00,0.75]	0.00[0.00,0.90]	0.00[0.00,0.50]	0.208
	CH4	0.00[0.00,0.00]	0.40[0.09,0.54]	1.31[0.30,1.45]	1.55[0.31,7.79]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.208
	H2	0.00[0.00,0.00]	1.68[0.79,2.26]	17.92[7.51,22.45]	8.05[1.96,13.25]	0.00[0.00,0.00]	1.76[1.16,1.93]	5.75[5.27,14.40]	7.38[6.55,13.13]	0.320
Inulin	Acetate	0.00[0.00,0.88]	5.70[5.38,6.14]	33.81[29.39,34.34]	37.69[34.28,55.32]	1.11[0.89,1.48]	5.64[5.50,21.88]	53.37[35.64,57.52]	50.91[48.09,54.55]	0.293
	Propionate	2.49[0.58,2.95]	2.69[2.20,3.89]	8.54[8.31,9.99]	17.53[15.05,30.01]	2.33[2.28,2.65]	4.05[3.26,4.17]	8.89[5.47,12.90]	11.56[6.88,16.43]	0.136
	Butyrate	0.00[0.00,0.00]	0.50[0.00,1.25]	30.08[18.31,30.71]	52.44[49.71,62.14]	0.00[0.00,0.00]	1.51[1.12,3.30]	17.74[14.13,27.62]	42.21[39.61,43.39]	0.195
	Lactate	0.67[0.58,0.76]	1.18[1.02,1.97]	2.05[1.66,3.32]	0.00[0.00,0.00]	0.46 [0.10,0.96]	1.59[1.179,6.49]	8.11[3.64,8.91]	0.45[0.37,1.19]	0.208
	Succinate	0.60[0.41,1.24]	1.19[1.09,1.70]	7.64[7.37,9.73]	6.53[3.89,9.78]	1.28[0.32,1.38]	2.45[0.59,4.51]	6.90[2.00,10.79]	7.04[1.60,11.49]	0.439
	Isobutyrate	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.63]	0.00[0.00,1.007]	0.00[0.00,0.87]	0.00[0.00,0.69]	0.317
	CH4	0.00[0.00,0.00]	**0.30[0.07,0.70]***	**1.29[0.32,3.52]***	**6.50[1.24,9.00]***	0.00[0.00,0.00]	**0.00[0.00,0.00]**	**0.00[0.00,0.00]**	**0.00[0.00,0.00]**	0.102
	H2	0.00[0.00,0.00]	0.83[0.45,1.74]	20.34[9.12,25.20]	1.66[0.09,12.31]	0.00[0.00,0.00]	1.51[0.91,1.98]	9.76[6.07,17.08]	11.12[9.29,12.62]	0.208
IMMP	Acetate	0.00[0.00,0.85]	5.38[5.25,5.97]	32.960[30.61,37.44]	60.34[52.14,68.05]	1.10[0.78,1.23]	6.45[6.10,7.63]	35.55[32.04,38.50]	58.93[57.45,61.55]	0.676
	Propionate	2.49[0.59,2.94]	4.51[2.72,5.21]	15.76[13.67,16.71]	28.53[24.93,31.53]	2.62[2.54,2.68]	4.69[3.69,5.201]	11.90[8.38,18.93]	18.10[8.71,34.30]	0.342
	Butyrate	0.00[0.00,0.00]	0.00[0.00,1.43]	8.41[5.87,10.42]	**29.37[26.96,41.61]***	0.00[0.00,0.38]	1.22[0.69,2.04]	5.79[5.59,7.02]	**20.84[15.46,23.72]**	0.080
	Lactate	0.73[0.59,0.82]	1.02[0.86,1.173]	0.060[0.00,0.57]	0.00[0.00,0.00]	0.32[0.24,0.53]	1.14[0.52,1.64]	0.00[0.00,0.16]	0.00[0.00,0.00]	0.469
	Succinate	1.26[0.84,1.46]	2.18[1.28,2.43]	9.20[8.37,20.06]	10.34[8.626,21.64]	0.97[0.69,1.28]	2.84[1.03,3.50]	22.61[11.67,24.55]	28.07[14.28,32.11]	0.317
	Isobutyrate	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,0.00]	0.00[0.00,1.50]	0.00[0.00,0.60]	0.00[0.00,0.00]	0.223
	CH4	0.00[0.00,0.00]	**0.37[0.15,0.55]***	**1.32[0.29,1.77]***	**2.02[0.56,2.08]***	0.00[0.00,0.00]	**0.02[0.00,0.07]**	**0.00[0.00,0.07]**	**0.00[0.00,0.06]**	0.080
	H2	0.00[0.00,0.00]	0.17[0.11,0.68]	0.17[0.09,0.24]	0.42[0.13,1.09]	0.00[0.00,0.00]	0.67[0.589,1.93]	0.40 [0.36,1.54]	0.09[0.05,0.63]	0.080

Data are expressed as median [Q1, Q3] (IQR). Changes in the concentration of each metabolites over time were analysed using two-way mixed ANOVA, with one between-subjects' factor (age group) and one within subject factor (incubation time), to uncover the effect of age group (adult vs elderly) over the incubation time period. P-values were corrected for multiple testing by false-discovery-rate (FDR) according to Benjamini-Hochberg procedure. * significant between age groups at corresponding time point

As for FOS, the concentrations of propionate and butyrate were significantly higher in elderly after 24 h of incubation, compared to those in adults (Table 3). Concurrently, the concentration of acetate did not differ between elderly and adults.

For incubations with inulin and IMMP, the concentration of CH_4 was significantly higher in elderly at 4 h, 10 h and 24 h compared to that of adults. Additionally, the concentration of butyrate at 24 h was significantly higher in elderly compared to that in adults when IMMP was used.

Despite the differences in carbohydrate structure, incubations with microbiota of adults and elderly did not differ significantly in the concentration of lactate, succinate or isobutyrate (Table 2&3). However, compared to the other carbohydrates, the concentration of succinate was higher in response to IMMP (Table 2&3), which coincided with predominance of Bacteroides in the microbiota (Fig. 2C and Fig. 3C), indicating succinate as the main product of IMMP utilization by Bacteroides. Furthermore, only methanogens, *i.e. Methanobrevibacter* in the faecal microbiota of two out of six adults and three out of six elderly, demonstrated production of CH_4 (Fig. S9).

4. Discussion

In the current study, we compared the metabolic capacity of faecal microbiota obtained from adults and pre-frail elderly during an *in vitro* incubation study in medium mimicking the intestinal lumen and using carbohydrates varying in molecular structure as carbon and energy source. We hypothesised that the microbiota of pre-frail elderly is less efficient in carbohydrate degradation and metabolite production. We found that a significant fraction of the microbiota variation and metabolite production could be explained by age group, although the subject- and carbohydrate-specificity explained most of the variation. Our data on microbiota composition and metabolite production supported the notion that the contribution of bifidobacteria to the incubation process in pre-frail elderly is lower than that in adults, which was accompanied by a decreased effectivity in the degradation of typical bifidogenic components, such as GOS, 2'-FL, FOS and inulin.

Although the overall dietary or fibre intake and the health status of the pre-frail elderly was similar to that of adults based on comorbidity, immune and oxidative stress markers [5], the lower relative abundance of *Bifidobacterium* in elderly was evident in the current study cohort. This is in line with earlier observations that the microbiota of elderly is characterized by lower relative abundance of *Bifidobacterium* compared to that of healthy adults as recently reviewed [4]. The genomes of *Bifidobacterium* spp. contain a large number of genes encoding carbohydrate modification enzymes, such as, glycosyl hydrolases, ABC transporters and the phosphoenolpyruvate-phosphotransferase system, and hence, bifidobacteria act actively in carbohydrate degradation and utilization [27]. In the current study, decreased relative abundance of *Bifidobacterium* in the microbiota of pre-frail elderly at the start of the incubation, as well as a less uniform/consistent increase in its relative abundance during incubation compared to that in healthy adults (Fig. 3A), could have contributed to the declined efficiency in the carbohydrate degradation (Fig. 5&6), especially for GOS [28], 2'-FL [29] and inulin [30], which are known as bifidogenic components. Collectively, we speculate based on our *in vitro* observations that a lower efficiency in degradation of bifidogenic components could be a sign of changed intestinal conditions in pre-frail elderly. Nevertheless, the impact of lower *Bifidobacterium* (relative) abundance on the intestinal physiology during the ageing process remains to be explored.

We also observed differences in altered genera during the incubation of different carbohydrates (Fig. 2). Although in line with other studied carbohydrates that the level of *Bifidobacterium* increased during the incubation, in the presence of IMMP the increase of Bacteroides was most pronounced and accompanied with increased concentration of succinate in both age groups. Consistently, in addition to the increase in the relative abundance of *Bifidobacterium*, Gu *et al.,*observed an increase in the relative abundance of Bacteroides and the concentration of succinate during *in vitro* incubation with adult faecal microbiota [14]. Generally, only low concentrations of succinate are observed in the human intestine [31] as an intermediate in the synthesis of propionate, a common product of Bacteroides or Veillonellaceae through the succinate pathway [32]. In the current study, a significant increase in the relative abundance of *Bacteroides* resulted in the accumulation of succinate, suggesting a high concentration of carbon dioxide in the incubation system which suppressed the conversion of s-methylmalony-CoA to propionyl-CoA (*i.e.* a

propionate precursor) [33]. Moreover, as reviewed by Fernandez-Veledo *et al.* [34], several recent studies in human and in mice have suggested to treat obesity and related co-morbidities via modulating the succinate level in the intestine. Hence, the IMMP studied here could be a potential candidate for intervention studies to investigate its direct and indirect impact on human health.

Consistent with the observations with respect to carbohydrate degradation, the dynamics of metabolite production over time differed significantly between adults and pre-frail elderly, in addition to the effect of carbohydrate- and subject-specificity. On one hand, this is in line with the high individuality shown in the microbiota composition. For instance, only in incubations with faecal inocula obtained from subjects with detectable levels of methanogens, *i.e. Methanobrevibacter*, demonstrated CH_4 production, further reinforcing individual differences in metabolic capacity of the microbiota. One the other hand, this emphasised the differences in microbial composition between age groups. Compared to healthy adults, the microbiota of pre-frail elderly had lower levels of *Bifidobacterium*, and as such was slower in 2'-FL utilization and acetate production at the beginning of the incubation (*i.e.* 4 h and 10 h after inoculation) [35]. In later time points, compared to healthy adults, incubation with microbiota of pre-frail elderly had higher concentration of propionate and butyrate, indicating the presence of propionate and butyrate producing microbes like *Coprococcus* [35]. Its relative abundance was also higher (classified as *Coprococcus* 1 and *Coprococcus* 2 in the current study) in pre-fail elderly than in adults at the start of the incubation (but not in case of *Coprococcus* 3). Furthermore, the differences in metabolites between age groups varied for the different carbohydrates, which could in part be attributed to the differences in carbohydrates' physical and chemical properties [13], although it remains challenging to explain comprehensibly how carbohydrate properties affect microbiota composition, metabolite production, as well as the corresponding direct/indirect impact on health status.

In the current study, we performed the *in vitro* incubation of different carbohydrates using faecal inocula of a subgroup of subjects involved in a GOS intervention study and revealed considerable similarity between *in vivo* and *in vitro* with respect to alterations in microbiota composition. Moreover, we demonstrated a large contribution of inter-individual differences in microbiota composition, and microbial capacity with respect to carbohydrate degradation and metabolite production, collectively underscored the importance of individual specific differences in future studies.

5. Conclusion

In the current study, the bifidobacterial contribution to the incubation process in pre-frail elderly was less than in adults and hence, the efficacy of typical bifidogenic components (GOS, 2'-FL, FOS and inulin) was lower. Although the GOS intervention study showed no physical and immune decline yet in pre-frail elderly [5], the lower level of *Bifidobacterium* observed in the GOS intervention and their lower involvement in this study suggests that a microbial change with declined efficacy of certain prebiotic carbohydrates is visible in these subjects. The impact of this on the progress of frailty or other health parameters and whether specific (dietary) interventions can postpone this, needs to be addressed in follow-up studies.

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Author contribution: The authors contributions were as follows: Conceptualization, E.G.Z., H.S., D.M.A.E.J., A.A.M.M and R.A.; Funding acquisition, E.G.Z., H.S., D.M.A.E.J. and A.A.M.M.; Resources, A.A.M.M., H.S. and H.A.S; Investigation, R.A. and E.W.; Methodology, M.J.L and M.P.H.T; Writing original draft, R.A.

Supplementary Material:

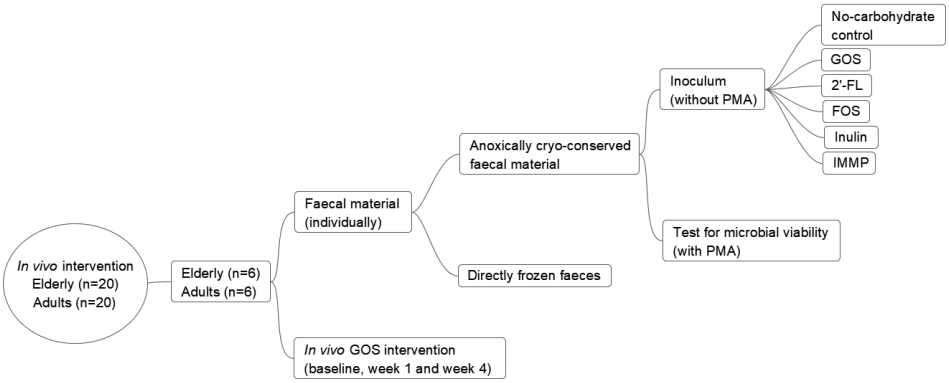

Figure S1. Flow diagram illustrating the study setup. GOS, galacto-oligosaccharides; 2'-FL, 2'-fucosyllactose; IMMP, isomalto/malto-polysaccharides; FOS, fructo-oligosaccharides.

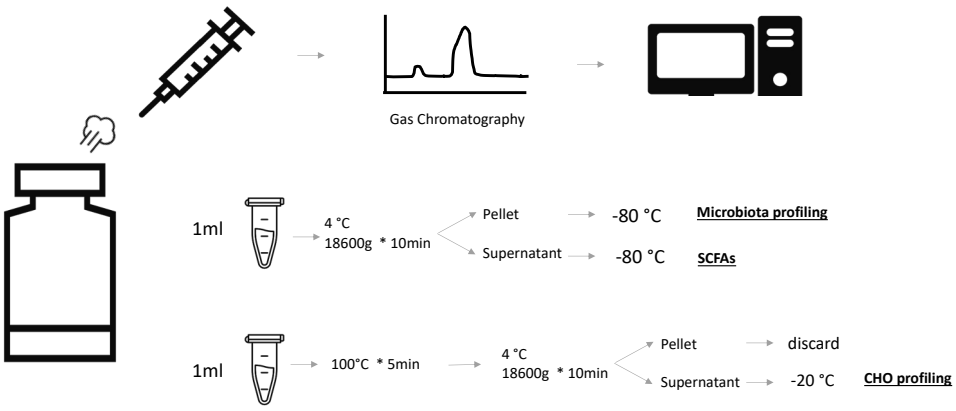

Figure S2. Schematic representation of sample collection during *in vitro* incubation for different analyses. Incubation lasted for 24 h. Samples were taken at 0 h, 4 h, 10 h and 24 h after inoculation.

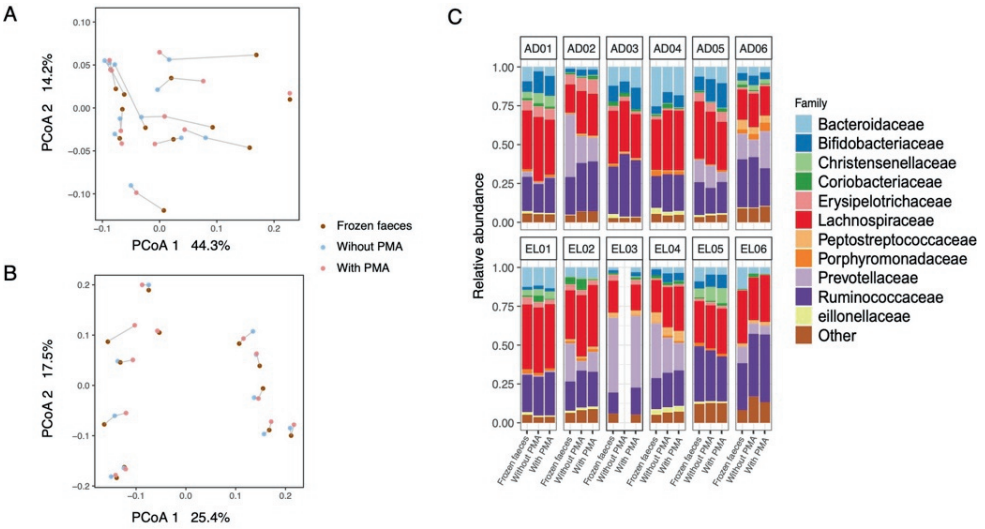

Figure S3. PCoA based on (A) weighted UniFrac and (B) unweighted UniFrac distance matrices. Samples from the same subject are linked with grey lines. (C) Relative abundance of different bacterial families (top 12, ranked base on the average relative abundance across the entire dataset) in directly frozen faeces and anoxically cryo-conserved faeces from six adults and six elderly. Top 12 microbial families are listed in the legend. Other families are summarized as "Other". Each column represents corresponding type of sample from one subject. The anoxic cryo-conserved faeces samples are further divided into with and without PMA treatment. The empty column of EL03 is due to repeated failure in DNA isolation from this specific sample. PCoA; Principal coordinate analysis. AD: adult; EL: elderly

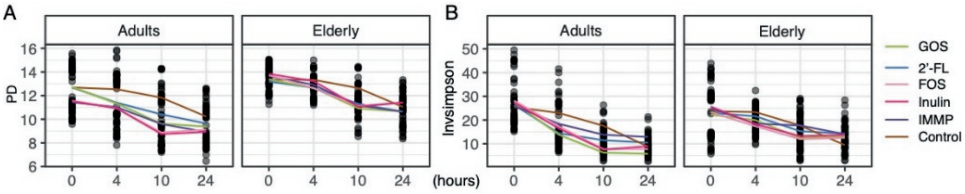

Figure S4. Microbial richness (A) and diversity (B) at different time points of incubation with microbiota from adults and pre-frail elderly, in presence of different carbohydrates and no-carbohydrate control. Incubation lasted for 24 h. Samples were taken at 0 h, 4 h, 10 h and 24 h. AD: adult; EL: elderly. GOS: galacto-oligosaccharides. 2'-FL: 2'-fucosyllactose. FOS: fructo-oligosaccharides. IMMP: isomalto/malto-polysaccharides.

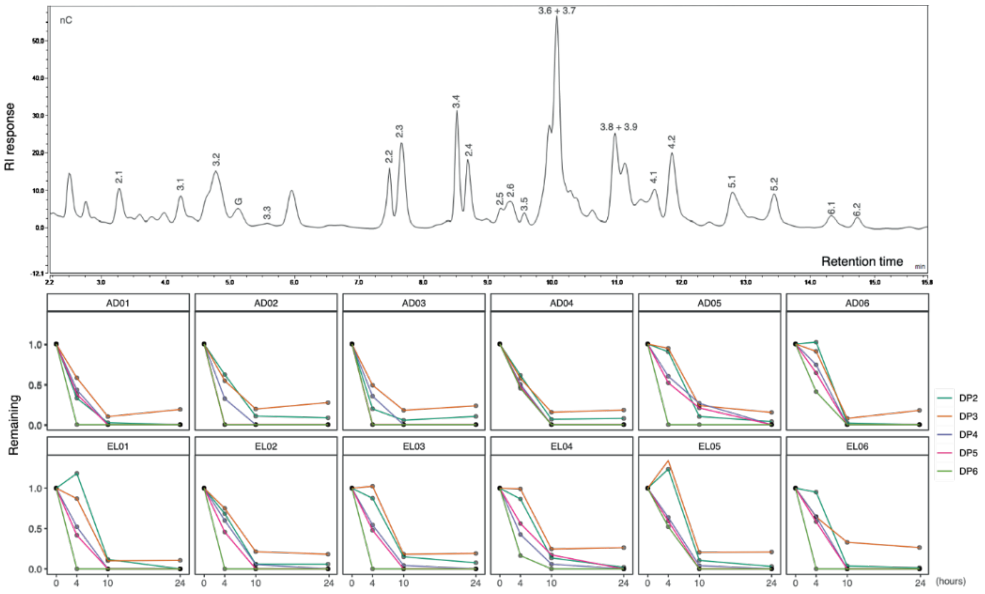

Figure S5. High performance anion exchange chromatography (HPAEC) elution pattern of GOS and percentage of remaining total GOS and of individual DPs present in GOS per subject. Starting concentrations per DP in GOS were set to 1.0. DP2 is annotated as 2.1-2.6. DP3 is annotated as 3.1-3.9. DP4 is annotated as 4.1 and 4.2. DP5 is annotated as 5.1 and 5.2. DP6 is annotated as 6.1 and 6.2. DP: degree of polymerization. AD: adult; EL: elderly.

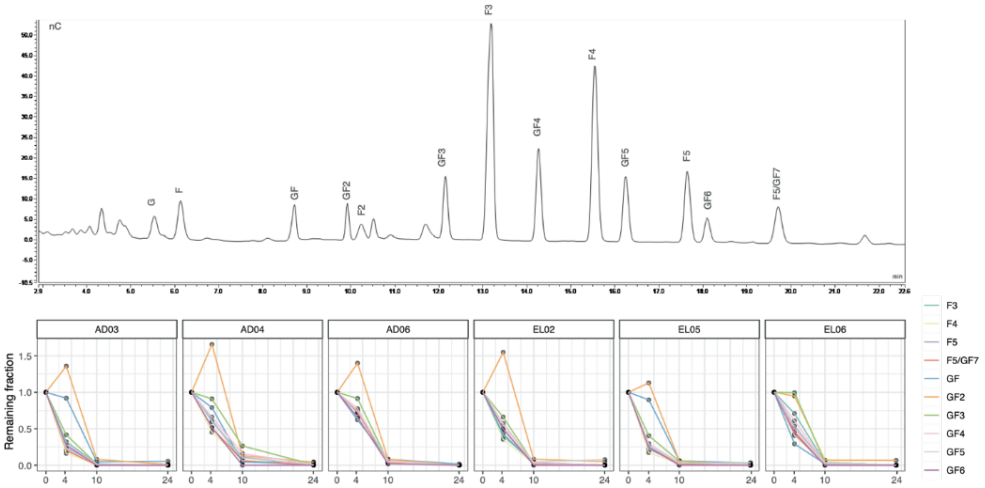

Figure S6. High performance anion exchange chromatography (HPAEC) elution pattern of FOS and percentage of remaining oligosaccharides from FOS per subject. F: fructose, G: glucose. AD: adult; EL: elderly.

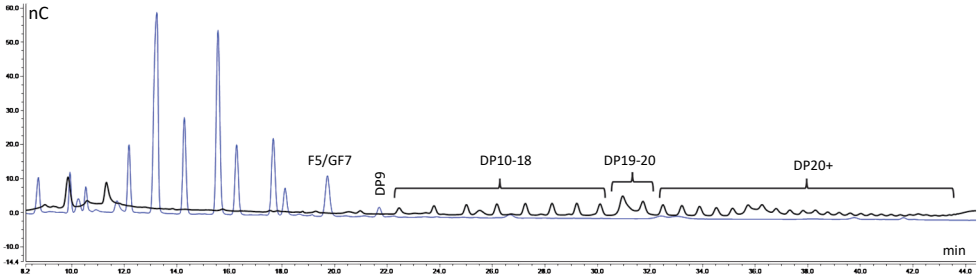

Figure S7. High performance anion exchange chromatography (HPAEC) elution pattern of FOS (blue line) and inulin (black line). Distribution of DPs over retention time. DP: degree of polymerization.

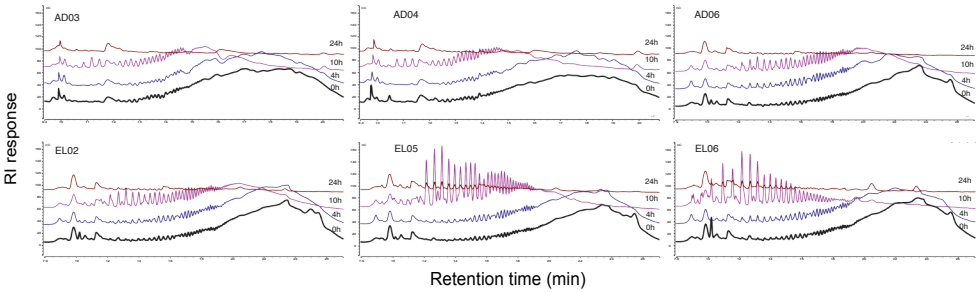

Figure S8. HPAEC elution chromatograms of IMMP before and after fermentation using faecal microbiota of three adults and three elderly. AD: adult; EL: elderly; IMMP: isomalto/malto-polysaccharides. Incubation lasted for 24 h.

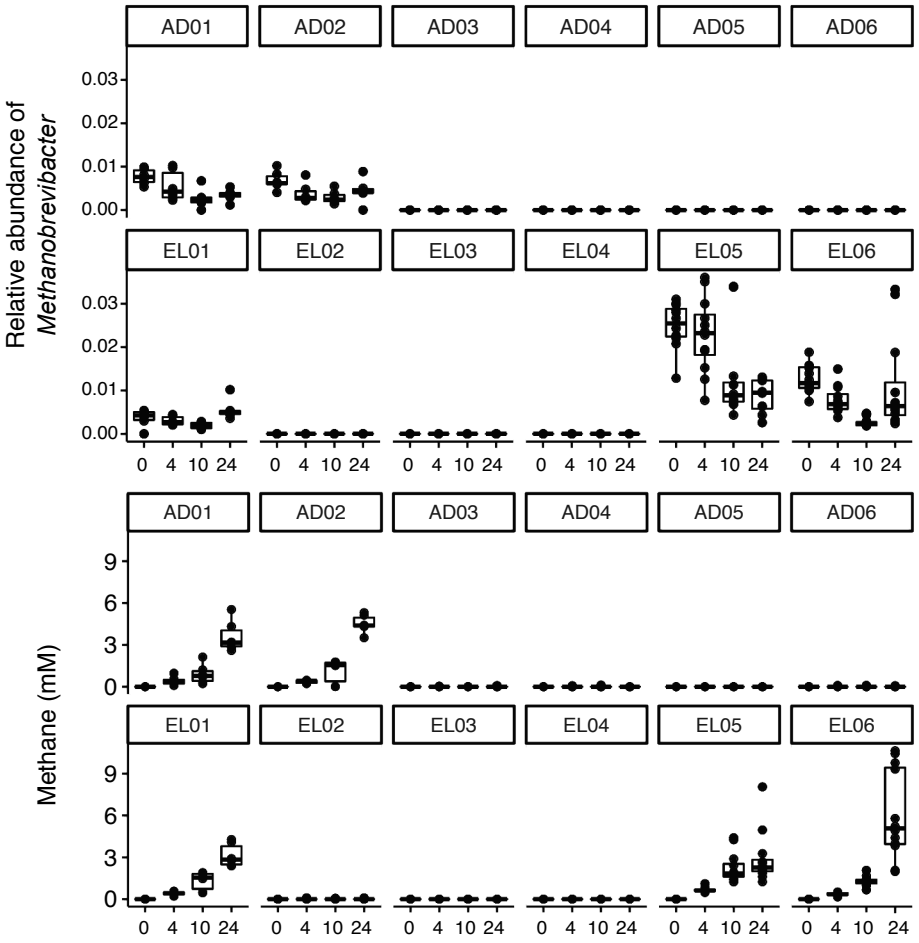

Figure S9. Changes in the relative abundance of *Methanobrevibacter* and concentration of methane over the incubation time period, using faecal inoculum from each subject. AD: adult; EL: elderly.

Table S1: Remaining fraction of FOS derived oligosaccharides over incubation period.

Subject	Time	F	F2	GF2	GF	GF3	F3	GF4	F4	GF5	F5	GF6	F5/GF7
AD03	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AD03	4	8.43	3.60	1.36	0.92	0.42	0.32	0.29	0.16	0.22	0.24	0.27	0.20
AD03	10	0.27	0.19	0.08	0.05	0.02	0.02	0.02	0.01	0.02	0.01	0.00	0.00
AD03	24	0.00	0.00	0.01	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AD04	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AD04	4	8.57	5.52	1.66	0.79	0.91	0.62	0.66	0.45	0.59	0.61	0.52	0.51
AD04	10	0.21	0.14	0.13	0.04	0.26	0.05	0.16	0.07	0.10	0.11	0.00	0.06
AD04	24	0.00	0.00	0.04	0.04	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00
AD06	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AD06	4	6.33	5.09	1.40	0.62	0.92	0.72	0.77	0.65	0.74	0.73	0.66	0.73
AD06	10	0.52	0.13	0.08	0.08	0.05	0.05	0.05	0.04	0.06	0.04	0.02	0.04
AD06	24	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EL02	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
EL02	4	11.51	6.09	1.55	0.41	0.66	0.43	0.57	0.36	0.54	0.50	0.50	0.50
EL02	10	0.28	0.15	0.08	0.04	0.03	0.03	0.03	0.02	0.05	0.01	0.00	0.02
EL02	24	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00
EL05	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
EL05	4	971.80	3.80	1.13	0.90	0.40	0.26	0.30	0.17	0.27	0.22	0.24	0.24
EL05	10	26.60	0.06	0.06	0.06	0.05	0.02	0.04	0.02	0.03	0.02	0.00	0.02
EL05	24	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00
EL06	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
EL06	4	5.53	5.01	0.95	0.29	0.99	0.71	0.61	0.41	0.50	0.54	0.43	0.46
EL06	10	0.11	0.16	0.07	0.02	0.04	0.04	0.03	0.02	0.03	0.03	0.00	0.00
EL06	24	0.00	0.01	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Data is expressed as fraction of residual substrate as compared to the initial concentration of FOS. F: fructose, G: glucose. FOS: fructo-oligosaccharides. AD: adult; EL: elderly.

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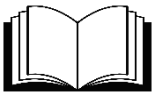
7

General Discussion



General discussion

The human intestinal tract plays an important role in human health, especially in food digestion and fermentation, as well as in nutrient absorption and immune modulation. Along its entire length the intestinal tract is inhabited by a large number of microbes, collectively called intestinal microbiota [1]. The intestinal microbiota, composed of bacteria, archaea, viruses and eukaryotes (*e.g.* fungi and protozoa), interacts with the host through many ways, including (but not limit to) immune modulation and metabolite production (*e.g.* short chain fatty acids and vitamins), and hence, plays an important role in human health [2]. In the research described in this thesis, I aimed to decode the composition and function of the intestinal microbiota in adults, especially elderly, as well as its response to indigestible carbohydrates and how this is related to health. In this section, I will discuss findings of my thesis in detail and highlight challenges as well as future perspectives.

Alterations in microbiota during ageing are pronounced in elderly with deteriorated health

Globally increased life expectancy, which relies largely on modern health care, enlarged the interest to delay the onset of ageing related diseases. Generally, during ageing the adaptive capacity and physiological function decline, which includes immune dysregulation (*i.e.* inflammageing) [3], impaired masticatory function and decreased compliance of the oesophagus [4, 5]. These alterations could possibly in part contribute to the onset of ageing related diseases such as cardiovascular disease [6] and Alzheimer's disease [7], or increased frailty [8]. Moreover, alterations in intestinal physiology and function affect the type and amount of nutrients to be delivered to the small and large intestine, thereby potentially impacting microbiota composition and function in these segments. Nevertheless, the underlying role of intestinal microbiota in healthy ageing remains poorly understood.

A large number of studies over the past decades investigated ageing related changes in microbiota composition via comparisons between adults and elderly, which we critically reviewed in **Chapter 2**. Specifically, it has frequently been reported that the microbiota of elderly has lower levels of bifidobacteria, but higher levels of streptococci and Enterobacteriaceae in comparison to young adults. An exception to this is healthy centenarians who have an intestinal microbiota that is more similar to that of younger adults. Indeed, microbiota studies that included centenarians and different measurements of frailty levels hint towards the importance of alterations in physiological functionality (*i.e.* biological ageing), instead of chronological ageing. Furthermore, we highlighted the possibilities and challenges that need to be addressed in future studies aiming to modulate the microbiota in elderly and ultimately achieve healthy ageing, which include the use of supplements with carbohydrates with the aim to specifically target certain groups of microbes such as bifidobacteria (Fig. 1)

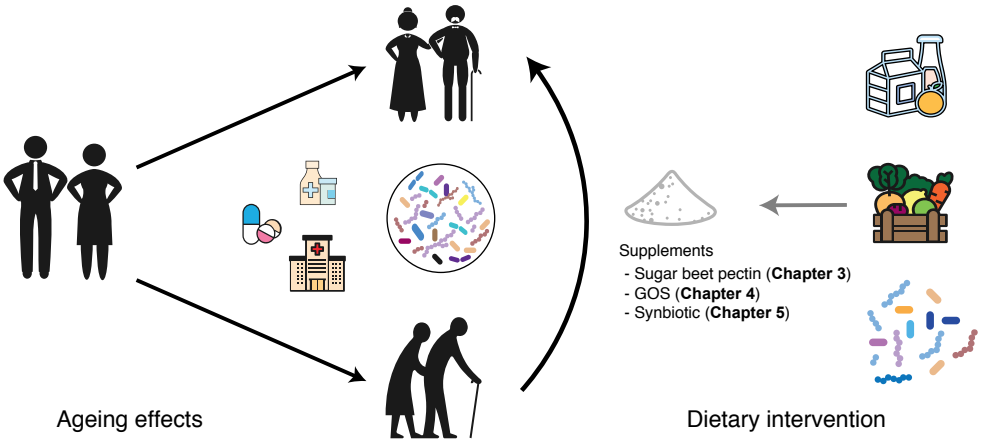


Figure 1. Key factors affecting the microbiota composition during ageing and dietary interventions performed in the current thesis. Chapter 5, synbiotic was only applied in adults.

In **Chapter 3**, I compared the microbiota of healthy adults (18-40 yrs) and healthy elderly (65-75 yrs), which demonstrated high similarity between age groups. Five genera differed significantly in relative abundance at baseline, and four of them (*i.e. Enterorhabdus*, uncultured Coriobacteriaceae, Lachnospiraceae UCG-008 and *Mogibacterium*) remained different at the second sampling timepoint. On one hand, these observations indicate the existence of microbial signatures that are different between the age groups. One the other hand, subjects in both age groups were healthy [9] and did not differ in faecal or breath metabolites (**Chapter 3**). The cause for the differences in microbial composition and the implication thereof still remains ambiguous. Similarly, in **Chapter 4**, no significant difference was found in faecal and breath metabolites, nor immune or oxidative stress markers between pre-frail elderly (70-85 yrs) and healthy adults (25-50 yrs), although the relative abundance of *Bifidobacterium* was lower in the microbiota of pre-frail elderly compared to that in healthy adults. Members of the genus *Bifidobacterium* are among the first microbes colonizing the human intestinal tract after birth and remain as key member of intestinal microbiota throughout life, conferring positive effects to human health [10]. Moreover, it has been reported that a decreased level of *Bifidobacterium* [11] could in part contribute to an increased oro-caecal transit time in elderly [12, 13]. Concurrently, comparative analysis in **Chapter 6** suggested a lower bifidobacterial contribution to large intestinal fermentation processes in pre-frail elderly compared to those in adults, which was coupled with decreased efficacy to degrade typical bifidogenic components such as GOS [14], inulin [15] and 2FL [16]. Whether a decreased metabolic capacity associated to bifidobacteria is a first indication of a decline in physiological function in these pre-frail elderly remains speculative. Hence, it is evident that the impact of this on the progress of frailty and/or other health parameters needs to be studied in the future.

It is of note that there is an inconsistent definition of elderly, not only exemplified in this thesis (**Chapter 3**&**4**) but ubiquitously in literature (*e.g.* 60 yrs+, 65 yrs+, 70 yrs+, 80 yrs+). This hampers comparative analyses between studies, and hence stresses the need for better definition of elderly and intestinal health as well as better biomarkers for health decline in elderly. As such, longitudinal studies following-up alterations in elderly microbiota

composition over time in large-scale population cohorts, as it has been done in adults [17, 18], could ultimately shed light into the role of intestinal microbiota in ageing, frailty and comorbid conditions

Microbial diversity decreases from upper, mid to lower section of the small intestine

Although it is generally accepted that faecal microbiota analyses provide relevant and representative information for distal colonic content (as used in **Chapter 2, 3, 4, 6**), faecal microbiota differs from that of the small intestine (**Chapter 5**) as reviewed previously [19]. As the longest part of the gastrointestinal tract, the small intestine plays an essential role in nutrient absorption, water and electrolyte balance maintenance, as well as endocrine secretion [20]. The small intestine can be divided into upper, mid and lower sections, namely duodenum (*ca.* 25 cm), jejunum (*ca.* 160 cm) and ileum (*ca.* 215 cm). These different sections are accompanied with physiologically relevant gradients of *e.g.* oxygen level and nutrient availability, which could act as selective force for inhabiting microbes [21]. In the study described in **Chapter 5**, we found that the spatial and physiological differences within the small intestine were reflected in the microbiota composition of duodenum, jejunum and proximal ileum. Remarkably, opposite to what is believed in general, *i.e.* increasing microbial diversity from the stomach to the colon [22], we observed a decrease of microbial diversity and richness from duodenum, jejunum to proximal ileum in **Chapter 5**. Up to now, available studies investigating the spatial differences in small intestinal microbiota composition are based on only one/a few subjects, or comparing samples from different locations that are derived from different subjects [23-26]. This issue could be mainly attributed to the difficulties in sample collection, especially in healthy subjects [27]. Early on, small intestinal microbiota research was based on small intestinal specimen of sudden death subjects, which are often precious (hard to obtain), limited in amount and likely not representative [28]. Alternatively, collection of ileostoma effluent from ileostomists, *i.e.* subjects without a colon, which enables repeated sampling in a non-invasive way, has been used to study the microbiota in the small intestine [24-26]. Collected ileostoma effluent, however, cannot be used to study the spatial differences in microbial composition and function along the small intestine. Over the past few decades, the development of new technologies, including IntelliCap capsules, endoscopy and aspiration catheters, brought more possibilities to obtain samples from healthy subjects for studying small intestinal microbiota [27]. In **Chapter 5**, we used a multichannel aspiration catheter and collected samples from duodenum, jejunum and proximal ileum at once from a total of 20 subjects. As such, this method allowed for comparative analysis among different segments of the small intestine within the same individual. In line with the consensus that small intestinal microbiota is highly dynamic [29], we observed changes in small intestinal microbiota composition within approximately two hours, whereas differences in microbial diversity (duodenum > jejunum > proximal ileum) were conserved at different sampling timepoints (**Chapter 5**). Nevertheless, the implications of the gradual decrease in microbial diversity, as well as the changes in microbial composition (possibly also function), from upper to lower sections of the small intestine on human health, remain to be uncovered.

Along the intestinal tract, food components that escaped from digestion and absorption in the small intestine, reach the large intestine which is *ca.* 1.5 m long and *ca.* 6-7 cm in diameter

[30]. It comprises the caecum, colon, rectum and anus. Among all segments, the colon is the longest segment in the large intestine, and can be divided roughly into three sections, namely ascending colon, transverse colon and descending colon [30]. However, comparing to the small intestine, the transit of chyme residues through the colon is much slower, which could in part contribute to the growth of microbes therein. Nevertheless, studies investigating the spatial differences in microbial composition and functionality along the colon are still sparse and face challenges in sample collection. However, with the development of new technologies, like sigmoidoscopy and IntelliCap capsules, the veil of spatio-temporal colonic microbiome dynamics will be uncovered in the near further

Use of different indigestible carbohydrates by microbiota in adults and elderly

Considering the importance of intestinal microbiota to human health [2] and of diet in shaping the intestinal microbiota [31], modulating intestinal microbiota composition and function through dietary changes is considered as a promising approach. In the current thesis, faecal microbiota of elderly and adults did not change significantly after supplementation with sugar beet pectin (**Chapter 3**) nor with a synbiotic combination of the probiotic mixture Ecologic®825 plus scFOS (**Chapter 5**). In contrast, significant changes in microbiota composition were observed after one week and four weeks of GOS supplementation (**Chapter 4**) leading to an increase in the (relative) abundance of *Bifidobacterium*. Nevertheless, the increase in the level of *Bifidobacterium* did not come along with changes in faecal and breath metabolites, or in immune and oxidative stress markers in adults or elderly. This is in line with a previous study that showed that 12 weeks of GOS supplementation increased the relative abundance of *Bifidobacterium* in prediabetic subjects but did not affect the concentration of faecal and plasma metabolites, immune markers or insulin sensitivity [32]. In another study, in contrast, ten weeks of GOS supplementation not only increased the level of *Bifidobacterium*, but also decreased some systemic inflammatory markers in healthy elderly (65-80 yrs) [33]. Interestingly, in this study, the production of inflammatory markers, *i.e.* cytokines IL-6 [34] and TNF-α [35], was not affected by the supplementation of GOS. One of the possible explanations for the inconsistency between studies could be differences in cohort characteristics [36]. The pre-frail elderly recruited in **Chapter 4** based on Fried criteria were still relatively healthy, as no significant differences in immune and oxidative stress markers between age groups were found. Therefore, opportunities for further improvement of immune and stress markers by GOS supplementation were limited. Future studies are warranted to find better biomarkers to define elderly health status and to investigate their response to dietary supplementation in comparison with healthy adults.

I also observed that incubation of faecal microbiota in the presence of different carbohydrates *in vitro* at some level reflected the *in vivo* observations with respect to alterations in microbiota composition (**Chapter 6**). Moreover, in line with the notion that differences in physical and/or chemical properties of carbohydrates can affect their effect on microbiota composition [47], type of added carbohydrates contributed to the overall microbiota variation observed in **Chapter 6**. For instance, in presence of IMMP, we observed a pronounced increase in the relative abundance of Bacteroides, which was accompanied with an increased concentration of succinate in both age groups (**Chapter 6**), being in line with an earlier *in vitro* incubation study using a pooled adult faecal inoculum [46].

Simultaneously, although altered genera differed per carbohydrate in **Chapter 6**, the increase in the relative abundance of *Bifidobacterium* was most pronounced in response to most bifidogenic carbohydrates, *i.e.* GOS, inulin and 2FL, in line with earlier research [40-43]. Nevertheless, the increase in the relative abundance of *Bifidobacterium* was largest in response to GOS comparing to the other carbohydrates and was more pronounced in the microbiota of adults than in that of elderly. The microbiota of one elderly (out of six) even did not have a measurable involvement of *Bifidobacterium* in the degradation of typical bifidogenic carbohydrates, which matched to the intervention observations for the same subject (**Chapter 4**&**6**). Considering *Bifidobacterium* as one of the key microbes involved in carbohydrate degradation [44], decreased levels of *Bifidobacterium* at the start of the incubation could in part explain the reduced degradation rate of the bifidogenic compounds observed in **Chapter 6**. This is also in line with the previously observed decreased saccharolytic potential of microbiota in elderly, as compared to that in adults [45]. Collectively, it is evident that in order to fully understand the mechanisms underlying *in vivo* observations, it is essential to integrate *in vivo* longitudinal studies and *in vitro* mechanistic studies in the future as was done in this thesis.

In addition to the impact of diet on faecal microbiota, the small intestinal microbiota was suggested to be also amenable to dietary supplements [48]. In **Chapter 5**, aside from decreased microbial diversity in the duodenum, two weeks of synbiotic (composed of scFOS in combination with strains of *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*) supplementation did neither alter microbiota composition of the small intestine nor that of faeces, while passage through the small intestine of genera to which the probiotic strains belong was shown within a day in four out of ten subjects. This is in line with the lack of significant effects on intestinal permeability, immune function or intestinal symptoms, as reported earlier [49]. Our data are, however, are in contrast to the previously raised hypothesis that considering the decreased microbial density in the small intestine compared to the large intestine, ingestion of probiotics could induce major changes in small intestinal microbiota whereas the alterations remain minor in colon [50]. This inconsistency could in part be attributed to the fact that abiotic and biotic characteristics shaping the small intestinal ecosystem are not sufficiently taken into consideration. For instance, the high flux and thus increased shear force [20] could result in ingested microbes being readily washed out from the small intestine. Moreover, ingested members of *Bifidobacterium* only grow in the absence of oxygen, and thus residual amounts of oxygen in the small intestine would not favour *Bifidobacterium* growth. Additionally, in **Chapter 5**, small intestinal microbiota demonstrated high instability, and the ingestion of probiotic bacteria induced a short-term spike in the relative abundance of corresponding genera within two hours after ingestion. The impact/activity of these increased genera in the small intestine and/or immune parameters remain to be studied in the near future. Last but not least, further down from the small intestine, *i.e.* in the distal ileum and colon, decreased concentrations of simple sugars, oxygen, antimicrobial compounds and transit rate favour the growth of complex carbohydrate utilizing microbes [14]. Thereby the number of inhabiting microbes increases largely compared to that in the upper small intestine [19], making it an interesting site for future studies investigating microbiota composition and activity, as well as their response to dietary supplementations and corresponding implications for human health.

Future perspectives

Findings of this thesis (**Chapter 2, 3, 4, 6**) reinforced the need for better definition of elderly and intestinal health as well as better biomarkers for health decline in the elderly. Although no difference was shown in immune and oxidative stress markers between age groups, the microbiota of pre-frail elderly revealed decreased efficiency in carbohydrate degradation (**Chapter 4**&**6**). At the same time, the oro-caecal transit time of elderly is longer comparing to that of adults [13], and no significant differences between frail and non-frail elderly in transit time were found [12]. Therefore, increased transit time could have partially compensated for the decreased metabolic efficiency, or vice versa. The balance between increased transit time and decreased metabolic activity in the microbiota of elderly, as well as to what extent the transit time is associated to microbiota functionality remains to be uncovered during ageing, especially for frail and/or comorbid elderly, or elderly under medication. Adding to that, the microbiota could affect non-antibiotic drug metabolism and its curative effects [51]. Specifically, Zimmermann *et al*. revealed remarkable and individual-specific contributions of the microbiome to the efficiency of drug treatments [52]. In reverse, aside from well-known effects of antibiotics on intestinal microbiota, the use of non-antibiotic drugs was shown to affect the microbiota composition as well as their metabolic function [53]. Notably, frail and/or comorbid elderly have a high prevalence in non-antibiotic drug use, as such being prone to the effects of these drugs on their microbiome. In reverse, altered microbiota composition and/or functionality could impact on the transformation of ingested drugs as well as their efficacy [54]. Future research could pay attention to non-antibiotic drugs and investigate the drug-microbiome interactions in subgroups of elderly, as well as investigating methods to manipulate the microbiota with the goal to increase treatment efficiency.

Aside from non-antibiotic drugs, elderly was found to use 50% more antibiotics per capita compared to adults [55]. Concurrently, the intake of antibiotics is associated with side effects, for instance increased fungal growth [56]. While research described in this thesis only focused on the bacteria and methanogenic archaea, other microorganisms like fungi (the mycobiome) as well as viruses (the virome), including prokaryotic viruses (the phageome), could play an important role in human health, [57]. Studies focusing on the mycobiome, however, still face different methodological challenges, similar to those that apply to microbiome research in general [58]. For instance, it is still challenging to separate fungal genomes from the genomes of predominant bacteria and archaea [59], especially considering the low abundance (0.1% total microbiome) of fungi in the human intestine [60]. As for the virome/phageome, mostly comprising bacteriophages [61], recently a few studies shed light on the human intestinal virome [62], demonstrating high stability and strong individuality [63]. Nevertheless, the role of the virome in shaping the microbiome [64] as well as in human health remains largely to be explored, especially in well-defined subgroups of subjects, like frail and comorbid elderly. Furthermore, considering the great implication of fungal disease, *i.e.* high mortality, intestinal mycobiome studies in comorbid elderly or elderly before and after antibiotic therapy, could ultimately help us gain insights into the host-fungal interaction.

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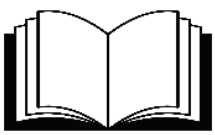
Summary



Summary

Research described in this thesis aimed to decipher the composition and function of the intestinal microbiota in adults, especially elderly, as well as the response of their microbiota to different carbohydrate supplementations and how this is related to health. In **Chapter 1**, I provided a brief overview of the current knowledge about human intestinal microbiota and pointed out the motivation for this thesis. **Chapter 2** dived into the literature and provided a detailed description of changes in the intestinal microbiota in the ageing population, highlighting the importance of health status. Based on the available literature, we hypothesised that observed changes in intestinal microbiota are not based on chronological ageing, but alterations in health status during ageing.

In **Chapter 3**, we observed high similarity in the microbiota of healthy adults and that of healthy elderly, aside from a few genera that differed in relative abundance. Therefore, this study supported the first part of the hypothesis that alteration in intestinal microbiota is not based on chronological ageing. To address the importance of the health status, **Chapter 4** compared the microbiota of healthy adults and pre-frail elderly and revealed significant differences between age groups with the relative abundance of *Bifidobacterium* being lower in elderly. Remarkably, immune and oxidative stress markers of recruited pre-frail elderly included in the study described in **Chapter 4** did not differ from those of healthy adults. In contrast, as shown in **Chapter 6**, the microbiota of pre-frail elderly differed from that of healthy adults in carbohydrate degradation efficiency and metabolite production based on a subgroup of subjects in **Chapter 4**. Accordingly, these studies reinforced the need for better biomarkers for health decline in elderly, and future studies are needed to uncover the contribution of health status to microbiota composition and functionality in the ageing population.

Dietary interventions are often used in studies aiming to change microbiota composition. In **Chapter 3**, the supplementation of sugar beet pectin, however, did not alter faecal microbiota composition or microbial diversity in healthy adults and healthy elderly. In **Chapter 4**, the supplementation of GOS changed the microbiota of healthy adults and pre-frail elderly with significant increases in the relative abundance of *Bifidobacterium* and decreases in microbial diversity in both age groups. These changes did not result in alterations in faecal and breath metabolites, nor in immune and oxidative stress markers. This could attribute to the fact that recruited pre-frail elderly are still healthy and hence leave little room for improvements. Using *in vitro* incubation experiments described in **Chapter 6**, we found that a large number of microbes changed in their relative abundance in response to different carbohydrates, with altered genera depending on the type of carbohydrate. Moreover, the increase in the relative abundance of *Bifidobacterium* was most pronounced in the microbiota of adults, compared to pre-frail elderly.

Although faeces are generally considered being representative for colonic microbiota, considering the physiological changes along the GI tract, studies taking into account spatial gradients are warranted. In **Chapter 5**, we identified significant differences between the microbiota of the small intestine and that of faeces. Moreover, the microbiota composition changed gradually from upper, middle to lower sections of the small intestine. Aside from

decreased microbial diversity in duodenum, two weeks of synbiotic supplementation did not alter overall microbiota composition in the small intestine and faeces, whereas within a day, the synbiotic ingestion induced remarkable transient changes in small intestinal microbiota composition.

Finally, in **Chapter 7**, I discussed all findings in this thesis in detail and highlighted remaining challenges and perspectives for future research.

Appendices

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Doing a PhD, different people may have different understandings. People may say “it is a training process to be an independent researcher” or “it is chance to systematically develop yourself and, in this process, get to yourself better (although, *i.e.* get to know yourself, can be a lifelong question)”. No matter which definition you believe, getting the PhD is something worth to be proud of. With this, I want to thank my promotor Hauke and co-promotor Erwin for giving me the chance to start this journey. Dear **Hauke**, it has been great to have you as my promotor. Your way of thinking always enlightens me and extends my understanding of scientific outcomes. You are always kind and eager to help. I am impressed by your warm heart. In the time I am lost in minutiae or distracted by different research questions, you can always guide me back to the right route. In addition to that, by the time I am looking for new positions, you shared your experience and giving me suggestions, which helped me to make the right decision. Dear **Erwin**, I am lucky to have you as my supervisor. You have always been available when I need your help. In the past years, you not only guided me in science but also in the real life. We have travelled many times together to the CCC meetings. On the way to those meetings, I talked many confusions in my life, and you give me many suggestions and advice! They did help me a lot to understand the world and especially understand myself and ultimately make the right decision or at least trying in the way towards it. You are my mentor not only in academia but also in the real life.

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Finding out a research question is one thing but figuring out how to do it is another thing. In the past a few years, many works wouldn't have been feasible without the technician team. With this I want to thank you, **Ineke**, **Steven**, **Laura**, **Ton, Merlijn, Hans** and **Sjon**. Dear Ineke, you were always there when I faced problems (my precious small intestine samples), thank you for your time and patience. Also I want to thank you for your hug when I was down in 2019. Dear Steven, without you, my *in vitro* fermentation study (and many more other projects) would have been much harder. I still remember in July 2019 you helped to fix the anaerobic chamber super early in the morning and saved my day! Great thanks for your help. Dear Laura, by the end of 2019, I was super stressed with my samples to send them to the sequencing company but didn't have enough time. By then, you offer to help me with those samples which really release my stress.

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To the end, I would like to thank my beloved **Xiao Xue** for your support over the past years! Without your support, I would not be able to get through it. In the coming years, let's experience and feel the world together.

Ran An

2020 12 10

About the author

Ran An was born in 1990 in Shandong, China. After graduating from Northwest Agriculture and Forestry University in China in 2014, she continued her study in Wageningen University and Research, specialized in the Food Biotechnology. She did her major thesis in the lab of Food Microbiology (WUR), followed by a joint internship in Danone Nutricia research and the lab of Microbiology (WUR). Her internship was about dietary intervention on the early life gut-brain axis, focusing on the microbiome side. Directly after gaining her master in 2016, she started as a PhD candidate under the supervision of Erwin Zoetendal and Hauke Smid in the lab of Microbiology, in Wageningen University and Research. In her PhD time, she continued her work in microbiome, but focused on the microbiome of adults especially the elderly. Currently, Ran works as a postdoc together with Clara Belzer and Jan Knol. Thereby she is involved in early life microbiome research again.

List of publications

1 **Ran An***, Wilms, E.*, Masclee, A. A., Smidt, H., Zoetendal, E. G., & Jonkers, D. Age-dependent changes in GI physiology and microbiota: time to reconsider? *Gut*, **2018**, 67(12), 2213-2222

2 **Ran An***, Wilms, E.*, Agnieszka Smolinska, Gerben D.A. Hermes, Ad A.M. Masclee, Paul de Vos, Henk A. Schols, Frederik J. van Schooten, Hauke Smidt, Daisy M.A.E. Jonkers, Erwin G. Zoetendal and Freddy J. Troost. Sugar beet pectin supplementation did not alter profiles of fecal microbiota and exhaled breath in healthy young adults and healthy Elderly. *Nutrients*. **2019**;11(9):2193

3 Kirsten Szklany, Harm Wopereis, Cindy de Waard, Thecla van Wageningen, **Ran An**, Kees van Limpt, Jan Knol, Johan Garssen, Leon M. J. Knippels, Clara Belzer & Aletta D. Kraneveld. Supplementation of dietary non-digestible oligosaccharides from birth onwards improve social and reduce anxiety-like behaviour in male BALB/c mice. *Nutrition Neuroscience*, **2019**: p. 1-15.

4 Renate Akkerman*, Logtenberg MJ *, **Ran An,** Marco A. Van Den Berg, Bart J. de Haan, Marijke M. Faas, Erwin Zoetendal, Paul de Vos and Henk A. Schols. Endo-1,3(4)-beta-glucanase-treatment of oat beta-glucan enhances fermentability by infant fecal microbiota, stimulates dectin-1 activation and attenuates inflammatory responses in immature dendritic cells. *Nutrients*, **2020**. 12(6).

5 Logtenberg MJ *, Renate Akkerman*, **Ran An**, Gerben D. A. Hermes, Bart J. de Haan, Marijke M. Faas, Erwin G. Zoetendal, Henk A. Schols, and Paul de Vos. Fermentation of chicory fructo-oligosaccharides and native inulin by infant fecal microbiota attenuates pro-inflammatory responses in immature dendritic cells in an infant-age-dependent and fructan-specific way. *Molecular Nutrition & Food Research*, **2020**. 64(13): e2000068.

6 Logtenberg MJ, Jolien C.M. Vink, Renske M. Serierse, **Ran An**, Gerben D. A. Hermes, Hauke Smidt, Henk A. Schols. Pooled faecal inoculum can predict infant fiber fermentability despite high inter-individual variability of microbiota composition. *Bioactive Carbohydrates and Dietary Fibre*, **2020**: 100235

7 Van Trijp MP, Rösch C, **Ran An**, Keshtkar S, Logtenberg MJ, Hermes GD, et al. Fermentation Kinetics of Selected Dietary Fibers by Human Small Intestinal Microbiota Depend on the Type of Fiber and Subject. *Molecular Nutrition & Food Research*. **2020**:2000455**.**

8 Wilms, E*, **Ran An***, Agnieszka Smolinska, Yala Stevens, Antje R. Weseler, Montserrat Elizalde, Marie-José Drittij, Athanasia Ioannou, Frederik J. van Schooten, Hauke Smidt, Ad A.M. Masclee, Erwin G. Zoetendal, Daisy M.A.E. Jonkers. Galacto-oligosaccharides supplementation in prefrail elderly and healthy adults increased faecal bifidobacteria,but did not impact immune function and oxidative stress. *(submitted for potential publication to Clinical Nutrition).*

9 Melany Rios-Morales; van Trijp MP; Christiane Rösch; **Ran An**; Theo Boer; Albert Gerding; Naomi de Ruiter; Martijn Koehorst; M. Rebecca Heiner-Fokkema; Henk A. Schols; Dirk-Jan Reijngoud; Guido J.E.J. Hooiveld; Barbara Bakker. A toolbox for the complete analysis of human intestinal content obtained by gastrointestinal sampling capsules. *(submitted for potential publication to Scientific Reports).*

10 **Ran An**, Wilms, E, Logtenberg MJ, van Trijp MP, Henk A. Schols, Ad A.M. Masclee, Hauke Smidt, Daisy M.A.E. Jonkers and Erwin G. Zoetendal. The faecal microbiota of pre-frail elderly has a less efficient metabolic capacity for typical bifidogenic carbohydrates compared to that of adults. *(manuscript in preparation).*

11 **Ran An**, Wilms, E, Jacoline Gerritsen, Celia Seguí Pérez, Isolde Besseling-van der Vaart, Daisy M.A.E. Jonkers, Ger T. Rijkers, Willem M. de Vos, Ad A.M. Masclee, Erwin G. Zoetendal, Freddy J. Troost, Hauke Smidt. Tracing the effects of ingested probiotics in combination with short-chain fructo-oligosaccharides through longitudinal sampling from different regions of the small intestine. *(manuscript in preparation).*

* These authors contributed equally to the work

Overview of completed training activities

Discipline specific activities	Organizing institute (s)	Year
Summer Course Glycosciences	VLAG	2016
KNVM fall meeting	KNVM	2016
GUT DAY	Maastricht University & gut flora fundation	2016
The intestinal microbiome and diet in human and animal health	VLAG	2017
KNVM Spring meeting	KNVM	2017
GUT DAY	University College Roosevelt	2017
INRA meeting	University of Aberdeen	2018
GUT DAY	Wageningen University	2018
KNVM Spring meeting	KNVM	2019
Metagenomics, metatranscriptomics, and multi'omics for microbial community studies	Physalia Courses	2019
Summer school on Microbial Community Modelling	KU Leuven	2019
NWO-CCC symposiums	CCC	2016-2020
General courses	**Organizing institute (s)**	**Year**
VLAG PhD week	VLAG	2016
Competence Assessment	WGS	2016
Efficient Writing Strategies	WGS	2017
Teaching and supervising Thesis students	WGS	2017
Project and time management	WGS	2018
Reviewing a Scientific Paper	WGS	2018
Presenting with impact	WGS	2019
Scientific Integrity	WGS	2019
Mixed Linear Models	PERC & Wimek	2019
Multivariate Analysis	PERC & Wimek	2020
Optionals	**Organizing institute (s)**	**Year**
Preparation of research proposal	VLAG	2016
CSA-50306 Ecological Modelling and Data Analysis in R	WUR	2016
PhD Trip 2017 Lab. of Microbiology	Lab. of Microbiology	2017
PhD Trip 2019 Lab. of Microbiology	Lab. of Microbiology	2019
AIO/Postdoc/PhD meeting	Lab. of Microbiology	2016-2020

VLAG: Graduate School for nutrition, food technology, Agrobiotechnology and Health Science; CCC: Carbohydrate Competence Center; WGS: Wageningen graduate school; KNVM: Koninklijke Nederlandse Vereniging voor Microbiologie; PERC: The Graduate School for Production Ecology & Resource Conservation; WUR: Wageningen University and research.

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