Role of nutritional interventions and aging in intestinal health

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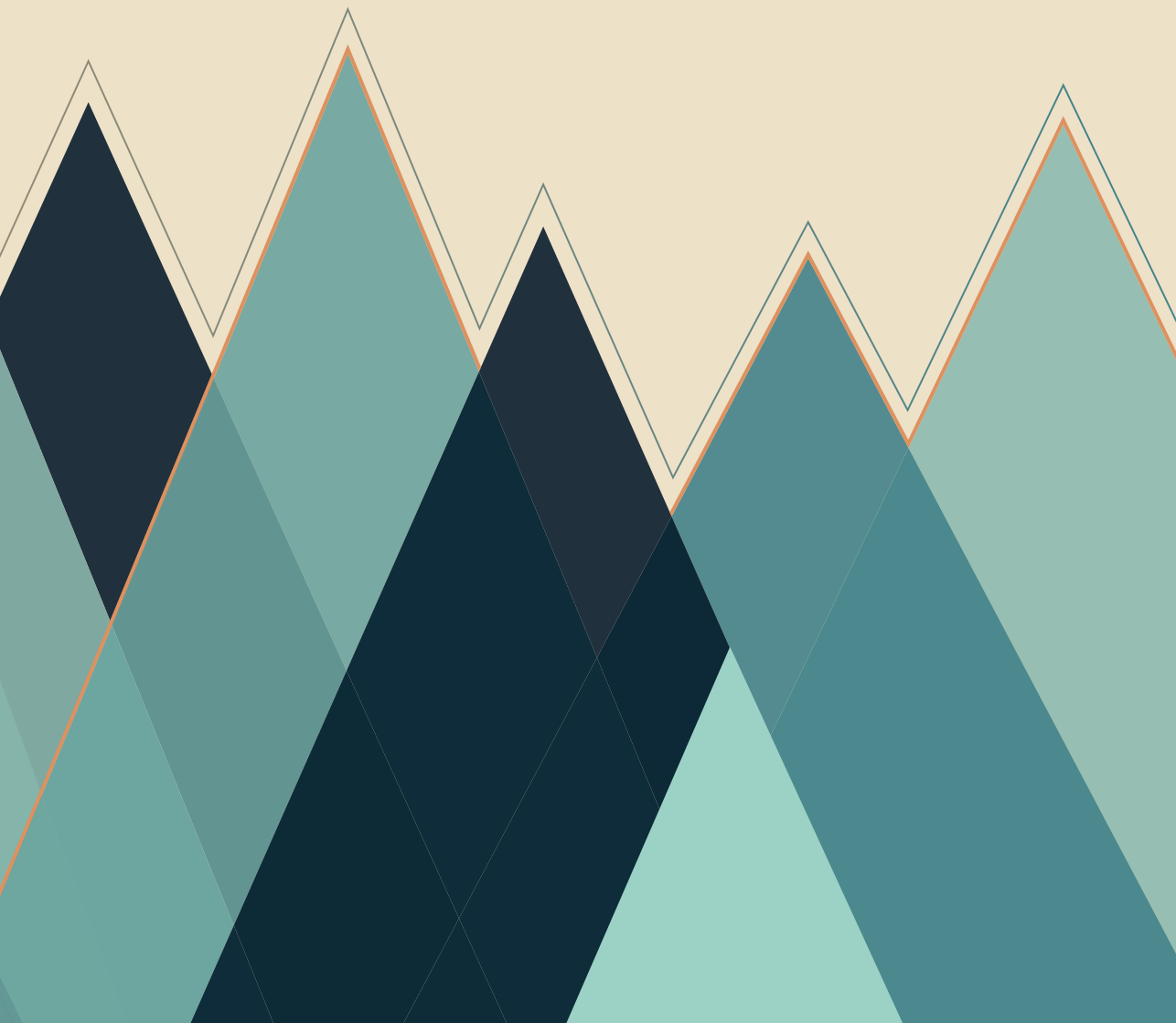
Ellen Wilms



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Role of nutritional interventions and aging in intestinal health

PROEFSCHRIFT

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Voor mam

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Chapter 1

General introduction

Aging

The aging population is growing, being a big challenge for society. Population aging is the result of a declining birth rate together with an increase in overall life expectancy, mainly due to decreased mortality by improved health care. Although geographical differences exist, this trend is observed worldwide (1). It was estimated that the number of individuals aged 65 years and over increases from 8.5% of the total population in 2015 to 16.7% in 2050 (1). Biological aging is associated with a decreased physiological resilience in response to stress, as a consequence of lifelong molecular and cellular stress, contributing to age-related conditions such as frailty and (co)morbidity (2, 3). Therefore, aging imposes a serious burden on the health care system and has economic consequences in terms of increasing health care costs (1). In the Netherlands, the absolute number of individuals over 65 years of age and the costs associated with care for the elderly, are projected to increase substantially in the next 20 years (Figure 1.1) (4).

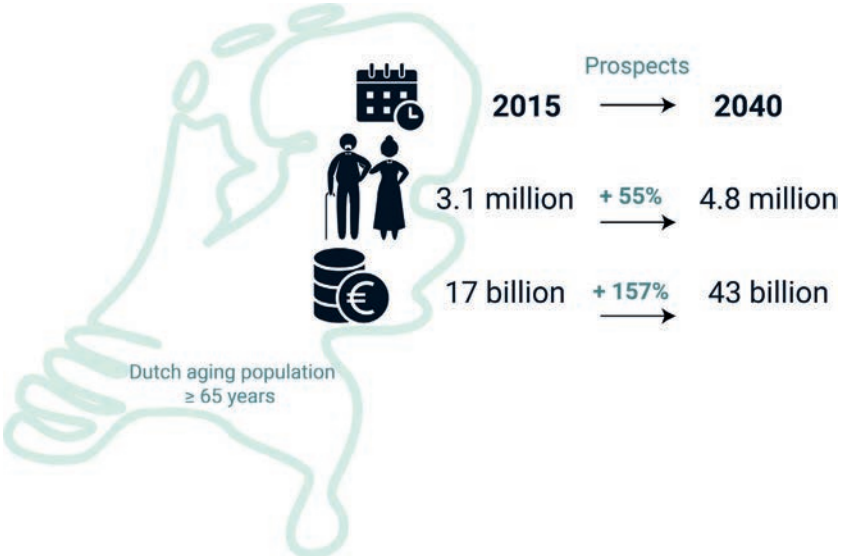

Figure 1.1 - Dutch aging population and elderly care costs prospects (numbers based on Hilderink & Verschuuren (4)).

Various factors such as host genetics (5), psychosocial factors (*e.g.* living situation, social support) (6) and lifestyle (*e.g.* diet, physical activity) (7) have been shown to impact the aging process, and are instrumental to the heterogeneous character of the elderly population. The relative contribution of these factors still remains to be established. Nevertheless, nutritional interventions are widely considered to improve and/or maintain healthy aging, thereby contributing to quality of life in elderly populations.

Intestinal health

Intestinal health is increasingly considered as an important target to improve overall health and well-being. Although a clear definition is lacking, one might define intestinal health as a state of physical and mental well-being in the absence of intestinal complaints and/or diseases (8). On one hand, intestinal function comprizes an effective digestion and absorption of food, but also an adequate intestinal barrier and immune function, as well as normal and stable intestinal microbiome are suggested to contribute to intestinal health (9-12).

Intestinal barrier function

The intestinal barrier has an important protective function against the external environment and consists of various components, as shown in figure 1.2A. From the luminal side the intestinal barrier includes *e.g.* the commensal intestinal microbiota, the mucus layer, anti-microbial peptides (AMPs) and secretory immunoglobulin A (sIgA) (11, 13). The mucus layer is composed of glycosylated mucin proteins, thereby forming a viscous structure to protect the intestinal epithelial cells (IECs) (11). AMPs (*e.g.* defensins and cathelicidins) and sIgA have antimicrobial properties and can prevent microbes from reaching and penetrating the IECs (13, 14). The intestinal mucosa further consists of a single layer of IECs including enterocytes, goblet cells, Paneth cells (in the small intestine) and neuroendocrine cells (15). IECs play an important role in nutrient absorption and secretion of *e.g.* mucus, antimicrobial peptides, digestive proteins and hormones (15). IECs form a barrier to prevent permeation of potentially noxious substances, but allow selective transport by two routes (Figure 1.2A). First, transcellular transport comprizes mainly the absorption of nutrients, a process which is actively and passively regulated by

transporters and ion channels on the cell membranes (16). Second, paracellular transport involves the passive passage of solutes through the intercellular space (*i.e.* between adjacent IECs), and is primarily regulated by tight junctions (TJs) and adherence junctions (AJs) as part of the junctional complex (16) (Figure 1.2B). TJs are multi-protein complexes of transmembrane proteins (*e.g.* occludin and claudins), which interact with the intracellular actin and myosin cytoskeleton via peripheral membrane proteins (*e.g.* zonula occludens) (10, 16). TJs can be regulated *e.g.* by myosin light chain kinase (MLCK) (10). Activation of MLCK leads to phosphorylation of myosin II regulatory light chain in the actomyosin ring (17). As a result, the TJs architecture can show either disruption (18) or dislocation and thereby opens the barrier (19). While TJs prevent passage of potential harmful substances, AJs (*e.g.* cadherins) and desmosomes maintain adhesive bonds between IECs and facilitate intercellular communication (16) (Figure 1.2B). An adequately functioning intestinal barrier protects the host from potential noxious substances (*e.g.* microbes and toxins). Impairments in barrier function may lead to permeation of these substances from the lumen into the lamina propria, activating the mucosal immune system (16). The intestinal immune system comprises a large number of immune cells including amongst others macrophages, dendritic cells and T-cells (Figure 1.2A). Many immune cells, especially of innate immunity, but also IECs contain pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) to recognize pathogen-associated molecular patterns (PAMPs) (20). Recognition of microbes by TLRs can lead to a variety of signal transduction pathways resulting in the production of pro- and anti-inflammatory cytokines (21).

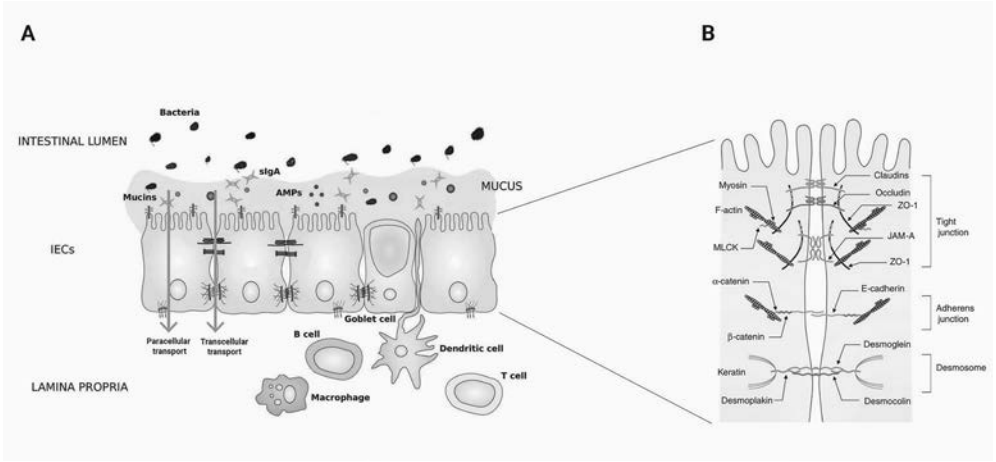

Figure 1.2 - Representation of key components related to intestinal barrier function. A. Bacteria reside in the intestinal lumen as well as in the mucus layer. The mucus layer covers the intestinal epithelial cells, is composed of gel-forming mucins, and contains substances like antimicrobial peptides (AMPs) and secretory immunoglobulin A (sIgA). Adjacent intestinal epithelial cells form a selective barrier and allow passage of *e.g.* nutrients for paracellular or transcellular transport. Impaired intestinal barrier function leads to passage of potential noxious substances which may activate immune cells (*e.g.* T-cells, macrophages, dendritic cells) located in the lamina propria. B. Molecular structures of the intercellular junctional complex between intestinal epithelial cells including tight junctions, adherence junctions and desmosomes. Adapted by permission from Vancamelbeke & Vermeire (11) and Suzuki (16).

Intestinal barrier function can be measured by intestinal permeability *in vivo* via oral ingestion of small size molecules (*e.g.* sucrose, L-rhamnose, mannitol, erythritol), larger size molecules (*e.g.* lactulose, sucralose, high molecular weight polyethylene glycol), or radio-labeled probes (^{51}Cr-Ethylenediaminetetraacetic acid), which can be determined in blood or urine (22). Van Wyck *et al.* (23, 24) have developed a multi-sugar test to determine functional segment-specific as well as whole intestinal permeability. Measuring urinary excretion ratios of large/small molecules (*e.g.* lactulose/L-rhamnose for small intestinal permeability) enables correction for potential differences in for example transit time or renal clearance. Other methods to determine intestinal barrier function include e*x vivo* intestinal permeability analyses by Ussing chamber experiments. This requires invasive methods to collect biopsy or surgical specimens, and allows for electrical as well as paracellular flux measurements to determine intestinal integrity while taking genetic host susceptibility into account (22, 25). Moreover, diverse stressors to increase intestinal permeability can be applied in these experiments, which is relevant in intervention studies focusing on prevention of or strengthening impaired barrier function. Biopsy or surgical specimens can also be used for complementary TJs or AJs protein- or gene expression analyses as well as

underlying signaling pathways, thereby providing more mechanistic insights. In addition, interest is growing in biomarkers of intestinal barrier function, such as serum zonulin (26), plasma or urinary intestinal fatty-acid binding protein, and plasma citrulline (22). Impaired intestinal barrier function, including intestinal epithelial disruption and immune activation, has been suggested to be associated in the pathophysiology of several intestinal diseases like inflammatory bowel syndrome (IBS) and inflammatory bowel diseases (IBD) as well as in metabolic diseases such as type 1 diabetes (11, 12, 27, 28).

Intestinal microbiota

The intestinal microbiota provides colonization resistance, thereby contributing to the intestinal barrier function. The intestinal microbiota plays also an important role in the development and well-functioning of the epithelial barrier and immune system (29). In humans, the intestinal microbiota is composed of a complex community of microbes, with highest numbers (*i.e.* up to 10^{12} cells/g of luminal content) in the colon (30). In healthy adults, the bacterial ecosystem is dominated by Bacteroidetes and Firmicutes, and smaller abundances of Actinobacteria, Proteobacteria and Verrucomicrobia (31). At the phylum level, the intestinal microbiota shows large overlap between individuals. At species or subspecies (*i.e.* strain) level, however, there are large inter-individual differences, which is reflected in a distinctive microbiota composition, comparable to the uniqueness of a fingerprint (31). It has been estimated that the human intestinal microbiota of an individual consists of 300-500 different species (30). The intestinal microbiota plays an important role in maintaining intestinal homeostasis, *e.g.* by regulating the maturation of intestinal epithelium and the immune system, and by its large metabolic capacity which results in the production of vitamins, and causes conversion in metabolism of bile salts and xenobiotics (31, 32). Bacterial fermentation of undigested carbohydrates results in the production of short-chain fatty acids (SCFAs) including acetate, propionate and butyrate. As the microbiota prefers carbohydrate over protein fermentation, SCFAs are mostly produced in the proximal colon and important for intestinal health by serving as energy substrate for the epithelium, reinforcement of the epithelial barrier, as well as having amongst others anti-inflammatory and anti-oxidative effects (30). In contrast, protein fermentation (*i.e.* in general more pronounced in the distal colon) results in the production of merely toxic metabolites (33).

In the last decade, technological developments boosted the insights into the intestinal microbiome in health and disease by application of next generation sequencing approaches based on the 16S rRNA gene, proteomics and metabolomics, as well as advanced computational techniques such as principal component analysis, discriminant and redundancy analyses. Findings have led to the emerging importance of investigating the activity of those microbes in addition to the composition. In this context, the term microbiome refers to the collection of all genomes of microbes with an ecosystem, while microbiota are the microbes hat collectively inhabit a specific ecosystem (31). Whereas, the intestinal microbiota composition differs largely between individuals, a rather stable functional core has been found in the healthy individuals (34). The molecular function of the intestinal microbiota is still largely unknown. Up to 50% of the microbial gene families remain functionally uncharacterized (34). In addition to host genetics, multiple environmental factors such as lifestyle and medication use have been found to influence both the human intestinal microbiota composition and activity (31). By investigating many of these factors (*i.e.* related to lifestyle, medication use and health status) in a large population-based cohort, Zhernakova *et al.* (35) showed these together explained 18.7% of the variation in the microbiota composition. In addition, several studies link perturbations in the intestinal microbiota composition and -activity to a wide variety of diseases (12, 31), whereas the exact underlying mechanisms remain to be elucidated. Still, the intestinal microbiome is considered a promising target as it clearly links to many host functions, and rapidly responds to major changes in dietary intake (*e.g.* high-fat diet versus high-fiber diet) (36, 37).

Intestinal health in aging

Adequate nutrient digestion and absorption together with a well-functioning intestinal barrier, immune function and microbiome, are considered key features of intestinal health, which can impact the gastrointestinal tract as well as extra-intestinal organs. Thereby, proper intestinal functioning contributes to healthy aging, and may prevent and/or delay health impairments (*e.g.* frailty). Age-related declines in intestinal physiology can induce symptoms and (co)morbidity, leading to impaired quality of life (38). Age-related changes in GI functions, such as (digestive) secretions and absorption, are however not well described. A limited number of studies that investigated the impact of

aging on the intestinal barrier has shown contrasting results. Animal studies indicated that older animals have an impaired intestinal barrier function compared with young animals, reflected by an increased paracellular permeability (39-41). However, previous human studies investigating the intestinal barrier of specific intestinal segments (*i.e.* small intestine or colon), showed no differences in intestinal permeability between age groups (42-45). Furthermore, the effects of aging on the intestinal microbiota have been studied in several cohorts and intervention studies. Most of these studies focus on intestinal microbiota composition, showing changes during aging. However, the reported perturbations varied widely, possibly due to differences in study populations (*e.g.* ethnicity, age, health status) as well as the applied methods to analyze the intestinal microbiota. Despite the current lack of consensus on the importance of intestinal barrier function in age-associated health concerns, further insight in possible changes in intestinal barrier function, immune function and the intestinal microbiota in the aging population in general and or (pre)frail elderly in particular, may aid to identify targets for interventions in the prevention of infections and age-related diseases.

Probiotics and prebiotics

A strategy to improve health in aging populations is via dietary interventions using probiotics and prebiotics (46). According to the most recent consensus definition, probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the hosts (47). This can be achieved by alterations in the intestinal microbiota (*e.g.* by competition for nutrients, antagonism and cross-feeding), but also via interactions with epithelial cells and immune cells (46). Widely used probiotics include strains of *Lactobacillus*, *Bifidobacterium* and *Saccharomyces*, but also some *Akkermansia*, *Roseburia* and *Faecalibacterium* species are suggested as promising probiotics (46). The effects of probiotics on intestinal barrier function are mostly studied in intestinal cell lines *in vitro*. For example, some *Lactobacillus* and *Bifidobacterium* strains have been shown to increase transepithelial electrical resistance and expression of several TJ proteins, and thereby may have the potential to improve intestinal permeability *in vivo* (48). It should be noted that probiotic effects are strain- and/or species specific. As a result, probiotics can be administered as single strains, but can also be combined in mixed products to target multiple mechanisms.

Prebiotics, which are predominantly carbohydrate-based, are defined as substrates that are selectively utilized by host microorganisms conferring a health benefit (46, 49). Prebiotics are fermented by intestinal microbes, resulting in increased bacterial growth and functionality (*i.e.* SCFAs production),contributing to defense against pathogens, immune modulation, improved bowel function and/or improved intestinal barrier function (46). Extensively studied and classified prebiotics are inulin-type fructans (*i.e.* inulin and fructo-oligosaccharides (FOS)) and galacto-oligosaccharides (GOS) (50). Inulin-type fructans are polysaccharides consisting of fructose with β-(2,1) fructosyl-fructose linkages, and are mostly derived from plant extracts (51). GOS is composed of galactose polymers with a terminal glucose monomer, produced by enzymatic transferase activity of β-galactosidases on lactose (50). Both inulin-type fructans and GOS resist hydrolysis by human small intestinal digestive enzymes and are rather easily fermentable and will mainly be metabolized in the proximal colon. More complex dietary fibers such as pectins and cellulose are candidate prebiotics based on the demonstrated impact on the microbiota, but health benefits are less well-studied (46). Pectins are especially interesting as they are composed of galacturonic acid, of which the residues are substituted with methyl esters at the C6-carboxyl group and rhamnogalacturonan (52). Various bacterial enzymes are needed for the fermentation of pectin, and is therefore suggested to delay it's fermentation rate. Prolonged saccharolytic fermentation towards the distal colon may reduce proteolytic fermentation, including a reduced production of potentially toxic metabolites (53).

Despite the anticipated prebiotic effects of several dietary fibers, it is important to consider that potential prebiotic effects may be source, dose and host specific (54). Additionally, they can be given as synbiotics, which is a combination of probiotics and prebiotics (55). In the past decades, much effort has been put in identification and selection of probiotics and prebiotics, mainly by *in vitro* and animal studies. To date, the number of well-designed studies investigated the effects of probiotics and prebiotics on intestinal barrier function and immune function *in vivo* in elderly is limited (56, 57). In a recent review, Sanders *et al.* (46) concluded that more high-quality randomized controlled human trials are needed to test potential health effects of these probiotics and prebiotics, and improve translation from *in vitro* and animal studies to the clinic.

Aim and outline of the thesis

The aging population is growing worldwide, posing an increasing burden on health care system and society. Further insight into modifiable factors to improve intestinal health are highly relevant. The aim of this thesis was to investigate the impact of aging and nutritional interventions (*i.e.* synbiotics and (candidate) prebiotics) on intestinal health, with a strong focus on intestinal barrier function and the intestinal microbiota, as human studies in these areas are limited. Figure 1.3 shows a non-exclusive overview of the topics addressed in this thesis, with the corresponding chapters.

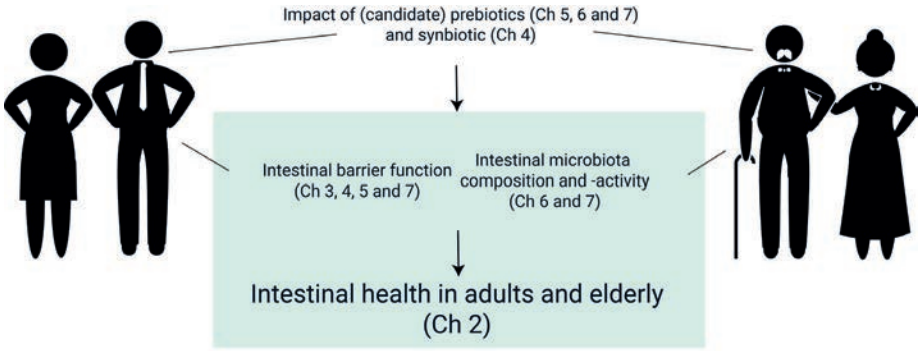

Figure 1.3 - Overview of the topics as presented in this thesis, and the corresponding chapters.

In **Chapter 2**, the existing evidence on the effects of human aging, and contributing factors such as frailty and medication use on gastrointestinal physiology and on the intestinal microbiota have been summarized. Although evidence is limited and largely based on animal research, intestinal barrier function is often reported to be impaired in the elderly. Therefore, the effects of aging on intestinal barrier function, as investigated in healthy subjects and irritable bowel syndrome patients by combined *in vivo* and *ex vivo* experiments, are described in **chapter 3**. To investigate the modifiable potential of the intestinal barrier, we evaluated the impact of two weeks synbiotic supplementation (*i.e.* multispecies probiotic mixture with FOS) on intestinal barrier function in healthy adults in unstressed and unstressed conditions in **chapter 4**. In the subsequent studies we investigate the effects of two different (candidate) prebiotics in randomized controlled trials in both adults and elderly. Pectin was selected as a complex dietary fiber, thereby assuming more distal colonic

fermentation. The effects of four weeks pectin supplementation on intestinal barrier function, and on profiles of the fecal microbiota and exhaled breath, have been presented in **chapters 5 and 6,** respectively**.** Frailty seems to play an important role in age-related changes in immune functioning and the intestinal microbiota composition, and preventive strategies are warranted. We describe an intervention study on the impact of four weeks GOS supplementation on immune- and microbial parameters in pre-frail elderly and healthy adults in **chapter 7**. To conclude, **Chapter 8** summarizes the main findings of the studies presented in this thesis, and discuss the outcomes in terms of potential implications for future research.

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Chapter 2

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 aging population. Aging 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 aging. Since aging 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 aging and frailty, how these are associated, and how intestinal microbiota-targeted interventions may counteract these changes.

Introduction

Although geographical differences exist, the overall life expectancy is increasing worldwide, leading to a steady rise in the aging 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). Aging 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 aging is decreasing, and cut-offs as well as age definitions vary widely. In scientific research and 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 operationalized 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, which is 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 aging population. Nutritional interventions, aiming to modulate intestinal microbiota composition and functionality, may contribute to intestinal health and general well-being (of the aging population). Therefore, our aim was to review 1) the current knowledge on the effect of aging on gastrointestinal (GI) physiology and on intestinal microbiota, 2) whether other factors besides aging, such as frailty, affect GI physiology and intestinal microbiota, and 3) potential targets to counteract the changes in GI physiology and intestinal 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 aging and frailty will be summarized, paying specific attention to age-definitions and associated health status.

Physiology and function of the aging 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 2.1).

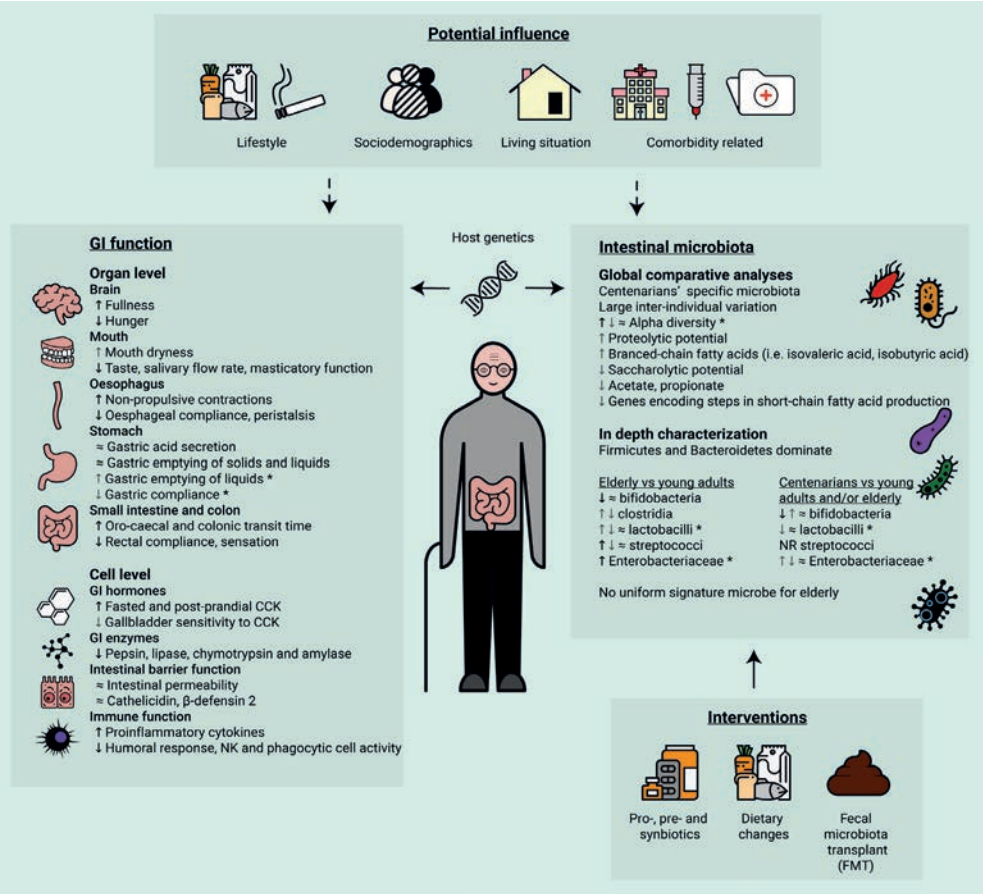

Figure 2.1 - Non-exclusive listing of key changes in gastrointestinal function and the intestinal microbiota during aging, including potential influencing factors as well as interventions to beneficially manipulate the intestinal microbiota. * Frailty related; ≈ No significant difference; NR Not Reported; ↑ 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.

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. 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

aging, 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. 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 aging 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-cecal 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-cecal and colonic transit time than healthy young adults (22). The decreased rectal compliance and decreased rectal sensation associates with healthy aging, and may contribute to complaints such as constipation (18). Several studies have provided evidence for alterations in motility associated with healthy aging, 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.

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 aging 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 aging (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 aging 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 standardized 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 aging 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, aging 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). Although not specifically studied in elderly, factors associated with aging such as non-steroidal 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 fecal 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 aging on the intestinal immune response. It has repeatedly been shown that the human intestinal mucosal immune system is compromized with aging, as nicely reviewed by Mabbott *et al*. (36) Alterations in dendritic cell subsets have been reported in elderly in general. Further, healthy aging is characterized amongst others by a pro-inflammatory cytokine profile ('inflammaging', *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 by 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).

Intestinal microbiota in elderly

Aging 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 harbor distinct microbial communities (39), but determination of the bacterial composition is mostly performed on fecal 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 fecal 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 fecal 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.

Global fecal microbiota comparative analyses

A wide variety of studies have compared the fecal microbiota composition in elderly versus young adults and centenarians. In supplementary table 2.S1, a non-exclusive overview of studies investigating the fecal microbiota profile of elderly is given. Their overall microbiota composition was generally visualized and verified by ordination and multivariate analysis, for example, significant age-group-based separation in diagrams. At phylum level, the fecal 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 fecal microbiota of centenarians, who are reported to have a lower incidence (46) of chronic illnesses than 80-99 years old elderly and considered a "successful" aging 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 20-59yrs (48). The effect of smoking in combination with aging has not been reported to date. Overall, the microbiota of elderly is highly variable (45). Therefore,

it is hard to define a typical microbiota of elderly and that of centenarians.

Observations with regard to the effect of aging 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 2.1). Initially, alpha diversity was found to decline during aging (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 frailty (53, 55, 56) (Table 2.2), which suggests that the health status of elderly rather than aging itself is associated with a lower alpha diversity of the fecal microbiota.

Table 2.1 - Aging associated changes in alpha diversity of fecal microbiota in elderly and centenarians.

Study and country of study cohort (Ref)	Young adults		Elderly		Group being compared	Microbiota profiling[a]	Elderly[b]		Centenarians[c]	
	Nr.	Age range	Nr.	Age range			Diversity	Richness	Diversity	Richness
Zwielehner 2009, Austria (49)	17	18-31y	17	78-94y	Young adults vs institutionalized elderly	PCR-DGGE, qPCR, clone libraries	↓	n.r.	n.a.	n.a.
Biagi 2010, Italy (42)	20	25-40y	43 21	59-78y 99-104y	Young adults vs elderly vs centenarians	Phylogenetic microarray & qPCR	≈	n.r.	↓	n.r.
Kong 2016, China (58)	47	24-64y	54 67	65-83y 90-102y	Nonagenarians and centenarians vs young adults and elderly	Illumina MiSeq	n.r.	n.r.	↑	↑
Biagi 2016, Italy (43)	15	22-48y	15 39	65-75y 99-109y	Young adults vs elderly vs centenarians	Illumina MiSeq	n.r.	n.r.	↑	↑
Wang 2015, China (59)			16 8	80-99y 100-108y	Elderly vs centenarians	Illumina MiSeq	n.a.	n.a.	≈	≈ or ↑[d]
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 2.2 - Summary of studies associating fecal microbiota profiles with frailty.

Study and country of study cohort (Ref)	Young adults Nr.	Young adults Age range	Elderly Nr.	Elderly Age range	Determination of frailty	16S rRNA approach	Main findings with increased frailty
van Tongeren 2005, The Netherlands (62)			23	70-100y	Groningen Frailty Indicator	FISH	↓lactobacilli/ *Enterococcus* group ↓*Bacteroides/Prevotella* group ↓*Faecalibacterium prausnitzii* ↑Enterobacteriaceae
Claesson 2012, Ireland (63)	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 25	71-97y 65-87y	Rockwood Frailty Index	Illumina MiSeq	≈ microbial richness ↑unclassified member of Clostridiaceae 1 family ↑unclassified member of Lachnospiraceae family ↑*Oscillospira, ↑Peptococcus, ↑Porphyromonas* ↑*Fonticella, ↑Prevotella*
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 hybridization; 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.

In depth characterization of fecal microbiota composition

The first paper that associated the dynamics of several bacterial genera during life was published by Mitsuoka (64) in 1990. This culture-based hallmark paper described that in comparison to young adults, the fecal microbiota of elderly comprized 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 aging-associated bacterial groups.

The reduction in the abundance of bifidobacteria in the fecal microbiota of elderly, has been confirmed in many studies (40, 43, 49, 65-67), irrespective of elderly or frailty definitions, though not exclusively (42, 43, 68). Surprisingly, a decreased abundance of bifidobacteria has also been reported in centenarians (*i.e.* 99-104yrs), compared to young adults (67) 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 (66) and in the subgroup of *Clostridium difficile* associated disease (CDAD) elderly (65), 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 aging, 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, 69, 70) or Bacteroidetes (44, 45, 59) is the most dominant phylum, differs between studies.

The number of studies that has reported an effect of aging 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 fecal microbiota of elderly versus young adults (66, 71), comparative analyses between studies is hampered by this continuous reclassification.

The reported increase in abundance of lactobacilli during aging by Mitsuoka *et al.* (64) could not be confirmed in other culture-based studies (65, 66, 72), whereas it was confirmed in several culture-independent studies (40, 71, 73). The abundance of lactobacilli in centenarians did not differ from that of young adults in the above mentioned Italian cohort (67). However, in a Chinese cohort, the abundance of lactobacilli in rural centenarians (consuming a high fiber diet) was not different from that in healthy urban elderly (consuming a low carbohydrate and low fiber diet), but was lower than that in healthy rural elderly (consuming a high fiber diet) (59). Moreover, compared to healthy elderly the abundance of lactobacilli was found to be higher in the subgroup of elderly with CDAD (65, 72) and in hospitalized elderly (66). 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.* (62) found that decreased abundance of lactobacilli was associated with deteriorated health status (increased frailty). 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, 66, 68, 71), 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 (66), whereas NSAID use had no effect (71).

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 fecal samples of healthy elderly than that of young adults from different countries (40, 66). Remarkably, a decreased abundance was reported in centenarians compared to young adults in an Italian cohort (67). 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 (74), a higher abundance of Enterobacteriaceae was found in the subgroup of elderly carrying *Clostridium difficile* compared to *Clostridium difficile* negative elderly (75). Moreover, increased Enterobacteriaceae abundance was associated with increased frailty (53, 62). 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 aging-associated by Mitsuoka (64), indicates that in comparison to young adults, the intestinal microbiota of elderly comprized 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 aging (Table 2.S1). Moreover, studies that included centenarians or different frailty phenotypes (Table 2.2) hint towards the observation that a decline in health status rather than aging itself is associated to changes in intestinal microbiota composition. Comparative analyses are further hampered by the myriad of methods (38, 76, 77) used to study the fecal microbiota composition as well as the lack of consistent definitions of frailty (53, 55, 61-63) and age for elderly (40, 43-45, 59, 62, 66), which could be 80yrs+ (59), 70yrs+ (44, 62), 65yrs+ (43, 45, 66) 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), will provide the ideal setting to study intestinal microbiota dynamics during aging. These studies are obviously long-lasting, adding to complexity, solid scientific infrastructure and costs. Nevertheless, several large scale population cohorts have already been initiated (69, 70). Follow up of such cohorts may ultimately provide insight into long-term intestinal microbiota dynamics

and their relation to aging, frailty and comorbid conditions.

Overall, we clearly observed that intestinal microbiota compositional changes during aging are more likely to be associated with health status of the elderly and confounding factors than with aging itself. This is summarized in Figure 2.1 and Table 2.2 (See also Table 2.S1 for details per study). It has to be taken into account that most studies focusing on the intestinal microbiota in elderly describe the fecal 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 fecal 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 fecal 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 fecal dry matter content and concentrations of branched-chain fatty acids (*i.e.* isovaleric acid, isobutyric 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 fecal metabolites profile, with higher concentrations of acetate, propionate and valerate in community-dwellers (63). A recent study investigated the functional capacity and activity of the fecal 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 (69, 70). It has been reported that short-term dietary changes can have a drastic impact on microbial metabolite production and host physiology without drastically changing fecal microbiota composition (81). Hence, we argue that approaches addressing functional capacity and activity of the fecal microbiota are crucial to further unravel the role of the microbiota in host physiology of the aging and/or frail population.

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 2.1). Several studies have been performed on the effect of probiotics in elderly, of which many focus on the risk of infections and immunosenescence. In a systematic review, including 15 randomized clinical trials in 5916 patients with a mean age of 75 years, Wachholz *et al.* (82) 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.* (83) concluded in a recent review that the evidence for the efficacy of probiotics in elderly is limited and/or inconsistent. Several elderly studies did, however, show changes in fecal 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*. (89) 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 fiber, when compared to soluble corn fiber alone. Furthermore, different pre- and synbiotic food supplements studies have been shown to lead to an increased abundance of fecal 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 to be targeted (84). 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, 90, 91). 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 are ongoing, investigating the effect of major diet changes (Mediterranean diet, 1 year randomized, single-blind controlled trial) on the intestinal health in 1250 elderly subjects (92).

Other strategies to manipulate the intestinal microbiota include fecal microbiota transplantation (FMT). The success rate is variable, and largely depends on the disease or disorder to be treated (93). FMT efficacy is most convincing for treating *Clostridium difficile* infection (93) which has a rather high incidence in the elderly, also because of the high antibiotic use (94). In a specific review focusing on elderly, Cheng *et al.* (95) 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. 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.

Summary and conclusions

Several small, age-related declines in the physiology of the GI tract have been reported for aging in general, whereas frailty and impaired health status seem to play a role in the (further) decline (Figure 2.1). Studies on GI physiology and function in subjects with age-related health decline or being resilient to diseases while aging, *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 aging 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-characterized 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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Supplementary table 2.S1

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Gavini 2001 (1)	15 children (3-15y)	Not reported	Not reported	Not reported	Not reported	Culture	↓*Bifidobacterium* prevalence
France	17 young adults (30-46y)						↓*Bifidobacterium pseudocatenulatum* prevalence
	22 elderly (69-89y)						↑*Bifidobacterium adolescentis* prevalence,↑*Proteus* prevalence, ↑*Citrobacter freundii* prevalence
Hopkins 2001 (2)	10 children (16 months-7y)	Not reported	No gastrointestinal complaints < 2 months	Metronidazole *(4 CDAD elderly)*	Not reported	Culture	↓total anaerobes, ↓*Bifidobacterium*, ↓*Lactobacillus*
UK	7 young adults (21-34 y)		No antibiotics < 2 months			Cellular fatty acid profiling	↑enterobacteria
	5 elderly (67-88y)					16S rRNA abundance measurement	CDAD vs healthy elderly, ↓total bacteria, ↓*Bifidobacterium*
	4 CDAD elderly (68-73y)						CDAD vs healthy elderly, ↑enterobacteria, ↑*Clostridium*, ↑*Lactobacillus*, ↑enterococci
Hopkins 2002 (3)	7 young adults (21-34y)	Not reported	No gastrointestinal complaints < 2 months	Metronidazole *(4 CDAD elderly)*	Not reported	Culture	↓*Bifidobacterium*, ↓*Lactobacillus*
UK	4 elderly (67-88y)		No antibiotics < 2 months			Cellular fatty acid profiling	CDAD vs healthy elderly, ↓ *Bifidobacterium*, ↓*Prevotella*, ↓*Bacteroides*
	4 CDAD elderly (67-73y)						CDAD vs healthy elderly, ↑*Clostridium* diversity, ↑*Lactobacillus* diversity
Bartosch 2004 (4)	35 elderly (63-90y)	Hospitalized elderly consuming hospital diet	Antibiotic & hospitalized elderly: nongut infections	One or multi-antibiotics (21 antibiotic treated elderly)	Not reported	16S rRNA gene-based qPCR	Hospitalized vs healthy elderly, ↓total bacteria, ↓ *Bacteroides-Prevotella* group
UK	38 hospitalized elderly (66-103y)	Community elderly consuming their habitual diet	Hospitalized elderly: no antibiotics < 2 months				Hospitalized vs healthy elderly, ↓*Clostridium clostridiiforme*, ↓*Faecalibacterium prausnitzii*
	21 antibiotic & hospitalized elderly (65-100y)		No gastrointestinal disease history				Antibiotic treated vs healthy elderly, ↑*Enterococcus faecalis*
							Antibiotic treated vs healthy elderly, ↓total bacteria, ↓*Bacteroides-Prevotella* group
							Antibiotic treated vs healthy and hospitalized elderly, ↓*Bifidobacterium*, ↓*Desulfovibrio*
							Antibiotic treated vs healthy and hospitalized elderly, ↓*Clostridium butyricum*, ↓*Clostridium clostridiiforme*
							Antibiotic treated vs healthy and hospitalized elderly, ↓*Ruminococcus albus*, ↓*Faecalibacterium prausnitzii*
							Enterobacteriaceae, healthy elderly ≈ hospitalized elderly ≈ antibiotic & hospitalized elderly

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Woodmansey 2004 (5)	12 young adults (19-35y)	Not reported	No steroids, immunosuppressives, acetylsalicylic acid or motility stimulating drugs	Not reported	Not reported	Culture	↑*Streptococcus*, ↑eubacteria
UK	6 elderly (67-75y)		Not terminally ill, dysphagia, severe dementia			Cellular fatty acid profiling	↓*Bacteroides*, ↓*Prevotella*, ↓*Lactobacillus*
	10 hospitalized elderly (73-101y)		No antibiotics < 8 weeks				↓*Clostridium*, ↓*Bifidobacterium*, ↓number of amylolytic bacteria *Total anaerobes, young adults ≈ healthy elderly ≈ hospitalized elderly* enterobacteria, young adults ≈ hospitalized elderly < healthy elderly Hospitalized vs elderly, ↓*Bifidobacterium*, ↓*Streptococcus* diversity Hospitalized vs elderly, ↑*Lactobacillus*, ↑*Clostridium diversity*, ↑*facultative anaerobes* Level of ammonia, healthy young > healthy elderly > hospitalized elderly
van Tongeren 2005 (6)	23 frail elderly (70-100y) including :	All volunteers were supplied with the same food menu	No antibiotics < 4 weeks	Not reported	Groningen frailty indicator	16S rRNA-based FISH	Increased frailty, ↓*Lactobacillus/ Enterococcus* group, ↓*Faecalibacterium prausnitzii*
The Netherlands	13 low frailty score (1-4)						Increased frailty, ↓*Bacteroides/Prevotella* group
	10 high frailty score (>5)						Increased frailty, ↑*Enterobacteriaceae*
Mueller 2006 (7)	France: 22 young adults (20-50y); 26 elderly (>60y)	No extreme diet (vegan, total fasting or high alcohol)	No antibiotics, sulfonamides, corticoid < 3 months	Not reported	Not reported	16S rRNA-based FISH + flow cytometry	↓*Bifidobacterium* (location independent)
France	Germany: 22 young adults (20-50y); 37 elderly (>60y)		No immunosuppressive agents < 3 months				↑enterobacteria (location independent)
Germany	Italy: 20 young adults (20-50y); 39 elderly (>60y)		No gastrointestinal disorders				*Lactobacillus-Enterococcus*, France (↑), Germany(↑), Italian (≈) and Sweden (≈)
Italy	Sweden: 20 young adults(20-50y); 39 elderly(>60y)		No hypercholesterolemia				*Streptococcus*, Germany (↑), Italy (↓), Sweden (≈), France (≈)
Sweden							*Bacteroides*, Italian (↓), France (↓), Sweden (↓), German (↑)
Collado 2007 (8)	150 infants (1 -12months)	Not reported	Not reported	Not reported	Not reported	16S rRNA-based FISH + flow cytometry	↓*Akkermansia muciniphila*
Finland	54 young adults (25-35y)					16S rRNA gene-based qPCR	
	45 elderly (80-82y)						

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Tiihonen 2008 (9)	14 young adults (21-39y)	Habitual diet	No critical illness, IBD and major malignancies	NSAID *(26 NSAID elderly)*	Not reported	Culture	No significant ageing effect on *Bacteroides, Bifidobacterium, Faecalibacterium prausnitzii* level
Finland	26 elderly NSAID users (70-88y)	Provided meal service at least once per day (most of volunteers)	No probiotic or prebiotic during the study	NSAIDs (> 2 times per week)		Flow cytometry	NSAID elderly vs young adults: ↓total SCFAs, ↓butyric acid, ↓propionic acid
	29 elderly non-NSAID users (68-84y)		No antibiotics < 2 months			16S rRNA gene-based qPCR	NSAID elderly vs young adults: ↓*Clostridium coccoides-Eubacterium rectale*
						16S rRNA-based FISH	NSAID vs non-NSAID elderly: ↓dry matter, ↓isovaleric acid, ↓isobutyric acid ↑isobutyric acid, ↑Isovaleric acid, ↑isocapronic acid ↓propionic acid
Mariat 2009 (10)	21 infants (3 weeks-10 months)	Unrestricted western diet (adult and elderly)	No antibiotics < 3 months (adult and elderly)	Not reported	Not reported	16S rRNA gene-based qPCR	↓*Escherichia coli*
France	21 young adults (25-45y)	Fomula feeding (7 infants)	No drugs influencing the microbiota < 3 months				↑*Clostridium leptum*, ↑*Clostridium coccoides*
	20 elderly (70-90y)	Breast feeding (16 infants)	No metabolic and gastrointestinal diseases				≈ *Bifidobacterium*
			No history of antibiotics (infants)				Firmicutes/Bacteroidetes ratio in infants, adults and elderly respectively (0.4, 10.9 and 0.6)
Zwielehner 2009 (11)	17 young adults (18-31y)	Typical central European diet (young aults)	No antibiotics or chemotherapeutic treatment < 3 months	NSAIDs (elderly, on demand)	Not reported	PCR-DGGE	Institutionalized elderly: ↓total bacteria, ↓bacterial diversity, ↓*Bifidobacterium*, ↓*Clostridium cluster* IV
Austria	17 institutionalized elderly (78-94y)	Not reported (elderly)	No gastrointestinal disease			16S rRNA gene-based qPCR	Institutionalized elderly: ↑*Bacteroides*
			No pregnancy			16S rRNA gene cloning and sequencing	
Rajilic 2009 (12)	5 young adults (mean 33y)	Not reported	Not reported	Not reported	Not reported	Phylogenetic microarray	Overall microbiota composition of your adults and elderly were significantly different
Not reported	5 elderly (mean 71y)					16S rRNA-based FISH	↓Bacteroidetes *(Allistipes, Bacteroides ovatus et rel., ,Bacteroides splachnicus et rel , Parabacteroides)* ↓Bacteroidetes *(Prevotella ruminicola et rel, Eubacterium hallii et rel)* ↑Bacilli *(Lactobacillus salivarius et rel., Aerococcus, Granulicatella)* ↑Bacilli *(Streptococcus bovis et rel, Streptococcus intermedius et rel.)*

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Biagi 2010 (13)	20 young adults (25-40y)	Not reported	No malignant neoplasis and/or immunosuppressive drugs	Medication use *(43 elderly and 21 centenarians)*	Not reported	Phylogenetic microarray	Age groups explained 6.1% of the total microbiota variation
Italy	22 elderly (63-76y) offspring of mean age 59.3y parents		No antibiotics < 1 month			16S rRNA gene-based qPCR	Differences between centenarians and other groups were bigger than differences between young and elderly
	21 elderly (59-78y) offspring of centenarians		Medication free (young adults)				*Bifidobacterium*: centenarians ≈ elderly < young adults (qPCR); centenarians ≈ elderly ≈ young adults (HitChip)
	21 centenarians (99-104y)						↑Proteobacteria, ↑*Eubacterium limosum*,↑Bacilli (no significant differences beetween young and elderly)
							↓microbial diversity (no significant differences between young and elderly)
							↓*Faecalibacterium prausnitzii* (no significant differeces between young and elderly)
							↓*Clostridium* cluster XIVa (no significant differences between young and elderly)
							Centenarians and their offsprings vs elderly and young adults, ↑Fusobacteria and Preoteobacteria prevalence
Mäkivuokko 2010 (14)	14 young adults (21-39y)	Habitual diet	No diarrhoea, IBD, coeliac disease, major malignancies	NSAIDs (> 2 times per week)	Not reported	16S rRNA gene cloning and sequencing	↓ Firmicutes, ↓*Rumicococcus*, ↓*Roseburia*, ↓*Coprobacillus*, ↓*Dialister*
Finland	9 elderly NSAID users (77-85y)	Provided meal service at least once per day (most of volunteers)		Medications use (18 elderly)		G+C% profiling	↑Bacteroidetes, ↑*Oscillospira*, ↑*Lactobacillus*, ↑*Clostridium*, ↑*Streptococcus*, ↑*Bacteroides*
	9 elderly non-NSAID users (70-83y)						NSAID vs non-NSAID elderly, ↓*Lactobacillus*, ↓Actinobacteria, ↓*Collinsella*,↑unclassified Firmicutes
Štšepetova 2011 (15)	24 young adults (23-31.5y)	Western type diet	No NSAID drugs, alcohol abuse, special diet, diabetes, malignancy	Not reported	Not reported	Culture	↑*Lactobacillus*
Estonia	37 elderly (68-75.2y)		No gastrointestinal disorders, antibiotics < 2 months			qPCR assays	↑*Lactobacillus paracasei* prevalence, ↑*Lactobacillus reuteri* prevalence, ↑*Lactobacillus plantarum* prevalence
			No gastrointestinal surgery < 4 weeks				↓*Lactobacillus acidopbilus,* ↓*Lactobacillus helveticus* prevalence
			No chronic renal or hepatic failure				*Lactobacillus rhamnosus* were not detected in elderly
Vollmerhausen 2011 (16)	30 young adults (20-45y)	Not reported	No symptomatic urinary tract infection	Not reported	Not reported	Phenotype characticationRAPD	↑*Escherichia coli*
Australia	29 elderly (65-79y)		No antibiotics or probiotics < 3 month				↑*Escherichia coli* with uropathogenic property prevalence
			No pregnancy				

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Hippe 2011 (17) Austria	15 young vegetarians (26±5y) 17 young omnivores (24±2.5y) 15 institutionalized elderly (86±8y)	Central European diet	Not reported	Not reported	Not reported	qPCR assays	↓*Clostridium cluster XIVa* ↓butyryl-CoA:acetate CoA-transferase gene number
Claesson 2011 (18)	9 young adults (28-46y)	Not reported	No advanced organic disease	Antibiotics (43 out of 161 elderly)	Not reported	16S rRNA gene based pyrosequencing	↓*Clostridium* XIVa
Ireland	161 elderly (65-96y)		No alcohol abuse				↑*Faecalibacterium*, ↑*Allistipes*, ↑*Roseburia*, ↑*Parabacteroides*, ↑*Clostridium* IV
Claesson 2012 (19)	13 young adults (28-46y)	Habitual diet (assessed by food frequency questionnaire)	Not involved in medication intervention	Long and short-term hospitalized and	Functional independence measure	16S rRNA gene based pyrosequencing	Clustering of subjects by diet, residence location, or thealth status
Ireland	178 elderly (64-102y) including:		No antibiotics < 30 days	outpatients elderly received medication	Barthel index	Shortgun metagenomic sequencing	Diversity of the microbiota is highest in community dwellers, followed by institutionalizedelderly, lowest in long-term hospitalized
	83 community dwellers		No alcohol abuse			NMR spectroscopy	Increased frailty, ↓*Ruminococcus* CAG, ↓*Prevotella*, ↓*Oscillibacter* CAG
	20 outpatients elderly						Increased frailty, ↓acetate, ↓butyrate, ↓propionate
	15 short-term hospitalized 60 long-term hospitalized						Increased frailty, ↑*Bacteroides* CAG
Rea 2012 (20)	123 community dwellers (>65y)	Not reported	Not involved in medication intervention	Antibiotics < 4 weeks	Not reported	Culture	Carriage rate of Clostridium difficile, long-term hospitalized > outpatients > short-term hospitalized > community dweller
Ireland	43 outpatients (>65y)		No advanced organic disease			16S rRNA gene based pyrosequencing	*Clostridium difficile* positive vs negative elderly, ↓*Enterococcus*, ↓*Leuconostoc*
	48 short-term hospitalized (>65y)		No alcohol abuse				*Clostridium difficile* positive vs negative elderly, ↑Enterobacteriaceae, ↑*Aerococcus*, ↑*Weeksella*
	103 long-term hospitalized (>65y)						*Clostridium difficile* positive vs negative elderly, ↑*Anaerococcus*, ↑*Helcococcus*

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Drago 2012 (21)	10 young adults (24-57y)	Not reported	No proton-pump inhibitors use < 1 month	Not reported	Not reported	Culture	Centenarians vs young adults, ↓total anaerobes, ↓Enterobacteriaceae, ↓*Bifidobacterium*, ↓*Bacteroides*
Italy	14 centenarians (100-104y)		No antibiotics, antidiarrheric medicines			16S rRNA gene based pyrosequencing	Centenarians vs young adults, ↑*Clostridium*
			No IBD or metabolic diseases				Level of *Lactobacillus*, centenarians ≈ young adults
			No probiotics < 1 month				*Lactobacillus reuteri, Lactobacillus johnsonni, Lactobacillus rhamnosus* in centernarians not in adults
							Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus paralimentarius in adults, not in centernarians.
							Bifidobacterium pseudocatenulatum, Bifidobacterium catenulatum in adults, not in centernarians
O'Sullivan 2012 (22)	73 community dwellers (65-95y)	Habitual diet (assessed by food frequency questionnaire)	Long-term hospitalized (> 6 weeks)	42 subjects receiving antibiotics < 1 month	Not reported	Culture	↓*Bifidobacterium* richness after antibiotic intake, especially after nucleic acid synthesis inhibitors intake
Ireland	40 outpatients elderly (65-95y)		Short-term hospitalized (< 6 weeks)	Long-term stay: 16 of 48 received antibiotics		16S rRNA gene based pyrosequencing	Antibiotic treated elderly, ↓*Faecalibacterium*, ↑Bacteroidetes, vs non-antibiotic elderly
	24 short-term hospitalized (65-95y)			Short-term stay: 10 of 24 received antibiotics			Short-term hospitalized vs non-antibiotic elderly: antibiotic treated elderly, ↓*Blautia*, ↓*Faecalibacterium*
	48 long-term hospitalized (65-95y)			Outpatients elderly: 7 of 40 receivedd antibiotics			Short-term hospitalized vs non-antibiotic elderly: antibiotic treated elderly, ↑*Lactobacillus*, ↑*Mogibacterium*, ↑*Moryella*
				Community dwellers: 9 of 75 received antibiotics			Short-term hospitalized vs non-antibiotic elderly: antibiotic treated elderly, ↑*Peptoniphilus*, ↑*Victivallis*, ↑*Weissella*
							Community vs non-antibiotic elderly: antibiotic treated elderly, ↑*Euryarchaeota*, ↑Rhodocyclaceae
							Community vs non-antibiotic elderly: antibiotic treated elderly, ↓Firmicutes, ↓Proteobacteria
							Long-term hospitalized vs non-antibiotic elderly: antibiotic treated elderly, ↓Lachnospiraceae

Ref.	Subjects	Dietary information	In/exclusion criteria	*Medication use (number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Rampelli 2013 (23) Italy	1 young adults (38y) 5 elderly (59-75y) 3 centenarians (99-102y)	Not reported	No malignant neoplasia and/or immunosuppressives Medication free (young adults) No antibiotics < 1 month	Elderly and centenarians received medication treatment	Not reported	Phylogenetic microarray Shortgun metagenomic sequencing NMR/MS	Elderly vs centenarians and young adults, ↑*Faecalibacterium*, ↑*Eubacterium*, ↑*Bifidobacterium* Centenarians vs young adults and elderly, ↑*Escherichia*, ↑*Ruminococcus* ↓genes for short chain fatty acid production, ↓saccharolytic potential ↑proteolytic functions
Wang 2015 (24) China	8 centenarians (100-108y) 8 rural elderly (85-99y) 8 urban elderly (80-92y)	Rural elderly and centenarians had high fiber diet Urban elderly had low carbohydrate and low fiber diet	No gastrointestinal disorders, hypertension, diabetes No antibiotics or probiotics < 1 month No systemic diseases	Not reported	Not reported	16S rRNA gene based Illumina MiSeq	Intestinal microbiota was dominated by Bacteroidetes Microbial diversity: centenarians ≈ rural elderly ≈ urban elderly Microbial richness: centenarians ≈ rural elderly > urban elderly Enterobacteriaceae and *Escherichia*, centenarians ≈ rural elderly > urban elderly *Lactobacillus*, rural elderly > urban elderly ≈ centenarians *Faecalibacterium*, rural elderly ≈ urban elderly > centenarians *Mitsuokella*, rural elderly > urban elderly > centenarians
Odamaki 2016 (25) Japan	367 community dwellers (0-104y)	Not reported	All volunteers are community dwellers (except 2)	Not reported	Not reported	16S rRNA gene based Illumina MiSeq	Elderly intestinal microbiota was dominited by Firmicutes ↓Firmicutes, ↓Actinobacteria ↑alpha diversity and richness until centenarians (with large inter-individual differences) ↑Bacteroidetes, ↑Proteobacteria, ↑Bacteroides and [Eubacterium] CAG

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Biagi 2016 (26)	15 young adults (22-48y)	Habitual diet ((super) centenarians)	Physical and cognitive healthy ((super) centenarians)	(super)Centenarians received a variety of medications	Not reported	16S rRNA gene based Illumina MiSeq	Four age groups significantly separate from each other, except for cetenarians and super cetenarians
Italy	15 elderly (65-75y)		Physical and cognitive healthy (centenarians)				↓dominating families (Bacteroidaceae, Lachnospiraceae and Ruminococcaceae) accumulative abundance
	15 centenarians (99-104y)		Healthy and medication free (young adults)				↓*Bifidobacterium*, except for centenarians aged > 105y (↑)
	24 (super)centenarians (105-109y)						↓*Coprococcus*, *Roseburia* and *Faecalibacterium* ↑*Akkermansia* and Christensenellaceae
Jackson 2016 (27)	728 community dwellers (42-86y)	Detailed informaiton provided in a separate article	Detailed informaiton provided in a separate article	Detailed information provided in an separate article	Rockwood frailty Index	16S rRNA gene based Illumina MiSeq	↓microbial diversity and richness,
UK							Increased frailty: ↓*Faecalibacterium*, especially *Faecalibacterium prausnitzii* Increased frailty: ↓a sub-set of Lachnospiraceae OTUs Increased frailty: ↑*Eubacterium*,↑*Eggerthella*, ↑*Eubacterium dolichum* Increased frailty: ↑*Eggerthella lenta*, ↑13 Enterobacteriaceae OTUs
Kong 2016 (28)	47 young adults (24-64y)	Not reported	None of volunteers were hospitalized	Not reported	Functional independence measure	16S rRNA gene based Illumina MiSeq	Long living vs young adults and elderly, ↑microbial richness
China	54 elderly (65-83y)						Long living vs young adults and elderly, ↑*Blautia*, *Clostridium* XIVa, *Faecalibacterium*, *Escherichia Shibacterium*
	67 long living elderly (90-102y)						Long living vs young adults and elderly, ↑*Ruminococcaceae*, unclassified Lachnospiraceae and Ersipelotrichaceae
Jeffery 2016 (29)	13 young adults (28-46y)	Habitual diet (assessed by food frequency questionnaire)	Not involved in medication intervention	Antibiotic intake were recorded, but not reported	Functional independence measure	16S rRNA gene based pyrosequencing	The microbiota of community dwellers were disturbanced by antibiotics stronger than that of long-term hospitalized elderly
Ireland	371 elderly (64-102y)		No antibiotics < 1 month (young adults)		Barthel index		Long-term hospitalized vs community-living elderly, after antibiotic intake: less recovery
			No advanced organic disease No alcohol abuse				Increased frailty: ↓alpha diversity Increased frailty: ↑alpha diversity

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Ticinesi 2017 (30) Italy	76 hospitalized elderly (71-97y) 25 elderly (65-87y)	Hospitalized elderly have the same hospital-based diet	No expected survival < 30 days or having artifical nutrition No patients received antibiotics continuously > 3 days Healthy elderly : no polypharmacy (< 5 drugs) Hospitalized elderly have multimorbidity burden No acute or chronic digestive diseases	Hospitalized elderly: all polypharmacy (> 4 medications) Control elderly: no polypharmacy (< 5 medications) 40 of 76 received antibiotics	Rockwood frailty Index	16S rRNA gene based Illumina MiSeq	Frailty was not associated with microbial richness Increased microbial richness, ↓drugs numbers, ↓motality Increased frailty, ↑*Fonticella*,↑*Prevotella* Increased frailty, ↑unclassified member of Clostridiaceae 1 family Increased frailty, ↑*Oscillospira*, ↑*Peptococcus*, ↑*Porphyromonas* Increased frailty, ↑unclassified member of Lachnospiraceae family
Bian 2017 (31) China	378 children (3-14y) 212 soldiers (19-24y) 221 young adults (19-50y) 86 elderly (60-79y) 198 long living elderly (>94y)	Not reported	Young adults have long living parents or grandparents (>80y or 85y) No prescription medication or antibiotics < 3 months No cardiovascular, gastrointestinal diesease history No metabolic, respiratory diseases history No smoking and alcohol No onset disease	Not reported	Not reported	16S rRNA gene based Illumina MiSeq	No clear difference found between young adults (30y+) and centenarians ↑microbial diversity between 30-79 years old Inter-individual difference: male > female Larger inter individual differences in microbial diversity
Kato 2017 (32) Japan	441 individuals (0-104y)	Not reported	Volunteers (> 80y) were community dwellers Yougurt consumption < 2 times a week	Not reported	Not reported	16S rRNA gene-based qPCR	*Bifidobacterium longum* present across age *Bifidobacterium dentium* had higher prevalence in the elderly than the others

Ref.	Subjects	Dietary information	In/exclusion criteria	Medication use *(number of subjects)*	Determination of frailty	Microbiological methods	Main findings with increased age
Maffei 2017 (33)	85 community dwellers (43-79y)	Not reported	Offsprings of nonagenarians	7 volunteers had antibiotic treatment < 6 months	34-item frailty index	16S rRNA gene based Illumina MiSeq	Chronological age did not correlate with microbial richness and eveness
US				13 volunteers had no antibiotic information < 6 months			Increased frailty, ↓microbial richness
							Increased frailty, ↓*Paraprevotella*, ↓*Sutterella*, ↓*Rikenellaceae* family OTU
							Increased frailty, ↑TM7 candidate-phylum OTU, ↑*Coprobacillu*, ↑*Dialister*

Elderly without addition refers to healthy elderly
↓ significant decrease
↑ significant increase
≈ not significantly different
y: years of age

CAG: co-abundance group
CDAD: *Clostridium difficile*-associated diarrhoea
FISH: fluorescence in situ hybridization
NMR: Nuclear magnetic resonance
NSAID: non-steroidal anti-inflammatory drugs
PCR-DGGE: Polymerase chain reaction-denaturing gradient gel electrophoresis
RAPD: Randomly amplified polymorphic DNA analysis
UK: United Kingdom
US: United States

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Chapter 3

Intestinal barrier function is maintained with aging – a comprehensive study in healthy individuals and irritable bowel syndrome patients

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Abstract

Animal studies have shown that intestinal barrier function is compromized with aging. We aimed to assess the effects of aging on intestinal barrier function in humans *in vivo* and *ex vivo*. In this cross-sectional study, healthy subjects and subjects with irritable bowel syndrome (IBS) of older (65-75 years) and young adult age (18-40 years) were compared. *In vivo* gastrointestinal site-specific permeability was assessed by a multi-sugar test, taking into account potential confounders. Sigmoid biopsies were collected from subgroups of healthy young adults and elderly for *ex vivo* Ussing chamber experiments, gene transcription of barrier-related genes and staining of junctional proteins. No significant differences between healthy young adults and elderly were found for small intestinal, colonic and whole gut permeability ($P \geq 0.142$). In IBS patients, gastroduodenal and colonic permeability did not differ significantly ($P \geq 0.400$), but small intestinal and whole gut permeability were higher in elderly versus young adults ($P \leq 0.009$), mainly driven by the IBS-diarrhea subtype. Ussing chamber experiments with or without stressor ($P \geq 0.052$), and relative expression of intestinal barrier-related genes ($P \geq 0.264$) showed no significant differences between healthy elderly and young adults, as confirmed by immunofluorescent stainings. Overall, the functional capacity of the intestinal barrier is maintained in elderly.

Introduction

Along with the rising life expectancy, the aging population is steadily increasing worldwide. In 2010, 8% of the world population was aged 65 years or older, and this proportion is expected to reach 16% by 2050, leading to substantial increases in direct and indirect health care costs (1). The associated functional decline of several organs and tissues, including those of the gastrointestinal (GI) tract and the immune system, contributes to higher vulnerability to infections with aging and age-related co-morbidities (2, 3). With respect to GI physiology and function, a recent review by our group showed that the aging process is associated with small, subtle alterations at both the organ and cellular level (4). Moreover, the GI mucosal immune function has been found to decline with aging (5). Based on data of mice, rat and baboon studies it has been stated that intestinal barrier function also decreases with aging, as reflected by an increased paracellular intestinal permeability (6-9). Intestinal permeability is an important functional feature of the intestinal epithelial barrier (10). Increased intestinal permeability may lead to permeation of noxious luminal substances into the intestinal mucosa, inducing local and systemic immune activation, and may contribute to *e.g.* an increased infection risk, inflammation, and GI symptoms. So far, a few human studies have investigated the effects of aging on small intestinal or colonic barrier function. In these studies, most of which addressed the small intestine, no differences in sugar excretion ratios between age groups were found (11-14). However, the impact of potential confounders such as medication use (*e.g.* proton pump inhibitors (PPIs) and non-steroidal anti-inflammatory drugs (NSAIDs)) have not been considered in previous studies, while these drugs are widely used by elderly and less in younger adults. Futhermore, most studies on aging focused on healthy subjects, while it may be also valuable to get insight in barrier function in subjects with mild disturbances in GI health, such as irritable bowel syndrome (IBS) patients, in whom intestinal barrier disfunction has been shown previously (15), but has not been studies with regard to age. Paracellular permeability is regulated by intercellular junctional complexes (16). At present, data from human studies with regard to aging on the expression and function of tight junction proteins, sealing the epithelial barrier, are not available.

In summary, available data on the effects of aging on the intestinal barrier in humans are very limited and when present, often conflicting (17). Therefore, a comprehensive study on the effects of aging on intestinal barrier function is needed. Our aim was

to study the effects of aging on human intestinal barrier function in combined *in vivo* and *ex vivo* experiments, by determining GI permeability and the expression of barrier related genes. We hypothesized that intestinal permeability is increased and the expression of intestinal barrier related genes is lower in elderly compared with young adults, in both healthy subjects and IBS patients.

Methods

Study design and participants

In this cross-sectional study, baseline data of healthy individuals who participated in a human intervention study, and of IBS patients of the Maastricht IBS cohort (15) were used. Both studies were approved by the Medical Ethics Committee of the University Hospital Maastricht and Maastricht University, were registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT02376270 and NCT00775060, respectively), and performed between November 2009 and April 2016, in accordance with the Declaration of Helsinki (latest amendment in Fortalesa, Brasil, 2013) and Dutch Regulations on Medical Research involving Human Subjects (1998). Healthy individuals were recruited by public advertisements and key exclusion criteria were GI diseases, abdominal surgery interfering with GI function, pregnancy, and use of medication influencing intestinal permeability such as NSAIDs. IBS patients were recruited via the Maastricht University Medical Center+ Gastroenterology-Hepatology outpatient clinic and via regional general practices, and were diagnosed and classified by the Rome III criteria with exclusion of organic diseases when indicated, as described previously (15). To assess the effects of aging, two groups were included in both the healthy and the IBS populations: *i.e.* elderly 65-75 years and young adults 18-40 years to create distinct age groups (Figure 3.1). All participants gave written informed consent before participation.

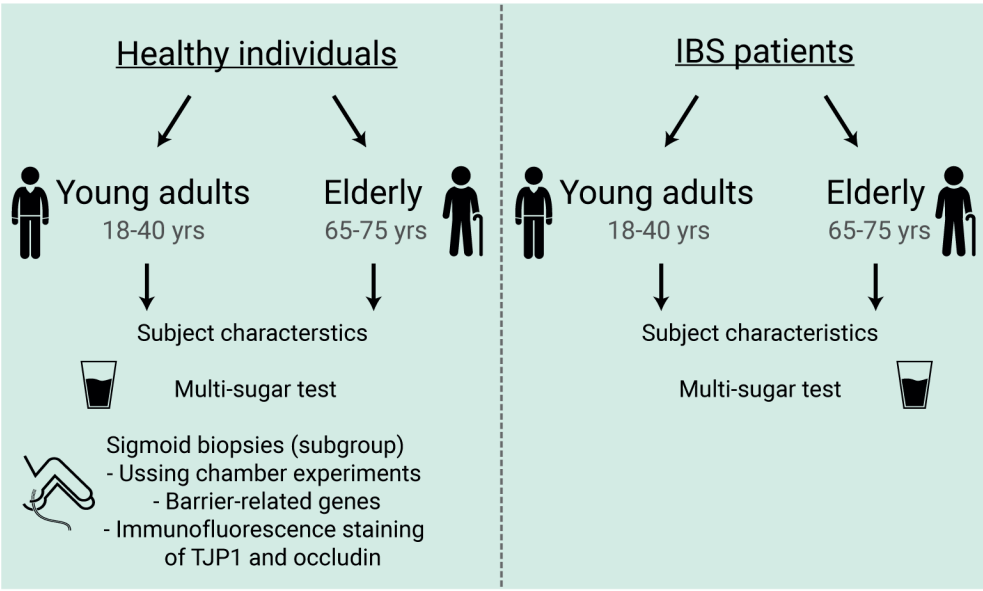

Figure 3.1 - Overview of the study population and measurements.

Outcome parameters

In the current study, various, different and complementary measurements of barrier function were measured (Figure 3.1). All healthy and IBS individuals were subjected to a standardized and validated multi-sugar urinary recovery test to assess GI segment-specific intestinal permeability *in vivo*. From a subgroup of the healthy subjects (10 elderly and 10 young adults) colonic biopsies were collected for *ex vivo* analyses of intestinal permeability by Ussing chamber experiments as well as for gene and protein analyses of intestinal barrier related proteins. These subjects underwent flexible sigmoidoscopy without bowel preparation. Biopsy specimens were taken with a jumbo biopsy forceps (Boston Scientific, Kerkrade, the Netherlands) from a standardized location, *i.e.* 30 cm proximal to the anus. Six tissue samples were immediately transported to the laboratory for Ussing chamber experiments. One tissue sample was snap frozen in liquid nitrogen and stored at -80°C for gene transcription analyses. One tissue sample was mounted in Tissue-Tek® optimal cutting temperature compound (Sakura, Finetek, Tokio, Japan), snap frozen in liquid nitrogen and stored at -80°C for immunofluorescent staining of Tight junction protein 1 (TJP1; *i.e.* Zona Occludens-1) and occludin.

Multi-sugar test for analysis of intestinal permeability *in vivo*

Segment-specific permeability of the GI tract was assessed with a validated multi-sugar test (18, 19). On the day prior to, as well as during the test, subjects were asked to refrain from excessive physical exercise and consumption of alcohol. After fasting overnight, a mix of water-soluble sugar probes were ingested. IBS patients ingested 1g sucrose (Van Gilse, Dinteloord, the Netherlands), 1 g lactulose (Centrafarm Services, Etten-Leur, the Netherlands), 0.5 g L-rhamnose (Danisco, Copenhagen, Denmark), 1 g sucralose (Tate and Lyle Ingredients Americas, Decatur, IL, USA) and 1 g erythritol (Now Foods, Bloomindale, IL, USA), dissolved in 200 ml tap water. Healthy individuals ingested the same mixture, except for 0.5 g mannitol (Roquette, Lestrem, France) instead of L-rhamnose. It was not possible to use L-rhamnose as permeation marker in that study, as the subsequent intervention from which these baseline data were obtained entailed supplementation with pectin, a non digestible carbohydrate that contains rhamnose residues. These residues would have interfered with a multisugar urinary recovery test containing L-rhamnose. Mannitol and L-rhamnose are both disaccharides with the same intestinal permeation characteristics, therefore we do not expect this to hinder comparability of the data. After ingestion, all participants collected 24 hours (h) urine in two separate fractions; 0-5 h and 5-24 h. During the first 5 h of urine collection, participants were asked to refrain from any food or drinks, except for water ad libitum. Thereafter, participants were allowed to eat and drink as preferred, except for sucralose containing foods. After the collection periods, volumes of urine fractions were determined and aliquots were frozen at -80 °C until analysis. Sugar probes were analyzed by isocratic ion-exchange High Performance Liquid Chromatography with mass spectrometry as described previously (18, 19). Gastroduodenal permeability was determined by sucrose excretion in 0-5 h urine, small intestinal permeability by 0-5h urine lactulose to mannitol (L/M) ratio in healthy individuals and lactulose to rhamnose (L/R) ratio in IBS patients, colonic permeability by sucralose to erythritol (S/E) ratio in 5-24 h urine, and whole gut permeability by sucralose to erythritol (S/E) ratio in 0-24 h urine.

***Ex vivo* Ussing chamber experiments**

Six tissue samples from the sigmoid colon were used for *ex vivo* Ussing chamber experiments as previously described by our group (20). Three tissue samples were stressed by adding mast cell degranulator Compound 48/80 (1 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) to the serosal compartment. Three non-exposed tissue samples

served as controls. At t=0, 1 mg/ml fluorescein (376 g/mol, Sigma-Aldrich, St. Louis, MO, USA) was added to the serosal compartment. Potential difference (PD), Transepithelial electrical resistance (TEER) and luminal fluorescein concentrations were determined at time point t=0, 30, 60, 80 and 120 min, respectively. TEER and PD were used as quality criteria for viability. Only samples with a baseline TEER above 20 Ω/cm^2, or those with baseline TEER between 15-20 Ω/cm^2 and PD below 0.5 mV, were included for analyses. Lower TEER values and higher fluorescein concentrations are indicators of impaired intestinal permeability.

Gene transcription of barrier-related genes

Transcription of junctional complex related genes as well as defense and immune related genes associated with barrier function or modulation thereof, were determined in colonic tissue samples. Nucleic acid extraction and purification, RNA isolation and reverse transcription were performed as previously described (21). Depending on the gene of interest, cDNA was diluted to final concentrations of 20 ng/µl, 40 ng/µl or 80 ng/µl (Supplementary Table 3.S1). Quantitative real-time polymerase chain reaction (qPCR) was performed as described previously (22). Expressions of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene.

Immunofluorescent staining of TJP1 and occludin

Sigmoid biopsy sections (10 µm) were used for immunofluorescent staining of TJP1 and occludin as previously described by Elamin *et al.* (23).

Statistical analyses

This is considered a first human study applying the multi-sugar test in combination with *ex vivo* analyses. We made use of existing data sets to investigate the effects of aging on intestinal permeability. The results of this study will enable future power calculations in this field, especially because of the complementary range of parameters to determine intestinal barrier function.

Missing data was not imputed, but reported upon in the results. We checked for normality of the data (histograms) and subsequently variables were summarized using median and interquartile range (IQR; 25-75th IQR) or means ± standard deviation for numerical variables, and percentages for categorical variables. Mann-Whitney U-tests and independent-samples T Tests were performed for numerical variables

and Chi-square tests for categorical variables to test for differences between age groups (elderly versus young adults). Factors potentially influencing intestinal permeability *in vivo* were tested by multivariable linear regression analysis. These regression models included age group, sex, body mass index (BMI), and PPI use for healthy individuals, and age group, PPI use, NSAID use, and IBS subtype for IBS patients. Differences in longitudinal trends in TEER and luminal fluorescein between age groups were assessed by random intercept linear mixed model analyses with age group (elderly and young adult), time (t=0, t=30, t=60, t=90, t=120 min) and 'age group x time' as fixed factors, and correction for t=0 values. All statistical analyses were performed using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY, USA: IBM Corp.). *P*-values ≤ 0.05 (two-sided) were considered statistically significant. Ussing chamber experiments and gene transcription *P*-values were corrected for multiple testing by the false-discovery-rate (FDR) of Benjamini-Hochberg.

Results

Intestinal permeability *in vivo*

Assessment of intestinal permeability *in vivo* by the multi-sugar test was performed in 100 healthy individuals including 48 elderly and 52 young adults, as well as in 48 IBS patients including 21 elderly and 27 young adults. Subject characteristics are shown in Table 3.1. BMI ($P < 0.001$) and PPI use ($P = 0.017$) were significantly higher in healthy elderly compared with healthy young adults.

Table 3.1 - Subject characteristics per age group of the healthy individuals and IBS patients undergoing the multi-sugar test for *in vivo* assessment of the intestinal permeability.

	Healthy individuals			IBS patients		
	Young adults (n=52)	Elderly (n=48)	*P*-value	Young adults (n=27)	Elderly (n=21)	*P*-value
Age (yrs, mean ± SD)	23.1 ± 4.3	69.7 ± 2.8	<0.001	29.4 ± 6.5	71.1 ± 4.0	<0.001
Female (%)	57.7	43.8	0.164	59.3	66.7	0.599
BMI (kg/m^2, mean ± SD)	22.9 ± 2.7	25.8 ± 2.7	<0.001	25.4 ± 5.2	25.5 ± 3.2[#]	0.896
Medication (%)						
PPI	0	10.4	0.017	26.9[#]	14.3	0.293
NSAID	N.A	N.A.	N.A.	7.7[#]	23.8	0.123
IBS subtype (%)						
IBS-C	N.A.	N.A.	N.A.	18.5	28.6	0.412
IBS-D				40.7	38.1	0.849
IBS-M				37.0	28.6	0.535
IBS-U				3.7	4.8	0.857

BMI: body mass index, IBS: irritable bowel syndrome, IBS-C: constipation-predominant irritable bowel syndrome, IBS-D: diarrhea-predominant irritable bowel syndrome, IBS-M: irritable bowel syndrome characterized by a mixed pattern, IBS-U: Unsubtyped irritable bowel syndrome, N.A: not applicable, NSAID: non-steroidal anti-inflammatory drugs, PPI: proton-pump inhibitors. Age and BMI were compared between age groups with the use of an independent samples t-test. Sex, medication and IBS subtype were compared between age groups with the use of a Pearson Chi-square test. [#] One missing value for this variable.

Gastroduodenal permeability as assessed by the 0-5h urinary sucrose excretion was lower in healthy elderly compared with young healthy adults (P = 0.025), but did not differ significantly between elderly and young IBS patients (P = 0.400) (Figure 3.2A and 3.2B, respectively). The 0-5h urinary L/M ratio reflecting small intestinal permeability in healthy subjects was not significantly different between elderly and young adults (P = 0.214) (Figure 3.2C), while in the IBS group, small intestinal permeability as determined by the 0-5h urinary L/R ratio was higher in elderly compared with young adults (P = 0.009) (Figure 3.2D).

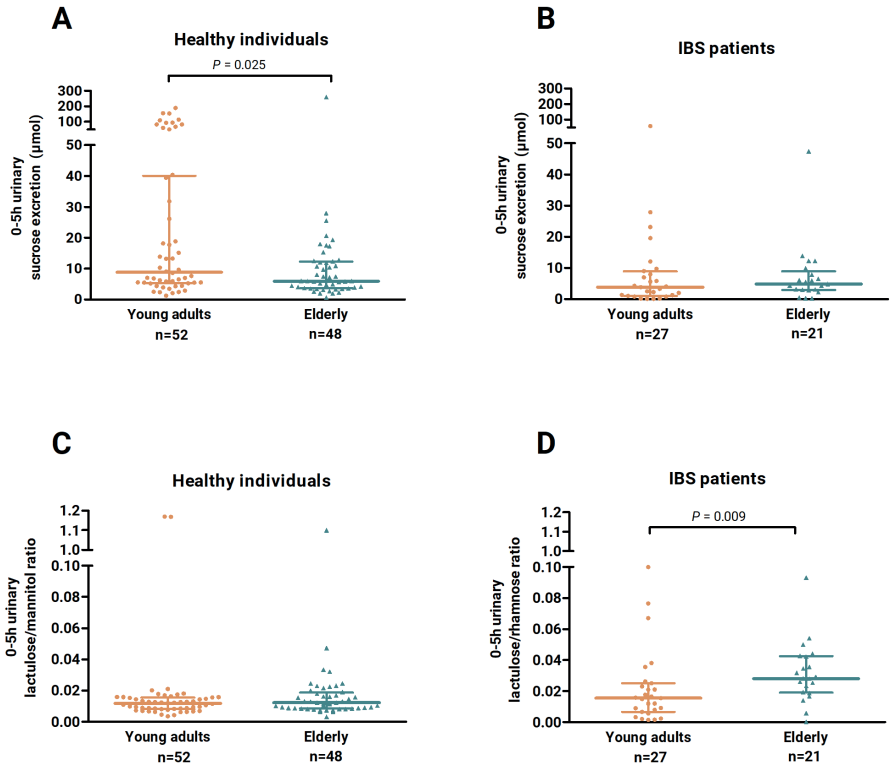

Figure 3.2 - Gastroduodenal and small intestinal permeability *in vivo* comparing young adults vs. elderly. A: 0-5h urinary sucrose excretion in healthy individuals. B: 0-5h urinary sucrose excretion in IBS patients. C: 0-5h urinary lactulose/mannitol ratio in healthy individuals. D: 0-5h urinary lactulose/rhamnose ratio in IBS patients. Values are presented in scatter plots with median line and IQR (25-75th interquartile range). Urinary sugar excretions and ratios were compared between age groups with the use of Mann-Whitney U-tests.

Colonic permeability *in vivo* as determined by the 5-24h urinary S/E ratio was not significantly different between healthy elderly and healthy young adults (P = 0.227), nor between elderly and young IBS patients (P = 0.664) (Figure 3.3A and 3.3B, respectively). The 0-24h urinary S/E ratio, as measure for whole gut permeability *in vivo*, is not significantly different between healthy elderly and healthy young adults (P = 0.061), whereas in IBS patients, the 0-24h urinary S/E ratio was higher in elderly compared with young adults (P = 0.003) (Figure 3.3C and 3.3D, respectively).

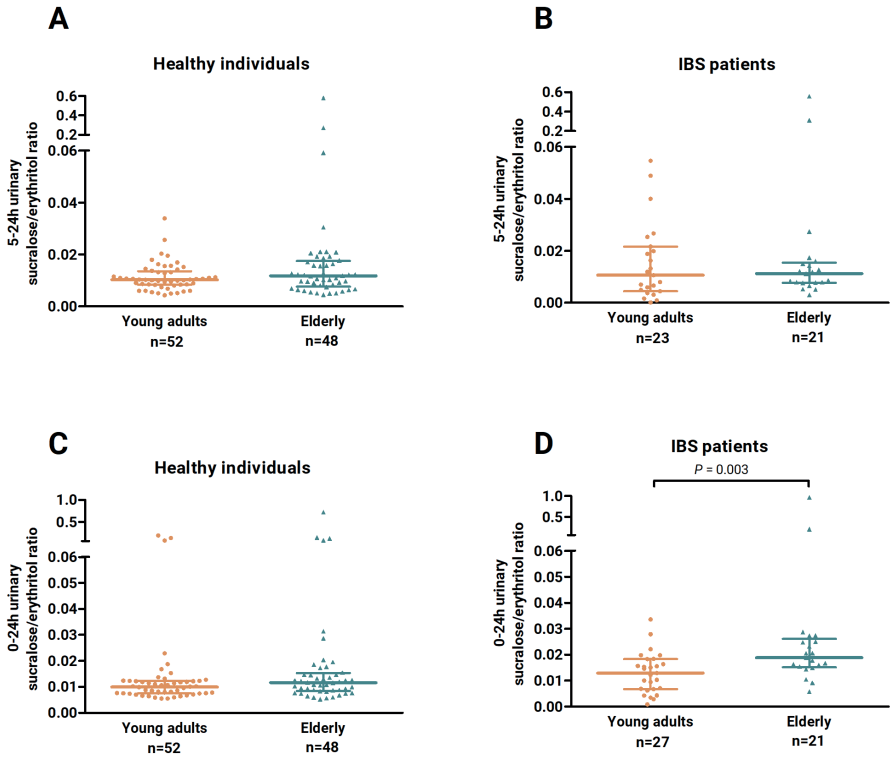

Figure 3.3 - Colonic and whole gut permeability *in vivo* comparing young adults vs. elderly. A: 5-24 urinary sucralose/erythritol ratio in healthy individuals. B: 5-24 urinary sucralose/erythritol ratio in IBS patients. C: 0-24 urinary sucralose/erythritol ratio in healthy individuals. D: 0-24 urinary sucralose/erythritol ratio in IBS patients. Values are presented in scatter plots with median line and IQR (25-75th interquartile range). Urinary sugar ratios were compared between age groups with the use of Mann-Whitney U-tests.

As demographics and medication use may influence intestinal permeability *in vivo,* their impact was accounted for by multivariable linear regression analysis as shown in Supplementary Tables 3.S2 (healthy individuals) and 3.S3 (IBS patients). In healthy individuals, the 0-5h urinary sucrose excretion (B = -16.430 (95% CI -35.855; 2.996)), 0-5h urinary L/M ratio (B = -0.051 (95% CI -0.140; 0.038)) and 0-24h urinary S/E ratio (B = -0.025 (95% CI -0.011; 0.060)) were not significantly affected by age group (elderly vs. young adults). However, healthy elderly had on average a significantly higher 5-24h urinary S/E ratio (B = 0.030 (95%CI 0.001; 0.058)) compared with healthy young adults. In IBS patients, the 0-5h urinary sucrose excretion (B = 1.391 (95%CI -5.549; 8.331)) as well as 0-5h urinary L/R ratio (B = 0.007 (95%CI -0.006;

0.020)), 5-24h urinary S/E ratio (B = 0.040 (95%CI -0.021; 0.101)) and 0-24h urinary S/E ratio (B = 0.066 (95%CI -0.021; 0.153)) were not significantly influenced by age group (elderly vs. young adults).
Sex, BMI and PPI use did not significantly affect urinary sugar excretions and ratios in the healthy subjects (Supplementary Table 3.S2). In IBS patients, the 0-5h urinary sucrose excretion, 5-24h urinary S/E ratio and 0-24h urinary S/E ratio were also not significantly influenced by PPI use, NSAID use or IBS subtype (Supplementary Table 3.S3). However, diarrhea-predominant irritable bowel syndrome (IBS-D) patients had on average a significantly higher 0-5h urinary L/R ratio (B = 0.018 (95%CI 0.004; 0.031)) compared with other subtypes.

Intestinal permeability *ex vivo*

Ussing chamber experiments were performed to study TEER and luminal fluorescein concentration as functional indicators of paracellular permeability, in unstressed and stressed biopsies of 10 healthy elderly (mean±SD: 70.7±2.8 yrs) and 10 healthy young adults (24.0±5.4 yrs). Mean BMI was found to be significantly higher in elderly (27.3±1.7 kg/m^2) compared with young adults (23.4±3.2 kg/m^2; P = 0.005). Sex, PPI use and NSAID use did not differ significantly between elderly (20% female, 20% PPI use, 0% NSAID use) and young adults (30.0% female, 0% PPI use, 0% NSAID use; all $P \geq 0.136$). After FDR correction for multiple time points, TEER did not significantly differ between elderly and young adults in unstressed (all $P \geq 0.208$), nor in stressed biopsies (all $P \geq 0.096$) (Figure 3.4A and 3.4B, respectively). In both unstressed biopsies (all $P \geq 0.052$) and stressed biopsies (all $P \geq 0.760$), luminal fluorescein concentration did not differ significantly between elderly and young adults (Figure 3.4C and 3.4D, respectively).

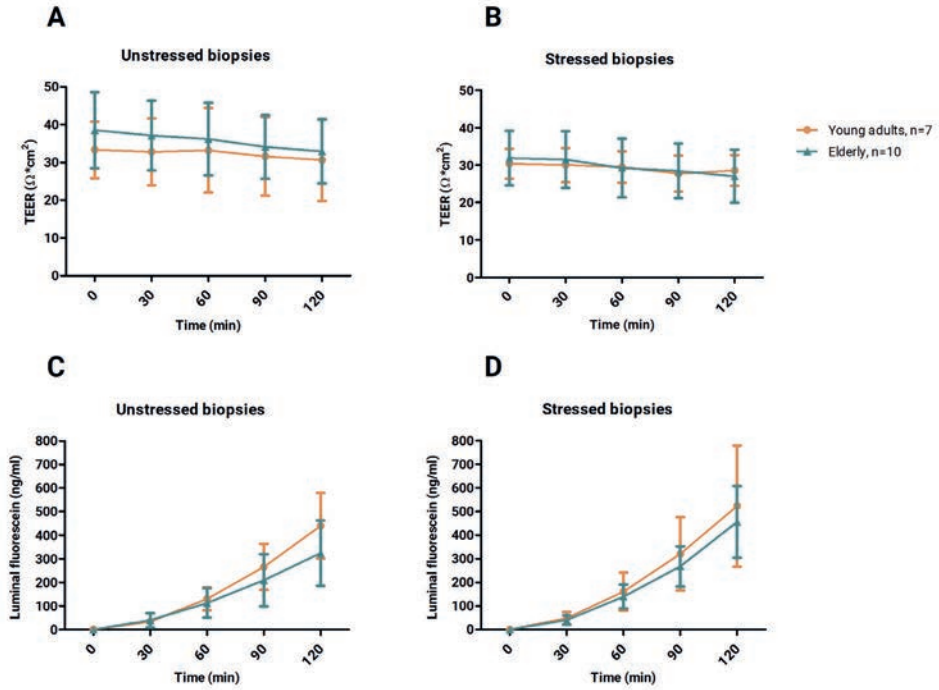

Figure 3.4 - Intestinal permeability *ex vivo* comparing healthy young adults (orange) vs. healthy elderly (green) by mounting fresh sigmoid colon biopsies in an Ussing chamber system, and assessing transepithelial electrical resistance (TEER) and luminal fluorescein concentration at t=0, t=30, t=60, t=90, t=120 min. A: TEER in unstressed biopsies. B: TEER in biopsies stressed by 1 µg/ml Compound 48/80 at t=0. C: Luminal fluorescein concentration in unstressed biopsies. D: Luminal fluorescein concentration in biopsies stressed by 1 µg/ml Compound 48/80 at t=0. In the young adult group, two subjects were removed from the unstressed biopsy analyses, and three subjects were removed from the stressed biopsy analyses because baseline values were not meeting the quality criteria for viability. Means and standard deviations are visualized. TEER and luminal fluorescein were compared between age groups using random intercept linear mixed model analyses including age group, time and age group x time as fixed factors and correction for t=0 values. *P*-values per time point were corrected for multiple testing by calculating the false-discovery-rate (FDR) of Benjamini-Hochberg.

Gene transcription of barrier related genes

Relative expression of junctional complex (*e.g.* tight junctions and adherens junctions), defense and immune-related (*e.g.* human defensins, cytokines and toll-like receptor) genes in sigmoid biopsies were studied because these are directly or indirectly related to paracellular permeability and barrier function in general. Before correcting for multiple testing, only cadherin 1 (P = 0.047) was higher and toll-like receptor 1 (P = 0.024) was lower in healthy elderly compared with healthy young

adults. After correcting for multiple testing, there were no significant differences (all $P \geq 0.264$) between healthy elderly and healthy young adults in the relative expression of junctional complex, defense and immune-related genes (Table 3.2).

Table 3.2 - Relative expression of junctional complex (*e.g.* tight junction related and adherens junctions), defense and immune related (*e.g.* human defensins, cytokines and toll-like receptor) genes in sigmoid biopsies of young adults and elderly.

Cluster	Gene name	Young adults			Elderly			*P*-value	Benjamini Hochberg *P*-value
		N	Mean	SD	N	Mean	SD		
Junctional complex related genes	TJP1 (ZO-1)	10	1.13	0.03	10	1.14	0.02	0.641	0.673
	OCLN	10	1.18	0.02	10	1.19	0.02	0.314	0.673
	CLDN2	5	1.36	0.03	6	1.32	0.07	0.269	0.673
	CLDN3	10	1.16	0.02	10	1.17	0.02	0.556	0.673
	CLDN4	10	1.10	0.02	10	1.11	0.02	0.673	0.673
	MLCK	10	1.15	0.03	10	1.15	0.03	0.534	0.673
	CDH1	10	1.15	0.01	10	1.17	0.02	0.047	0.376
	CTNNB1	10	1.12	0.01	10	1.13	0.02	0.386	0.673
Defense and immune related genes	CAMP	9	1.30	0.05	10	1.28	0.06	0.462	0.726
	DEFB1	10	1.15	0.03	10	1.16	0.03	0.951	0.951
	MUC2	10	1.01	0.03	10	1.01	0.02	0.559	0.769
	TFF3	10	0.98	0.04	10	0.98	0.04	0.898	0.951
	IL1B	10	1.35	0.05	9	1.32	0.04	0.233	0.667
	IL10	9	1.25	0.03	9	1.23	0.06	0.300	0.667
	TNF	9	1.35	0.06	5	1.34	0.04	0.805	0.951
	TLR1	10	1.18	0.04	10	1.13	0.04	0.024	0.264
	TLR2	9	1.26	0.05	10	1.23	0.06	0.354	0.667
	TLR4	10	1.21	0.03	10	1.19	0.03	0.063	0.347
	TLR6	8	1.29	0.04	7	1.27	0.06	0.364	0.667

TJP1 (ZO-1): Tight junction protein 1 (*i.e.* Zona Occludens-1), OCLN: Occludin, CLDN: Claudin, MLCK: Myosin light chain kinase, CDH1: Cadherin 1, CTNNB1: Catenin beta 1, CAMP: Cathelicidin antimicrobial peptide, DEFB1: Defensin beta 1, MUC2: Mucin 2, TFF3: Trefoil factor 3, IL: Interleukin, , TNF: Tumor necrosis factor, TLR: Toll like receptor. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. Values are presented as mean ± SD. Differences between age groups were tested by independent-samples T Tests. *P*-values were corrected for multiple testing by calculating the false-discovery-rate of Benjamini Hochberg per cluster.

Immunofluorescent stainings of TJP1 and occludin

Representative images of immunofluorescent staining TJP1 and occludin in sigmoid biopsy sections of a healthy elderly and healthy young adult are presented in Figure 3.5. TJP1 and occludin showed continuous staining without disruption along the villous epithelium. No apparent differences were noted between the elderly and young adults. These observations are in line with quantitative analyses of TJP1 and occludin gene transcription levels as reported in Table 3.2.

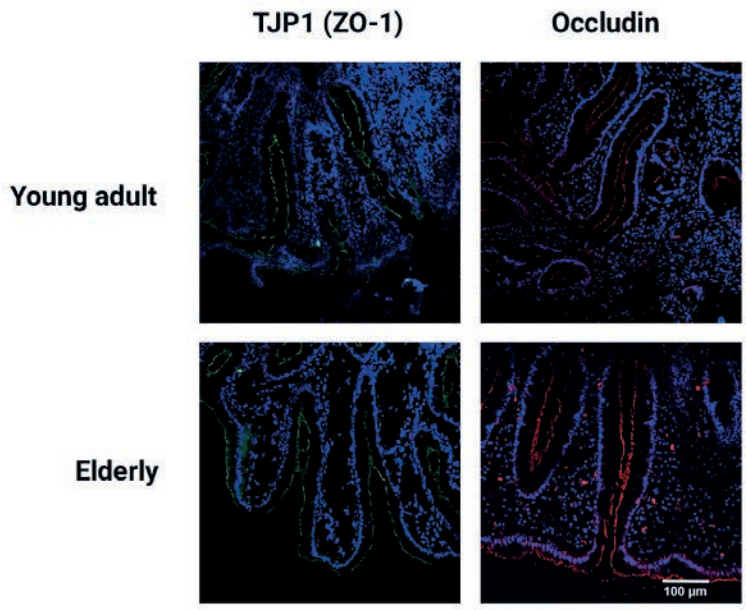

Figure 3.5 - Representative images of tight junction proteins TJP1 (green) and occludin (red) immunofluorescent stainings in sigmoid biopsy sections showing glandular epithelium of a healthy young adult and healthy elderly. Scale bar represents 100 µm. Blue counterstaining (DAPI) shows nuclei. TJP1: Tight junction protein 1.

Discussion

In this study, we evaluated various, different and complementary aspects of GI barrier function to assessed the effects of aging in humans. Overall, GI segment-specific permeability and expression of barrier-related genes were not significantly affected by aging in healthy individuals. In IBS, a condition which is generally considered to be associated with mild alterations in gut function, small intestinal permeability was higher in the elderly. This difference was partly explained by the IBS-D subtype. We therefore rejected the study hypothesis that intestinal barrier function and the expression of barrier related genes is altered with aging per se in healthy subjects and in mild GI disorders.

Up to now, previous studies on aging and intestinal permeability *in vivo* have been critically debated because of methodological issues. One study (11) included a heterogeneous group of patients and hospital staff. In two other studies (12, 13) high dosages of mannitol (5 g) and lactulose (10 g) were used, which is in this dosage considered a laxative, and therefore probably inducing osmotic effects (18). We applied the validated multi-sugar test, using low sugar dosages (0.5-1 g per sugar probe), to determine site-specific paracellular intestinal permeability *in vivo* in well defined groups of healthy individuals and IBS patients, taking into account factors potentially influencing these parameters. Gastroduodenal permeability was determined by assessing urinary sucrose excretion in the 0-5h fasting period of the multi-sugar test. Urinary sucrose excretions were significantly lower in the healthy elderly compared with healthy young adults. Although we cannot exclude that this may in part be due to more outliers in the young adults group than in the elderly, age group was not significant in the multivariable linear regression analysis by correcting for potential confounders. Therefore our findings are in line with previous findings in healthy adults (24, 25), and indicating the initial difference in gastroduodenal permeability should be interpreted with caution. Moreover, in elderly and young adult IBS patients we observed a comparable gastroduodenal permeability. To the best of our knowledge, this is the first study investigating gastroduodenal permeability and aging. A clear reason for the outliers in the healthy young adults is lacking, but it should be acknowledged that urinary sucrose excretion was not corrected for potential differences in for example transit time or renal clearance as no transcellar probe was available to correct for. Although some debates are ongoing on the optimal timing of urine collection, in the current study the 0-5 urinary L/R ratio and L/M ratio

used in the current study are considered to reflect small intestinal permeability in healthy and IBS individuals, respectively. As medians of 0-5h urinary L/R and L/M ratios were in the same range, the impact of using different disaccharides as a transcellar probe indeed seemed negligible. We showed that small intestinal permeability did not differ significantly between healthy elderly and healthy young adults, which is in line with previous observations in healthy individuals (12, 13). In IBS patients however, small intestinal permeability was higher in elderly compared with young adults. Furthermore, we showed that the IBS-D subtype was associated with an increased small intestinal permeability in these elderly, confirming previous observations of the Maastricht IBS cohort (15) and others (26, 27). This indicates that the observed increase was not due to the aging per se. Using univariate non-parametric comparisons (*i.e.* Mann-Whitney U-tests), no significant differences were found in colonic permeability as measured by the 5-24h urinary S/E ratio between elderly and young adults neither in healthy individuals, nor in IBS patients. These findings are largely in line with the comparable 5-24h urinary S/E ratios between elderly without GI symptoms and younger healthy individuals and as shown by Ganda Mall *et al.* (14). However, parametric, multivariable linear regression analyses showed that healthy elderly had on average a significantly higher 5-24h urinary S/E ratio compared with healthy young adults. This was caused by a higher sucralose flux (data not shown), pointing towards a slightly increased paracellular permeability. The colon harbors a complex dense environment of not only beneficial microbes, but also potentially harmful microbes and antigens. Maintaining a well-functioning colonic barrier function with aging is advantageous since it will limit permeation of such components into the intestinal mucosa, and prevent mucosal damage, local and systemic immune activation. The 0-24h urinary S/E ratio, reflecting whole gut permeability, did not differ significantly between elderly and healthy young adults, but in IBS patients a higher whole gut permeability was observed in elderly compared with young adults. This can at least partly be explained by the observed effect of aging on small intestinal permeability in the diarrhea-predominant IBS patients. In the current study, sugar ratios were used to correct for *e.g.* transit time and renal clearance. Additionally, to determine intestinal permeability *ex vivo* as well as the susceptibility to a stressed condition, Ussing chamber experiments with sigmoid biopsies of a subgroup of healthy individuals were conducted. We demonstrated that after FDR correction, TEER and luminal fluorescein concentrations were not significantly different between elderly and young adults. To our knowledge, one

other study compared sigmoid biopsies of elderly with GI symptoms versus young healthy controls, and found no significant difference in TEER, although fluorescein flux and horseradish peroxidase (HRP) were significantly higher in elderly with GI symptoms compared with healthy controls (14). Unfortunately, no biopsies of IBS patients were available for Ussing chamber experiments. Moreover, a human study by Man *et al*. (28) performed Ussing chamber experiments in unstressed ileum biopsies and found that TEER values were significantly lower in healthy elderly (67-77 yrs) compared with healthy adults (20-40 yrs), while HRP flux remained unchanged. TEER does also reflect transcellular ion transport and thereby does not necessarily indicated alterations in the junctional complex. Differences between the locations of tissue sampling between the previously reported observations and the current study, the ileum and the colon, respectively, further impede direct comparison of the results. We used sigmoid biopsies to limit the invasiveness for the subjects and as a tightly controlled permeability is considered especially important in the colon with its high bacterial load. We did apply a mild physiological relevant stressor (1 µg/ml Compound 48/80), which resulted in an average increase in luminal fluorescein concentrations of 28.7% and average decrease in TEER of 13.2% after 120 minutes (data not shown) to check for a potential increased susceptility to stressed conditions. In the by Compound 48/80 mildly stressed biopsies, no differences were observed between young adults and elderly in in TEER and luminal fluorescein concentrations. This point towards a maintained adaptive capacity of the tissue samples with aging. Relative expression levels of genes related to the junctional complex, innate and adaptive immunity and barrier function in general, as analyzed by qPCR, showed no significant differences between healthy adults and healthy elderly after FDR correction. These observations were supported by immunofluorescent stainings of TJP1 and occludin in representative colon biopsy sections, confirming that the junctional complex seems to remain intact with aging. In the study by Man *et al*. (28), claudin-2 and IL-6 expression levels were found to be higher in ileum biopsies of elderly (67-77 yrs) compared with young adults (20-40 yrs), but no changes were found in TJP1, occludin, JAMA-1, IFN-γ, IL-1β and TNF-α. In that study, while testing a relatively large number of genes the results were not corrected for multiple testing (28), and therefore should be interpreted with care. In the current study corrections for multiple testing were applied and we show no statistically signicant differences between age groups. Secondly, findings of the ileum and sigmoid colon are difficult to compare because junctional complexes are stronger in the colon compared with

small intestine. Lastly, the intestinal barrier is not static, but constantly remodeling in order to selectively regulate intestinal permeability. For example, TJP1 stabilizes claudin strands and tightens them to the actin cytoskeleton. Therefore, single gene expression needs to be interpreted with care. In the current study, we investigated a broad range of junctional complex-related genes showing no significant differences with aging. The intestinal barrier is not only formed by the epithelial layer, but includes overlying mucus and produces for example antimicrobial proteins. Therefore we analyzed innate defense and immune related genes, but these did not differ significantly between healthy adults and elderly. Overall, our findings in *ex vivo* experiments are in line with the measured *in vivo* intestinal permeability.

The strength of the current study is that the effects of aging on gastroduodenal, small intestinal and colonic permeability were investigated in distinct age groups of healthy individuals and IBS patients using a combined *in vivo* and *ex vivo* approach, enabling to study functional as well as structural aspects of intestinal barrier function. This study showed that intercellular junctional functionality regulating paracelular permeation, was maintained with aging in both healthy individuals and IBS patients. Only small intestinal permeability was increased in IBS-D, independent of age. Moreover, the expression of barrier related genes was comparable between healthy elderly and healthy young adults. Intact intestinal permeability regulation can prevent permeation of noxious luminal substances into the intestinal mucosa, with subsequent local and systemic immune activation. In conclusion, although age-related factors such as medication use and co-morbidities may impact barrier function, we did not find an indication for impaired intestinal permeability in aging per se.

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Supplementary material

Supplementary Table 3.S1 - Forward and reverse primer sequences and final cDNA concentrations of all target genes, as determined in sigmoid tissue samples.

Name	Description	Primer sequences	cDNA concentration
CAMP	Cathelicidin antimicrobial peptide	5'-AGGATTGTGACTTCAAGAAGGACG-3' 5'-GTTTATTTCTCAGAGCCCAGAAGC-3'	80 ng/µl
CDH1	Cadherin 1	5'-CACCTGGAGAGAGGCCGCGT -3' 5'-AACGGAGGCCTGATGGGGCG -3'	20 ng/µl
CLDN2	Claudin 2	5'-AACTACTACGATGCCTACC-3' 5'-GAACTCACTCTTGACTTTGG -3'	20 ng/µl
CLDN3	Claudin 3	5'-TTCATCGGCAGCAACATCATC-3' 5'-CGCCTGAAGGTCCTGTGG-3'	20 ng/µl
CLDN4	Claudin 4	5'-ACAGACAAGCCTTACTCC-3' 5'-GGAAGAACAAAGCAGAG-3'	20 ng/µl
CTNNB1	Catenin beta 1	5'-GTGCTATCTGTCTGCTCTAGTA -3' 5'-CTTCCTGTTTAGTTGCAGCATC -3'	20 ng/µl
DEFB1	Defensin beta 1	5'-CTCTGTCAGCTCAGCCTC-3' 5'-CTTGCAGCACTTGGCCTTCCC-3'	20 ng/µl
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	5'-TGCACCACCAACTGCTTAGC-3' 5'-GGCATGGACTGTGGTCATGAG-3'	20 ng/µl
IL1B	Interleukin 1 beta	5'-AAACAGATGAAGTGCTCCTTCCAGG-3' 5'-TGGAGAACACCACTTGTTGCTCCA-3'	40 ng/µl
IL10	Interleukin 10	5'-TCAGGGTGGCGACTCTAT-3' 5'-TGGGCTTCTTTCTAAATCGTTC-3'	80 ng/µl
MUC2	Mucin 2	5'-GTCAACCCTGCCGACACCTG-3' 5'-ACTCACACCAGTAGAAAGGACAGC-3'	20 ng/µl
MLCK	Myosin light chain kinase	5-GCCTGACCACGAATATAAGTT-3' 5'-GCTCCTTCTCATCATCATCTG-3'	20 ng/µl
OCLN	Occludin	5'-TCAGGGAATATCCACCTATCACTTCAG-3' 5'-CATCAGCAGCAGCCATGTACTCTTCAC-3'	20 ng/µl
TFF3	Trefoil factor 3	5'-CTTGCTGTCCTCCAGCTCT-3' 5'-CCGGTTGTTGCACTCCTT-3'	20 ng/µl
TJP1 (ZO-1)	Tight junction protein 1	5'-AGGGGCAGTGGTGGTTTTCTGTTCTTTC-3' 5'-GCAGAGGTCAAAGTTCAAGGCTCAAGAGG-3'	20 ng/µl
TLR1	Toll like receptor 1	5'-CAGTGTCTGGTACACGCATGGT-3' 5'-TTTCAAAAACCGTGTCTGTTAAGAGA-3'	80 ng/µl
TLR2	Toll like receptor 2	5'-GCCAAAGTCTTGATTGATTGG-3' 5'-TATACCACAGGCCATGGAAAC-3'	20 ng/µl
TLR4	Toll like receptor 4	5'-CCTGCGTGAGACCAGAAAGC-3' 5'-TCAGCTCCATGCATTGATAAGTAATA-3'	80 ng/µl
TLR6	Toll like receptor 6	5'-GAAGAAGAACAACCCTTTAGGATAGC-3' 5'-AGGCAAACAAAATGGAAGCTT-3'	20 ng/µl
TNF	Tumor Necrosis Factor	5'-CCGAGTGACAAGCCTGTAGC-3' 5'-GAGGACCTGGGAGTAGATGAG-3'	40 ng/µl

Supplementary Table 3.S2 - Multivariable regression analyses of intestinal permeability *in vivo* including 0-5h urinary sucrose excretion, 0-5h urinary L/M ratio, 5-24h urinary S/E ratio and 0-24h urinary S/E ratio in healthy individuals (n=100).

	Variable	Adjusted effects B	95% CI	*P*-value
0-5h urinary sucrose excretion	Age group (elderly vs. young adults)	-16.430	-35.855; 2.996	0.096
	Sex (female vs. male)	0.952	-16.560; 18.464	0.914
	BMI	-0.373	-3.631; 2.884	0.821
	PPI use (yes vs. no)	-4.334	-44.018; 35.351	0.829
0-5h urinary lactulose/ mannitol ratio	Age group (elderly vs. young adults)	-0.051	-0.140; 0.038	0.257
	Sex (female vs. male)	-0.060	-0.140; 0.020	0.140
	BMI	0.010	-0.005; 0.025	0.204
	PPI use (yes vs. no)	-0.045	-0.227; 0.137	0.623
5-24h urinary sucralose/erythritol ratio	Age group (elderly vs. young adults)	0.030	0.001; 0.058	0.040
	Sex (female vs. male)	0.013	-0.012; 0.039	0.301
	BMI	-0.002	-0.007; 0.002	0.323
	PPI use (yes vs. no)	-0.019	-0.077; 0.039	0.521
0-24h urinary sucralose/erythritol ratio	Age group (elderly vs. young adults)	0.025	-0.011; 0.060	0.168
	Sex (female vs. male)	0.003	-0.029; 0.035	0.853
	BMI	-0.002	-0.008; 0.004	0.540
	PPI use (yes vs. no)	-0.025	-0.097; 0.047	0.495

BMI: body mass index, PPI: proton pump inhibitors, B: unstandardized regression coefficient, 95% CI: 95% confidence interval. The multivariable models include age group, sex, BMI and PPI use as independent variables. Assumptions for linear regression were met since there were no influential outliers based on Cooks distance ≤ 0.883, and collinearity was met as indicated by variance inflation factor values ≤ 1.418. R squares of the models were 0.048 for 0-5h urinary sucrose excretion, 0.055 for 0-5h urinary lactulose/ mannitol ratio, 0.057 for 5-24h urinary sucralose/erythritol ratio and 0.023 for 0-24h urinary sucralose/ erythritol ratio.

Supplementary Table 3.S3 - Multivariable regression analyses of intestinal permeability *in vivo* including 0-5h urinary sucrose excretion, 0-5h urinary L/R ratio, 5-24h urinary S/E ratio and 0-24h urinary S/E ratio in IBS patients (n=47).

	Variable	Adjusted effects		
		B	95% CI	*P*-value
0-5h urinary sucrose excretion	Age group (elderly vs. young adults)	1.391	-5.549; 8.331	0.688
	PPI use (yes vs. no)	1.593	-6.641; 9.827	0.698
	NSAID use (yes vs. no)	-6.345	-16.568; 3.878	0.217
	IBS subtype (IBS-D vs. other subtypes)	-3.170	-10.357; 4.017	0.378
0-5h urinary lactulose/ rhamnose ratio	Age group (elderly vs. young adults)	0.007	-0.006; 0.020	0.316
	PPI use (yes vs. no)	-0.010	-0.025; 0.006	0.216
	NSAID use (yes vs. no)	0.015	-0.004; 0.034	0.126
	IBS subtype (IBS-D vs. other subtypes)	0.018	0.004; 0.031	0.010
5-24h urinary sucralose/erythritol ratio [#]	Age group (elderly vs. young adults)	0.040	-0.021; 0.101	0.188
	PPI use (yes vs. no)	-0.006	-0.077; 0.065	0.871
	NSAID use (yes vs. no)	-0.051	-0.138; 0.035	0.239
	IBS subtype (IBS-D vs. other subtypes)	-0.045	-0.109; 0.018	0.157
0-24h urinary sucralose/erythritol ratio	Age group (elderly vs. young adults)	0.066	-0.021; 0.153	0.134
	PPI use (yes vs. no)	-0.014	-0.118; 0.089	0.779
	NSAID use (yes vs. no)	-0.067	-0.196; 0.061	0.297
	IBS subtype (IBS-D vs. other subtypes)	-0.054	-0.144; 0.037	0.237

NSAID: non-steroidal anti-inflammatory drugs, IBS: irritable bowel syndrome, PPI: proton pump inhibitors, B: unstandardized regression coefficient, 95% CI: 95% confidence interval. The multivariable models include age group, PPI use, NSAID use and IBS subtype use as independent variables. Assumptions for linear regression were met since there were no influential outliers based on Cooks distance ≤ 0.736, and collinearity was met as indicated by variance inflation factor values ≤ 1.211. R squares of the models were 0.043 for 0-5h urinary sucrose excretion, 0.210 for 0-5h urinary lactulose/rhamnose ratio, 0.099 for 5-24h urinary sucralose/erythritol ratio and 0.089 for 0-24h urinary sucralose/erythritol ratio. [#] Four missing values for this variable.

4

Chapter 4

Effects of supplementation of the synbiotic Ecologic® 825/FOS P6 on intestinal barrier function in healthy humans: a randomized controlled trial

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Abstract

Probiotics, prebiotics and synbiotics have been suggested as dietary strategies to improve intestinal barrier function. This study aimed to assess the effect of two weeks synbiotic supplementation on intestinal permeability under basal and stressed conditions. Secondary aims were the assessment of two weeks synbiotic supplementation on systemic immune function and gastrointestinal symptoms including defecation pattern. Twenty healthy adults completed a double-blind, controlled, randomized, parallel design study. Groups either received synbiotic (1.5 × 1010 CFU Ecologic® 825 + 10 g fructo-oligosaccharides (FOS P6) per day) or control supplements for two weeks. Intestinal segment specific permeability was assessed non-invasively by oral administration of multiple sugar probes and, subsequently, assessing the excretion of these probes in urine. This test was conducted at baseline and at the end of intervention, in the absence and in the presence of an indomethacin challenge. Indomethacin was applied to induce a compromised gut state. Plasma zonulin, cytokines and chemokines were measured at baseline and at the end of intervention. Gastrointestinal symptoms and stool frequency were recorded at baseline and daily during intervention. Significantly more male subjects were in the synbiotic group compared to the control group ($P = 0.025$). Indomethacin significantly increased urinary lactulose/rhamnose ratio versus without indomethacin, both in the control group ($P = 0.005$) and in the synbiotic group ($P = 0.017$). Urinary sugar recoveries and ratios, plasma levels of zonulin, cytokines and chemokines, and gastrointestinal symptom scores were not significantly different after control or synbiotic intervention. Stool frequency within the synbiotic group was significantly increased during synbiotic intervention compared to baseline ($P = 0.039$) and higher compared to control intervention ($P = 0.045$). Two weeks Ecologic® 825/FOS P6 supplementation increased stool frequency, but did not affect intestinal permeability neither under basal nor under indomethacin-induced stressed conditions, immune function or gastrointestinal symptoms in healthy adults.

Introduction

Epithelium integrity of the gastrointestinal tract is pivotal for maintainance of the intestinal barrier. Apart from epithelial cells, the activation state of the immune system, intestinal microbiota and their metabolism, mucus production, secretion of antimicrobial peptides, tight junction proteins and the enteric nervous system all contribute to maintaining the intestinal barrier. Patients with intestinal diseases such as inflammatory bowel disease (IBD) (1, 2) or celiac disease (3, 4) show increased intestinal permeability. It is not clear whether increased intestinal permeability is a causal factor or a consequence of intestinal disorders, however, in a subset of relatives of symptom-free IBD patients intestinal permeability was found to be increased (5). These subjects have a significantly inceased risk to develop IBD, suggesting that changes in intestinal permeability precede the development of intestinal disease. Reinforcement of (disturbed) intestinal barrier may thus become an important target in prevention and treatment of intestinal disorders (6).

Prebiotics and probiotics have been proposed as promising interventions to improve intestinal barrier function. Indeed, in several studies the effects of probiotics and prebiotics on intestinal permeability have been investigated in healthy volunteers. While some human intervention studies found evidence for improvement in intestinal permeability after either probiotic (7, 8), prebiotic (9), or synbiotic (10) consumption, others did not observe any change in permeability with a prebiotic product (11).

Recently, *in vitro, ex vivo* and animal studies have shown positive effects of the multispecies probiotic mixture Ecologic® 825 on intestinal barrier function (12-14). Our aim was to evaluate the effect of this mixture Ecologic® 825 on intestinal barrier function in healthy volunteers. In addition we chose to fortify the multispecies probiotic mixture with fructo-oligosaccharides (FOS P6) to stimulate selective growth and activity of the probiotic strains. We hypothesized that a two-week synbiotic supplementation will decrease intestinal permeability in healthy adults, both under basal conditions and under conditions of mucosal stress, induced by administering the non-steroidal anti-inflammatory drug indomethacin. The primary aim was to assess the effect of two weeks synbiotic supplementation on intestinal permeability under basal and stressed conditions. Secondary aims were to evaluate the effect of two weeks synbiotic supplementation on systemic immune function, gastrointestinal symptoms and stool frequency.

Methods

This last version of the study protocol was approved by the Medical Ethics Committee of the Maastricht University Medical Center + at 13 November 2013, and performed in accordance with 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 was performed at the Maastricht University Medical Center + from 20 November 2013 to 28 May 2014. This study was part of a larger study which has been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, ID NCT02018900). The study protocol included a detailed analysis of microbiota composition and functionality along the gastrointestinal tract by sampling content from the duodenum, jejunum, ileum and feces. Those data will be published in a separate manuscript. All subjects gave written informed consent before screening.

Subjects

Healthy men and women were recruited by local advertisements. Inclusion criteria included age between 18 and 65 years, and body mass index (BMI) between 20 and 30 kg/m². Exclusion criteria included gastrointestinal symptoms, history of any chronic disorder, allergy, major surgery, self-reported human immunodeficiency virus, excessive alcohol consumption (>20 alcohol units per week), smoking, pregnancy, lactation, use of any medication or vitamin supplements 14 days prior to testing, use of antibiotics 90 days prior to testing, blood donation three months prior to testing, use of pro- or prebiotics 180 days prior testing, and a history of side effects towards pro- or prebiotic supplements.

Sample size calculation

The sample size calculation was based on the difference in urinary lactulose recovery between indomethacin ingestion and placebo ingestion as reported by van Wijck *et al*. (15). We assumed a difference between treatments of 3.04 µmol (20%), standard deviation of 2.10 µmol, an alpha of 0.05, and a power of 0.80. Based on this calculation, 9 participants per group were needed to complete the study to reach sufficient statistical power. We included 10 participants per group because of the estimated dropout rate of 10% (Figure 4.1).

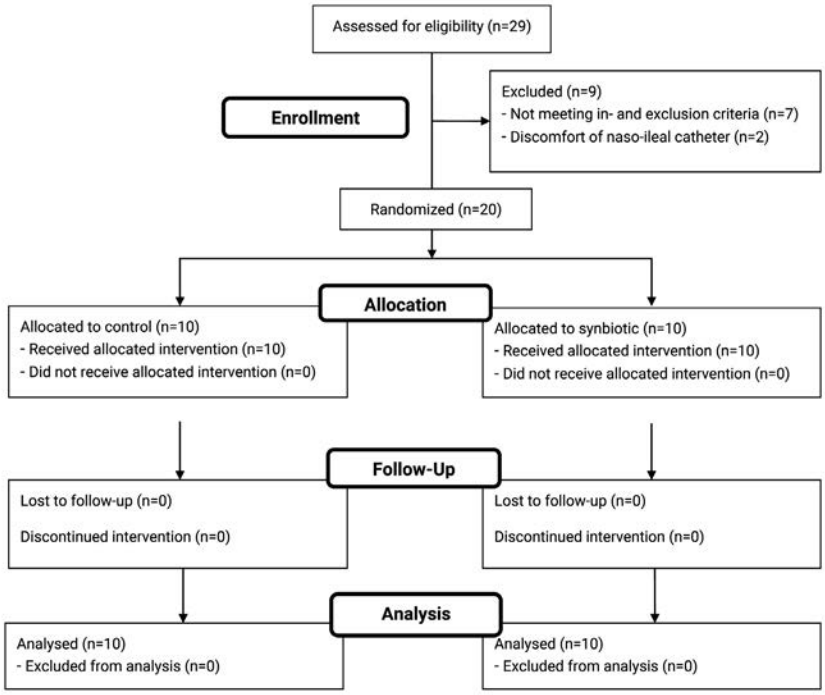

Figure 4.1 - Flow diagram of the study.

Study design

The study was a double-blind, randomized, controlled, parallel design study. Participants were randomly and equally assigned to the control or synbiotic group. The randomization list was generated by using a computerized procedure. All participants and investigators remained blinded to treatment until all analyses were completed. Subjects in the synbiotic group received synbiotic supplements that were composed of a multispecies probiotic mixture (Ecologic® 825, 6 g/day, $1.5*10^{10}$ colony-forming units/day); Winclove Probiotics BV, Amsterdam, the Netherlands) comprising *Bifidobacterium bifidum* (W23), *B. lactis* (W51), *B. lactis* (W52), *Lactobacillus acidophilus* (W22), *L. casei* (W56), *L. paracasei* (W20), *L. plantarum* (W62), *L. salivarius* (W24) and *Lactococcus lactis* (W19) combined with fructo-oligosaccharides (FOS P6, degree of polymerization between 3 and 5, 10 g/day; Winclove Probiotics BV, Amsterdam, the Netherlands). Subjects in the control group received the same carrier material as the multispecies probiotic mixture (6 g/day; Winclove Probiotics BV, Amsterdam, the Netherlands), but without probiotic strains, combined with maltodextrin (10 g/

day; Winclove Probiotics BV, Amsterdam, the Netherlands) instead of FOS. Carrier material comprised maize starch, maltodextrins, a mineral mix, inulin and FOS (P6; inulin and FOS comprised maximum 15% of the total carrier material). Synbiotic and control mixtures had an identical appearance and were supplied in duo sachets. Subjects ingested the mixtures every morning and evening at the same time, for two weeks. The total sachet content was dissolved in 200 ml lukewarm water, left for 10 min to mix and dissolve, stirred and subsequently ingested. The time of consumption was recorded in a diary. All empty and remaining duo sachets were returned to the investigator. At day -6 and day 14 a multi-lumen customized sampling catheter (Mui Scientific, Mississauga, Ontario, Canada) was placed in the small intestine for analysis of small intestinal microbiota composition and functionality (data will be published in a separate manuscript). To determine plasma zonulin, cytokine- and chemokine concentrations, blood samples were taken at day -5 and day 15. Intestinal permeability was examined by multi-sugar tests without indomethacin challenge at day -3 and day 17 and with indomethacin challenge at day -1 and day 19. Gastrointestinal symptom scores and stool frequency were recorded once daily at baseline and during 14 days of supplementation. (Figure 4.2)

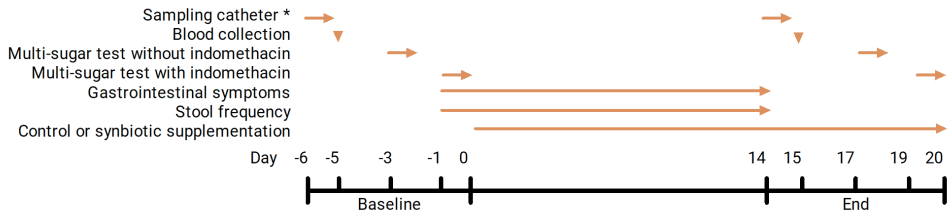

Figure 4.2 - Timeline of the intervention period. Sampling catheter, blood collection, multi-sugar tests, gastrointestinal symptoms, stool frequency, and control or synbiotic supplementation were completed at the days as indicated by arrows. * Data not in present manuscript.

Intestinal permeability

Permeability of different segments of the gastrointestinal tract was assessed non-invasively by multi-sugar tests as validated by van Wijck *et al*. (15, 16). One day prior to testing, and during all test days, excessive physical exercise and consumption of alcohol were not allowed. Water-soluble, non-degradable sugar probes were ingested after fasting overnight. Subjects ingested 1 g sucrose (Van Gilse, Dinteloord, the

Netherlands), 1 g lactulose (Centrafarm Services, Etten-Leur, the Netherlands), 0.5 g L-rhamnose (Danisco sweeteners, Thomson, IL, USA), 1 g sucralose (Tate and Lyle Ingredients Americas, Decatur, IL, USA) and 1 g erythritol (Now Foods, Bloomindale, IL, USA). Subjects collected 24 hours (h) urine in two separate containers; 0-5 h and 5-24 h after sugar ingestion. Subjects were not allowed to consume food or drinks, except for water *ad libitum*, during the first 5 h of urine collection. After these 5 h, subjects were allowed to eat and drink as preferred, with the exception of sucralose containing foods. Indomethacin was ingested to induce standardized, reversible damage to the healthy small intestine (15). Exactly nine hours and one hour prior to the intake of the multi-sugar drink, subjects ingested 75 mg and 50 mg of indomethacin Retard (Mylan, Bunschoten, the Netherlands), respectively. After urine collection, urine was handed in, volumes of urine fractions were determined and urine aliquots were frozen at -80 °C until analysis. Sugar probes were analyzed by isocratic ion-exchange High Performance Liquid Chromatography with mass spectrometry as described previously (15, 16). Gastroduodenal permeability was determined by sucrose excretion in 0-5 h urine, whereas small intestinal permeability was measured by lactulose excretion and the lactulose to rhamnose (L/R) ratio in 0-5 h urine. Sucralose excretion as well as the sucralose to erythritol (S/E) ratio in 5-24 h urine were used as indicators for colonic permeability. Rhamnose excretion in 0-5 h urine and erythritol excretion in 5-24 h urine were measured and reported, albeit these are no parameters of intestinal permeability. Further, participants fasted for at least ten hours before blood sampling. Blood was collected in BD Vacutainer® K_2EDTA tubes (BD, Breda, The Netherlands), and centrifuged at 3000 × g for 10 min at 4 °C. Plasma was stored at -80 °C until analysis. As biomarker of intestinal barrier disruption, active uncleaved zonulin was measured in blood plasma by using a standard Zonulin Enzyme-Linked ImmunoSorbent Assay Kit (K5601, Immundiagnostik AG, Bensheim, Germany) and expressed as ng/ml blood plasma.

Immune function

Plasma levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1b, IL-6, IL-8, IL-17, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1α) were measured by using the Bio-Plex Pro™ Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and expressed as pg/ml blood plasma. IL-17 and MIP-1α were excluded from statistical analyses because values were under the detection limit of the assay.

Gastrointestinal symptoms and stool frequency

At baseline and during the 14 days supplementation period participants completed a 'symptoms diary' at the end of each day. This non-validated questionnaire has been described before by Salden *et al*. (17). Feelings of abdominal discomfort, abdominal pain, abdominal distension, constipation, diarrhea, flatulence, eructation, nausea and total discomfort were assessed by scores from 1 (no symptoms) to 5 (pronounced symptoms). In addition, stool frequency was assessed as number of bowel movements per day.

Statistical analyses

The primary outcome of the study was the effect of two weeks synbiotic supplementation on intestinal permeability under basal and stressed conditions. Secondary outcomes were the effects of two weeks synbiotic supplementation on systemic immune function and on gastrointestinal symptoms including stool frequency. Statistical analyses were performed using IBM SPSS Statistics Version 21 (IBM Corporation, Armonk, NY, USA). A Shapiro-Wilk test was performed to test for normality. Gender was compared between intervention groups by a Chi-square test. Age, BMI, parameters of intestinal permeability, zonulin concentration, cytokine- and chemokine concentrations, and average symptom scores were compared non-parametrically. Mann-Whitney U tests and a Wilcoxon signed-rank tests were used to compare data between and within groups, respectively. Stool frequency was normally distributed. An independent samples t-test and a paired samples t-test were performed to compare between and within group data, respectively. For all analyses a two-sided test was performed and $P < 0.05$ was considered statistically significant.

Results

Subjects

As shown in the flow diagram (Figure 4.1), a total of 29 volunteers were recruited. Seven volunteers did not meet the in- and exclusion criteria. Two participants terminated the study before allocation of the intervention, due to discomfort of the naso-ileal catheter which was placed to sample small intestinal contents. Therefore, 20 participants completed the entire study protocol and were included in the analyses. In the control group (n=10) 30% were males, with a median age of 21.7 [20.0-24.0] years and median BMI of 24.1 [22.9-24.9] kg/m^2. In the synbiotic group (n=10) 80% were males, with a median age of 19.7 [19.1-21.8] years and median BMI of 22.9 [21.7-24.1] kg/m^2. (Table 4.1)

Table 4.1 - Baseline characteristics of the control group (n=10) and synbiotic group (n=10).

	Control (n=10)	Synbiotic (n=10)	*P*-value
Gender (male : female)	3 : 7	8 : 2	0.025
Age (yrs)	21.7 [20.0-24.0]	19.7 [19.1-21.8]	0.082
BMI (kg/m2)	24.1 [22.9-24.9]	22.9 [21.7-24.1]	0.226

Values are presented as median and IQR (25-75th interquartile range). Gender was compared between groups with the use of a Chi-square test. Age and BMI were compared between groups with the use of a Mann-Whitney U test. BMI, Body Mass Index.

Gastrointestinal permeability

Effect of indomethacin: Urinary sugar excretions and ratios at baseline are given in Table 4.2. Data of urinary sugar excretion after indomethacin challenge were compared to data of urinary sugar excretion without indomethacin challenge. In the synbiotic group, indomethacin significantly decreased urinary rhamnose excretion and significantly increased urinary L/R ratio. In the control group, indomethacin significantly increased urinary sucrose excretion, urinary lactulose excretion and urinary L/R ratio, pointing to increased gastroduodenal and small intestinal permeability. Urinary sucralose excretion, urinary erythritol excretion and urinary S/E ratio were not affected by indomethacin.

Table 4.2 - Urinary sugar excretions (µmol) and ratios of the control group (n=10) and synbiotic group (n=10) at baseline without and with indomethacin challenge.

	Control			Synbiotic		
	Without indomethacin	With indomethacin	*P*-value	Without indomethacin	With indomethacin	*P*-value
0-5 h sucrose	6.84 [5.65-9.12]	9.67 [8.50-18.94]	0.022	7.24 [6.11-10.89]	13.28 [6.03-19.50]	0.333
0-5 h lactulose	6.19 [5.14-7.24]	11.73 [9.30-16.27]	0.005	14.08 [6.30-23.03]	18.92 [11.45-31.76]	0.139
0-5 h rhamnose	287 [216-336]	252 [163-327]	0.285	395 [280-603]	213 [241-358]	0.037
0-5 h L/R ratio	0.023 [0.020-0.026]	0.061 [0.042-0.074]	0.005	0.032 [0.022-0.043]	0.064 [0.046-0.106]	0.017
5-24 h sucralose	39.48 [31.54-93.49]	47.00 [37.89-63.15]	0.799	59.29 [49.75-71.59]	54.29 [43.16-95.52]	0.445
5-24 h erythritol	3291 [2490-3523]	2731 [2164-3073]	0.093	3163 [2483-3460]	2845 [1700-3469]	0.285
5-24 h S/E ratio	0.016 [0.011-0.023]	0.021 [0.012-0.024]	0.646	0.019 [0.014-0.023]	0.023 [0.014-0.047]	0.241

Values are presented as median and IQR (25-75th interquartile range). Urinary sugar excretions and ratios without indomethacin vs. with indomethacin were compared with the use of a Wilcoxon signed-rank test; L/R, lactulose/rhamnose; S/E, sucralose/erythritol.

Effect of synbiotic supplementation: Within the synbiotic group, no significant differences were found when comparing values observed at baseline and after intervention in urinary sucrose excretion, urinary lactulose excretion, urinary rhamnose excretion, urinary L/R ratio, urinary sucralose excretion, urinary erythritol excretion or urinary S/E ratio, neither without nor with indomethacin challenge (Table 4.3). Besides the significantly higher urinary rhamnose excretion after intervention with indomethacin challenge, no significant differences were observed in the control group with respect to urinary sugar excretions and ratios (Table 4.4). Also, no significant differences were observed between the synbiotic and control group after the two-week supplementation period in urinary sucrose excretion, urinary lactulose excretion, urinary rhamnose excretion, urinary L/R ratio, urinary sucralose excretion, urinary erythritol excretion, or urinary S/E ratio. Overall, synbiotic supplementation did not influence gastroduodenal, small intestinal or colonic permeability.

Table 4.3 - Urinary sugar excretions (µmol) and ratios of the synbiotic group (n=10) at baseline and after two weeks synbiotic supplementation, without and with indomethacin challenge.

	Without indomethacin		*P*-value	With indomethacin		*P*-value
	Baseline	End		Baseline	End	
0-5 h sucrose	7.24 [6.11-10.89]	10.72 [7.02-19.53]	0.059	13.28 [6.03-19.50]	13.71 [9.93-21.06]	0.959
0-5 h lactulose	14.08 [6.30-23.03]	9.34 [6.92-18.68]	0.575	18.92 [11.45-31.76]	17.91 [9.17-24.97]	0.721
0-5 h rhamnose	395 [280-603]	383 [280-436]	0.139	213 [241-358]	360 [251-414]	0.799
0-5 h L/R ratio	0.032 [0.022-0.043]	0.031 [0.024-0.044]	0.878	0.064 [0.046-0.106]	0.055 [0.037-0.072]	0.203
5-24 h sucralose	59.29 [49.75-71.59]	40.10 [33.52-74.44]	0.285	54.29 [43.16-95.52]	51.95 [40.83-64.85]	0.646
5-24 h erythritol	3163 [2483-3460]	2864 [2604-3176]	0.333	2845 [1700-3469]	3316 [2276-3602]	0.169
5-24 h S/E ratio	0.019 [0.014-0.023]	0.014 [0.013-0.026]	0.508	0.023 [0.014-0.047]	0.016 [0.015-0.022]	0.169

Values are presented as median and IQR (25-75th interquartile range). Urinary sugar excretions and ratios at baseline vs. end were compared with the use of a Wilcoxon signed-rank test. L/R, lactulose/rhamnose; S/E, sucralose/erythritol.

Table 4.4 - Urinary sugar excretions (µmol) and ratios of the control group (n=10) at baseline and after two weeks intervention, without and with indomethacin challenge.

	Without indomethacin		*P*-value	With indomethacin		*P*-value
	Baseline	End		Baseline	End	
0-5 h sucrose	6.84 [5.65-9.12]	8.69 [6.76-10.94]	0.169	9.67 [8.50-18.94]	9.82 [6.89-18.70]	0.646
0-5 h lactulose	6.19 [5.14-7.24]	9.56 [6.59-13.29]	0.139	11.73 [9.30-16.27]	18.06 [10.77-25.39]	0.203
0-5 h rhamnose	287 [216-336]	341 [269-474]	0.285	252 [163-327]	312 [260-464]	0.037
0-5 h L/R ratio	0.023 [0.020-0.026]	0.025 [0.021-0.041]	0.093	0.061 [0.042-0.074]	0.055 [0.041-0.071]	0.799
5-24 h sucralose	39.48 [31.54-93.49]	44.43 [31.41-58.27]	0.333	47.00 [37.89-63.15]	53.51 [41.63-63.76]	0.386
5-24 h erythritol	3291 [2490-3523]	2565 [2035-2865]	0.059	2731 [2164-3073]	3052 [2386-3395]	0.285
5-24 h S/E ratio	0.016 [0.011-0.023]	0.021 [0.013-0.023]	0.203	0.021 [0.012-0.024]	0.018 [0.014-0.024]	0.959

Values are presented as median and IQR (25-75th interquartile range). Urinary sugar excretions and ratios at baseline vs. end were compared with the use of a Wilcoxon signed-rank test. L/R, lactulose/rhamnose; S/E, sucralose/erythritol.

Zonulin

Within the synbiotic group, plasma zonulin was 14.3 [12.6-17.1] ng/ml at baseline and 13.2 [10.4-17.2] ng/ml at the end of intervention (*P* = 0.721). Plasma zonulin was 14.8 [11.6-16.3] ng/ml at baseline and 14.7 [12.1-15.6] ng/ml at the end intervention in the control group (*P* = 0.959). Moreover, at the end of intervention, plasma zonulin concentrations were not significantly different between the control group and synbiotic group (*P* = 0.650).

Immune function

To study the effect of synbiotic supplementation on immune modulation, plasma cytokines and chemokines were determined before and after the intervention. TNF-α, IL-1β, IL-6, IL-8 and MCP-1 were not significantly different between the control and synbiotic group at baseline (all *P* > 0.174). After two weeks of supplementation, neither plasma TNF-α, IL-1β, IL-6, IL-8 nor MCP-1 differed between baseline versus end in the synbiotic or control group (Table 4.5).

Table 4.5 - Plasma cytokines and chemokines of the control group (n=10) and synbiotic group (n=10) at baseline and after two weeks.

	Control		*P*-value	Synbiotic		*P*-value
	Baseline	End		Baseline	End	
TNF-α (pg/ml)	1.75 [1.17-8.08]	1.78 [1.11-6.10]	0.374	3.55 [1.11-6.95]	3.36 [2.18-5.70]	0.799
IL-1β (pg/ml)	0.08 [0.05-0.11]	0.05 [0.03-0.08]	0.112	0.10 [0.05-0.15]	0.13 [0.09-0.17]	0.540
IL-6 (pg/ml)	0.25 [0.11-0.39]	0.22 [0.01-0.50]	0.249	0.50 [0.22-1.45]	0.31 [0.25-0.60]	0.208
IL-8 (pg/ml)	0.95 [0.72-1.40]	0.94 [0.69-1.53]	0.878	1.22 [0.93-1.69]	1.39 [1.00-1.91]	0.878
MCP-1 (pg/ml)	18.79 [12.60-29.40]	19.14 [14.57-27.31]	0.878	25.96 [18.87-31.23]	25.80 [22.66-28.73]	0.333

Values are presented as median and IQR (25-75th interquartile range). Plasma cytokines and chemokines at baseline vs. end were compared with the use of a Wilcoxon signed-rank test. TNF-α, Tumor necrosis factor-alpha; IL, Interleukin; MCP-1, Monocyte chemoattractant protein-1.

Gastrointestinal symptoms and stool frequency

At baseline and during the intervention period average gastrointestinal symptom scores were not significantly different between control and synbiotic supplementation (Table 4.6). Stool frequency did not significantly differ between the control group and synbiotic group at baseline (*P* = 0.177). Stool frequency within the synbiotic

group was 1.54 ± 0.59 bowel movements per day during the intervention, which was a significant increase compared to 1.00 ± 0.47 bowel movements at baseline (Figure 4.3), and significantly higher compared to 1.02 ± 0.47 bowel movements per day the intervention in the control group (Figure 4.3).

Table 4.6 - Gastrointestinal symptom scores at baseline and during control (n=10) and synbiotic (n=10) supplementation.

	Baseline		*P*-value	Average during supplementation		*P*-value
	Control	Synbiotic		Control	Synbiotic	
Abdominal discomfort	1.00 [1.00-2.00]	1.00 [1.00-2.00]	0.687	1.39 [1.00-1.80]	1.25 [1.00-1.77]	0.670
Abdominal pain	1.00 [1.00-1.25]	1.00 [1.00-2.00]	0.726	1.18 [1.00-1.29]	1.14 [1.00-1.39]	0.908
Abdominal distension	1.00 [1.00-1.25]	1.00 [1.00-1.25]	0.914	1.18 [1.00-1.68]	1.14 [1.00-2.00]	0.938
Constipation	1.00 [1.00-1.25]	1.00 [1.00-1.00]	0.146	1.00 [1.00-1.16]	1.07 [1.00-1.46]	0.361
Diarrhea	1.00 [1.00-1.00]	1.00 [1.00-1.00]	0.317	1.14 [1.05-1.23]	1.07 [1.00-1.23]	0.461
Flatulence	1.00 [1.00-2.00]	1.00 [1.00-1.25]	0.726	1.29 [1.07-2.14]	1.50 [1.14-2.14]	0.492
Eructation	1.00 [1.00-1.25]	1.00 [1.00-2.00]	0.451	1.00 [1.00-1.14]	1.18 [1.00-1.39]	0.225
Nausea	1.00 [1.00-1.00]	1.00 [1.00-1.00]	0.317	1.00 [1.00-1.02]	1.07 [1.00-1.32]	0.084
Total discomfort	1.00 [1.00-2.00]	1.00 [1.00-2.00]	0.888	1.25 [1.00-1.71]	1.36 [1.00-2.00]	0.938

Symptoms are scored on a five-point Likert scale. Values are presented as median and IQR (25-75th interquartile range). Average gastrointestinal symptom scores control vs. synbiotic were compared with the use of a Mann-Whitney U test.

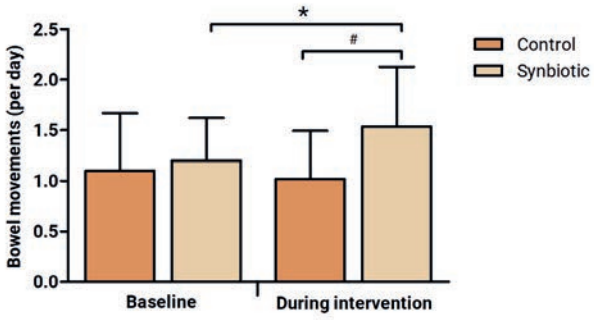

Figure 4.3 - Stool frequency indicated as bowel movements per day (mean ± SD) at baseline and during synbiotic (n=10) or control (n=10) supplementation. * Significantly increased (P = 0.045) based on paired samples t-test. # Significantly higher (P = 0.039) based on independent samples t-test.

Discussion

This study showed that supplementation with the synbiotic mixture Ecologic® 825/ FOS P6 did not affect intestinal permeability neither without nor with indomethacine challenge. In line with these findings, no effect of the synbiotic intervention on plasma levels of zonulin and proinflammatory cytokines and chemokines were observed. Administration of indomethacin resulted in an increase in gastroduodenal and small intestinal permeability, confirming previous observations that this serves as a reproducible model for a compromised gut (15). As expected, colonic permeability was not affected by indomethacin administration. In contrast to our hypothesis, two weeks synbiotic supplementation did not prevent or reduce gastroduodenal and small intestinal permeability, even under compromised conditions.

Indomethacin is known to inhibit cyclooxygenase (COX)-1 and COX-2 activity in the stomach and small intestine and subsequently can cause direct damage to the enterocyte by disruption of the mitochondrial process through uncoupling of oxidative phosphorylation, leading to reduced intracellular adenosine triphosphate levels (18, 19). Reductions in adenosine triphosphate-dependent actin organisation and myosin-dependent contractility will lead to an impaired tight junction complex integrity (19). An *in vitro* study showed that *L. rhamnosus* GG induced COX-2 expression in T84 cells, up to 48 h from the start of incubation (20). Moreover, a human intervention study in healthy volunteers has shown that intake of *L. rhamnosus* GG significantly reduced the indomethacin-induced alteration in gastric permeability as determined by the urinary sucrose excretion, but not intestinal permeability as determined by urinary lactulose/ mannitol ratio (8). Thus, probiotics exert strain-specific and strain-dependent effects. *L. rhamnosus* GG was not included in the synbiotic mixture used in the present study. Ecologic® 825 contains probiotic strains belonging to *B. bifidum*, *B. lactis*, *L. acidophilus* , *L. casei*, *L. paracasei*, *L. plantarum*, *L. salivarius* and *Lactococcus lactis*. Several *in vitro* studies showed that single strains of *B. bifidum* (21), *L. casei* (22, 23) and *L. plantarum* (24) were able to increase transepithelial electrical resistance in intestinal epithelial cells. Although Ecologic® 825 combined with FOS P6 did not reinforce intestinal barrier function in healthy adults in this study, a previous study has shown positive effects of eight weeks Ecologic® 825 supplementation on intestinal barrier function in IBD patients with active pouchitis, which also received antibiotic treatment before the start of the probiotic supplementation (12). In an Ussing chamber experiment using human ileum mucosa samples from pouchitis patients with an ileoanal pouch, the

transmucosal passage of *Escherichia coli* K12 and the permeability to horseradish peroxidase were lower after probiotic supplementation, when compared to intestinal mucosa after antibiotic treatment and when compared to intestinal mucosa of healthy individuals. Therefore, it was concluded that Ecologic® 825 restored the mucosal barrier in patients suffering from active pouchitis (12). It should be noticed that IBD patients have a disturbed barrier function, whereas here we investigated healthy volunteers in whom the barrier is considered to be intact although we attempted to mimic the compromised state with the indomathic stressor protocol. Further, Ecologic® 825 has been shown to exert protective effects on the colonic mucosal barrier in rat models of chronic stress. It has been suggested that these effects are modulated by a mast cell dependent pathway (13). Ecologic® 825 has been suggested to be able to modulate mast cells (14). Mast cell function or mast cell markers or metabolites have unfortunately not been evaluated in our study.

In our study, Ecologic® 825/FOS P6 supplementation did not affect plasma zonulin concentrations. Circulating zonulin is a marker of intestinal permeability, and levels are higher in cases of increased intestinal permeability (25, 26). Data on effects of probiotics or prebiotics on circulating zonulin levels are limited. In one randomized crossover study serum zonulin was found to be decreased by five weeks inulin-enriched pasta intake in healthy male subjects, indicating that the intestinal barrier function was improved (9). In a case study fecal zonulin has shown to be significantly decreased by eight weeks Ecologic® 825 supplementation in subjects who had an elevated zonulin level (>30 ng/ml) at baseline (27). Another randomized, double-blind, placebo controlled trial has shown a decrease of fecal zonulin levels by 14 weeks of probiotic mix Ecologic® Performance supplementation in trained men (28). It should be noted, however, that we did not measure fecal zonulin levels, and thus the outcomes of this study and the present are not directly comparable.

Studies investigating effects of probiotics and prebiotics on immune modulation by determining cytokines in healthy volunteers have reported variable outcomes. A twelve-week intervention with a multispecies probiotic containing *L. acidophilus, B. lactis and B. bifidum* combined with FOS, showed a significant decrease in proinfammatory cytokines IL-6 and IL-1β when peripheral blood mononuclear cells were isolated and cultured *ex vivo* in presence of lipopolysaccharide (29). In a randomized, double-blind, placebo-controlled trial three different probiotic strains were supplemented for 30 days. Serum levels of anti-inflammatory cytokines IL-4 and IL-10 significantly increased

in subjects that consumed with *L. rhamnosus*. Subjects receiving *L. rhamnosus* and *L. paracasei* showed a significantly increased IL-10/IL-12 ratio, which is considered as an anti-inflammatory index. Moreover, *L. rhamnosus* decreased proinflammatory index TNF-α/IL-10 ratio (30). *L. salivarus* supplementation for four weeks has also been shown to significantly increase plasma IL-10 levels (31). In contrast, no differences in cytokine expression were found in a double-blind, placebo-controlled study after two months of *L. reuteri* supplementation (32). Also six weeks *Bacteroides xylanisolvents* supplementation did not affect inflammatory markers IL-6, interferon-γ and C-reactive protein levels (33). Furthermore, four weeks prebiotic β2-1 fructan supplementation did not alter cytokine levels in *in vitro* restimulated blood (34). Taken together, previous studies on effects of probiotic and prebiotic supplementation on cytokine production *in vivo* did not provide consistent results. No changes in plasma cytokine- and chemokine levels as compared to baseline values have been observed in the present study. In future studies it might be considered to measure cytokine production after *in vitro* stimulation of peripheral blood mononuclear cells (35).

In this study symptom diaries were completed at baseline and during 14 days of supplementation with synbiotic or control. Average symptom scores were low, and ranged between 1.0 and 1.7 on a five-point Likert scale in both groups. No significant differences were observed in average symptom scores between control and Ecologic® 825/FOS P6 intervention. However, Ecologic® 825/FOS P6 supplementation increased stool frequency significantly. Several studies in healthy adults showed that *L. salivarius* (31), *L. rhamnosus* combined with *L. paracasei* (36), *L. acidophilus* combined with lactitol (37), *L. gasseri* combined with *L. coryniformis* (38), FOS (39) and arabinoxylan oligosaccharides (40) significantly increased stool frequency in healthy adults. In contrast, *L. casei Shirota* has been shown to decrease stool frequency in healthy adults with soft stools at baseline (41). In another study, three weeks of *L. rhamnosus* intake did not influence stool frequency (42). A stool frequency considered as normal may vary between one bowel movement per three days to three bowel movements per day. In both intervention groups of our study, stool frequency was within this range. We found evidence that supplementation with the synbiotic Ecologic® 825/FOS P6 increases stool frequency, possibly through acceleration of transit or through other mechanisms. In this respect it will be interesting to evaluate the effects of the synbiotic Ecologic® 825/FOS P6 in subjects with symptomatic constipation. Overall, intake of synbiotic Ecologic® 825/FOS P6 was well tolerated by human subjects in our study.

The generalisability of this study across populations and situations is high, as

healthy volunteers were recruited by broad in- and exclusion criteria, and subjects maintained there habitual lifestyle. Moreover, we used a stressor protocol to mimic a compromised state, as for instance in IBD or celiac patients (43). However, some limitations should be considered. Firstly, although the study was executed as a double-blind randomized controlled study, it had a parallel design. A cross-over study would have allowed comparison within subjects but also is prone to carry-over effects and would have extended the participation period for individual participants considerably, which hampers study compliance and completion rate. Secondly, we assumed that habitual diet in general, and fiber intake more specifically, was the same before and during intervention but we did not control for that. In future research a run-in period of for instance two weeks, in which subjects consume a standardized diet, should be considered in order to correct for differences in dietary fiber intake (9). Thirdly, the intervention period of two weeks may have been too short to demonstrate significant changes in intestinal permeability. Forthly, we chose to combine Ecologic® 825 with FOS P6 with the assumption to stimulate selective growth and activity of the probiotic strains. This assumption was based on *in vitro* pilot data, not on *in vivo* human data. Theoretically although unlikely, it may be that *in vivo* the mix of pro- and prebiotics may not enhance but possibly counteract beneficial strain specific effects. Fifth, a significant gender dysbalance was present between intervention groups. Finally, baseline urinary sucrose and lactulose excretions were significantly increased after indomethacin challenge in the control group but not in the Ecologic® 825/FOS P6 group. However, the lactulose/rhamnose ratio and sucralose/erythritol ratio are the most accurate parameters of intestinal permeability, because these parameters correct for pre-absorption factors such as gastric emptying, dilution by secretion and intestinal transit time, and post-absorption factors such as systemic distribution and renal clearance affecting both molecules of the ratio equally (44). Hence, these most important parameters were equally affected by indomethacin in the control and Ecologic® 825/FOS P6 intervention groups.

In conclusion, two weeks Ecologic® 825/FOS P6 supplementation did not reduce intestinal permeability under basal and under indomethacin-induced stressed conditions in healthy adults. Furthermore, two weeks Ecologic® 825/FOS P6 supplementation did not alter immune function and gastrointestinal symptoms in healthy adults. Stool frequency was increased by two weeks Ecologic® 825/FOS P6 supplementation.

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5

Chapter 5

The impact of pectin supplementation on intestinal barrier function in healthy young adults and healthy elderly

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Abstract

Intestinal barrier function is suggested to decrease with aging and may be improved by pectin intake. The aim of this study was to investigate the effects of four weeks pectin supplementation on gastrointestinal barrier function *in vivo* and *ex vivo* in different age groups. In a randomized, double-blind, placebo-controlled, parallel study, 52 healthy young adults (18–40 years) and 48 healthy elderly (65–75 years) received 15 g/day pectin or placebo for four weeks. Pre- and post-intervention, *in vivo* gastrointestinal permeability by a multisugar test, and defense capacity in mucosal samples were assessed. Sigmoid biopsies were collected post-intervention from subgroups for Ussing chamber experiments and gene transcription of barrier-related genes. Pectin intervention did not affect *in vivo* gastroduodenal, small intestinal, colonic, and whole gut permeability in young adults nor in elderly ($P \geq 0.130$). Salivary and fecal sIgA and serum IgA were not significantly different between pectin versus placebo in both age groups ($P \geq 0.128$). In both young adults and elderly, no differences in transepithelial electrical resistance and fluorescein flux ($P \geq 0.164$) and relative expression of genes analyzed ($P \geq 0.222$) were found between pectin versus placebo. In conclusion, intestinal barrier function was not affected by four weeks pectin supplementation neither in healthy young adults nor in healthy elderly.

Introduction

An intact epithelial barrier is important for intestinal health and general well-being (1, 2). The epithelial cells are sealed by the junctional complex, which permits selective entry of nutrients, ions and water while restricting permeation of bacteria and their products. An increased permeability can lead to translation of luminal antigens and thereby to intestinal and systemic inflammation. Consequently, intestinal barrier dysfunction has been associated with a variety of intestinal and systemic diseases (2) and with aging (3-5). Interest in nutritional interventions to improve intestinal barrier function is increasing. Functional foods, which can be applied in targeted nutrition strategies, are added foods or ingredients that may provide health benefits beyond basic nutritional impact and/or reduce the risk of disease (6). Examples of functional foods are food items enriched with dietary fibers. Pectin is a complex polysaccharide originating from cell walls of for example citrus peel, apple and sugar beet pulp (7, 8) and is composed of galacturonic acid of which the residues are substituted with methyl esters at the C6-carboxyl group and rhamnogalacturonan (9). In addition, sugar beet pectin as compared to for example citrus and apple pectins, comprizes acetylation of homogalacturonan. In the upper gastrointestinal (GI) tract, pectin is resistant to digestion and hydrolysis. Because of the complex structure, pectin serves as substrate for fermentation by the microbiota in both the proximal and distal colon, resulting in the production of beneficial short-chain fatty acids (SCFAs) (8, 10, 11). Pectin may impact the intestinal epithelial barrier indirectly by modulating the colonic microbial composition- and activity, and/or directly act on the epithelial cells (12, 13). Especially the SCFA butyrate has been shown to both protect and repair intestinal barrier function, possibly via beneficial effects on junctional proteins and underlying signaling cascades (14). Moreover, dietary fibers are suggested to reinforce intestinal barrier function through modulating the enteric immune system; it has been shown that prebiotics can be sensed by dendritic cells, and in some cases selectively be transferred to the lamina propria via specialized epithelial cells, *i.e.* microfold (M) cells, thus signaling to the gut associated lymphoid tissue (15). Pectin enhanced diets have been shown to improve intestinal barrier function as reflected by decreased small intestinal permeability in infants with persistent diarrhea (16) and rat studies (17, 18) compared with control diets, whereas data on colonic permeability and responses to a potential stressor are not available. Furthermore, studies on the effects of pectin on mucosal defense capacity in healthy

adults and elderly are lacking.
Within the development of functional foods to target specific health concerns, it is important to study the impact of nutrition in relevant subgroup(s) (19). For this reason, we included two different age groups, *i.e.* young adults and elderly, respectively. The purpose of this study was to investigate both the functional and structural effects of pectin on GI barrier function *in vivo* and *ex vivo* in young adults and elderly. The primary aim was to investigate the effects of four weeks pectin supplementation on segment-specific intestinal permeability *in vivo*, stratified for age group. Secondary, we aimed to investigate the effects of four weeks pectin supplementation on *ex vivo* stressed and unstressed intestinal barrier function, the expression of intestinal barrier related genes and mucosal defense parameters, all stratified for age group. We hypothesized that four weeks pectin supplementation improves intestinal barrier function and mucosal defense capacity in healthy subjects, while we expect effects to be most pronounced in the elderly.

Methods

The Medical Ethics Committee of the Maastricht University Medical Center+ approved this study, which has been designed and performed in accordance with the Declaration of Helsinki (latest amendment of 2013, Fortaleza, Brazil) and Dutch Regulations on Medical Research involving Human Subjects (1998). The study was performed at the Maastricht University Medical Center + from March 2015 until April 2016. The trial has been registered in the Clinical Trials register (NCT02376270). All participants gave written informed consent before prior to participation.

Subjects

Healthy men and women with a Body Mass Index (BMI) between 20-30 kg/m² were recruited from two age groups by advertising: young adults between 18-40 years of age and elderly between 65-75 years of age. Key exclusion criteria included the presence of GI symptoms, history of any chronic disorder or major surgery which potentially limited participation or completion of the study, abdominal surgery interfering GI function, self-reported human immunodeficiency virus, average alcohol consumption of >20 alcoholic units per week, smoking, pregnancy, lactation, blood donation 90 days prior to the study, use of antibiotics, antifungal medication,

probiotics or prebiotics 90 days before the start of the study, history of side effects towards pro- or prebiotic supplements and use of non-steroidal anti-inflammatory drugs. Use of other medication or dietary supplements was reviewed by a medical doctor, who decided on in- or exclusion based on the medications or supplements used. Included subjects using medication had to use a stable dose. Moreover, serum C-reactive protein concentrations were determined to exclude inflammation and infections, and measured by immunoturbidimetric assay using Cobas 6000 analyzer (Roche, Mannheim, Germany).

Study design

This study was designed as a randomized, double-blind, placebo-controlled, parallel-group study. Per age group, randomization was performed to assign participants to the placebo or the pectin intervention arm. An independent person generated both lists, for the young adults and the elderly, of random allocations using a computerized procedure. All study participants and investigators were blinded to intervention allocations until analyses were completed. Participants in the pectin group received 15 g/day of sugar beet derived pectin (GENU® BETA pectin, CP Kelco Germany GmbH, Grossenbrode, Germany) for four weeks. Participants in the placebo group received 15 g/day of maltodextrin (GLUCIDEX® IT 12, Roquette Frères, Lestrem, France) for four weeks. Fifteen grams daily were given as this is a considered a prebiotic dosage in the higher physiological range with a minimal risk of side effects (20, 21). Furthermore, four weeks is considered sufficient to strengthen the barrier function by direct effects or changes in intestinal microbiota composition and activity (22, 23). Both pectin and placebo were supplemented as dry powders free from off-flavors and odors, and packed in closed sachets of a single dose of 7.5 grams. Subjects were asked to ingest the supplements twice daily, before breakfast in the morning and before diner in the evening, being dissolved in approximately 200 ml of tap water and mixed with flavored syrup (Karvan Cévitam®, Koninklijke De Ruijter B.V., Zeist, the Netherlands). Time of consumption had to be recorded, and empty and remaining sachets were returned to the investigator. At baseline and after four weeks pectin or placebo supplementation, segment-specific gut permeability tests were performed and bio samples were collected (Figure 5.1). Fecal samples were collected at home, stored at -20 °C until arrival at the study site and immediately stored at -80 °C. After fasting overnight, venous blood and saliva samples were collected and stored at -80 °C until further use. Additionally, the GI symptom rating

scale (GSRS) was completed at baseline and at weekly intervals to check for GI tolerance. Due to the invasive character, a flexible sigmoidoscopy without bowel preparation was performed only at the end of each intervention and in subgroups of the young adults and elderly. A standard flexible colonoscope was inserted and 12 biopsy specimens were taken from the sigmoid colon region, with a jumbo biopsy forceps (Boston Scientific, Kerkrade, the Netherlands). Seven samples were kept viable in pre-oxygenated Krebs-Ringer bicarbonate (KRB) solution on melting ice and directly transported to the laboratory for Ussing chamber experiments. Five tissue samples were snap frozen in liquid nitrogen and stored at -80°C for later analyses.

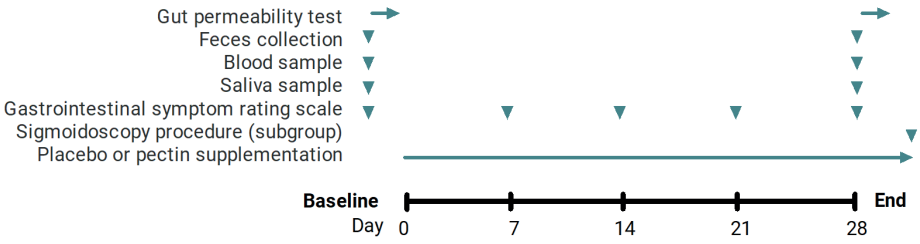

Figure 5.1 - Timeline of the intervention period. Gut permeability test, feces collection, blood and saliva sampling, gastrointestinal symptom rating scale, sigmoidoscopy procedure and placebo or pectin supplementation were completed at the days as indicated by arrows. Intake of supplements continued until all measurements were finished.

Gut permeability test

Segment-specific permeability of the GI tract was assessed by the multi-sugar test as validated by van Wijck *et al.* (24, 25). One day prior to testing as well as during the test, subjects were instructed to refrain from excessive physical exercise and alcohol consumption. After fasting overnight, a mix of water-soluble, non-degradable sugar probes were ingested, comprising 1 g sucrose (Van Gilse, Dinteloord, the Netherlands), 1 g lactulose (Centrafarm Services, Etten-Leur, the Netherlands), 0.5 g mannitol (Roquette, Lestrem, France), 1 g sucralose (Tate and Lyle Ingredients Americas, Decatur, IL, USA) and 1 g erythritol (Now Foods, Bloomindale, IL, USA), dissolved in 200 ml tap water. After ingestion, participants collected 24 hours (h) urine output in two separate fractions; 0-5 h and 5-24 h, respectively. During the first 5 h of urine collection, participants were asked to refrain from any food or drinks, except for water ad libitum. Thereafter, participants were allowed to eat and drink as preferred, except for sucralose containing foods. When urine was delivered to

the researcher, volumes of urine fractions were determined and urine aliquots were frozen at -80 °C until analysis. Sugar probes were analyzed by isocratic ion-exchange High Performance Liquid Chromatography with mass spectrometry as described previously (24, 25). Gastroduodenal permeability was determined by sucrose excretion in 0-5 h urine, whereas small intestinal permeability was measured by calculating the lactulose to mannitol (L/M) ratio in 0-5 h urine. Sucralose to erythritol (S/E) ratios in 5-24 h and 0-24 h urine were used as indicators for colonic- and whole gut permeability respectively.

Mucosal defense parameters

For total secretory immunoglobulin A (sIgA) determination, fecal samples were thawn, 1:5 diluted with sodium chloride, incubated for 96 hour and measured by radial immunodiffusion using a commercial test kit (Binding Site, Birmingham, United Kingdom). Immunoglobulin A (IgA) subclasses IgA1 and IgA2 in serum and saliva samples were quantified by enzyme-linked immunosorbent assay. To this end, high binding 96-well plates (Greiner Bio one 655061, Monroe, North Carolina, USA) were coated with goat anti-human IgA-antibody preparation (Southern Biotech, 2050-01, Birmingham, United Kingdom) which was diluted in phosphate-buffered saline (PBS) at a coating concentration of 1 µg/mL for serum detection and 0.1 µg/mL for saliva detection, and incubated overnight at 4°C. The plates were blocked with 5% fat-free milk powder in PBS, at 150 µL/well for 1-2h at room temperature (RT). After washing 3 times with wash buffer (PBS + 0.05% Tween-20, Merck, Darmstadt, Germany), a total of 100 µL IgA1 or IgA2 standards and test samples per well were applied on separate plates for 1h at 37°C. Standard curves were set up on each plate, ranging from 200 to 0.2 ng/mL for both IgA1 and IgA2. Serum samples were diluted in Universal Casein Diluent in PBS (PBSC) at 1:32,000 and 1:64,000 in IgA1 plates, and at 1:400 and 1:800 in IgA2 plates. Saliva samples were diluted in PBSC at 1:5000 and 1:10000 on IgA1 plates, and at 1:2000 and 1:10000 on IgA2 plates. After washing 4 times with wash buffer, secondary antibodies specific for human IgA1 (mouse anti-human IgA1 (at 1:5,000 in PBSC) or mouse anti-human IgA2 (at 1:2,000 in PBSC) (Southern Biotech, 9130-08 and 9140-08, respectively) were added at 100 µL/well and incubated for 1h at RT. After washing 4 times with wash buffer, 100 µL/well streptavidin-horseradish peroxidase (Southern Biotech, 7100-05), diluted in PBSC at 1:5,000 was added to the plates and incubated for 45 min at RT, while covered with aluminium foil. After washing 6 times with wash buffer, 100 µL/well 3,3',5,5'-tetramethylbenzidine

(TMB) substrate solution (SDT, Baesweiler, Germany) was added to the plates and incubated for 15 min at RT, while covered with aluminium foil. The reaction was stopped by adding 2%HCL solution and measured in a Filtermax microplate reader (Molecular Devices, San Jose, CA, USA) at 450 nm minus 620 nm as a reference value. After applying 5-parameter logistic transformation, the data were calculated according to best fit on the standard curve.

Gastrointestinal tolerance

The GSRS was completed at weekly intervals to check for GI tolerance. This instrument contains 15 items, each item was graded by using a seven-point Likert-type scale where 1 represents absence of troublesome symptoms and 7 represents very troublesome symptoms. The items were combined into five subscales depicting reflux, abdominal pain, indigestion, diarrhea and constipation (26).

Ussing chamber experiment

Six tissue samples from the sigmoid colon were used for *ex vivo* Ussing chamber experiments as previously described by our group (27). Three tissue samples were mildy stressed by adding 1 µg/ml of the mast cell degranulator Compound 48/80 (Sigma-Aldrich, St. Louis, MO, USA) to the serosal compartment. Three non-exposed tissue samples served as unstressed controls. At t=0, 1 mg/ml fluorescein (376 g/mol, Sigma-Aldrich, St. Louis, MO, USA) was added to the serosal compartment, for determination of fluorescein flux to the luminal compartment. From all tissue samples, potential difference (PD), transepithelial electrical resistance (TEER) and fluorescein concentrations were determined at time point t=0, 30, 60, 80 and 120 minutes. TEER and PD were used as quality criteria for viability. Only samples with a baseline TEER above 20 Ω/cm^2, or those with baseline TEER between 15-20 Ω/cm^2 and PD below 0.5 mV, were included for analyses. TEER and fluorescein concentrations are indicators of intestinal permeability.

Gene transcription of relevant proteins

Transcription of junctional complex related genes as well as defense and immune related genes associated with barrier function or modulation thereof, were determined in colonic tissue samples. Nucleic acid extraction and purification, RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR) were performed as previously described (28). Depending on the gene of

interest, cDNA was diluted to final concentrations of 20 ng/µl, 40 ng/µl or 80 ng/µl (Table 5.S1). Expressions of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (18S RNA) as reference genes (Table 5.S1).

Immunofluorescence staining of TJP1 and occludin

Sigmoid biopsy sections (10 µm) were used for immunofluorescent staining of TJP1 and occludin as previously described by our group (29).

Statistical analyses

The sample size calculation of the primary outcome (*i.e. in vivo* intestinal permeability) was based on the difference in urinary lactulose/mannitol ratio between inulin-enriched pasta and control pasta in young males as reported by Russo *et al.* (30). A difference between treatments of 0.02, standard deviation of 0.022, alpha of 0.025, and power of 0.80 were assumed. Thereby, a minimum of 24 completers per intervention group in each age group were needed.

Intention to treat analyses were performed. Normality of the data was checked by histograms and were summarized accordingly using median and interquartile range (IQR; 25-75th IQR) or means ± standard deviation for numerical variables, and percentages for categorical variables. Independent-samples T Tests were performed for numerical variables and Chi-square tests for categorical variables to test for differences between intervention groups (pectin versus placebo) in young adults and in elderly.

Within each age group, differences between interventions were assessed by unstructured linear mixed model analyses with intervention group (pectin and placebo), time (baseline and end) and 'intervention group x time' as fixed factors, and correction for baseline values. Differences in longitudinal trends in TEER and luminal fluorescein between intervention groups were assessed by random intercept linear mixed model analyses with intervention group (pectin and placebo), time (t=0, t=30, t=60, t=90, t=120 min) and 'intervention group x time' as fixed factors, and correction for t=0 values. All statistical analyses were performed for young 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 statistically significant. GI symptoms, Ussing chamber experiments and gene transcription *P*-values were corrected for multiple testing by the false-discovery-rate (FDR) of Benjamini-Hochberg.

Results

Study subjects

After assessment of eligibility, 52 young adults and 48 elderly were enrolled in the study. Three young adults dropped out, one because of overt non-compliance and two because of antibiotic use (Figure 5.2). Baseline characteristics of the young adults and elderly, undergoing either pectin or placebo intervention, are shown in Table 5.1. Moreover, a subgroup of 22 young adults and 22 elderly underwent a sigmoidoscopy after four weeks pectin or placebo intervention (Figure 5.2), of which baseline characteristics are shown in Table 5.S2.

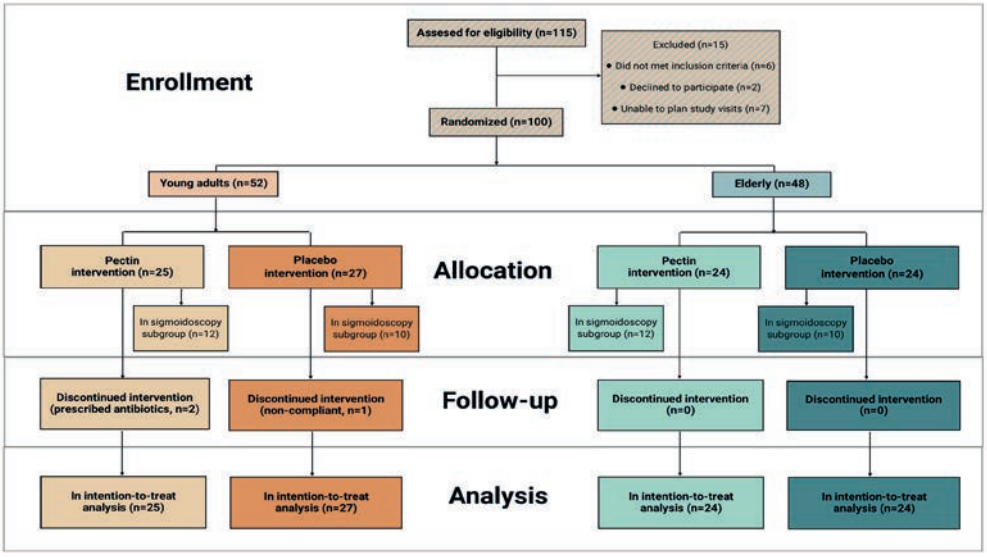

Figure 5.2 - Flow diagram of the study.

Table 5.1 - Baseline characteristics of the total sample of young adults (n=52) and elderly (n=48), undergoing either pectin or placebo intervention.

	Young adults (n=52)			Elderly (n=48)		
	Pectin (n=25)	Placebo (n=27)	*P*-value	Pectin (n=24)	Placebo (n=24)	*P*-value
Age (yrs, mean ± SD)	23.4 ± 4.5	22.8 ± 4.1	0.613	69.5 ± 3.1	69.8 ± 2.4	0.723
Sex (% female)	68.0	48.1	0.148	37.5	50.0	0.383
BMI (kg/m^2, mean ± SD)	23.2 ± 2.7	22.6 ± 2.7	0.444	25.5 ± 2.6	26.2 ± 2.8	0.334
Serum CRP (mg/L, mean ± SD)	1.7 ± 2.5	1.0 ± 1.2	0.161	1.1 ± 1.3	1.8 ± 2.1	0.203
Medication (%)						
PPI	N.A.	N.A.	N.A.	12.5	12.5	1.000
Statins				4.2	4.2	1.000
Antihypertensives				12.5	8.3	0.637
Alcohol consumption (units/week, mean ± SD)	3.5 ± 3.2	5.3 ± 5.4	0.165	8.4 ± 6.9	9.3 ± 7.1	0.667

BMI: body mass index, CRP: C-reactive protein, N.A: not applicable, PPI: proton-pump inhibitors. Age, BMI, CRP and alcohol consumption were compared between intervention groups with the use of an independent samples t-test. Sex and medication were compared between intervention groups with the use of a Pearson Chi-square test.

Intestinal permeability *in vivo*

Gastroduodenal and small intestinal permeability as assessed by the 0-5h urinary sucrose excretion and 0-5h urinary L/M ratio, respectively (Figure 5.3), did not differ significantly between four weeks pectin and placebo supplementation in the young adults nor in the elderly (all $P \geq 0.861$). The 5-24h urinary S/E ratio and 0-24h urinary S/E ratio (Figure 5.4), as measures of colonic and whole gut permeability, were not significantly different between four weeks pectin vs placebo supplementation in both young adults and elderly (all $P \geq 0.130$).

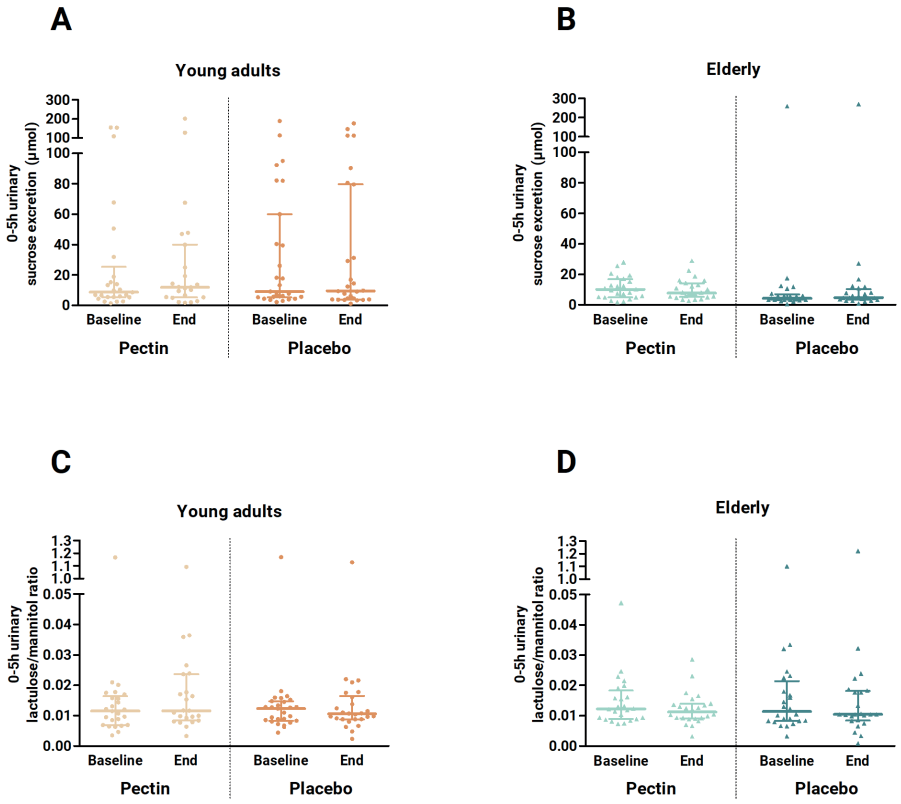

Figure 5.3 - Gastroduodenal and small intestinal permeability *in vivo* at baseline and after four weeks of pectin or placebo intervention in young adults and elderly. A: 0-5h urinary sucrose excretion (µmol) in young adults. B: 0-5h urinary sucrose excretion (µmol) in elderly. C: 0-5h urinary lactulose/mannitol ratio in young adults. D: 0-5h urinary lactulose/rhamnose ratio in elderly. Values are presented in scatter plots with median line and IQR (25-75th interquartile range). Sample size differences between baseline and end are due to drop-outs. Within age groups, urinary sugar excretions and ratios were compared between intervention groups with unstructured linear mixed model and correction for baseline values.

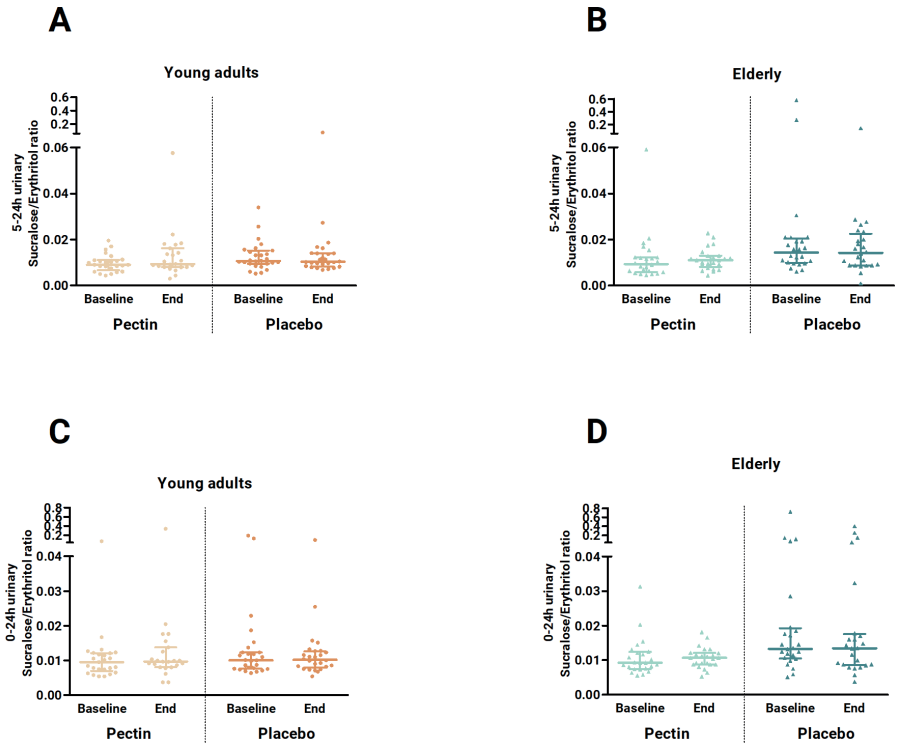

Figure 5.4 - Colonic and whole gut permeability *in vivo* at baseline and after four weeks of pectin or placebo intervention in young adults and elderly. A: 5-24 urinary sucralose/erythritol ratio in young adults. B: 5-24 urinary sucralose/erythritol ratio in elderly. C: 0-24 urinary sucralose/erythritol ratio in young adults. D: 0-24 urinary sucralose/erythritol ratio in elderly. Values are presented in scatter plots with median line and IQR (25-75th interquartile range). Sample size differences between baseline and end are due to drop-outs. Within age groups, urinary sugar ratios were compared between intervention groups with unstructured linear mixed models and correction for baseline values.

Mucosal defense parameters

No significant changes in salivary sIgA1 and sIgA2, serum IgA1 and IgA2, and fecal sIgA were observed between pectin or placebo intervention, neither in young adults, nor in elderly (all $P \geq 0.128$) (Figure 5.S1).

Gastrointestinal tolerance

GI tolerance was assessed weekly by completing the GSRS questionnaire. After FDR correction for multiple testing, GI symptom scores were not significantly different between pectin and placebo supplementation in young adults, nor in elderly (all $P \geq 0.054$) (Figure 5.S2 and Figure 5.S3, respectively). In young adults however, pectin

intervention induced significantly higher diarrhea score (P = 0.020) compared with placebo at week two only (Figure 5.S2).

Intestinal permeability *ex vivo*

Ussing chamber experiments were done to determine *ex vivo* TEER and luminal fluorescein concentration as indicators of paracellular permeability in unstressed and stressed conditions. After FDR correction for multiple time points, TEER in unstressed and stressed biopsies did not significantly differ between four weeks pectin versus placebo supplementation in elderly, nor in young adults (all $P \geq 0.226$) (Figure 5.5). In both young adults and elderly, luminal fluorescein concentrations in unstressed and stressed biopsies did not differ significantly between four weeks pectin vs placebo supplementation (all $P \geq 0.164$) (Figure 5.6).

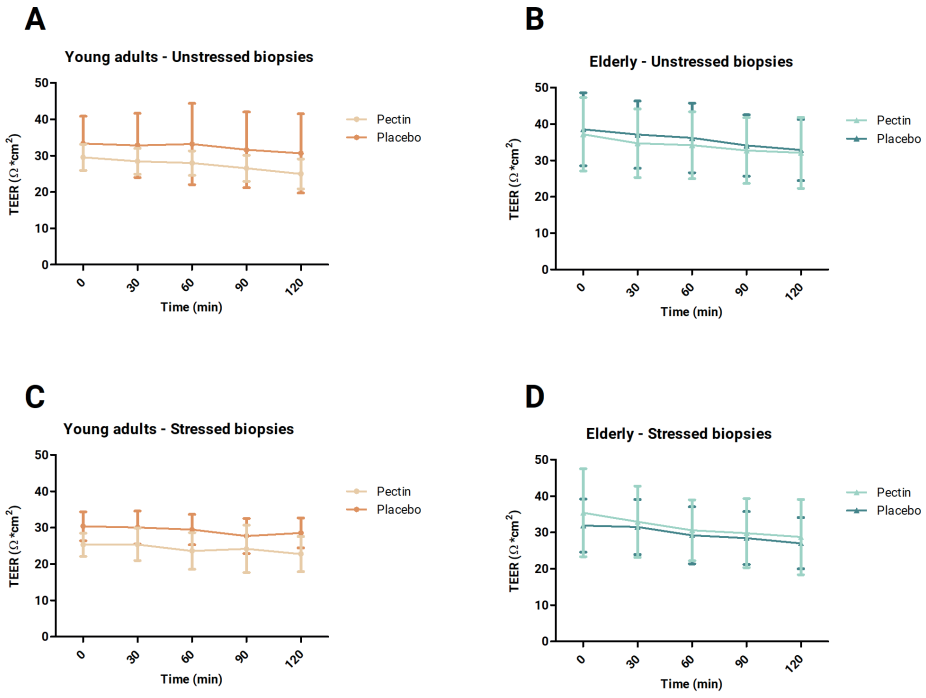

Figure 5.5 - Intestinal permeability *ex vivo* after four weeks of pectin (light lines) and placebo (dark lines) intervention in young adults and elderly, by mounting fresh sigmoid biopsies in an Ussing chamber system and assessing transepithelial electrical resistance (TEER) at t=0, t=30, t=60, t=90, t=120 min. A: TEER in young adults in unstressed biopsies. B: TEER in elderly in unstressed biopsies. C: TEER in young adults in biopsies stressed by 1 µg/ml Compound 48/80 at t=0. D: TEER in elderly in biopsies stressed by 1 µg/ml Compound 48/80 at t=0. Means and standard deviations are shown. Sample sizes vary because baseline values of some sigmoid biopsies were not meeting quality criteria for viability. Within age groups, TEER and luminal fluorescein were compared between intervention groups with random intercept linear mixed models and correction for baseline values. *P*-values per time point were corrected for multiple testing by calculating the false-discovery-rate (FDR) of Benjamini-Hochberg.

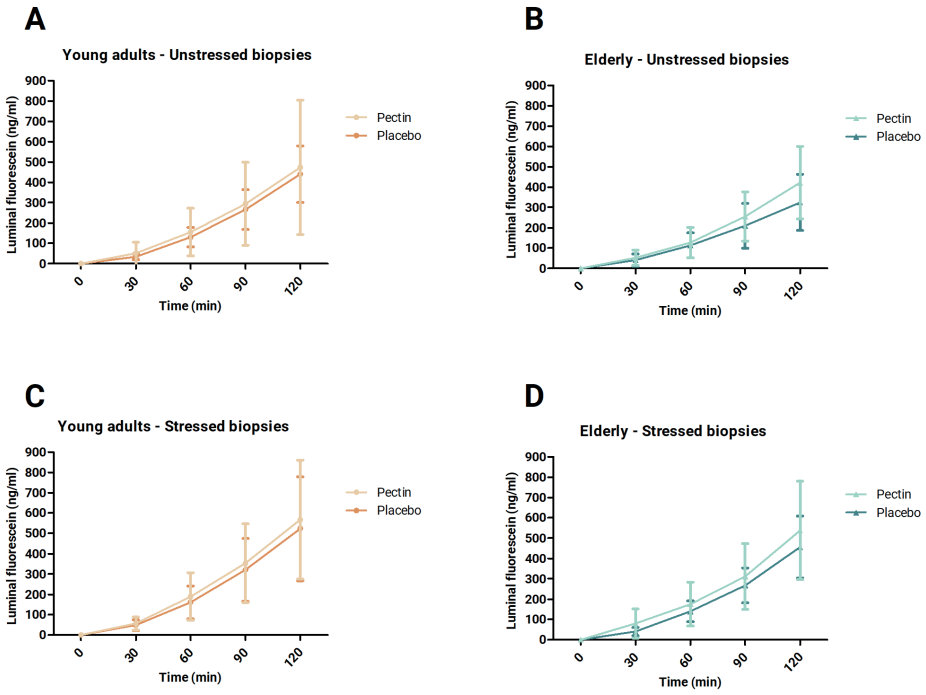

Figure 5.6 - Intestinal permeability *ex vivo* after four weeks of pectin (light lines) and placebo (dark lines) intervention in young adults and elderly, by mounting fresh sigmoid biopsies in an Ussing chamber system and assessing luminal fluorescein concentration at t=0, t=30, t=60, t=90, t=120 min. A: Luminal fluorescein concentration in young adults in unstressed biopsies. B: Luminal fluorescein concentration in elderly in unstressed biopsies. C: Luminal fluorescein concentration in young adults in biopsies stressed by 1 µg/ml Compound 48/80 at t=0. D: Luminal fluorescein concentration in elderly in biopsies stressed by 1 µg/ml Compound 48/80 at t=0. Means and standard deviations are shown. Sample sizes vary because baseline values of some sigmoid biopsies were not meeting quality criteria for viability. Within age groups, luminal fluorescein concentrations were compared between intervention groups with random intercept linear mixed models. *P*-values per time point were corrected for multiple testing by calculating the false-discovery-rate (FDR) of Benjamini-Hochberg.

Gene transcription of barrier related genes

Mean Cq values of both GAPDH and 18S RNA did not differ between pectin and placebo intervention. GAPDH normalized relative expression of junctional complex (*e.g.* tight junctions and adherens junctions), defense and immune-related (*e.g.* human defensins, cytokines and toll-like receptor) genes in sigmoid biopsies of young adults and elderly after four weeks pectin or placebo intervention are shown in Table 5.2. After FDR correction for multiple testing, in both young adults and elderly,

no significant differences were found between pectin vs placebo intervention (all *P* ≥ 0.222) in the relative expression of all genes analyzed. Moreover, analyses on 18S RNA normalized gene expressions resulted in the same conclusions.

Table 5.2 - Relative expression of junctional complex (*e.g.* tight junction related and adherens junctions), defense and immune related (*e.g.* human defensins, cytokines and toll-like receptor) genes in sigmoid biopsies of young adults and elderly after four weeks pectin or placebo intervention.

Cluster	Gene name	Young adults		*P*-value	Benjamini Hochberg *P*-value	Elderly		*P*-value	Benjamini Hochberg *P*-value
		Pectin	Placebo			Pectin	Placebo		
Junctional complex related genes	TJP1 (ZO-1)	1.15 ± 0.03	1.13 ± 0.03	0.313	0.417	1.15 ± 0.02	1.13 ± 0.02	0.195	0.260
	OCLN	1.19 ± 0.02	1.18 ± 0.02	0.128	0.417	1.20 ± 0.01	1.19 ± 0.02	0.184	0.260
	CLDN2	1.34 ± 0.07	1.36 ± 0.03	0.527	0.602	1.36 ± 0.05	1.32 ± 0.07	0.250	0.286
	CLDN3	1.17 ± 0.02	1.16 ± 0.02	0.245	0.417	1.18 ± 0.03	1.16 ± 0.02	0.079	0.222
	CLDN4	1.11 ± 0.03	1.10 ± 0.02	0.311	0.417	1.12 ± 0.02	1.10 ± 0.02	0.111	0.222
	MLCK	1.15 ± 0.02	1.15 ± 0.03	0.982	0.982	1.16 ± 0.02	1.14 ± 0.03	0.109	0.222
	CDH1	1.17 ± 0.02	1.15 ± 0.01	0.072	0.417	1.17 ± 0.03	1.17 ± 0.02	0.852	0.852
	CTNNB1	1.13 ± 0.01	1.12 ± 0.01	0.236	0.417	1.15 ± 0.02	1.12 ± 0.02	0.029	0.222
Defense and immune related genes	CAMP	1.29 ± 0.05	1.30 ± 0.05	0.630	0.770	1.32 ± 0.06	1.28 ± 0.06	0.179	0.405
	DEFB1	1.17 ± 0.05	1.15 ± 0.03	0.468	0.735	1.18 ± 0.05	1.16 ± 0.03	0.184	0.405
	MUC2	1.02 ± 0.03	1.01 ± 0.03	0.429	0.735	1.01 ± 0.03	1.01 ± 0.02	0.832	0.915
	TFF3	0.99 ± 0.03	0.98 ± 0.04	0.432	0.735	0.98 ± 0.05	0.98 ± 0.04	0.832	0.915
	IL1B	1.32 ± 0.05	1.35 ± 0.05	0.217	0.597	1.33 ± 0.04	1.31 ± 0.06	0.232	0.405
	IL10	1.25 ± 0.07	1.25 ± 0.03	0.856	0.856	1.27 ± 0.05	1.23 ± 0.06	0.141	0.405
	TNF	1.31 ± 0.05	1.35 ± 0.06	0.151	0.554	1.35 ± 0.04	1.35 ± 0.04	0.937	0.937
	TLR1	1.15 ± 0.05	1.18 ± 0.04	0.144	0.554	1.16 ± 0.05	1.13 ± 0.04	0.153	0.405
	TLR2	1.25 ± 0.06	1.26 ± 0.05	0.818	0.856	1.26 ± 0.06	1.23 ±.0.06	0.258	0.405
	TLR4	1.19 ± 0.03	1.21 ± 0.03	0.056	0.554	1.21 ± 0.03	1.19 ± 0.03	0.042	0.405
	TLR6	1.12 ± 0.06	1.29 ± 0.04	0.622	0.770	1.30 ± 0.07	1.27 ± 0.06	0.358	0.492

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene in this table, since analyses on 18S RNA normalized gene expressions resulted in the same conclusions. Values are presented as mean ± SD. For a limited number of genes, sample sizes may differ due to technical reasons. Within age groups, genes were compared between intervention groups by independent-samples T Tests. P-values were corrected for multiple testing by calculating the false discovery rate of Benjamini Hochberg per cluster. TJP1 (ZO-1): Tight junction protein 1 (*i.e.* Zona Occludens-1), OCLN: Occludin, CLDN: Claudin, MLCK: Myosin light chain kinase, CDH1: Cadherin 1, CTNNB1: Catenin beta 1, CAMP: Cathelicidin antimicrobial peptide, DEFB1: Defensin beta 1, MUC2: Mucin 2, TFF3: Trefoil factor 3, IL: Interleukin, TNF: Tumor necrosis factor, TLR: Toll like receptor.

Immunofluorescence staining of TJP1 and occludin

Visual inspection of representative immunofluorescence staining of TJP1 (Figure 5.S4) and occludin (Figure 5.S5) in sigmoid biopsy sections showed no apparent differences between four weeks pectin versus placebo supplementation in young adults, nor in elderly. These observations are in line with quantitative analyses of TJP1 and occludin gene transcription levels as reported in Table 5.2 as well as with the functional analyses performed.

Discussion

In the current study, the impact of pectin on the functional and structural GI barrier function in young adults and elderly has been investigated *in vivo* and *ex vivo*. We showed that GI segment-specific permeability, intestinal permeability *ex vivo,* expression of barrier-related genes and parameters of mucosal defense were not significantly improved by four weeks pectin supplementation neither in healthy young adults, nor in healthy elderly.

The present study was designed based on the previously described features of pectin intake. It may strengthen the highly dynamic epithelial barrier directly by interacting with tight junction proteins, and indirectly via modulating the colonic microbial composition- and activity, which is known to affect intestinal homeostasis and barrier function. An intervention period of four weeks should be adequate to both directly and indirectly modulate intestinal barrier function. Moreover, sugar beet pectin was chosen because of the complex structure, which causes it to be fermented in both the proximal and distal colon. Saccharolytic fermentation (*i.e.* fermentation of dietary fibers) may inhibit the fermentation of proteins in the distal colon due to substrate competition, thereby lowering the production of mostly toxic compounds that result from proteolytic fermentation. The only previous human study on pectin and intestinal permeability *in vivo* showed that one week supplementation with pectin (4 mg/kg body weight) improved small intestinal permeability (*i.e*. decreased 0-5h urinary L/M ratio) in infants with persistent diarrhea (16). In our study, we found no significant effects of four weeks pectin intake on small intestinal permeability, as determined by the 0-5h urinary L/M ratio, in healthy adults and healthy elderly. Because the type and dosage of pectin, intervention duration and target populations of both human studies differed, adequate comparison of these studies is difficult.

We also showed that gastroduodenal, colonic and whole gut permeability, as determined by the multi-sugar test, were not significantly affected by four weeks pectin supplementation. This may be due to the well-functioning intestinal barrier at baseline in both the healthy young adults and the elderly, although the intestinal epithelium is often exposed to stressors, such as alcohol, high fat diet, medication use, psychological and psychosocial stress, etc. In the current study, subjects were instructed to maintain their habitual diet. As we did not actually monitor food intake over the study period, we cannot exclude that the intake of 15 g/day pectin or placebo may have impacted food intake. Furthermore, it should be noted that the

inter-individual variations were rather high, although this was in accordance with previous observations (31). This stresses the importance of assessments within subjects as was done in the current study, with measurements of the intestinal barrier before and after the intervention period. As other dietary fibers (*i.e.* galacto-oligosaccharides) have been found to improve colonic permeability in obese subjects (32), based on our results we cannot exclude potential impact of pectin on intestinal barrier function in more susceptible (sub)groups of adults or elderly.

To further examine the effect of pectin in stressed conditions, sigmoid biopsies were collected at the end of each intervention in subgroups of the young adults and the elderly. The mucosal tissue samples were used to determine intestinal permeability *ex vivo* in Ussing chamber experiments. We used Compound 48/80 to induce a mild stress as reflected by an increase in luminal fluorescein concentrations. Though, tissue TEER and mucosal fluorescein permeation were not affected by the four weeks pectin versus placebo supplementation in the unstressed nor in the stressed condition. Ganda Mall *et al.* (33) exposed sigmoid biopsies of elderly with GI symptoms and of healthy adults with dietary fibers (*i.e.* yeast-derived beta-glucan and wheat-derived arabinoxylan) before adding Compound 48/80. Especially in elderly with GI symptoms, beta-glucan was found to attenuate the hyperpermeability induced by Compound 48/80 as reflected by both higher TEER and lower mucosal to serosal fluorescein concentrations. Differences with the current study may be explained by the more vulnerable elderly population (*i.e.* with GI symptoms) and exposure to beta-glucan *in vitro* rather than *in vivo*.

Relative expression levels of junctional complex, defense and immune related genes in sigmoid tissue samples also showed no significant differences between the sugar beet derived pectin and placebo supplementation in any of the age groups after FDR correction. This was further supported by immunofluorescence staining of TJP1 and occludin in representative sigmoid biopsy sections. Interestingly, in rats which were selected by their response to a high fat diet by gaining weight, subsequent high fat diet supplemented with apple-derived pectin versus normal high fat diet resulted in lower Interleukin (IL)-6, Tumor necrosis factor-α and TLR4, and higher IL-10 and claudin-1 mRNA levels in ileal tissue, suggestive for an anti-inflammatory activity of this pectin (18). However, possible disturbances in intestinal barrier function induced by the high fat diet, differences in pectin source, and no corrections for multiple testing may explain, at least in part, different effects of the rat study when compared to the current human intervention study. Furthermore, in a mice model

of acute pancreatitis, low-methoxyl pectin was found to upregulate occludin, TJP1 and defensin beta 1, and downregulate Tumor Necrosis Factor-α, IL-β and IL-6 relative mRNA levels in ileal and colonic tissue, pointing towards restoration of acute pancreatitis-associated disruption of the intestinal barrier (34). This is not in line with our observations, probably caused by the difference between acute pancreatitis induced animals versus healthy human participants and variation in degree of methylation.

The effects of four weeks pectin supplementation on mucosal defense capacity was further studied by assessing salivary sIgA1 and sIgA2, fecal sIgA and serum IgA1 and IgA2, demonstrating no significant effect of the pectin intervention in any of the age groups. Production of sIgA in the human intestine in absolute quantities exceeds that of all other antibody classes together (35), and IgA can be seen as a key antibody class for the first line of defense in mucous membranes. Human studies investigating the effects of other dietary fibers on human mucosal defense as assessed by salivary and fecal sIgA *in vivo* have been performed previously (36-39). However to our knowledge, this is the first human study investigating the effects of sugar beet pectin supplementation on IgA levels. In a rat study comparing pectin (unspecified origin) versus cellulose supplemented diets, higher serum IgA concentrations in pectin supplemented rats were found (40), although sIgA was not determined. Conflicting results between rat and human studies can be caused by differences in pectin source or normal physiological processing of the IgA molecule, and the fact that only humans have two isotypes of IgA that are differentially regulated and distributed.

GI symptoms were determined throughout the four weeks pectin supplementation period, to monitor GI tolerance. Although pectin, in comparison to placebo, did not alter any GI symptom score in the elderly, pectin caused an increase in the diarrhea score after two weeks pectin intake by young adults. After four weeks of pectin intervention, diarrhea decreased and was no longer significantly different compared to placebo, illustrating habituation to 15 g/day of pectin supplementation in young adults. This habituation period is in line with previous findings on dietary fibers and the occurrence of gastrointestinal symptoms (41, 42), and may in case of pectin supplementation be due to increased microbial fermentation and/or increased viscosity in the colonic lumen.

In conclusion, by using a combined *in vivo* and *ex vivo* approach, we consistently showed that intestinal barrier function was not affected by four weeks sugar beet pectin supplementation neither in healthy young adults, nor in healthy elderly. As there are clear leads in literature that dietary fibers may improve the intestinal barrier but clinical data are still limited, further human intervention studies are needed to explore potential effects of pectin and other dietary fibers in patients with an impaired intestinal barrier function.

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Supplementary material

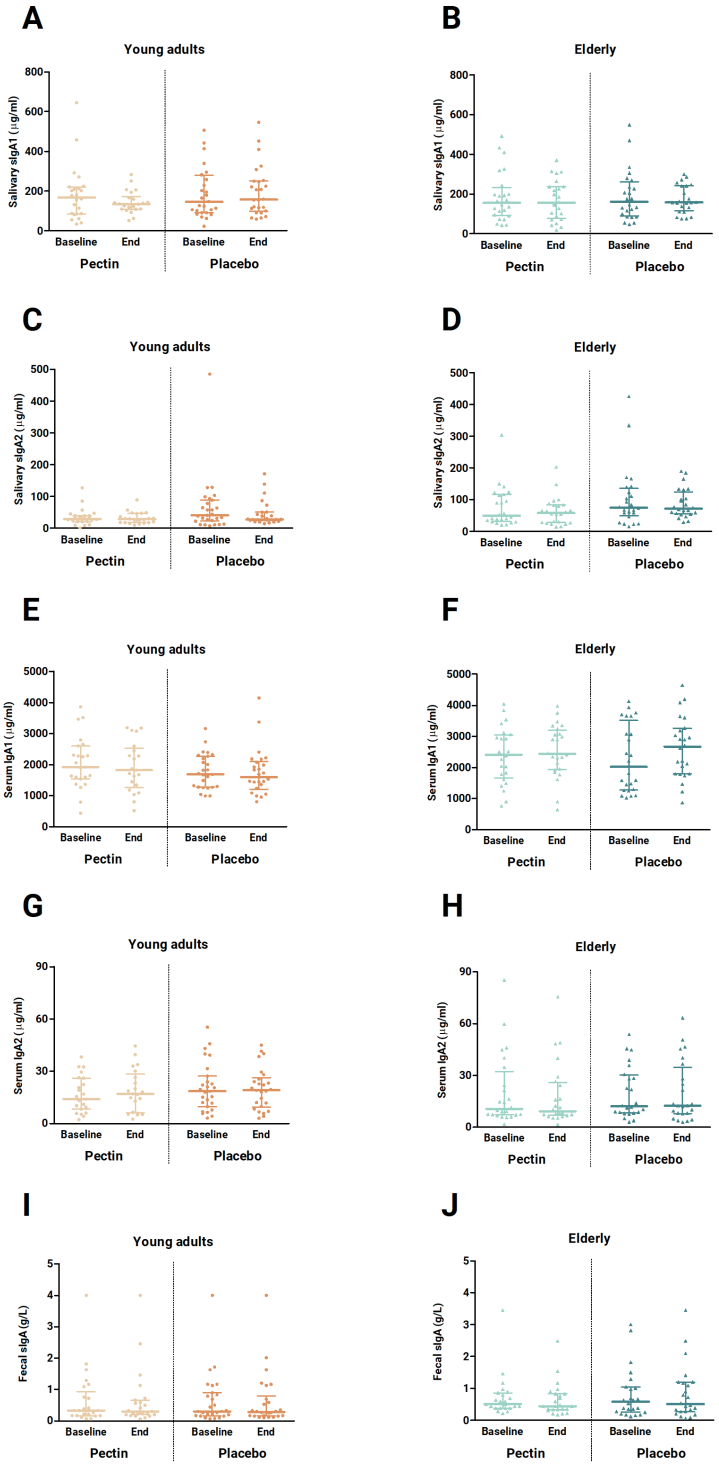
Table 5.S1 - Forward and reverse primer sequences and final cDNA concentrations of all target genes, as determined in sigmoid biopsies.

Name	Description	Primer sequences	cDNA concentration
CAMP	Cathelicidin antimicrobial peptide	5'-AGGATTGTGACTTCAAGAAGGACG-3' 5'-GTTTATTTCTCAGAGCCCAGAAGC-3'	80 ng/µl
CDH1	Cadherin 1	5'-CACCTGGAGAGAGGCCGCGT -3' 5'-AACGGAGGCCTGATGGGGCG -3'	20 ng/µl
CLDN2	Claudin 2	5'-AACTACTACGATGCCTACC-3' 5'-GAACTCACTCTTGACTTTGG -3'	20 ng/µl
CLDN3	Claudin 3	5'-TTCATCGGCAGCAACATCATC-3' 5'-CGCCTGAAGGTCCTGTGG-3'	20 ng/µl
CLDN4	Claudin 4	5'-ACAGACAAGCCTTACTCC-3' 5'-GGAAGAACAAAGCAGAG-3'	20 ng/µl
CTNNB1	Catenin beta 1	5'-GTGCTATCTGTCTGCTCTAGTA -3' 5'-CTTCCTGTTTAGTTGCAGCATC -3'	20 ng/µl
DEFB1	Defensin beta 1	5'-CTCTGTCAGCTCAGCCTC-3' 5'-CTTGCAGCACTTGGCCTTCCC-3'	20 ng/µl
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	5'-TGCACCACCAACTGCTTAGC-3' 5'-GGCATGGACTGTGGTCATGAG-3'	20 ng/µl
IL1B	Interleukin 1 beta	5'-AAACAGATGAAGTGCTCCTTCCAGG-3' 5'-TGGAGAACACCACTTGTTGCTCCA-3'	40 ng/µl
IL10	Interleukin 10	5'-TCAGGGTGGCGACTCTAT-3' 5'-TGGGCTTCTTTCTAAATCGTTC-3'	80 ng/µl
MUC2	Mucin 2	5'-GTCAACCCTGCCGACACCTG-3' 5'-ACTCACACCAGTAGAAAGGACAGC-3'	20 ng/µl
MLCK	Myosin light chain kinase	5-GCCTGACCACGAATATAAGTT-3' 5'-GCTCCTTCTCATCATCATCTG-3'	20 ng/µl
OCLN	Occludin	5'-TCAGGGAATATCCACCTATCACTTCAG-3' 5'-CATCAGCAGCAGCCATGTACTCTTCAC-3'	20 ng/µl
TFF3	Trefoil factor 3	5'-CTTGCTGTCCTCCAGCTCT-3' 5'-CCGGTTGTTGCACTCCTT-3'	20 ng/µl
TJP1 (ZO-1)	Tight junction protein 1	5'-AGGGGCAGTGGTGGTTTTCTGTTCTTTC-3' 5'-GCAGAGGTCAAAGTTCAAGGCTCAAGAGG-3'	20 ng/µl
TLR1	Toll like receptor 1	5'-CAGTGTCTGGTACACGCATGGT-3' 5'-TTTCAAAAACCGTGTCTGTTAAGAGA-3'	80 ng/µl
TLR2	Toll like receptor 2	5'-GCCAAAGTCTTGATTGATTGG-3' 5'-TATACCACAGGCCATGGAAAC-3'	20 ng/µl
TLR4	Toll like receptor 4	5'-CCTGCGTGAGACCAGAAAGC-3' 5'-TCAGCTCCATGCATTGATAAGTAATA-3'	80 ng/µl
TLR6	Toll like receptor 6	5'-GAAGAAGAACAACCCTTTAGGATAGC-3' 5'-AGGCAAACAAAATGGAAGCTT-3'	20 ng/µl
TNF	Tumor Necrosis Factor	5'-CCGAGTGACAAGCCTGTAGC-3' 5'-GAGGACCTGGGAGTAGATGAG-3'	40 ng/µl
18S RNA	18S ribosomal RNA	5'-GTAACCCGTTGAACCCCATT-3' 5'-CCATCCAATCGGTAGTAGCG-3'	20 ng/µl

Table 5.S2 - Baseline characteristics of the subgroups of young adults (n=22) and elderly (n=22), undergoing sigmoidoscopy after the pectin or placebo intervention.

	Young adults (n=22)		*P*-value	Elderly (n=22)		*P*-value
	Pectin (n=12)	Placebo (n=10)		Pectin (n=12)	Placebo (n=10)	
Age (yrs, mean ± SD)	23.9 ± 4.6	24.2 ± 5.4	0.893	70.4 ± 3.0	70.7 ± 2.8	0.784
Sex (% female)	58.3	30.0	0.184	41.7	20.0	0.277
BMI (kg/m^2, mean ± SD)	22.3 ± 2.8	23.7 ± 3.2	0.299	25.6 ± 2.6	27.3 ± 1.7	0.083
Serum CRP (mg/L, mean ± SD)	1.8 ± 1.8	0.6 ± 0.7	0.061	0.7 ± 0.6	1.8 ± 1.6	0.032
Medication (%)						
PPI	N.A.	N.A.	N.A.	8.3	20.0	0.427
Statins				8.3	0	0.350
Antihypertensives				0	10	0.262
Alcohol consumption (units/week, mean ± SD)	3.0 ± 2.7	6.3 ± 6.3	0.156	5.5 ± 5.0	10.8 ± 6.8	0.049

BMI: body mass index, CRP: C-reactive protein, N.A: not applicable, PPI: proton-pump inhibitors. Age, BMI, CRP and alcohol consumption were compared between intervention groups with the use of an independent samples t-test. Sex and medication were compared between intervention groups with the use of a Pearson Chi-square test.


A
Young adults
Salivary sIgA1 (µg/ml)
800
600
400
200
0
Baseline
End
Baseline
End
Pectin
Placebo
B
Elderly
Salivary sIgA1 (µg/ml)
800
600
400
200
0
Baseline
End
Baseline
End
Pectin
Placebo
C
Young adults
Salivary sIgA2 (µg/ml)
500
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300
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Baseline
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Pectin
Placebo
D
Elderly
Salivary sIgA2 (µg/ml)
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Placebo
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Young adults
Serum IgA1 (µg/ml)
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4000
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2000
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Baseline
End
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Pectin
Placebo
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Elderly
Serum IgA1 (µg/ml)
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0
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End
Pectin
Placebo
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Young adults
Serum IgA2 (µg/ml)
90
60
30
0
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Elderly
Serum IgA2 (µg/ml)
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Young adults
Fecal sIgA (g/L)
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Pectin
Placebo
J
Elderly
Fecal sIgA (g/L)
5
4
3
2
1
0
Baseline
End
Baseline
End
Pectin
Placebo

Figure 5.S1 - Mucosal defense parameters at baseline and after four weeks of pectin or placebo intervention in young adults and elderly. A: Salivary sIgA1 (µg/ml) in young adults. B: Salivary sIgA1 (µg/ml) in elderly. C: Salivary sIgA2 (µg/ml) in young adults. D: Salivary sIgA2 (µg/ml) in elderly. E: Serum IgA1 (µg/ml) in young adults. F: Serum IgA1 (µg/ml) in elderly. G: Serum IgA2 (µg/ml) in young adults. H: Serum IgA2 (µg/ml) in elderly. I: Fecal sIgA (g/L) in young adults. J: Fecal sIgA (g/L) in elderly. Values are presented in scatter plots with median line and IQR (25-75th interquartile range). Sample sizes vary due to drop-outs and technical reasons. Within age groups, mucosal defense parameters were compared between intervention groups with unstructured linear mixed models and correction for baseline values. IgA, Immunoglobulin A; sIgA, secretory Immunoglobulin A.

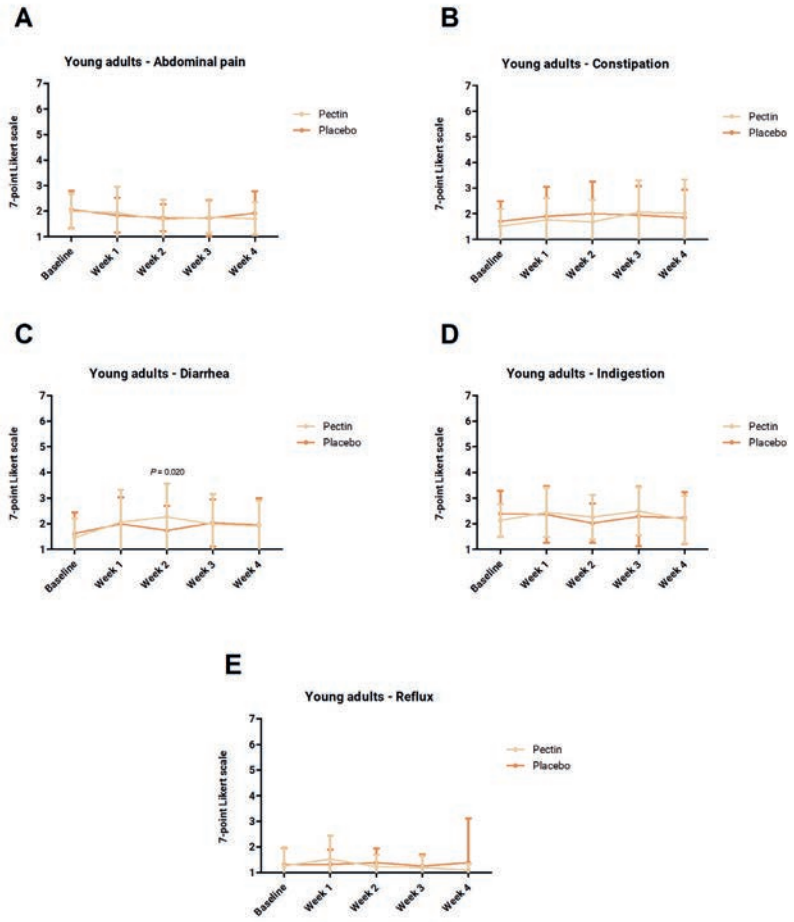

Figure 5.S2 - Gastrointestinal symptoms at baseline and every week of pectin (light lines) and placebo (dark lines) intervention in young adults. A: Abdominal pain scores. B: Constipation scores. C: Diarrhea scores. D: Indigestion scores. E: Reflux scores. Means and standard deviations are shown. Missing values at specific weeks were due to drop-outs. Gastrointestinal symptom scores were compared between intervention groups with random intercept linear mixed models and correction for baseline values. *P*-values per time point were corrected for multiple testing by calculating the false-discovery-rate (FDR) of Benjamini-Hochberg.

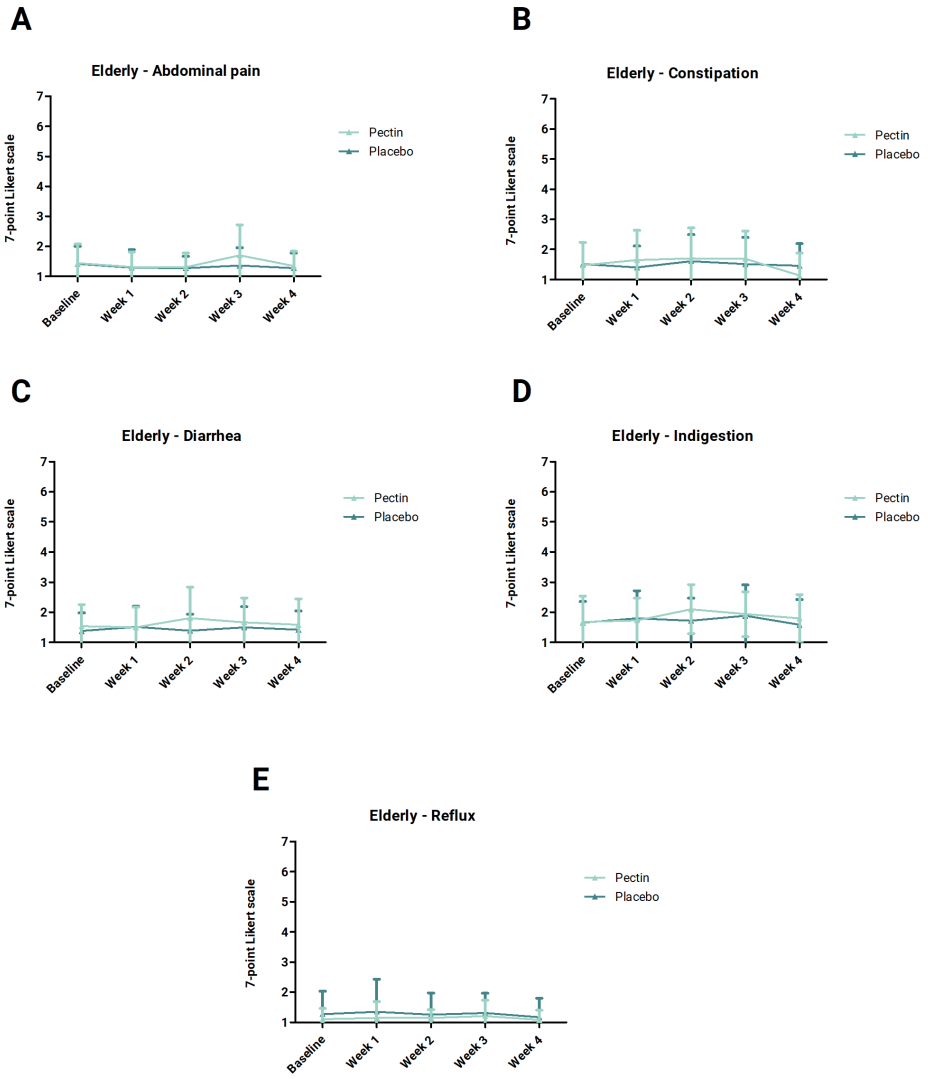

Figure 5.S3 - Gastrointestinal symptoms at baseline and every week of pectin (light lines) and placebo (dark lines) intervention in elderly. A: Abdominal pain scores. B: Constipation scores. C: Diarrhea scores. D: Indigestion scores. E: Reflux scores. Means and standard deviations are shown. Gastrointestinal symptom scores were compared between intervention groups with random intercept linear mixed models and correction for baseline values. *P*-values per time point were corrected for multiple testing by calculating the false-discovery-rate (FDR) of Benjamini-Hochberg.

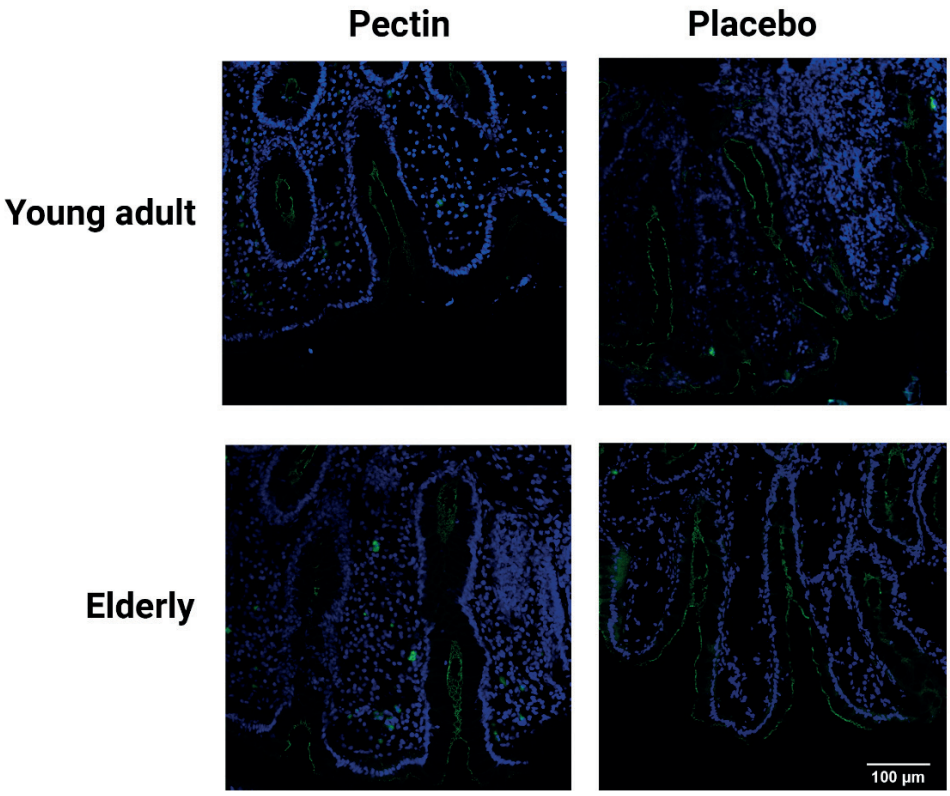

Figure 5.S4 - Representative images of tight junction protein TJP1 (green) immunofluorescence staining in sigmoid biopsy sections of a healthy young adult and healthy elderly after four weeks pectin or placebo intervention. Scale bar represents 100 µm. Blue counterstaining (DAPI) shows nuclei. TJP1: Tight junction protein 1.

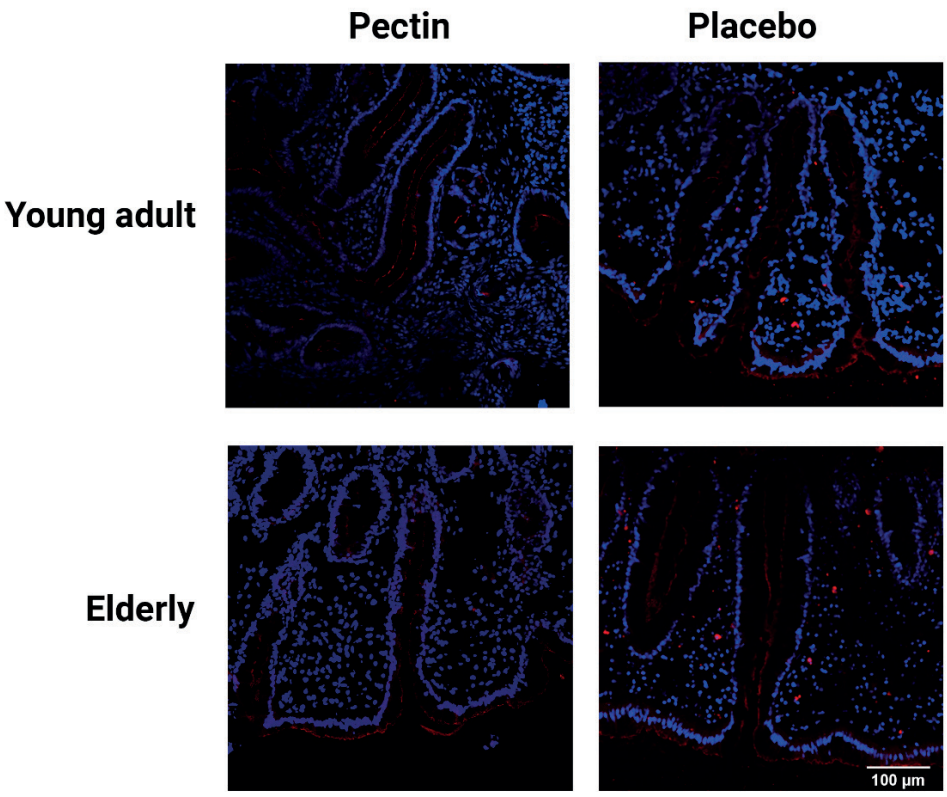

Figure 5.S5 - Representative images of tight junction protein occludin (red) immunofluorescence staining in sigmoid biopsy sections of a healthy young adult and healthy elderly after four weeks pectin or placebo intervention. Scale bar represents 100 µm. Blue counterstaining (DAPI) shows nuclei.

6

Chapter 6

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

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Abstract

Aging is accompanied with increased frailty and comorbidities, which is potentially associated with microbiome perturbations. Dietary fibers could contribute to healthy aging by beneficially impacting gut microbiota and metabolite profiles. We aimed to compare young adults with elderly and investigate the effect of pectin supplementation on fecal microbiota composition, short chain fatty acids (SCFAs), and exhaled volatile organic compounds (VOCs) while using a randomized, double-blind, placebo-controlled parallel design. Fifty-two young adults and 48 elderly consumed 15 g/day sugar beet pectin or maltodextrin for four weeks. Fecal and exhaled breath samples were collected before and after the intervention period. Fecal 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 fecal SCFA and exhaled VOC profiles. Additionally, fecal microbiota profiles were similar, with five genera significantly different in relative abundance. Pectin supplementation did not significantly alter fecal microbiota, SCFA or exhaled VOC profiles in elderly or young adults. In conclusion, aside from some minor differences in microbial composition, healthy elderly and young adults showed comparable fecal microbiota composition and activity, which were not altered by pectin supplementation.

Introduction

In line with the rising life expectancy, the aging population is increasing globally, leading to an increase in direct and indirect healthcare costs (1, 2). General health status may decline with aging 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 aging" (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 fibers, 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 fiber 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 fecal 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 fecal 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 GI 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 fecal 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 byproduct 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, comprizes 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 fecal microbiota composition, fecal 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 hypothesized that the intestinal microbiota and metabolite profiles in feces and breath differ between young adults and elderly, with a greater response to four weeks pectin supplementation in elderly versus young adults.

Methods

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 6.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.

Participants

Healthy young adults (18-40 years) and healthy elderly (65-75 years) with a body mass index between 20 and 30 kg/m^2 were recruited by public advertisements. Key exclusion criteria included GI diseases, abdominal surgery interfering with GI function, use of non-steroidal 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 fiber-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).

Dietary intervention

Each subject was randomly assigned to the pectin or placebo group (Figure 6.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 15 g/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 15 g/day maltodextrin (GLUCIDEX® IT 12, Roquette Freres, Lestrem, France). Both maltodextrin and pectin were supplemented as dry powders free from off-flavors and odors, 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 flavored 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.

Fecal samples and microbiota profiling

Fecal 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 fecal 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 fecal 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.

Fecal metabolite profiling

SCFAs were measured in the feces 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 feces 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 at 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 aging and by prebiotic intake), SCFA concentrations were expressed per gram dry matter. Dry matter content was determined by vacuum drying of 500 mg feces for five hours at 60 °C using a concentrator plus (Eppendorf, Hamburg, Germany).

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). 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, MA, 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 denoized, baseline corrected, aligned, normalized by probabilistic quotient normalization and scaled for further analyses (39).

Statistical analyses

Statistical analyses of baseline characteristics of study participants were 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.* fecal 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 analyzed 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 principle 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 analyzed 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 analyzed 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.)

Results

Subjects

For the current study 52 healthy young adults and 48 elderly were included, of whom the baseline characteristics are provided in Table 6.1. 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 fecal- and exhaled breath analyses but were not included in the post intervention measurements. DNA isolation failed for one fecal sample from a young adult (placebo group, post intervention), and hence was excluded for microbiota profiling.

Table 6.1 - Baseline characteristics of the young adults (n=52) and elderly (n=48) study populations.

	Young adults (n=52)			Elderly (n=48)			*P*-value
	Placebo (n=27)	Pectin (n=25)	*P*-value	Placebo (n=24)	Pectin (n=24)	*P*-value	all young adults vs. all elderly
Age (yrs, mean ± SD)	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, mean ± SD)	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.

Young adults and elderly showed similar fecal 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 6.1A). 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 6.1B). 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*,* uncultured genus within the *Coriobacteriaceae, Mogibacterium, Lachnospiraceae* UCG-008) out of 224 genera were significantly different between young adults and elderly before the intervention (Figure 6.2). In addition, no significant differences were found in their fecal microbiota alpha diversity at baseline (Figure 6.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.

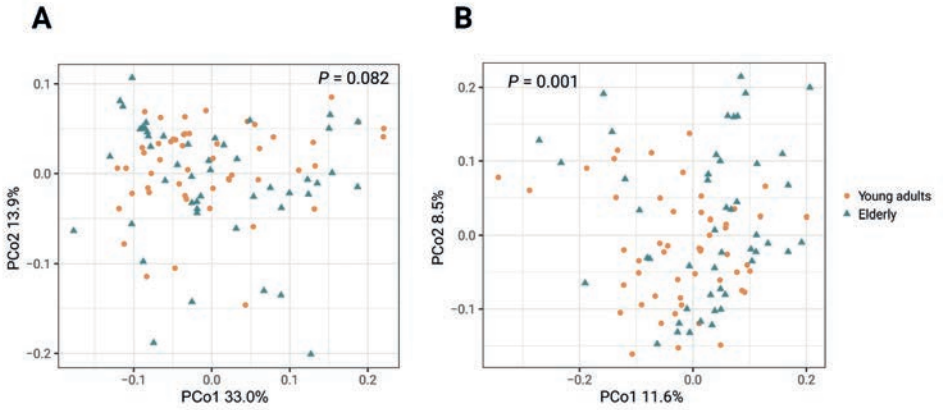

Figure 6.1 - Baseline PCoA plots based on A: weighted UniFrac and B: unweighted UniFrac pairwise distance matrices using amplicon sequence variant-level data, show overlapping fecal microbiota profiles of young adults and elderly. Significance of observed differences between groups was evaluated by PERMANOVA.

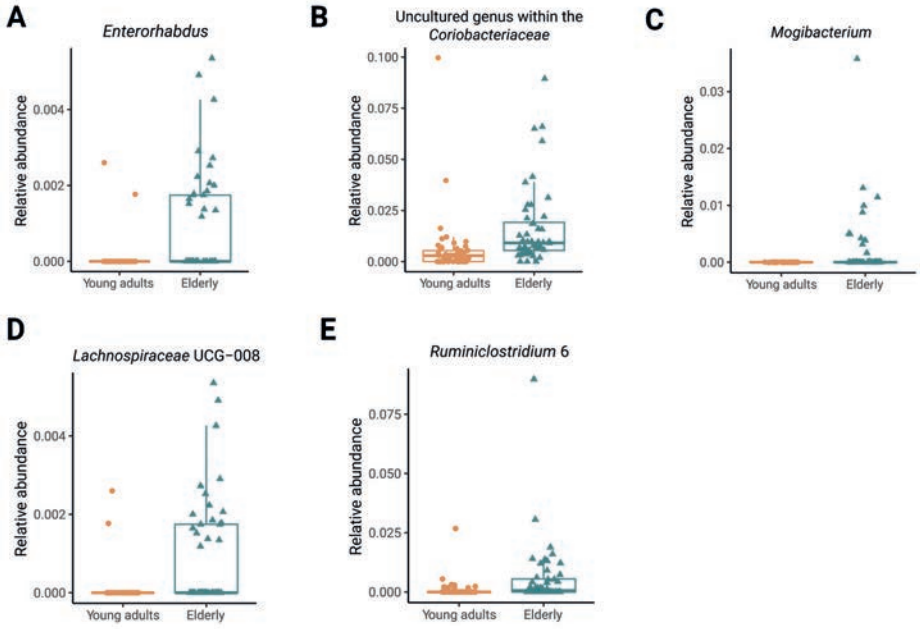

Figure 6.2 - Genus level taxa that significantly differed (FDR<0.05) in relative abundance between young adults and elderly at baseline. The relative abundance of each genera are shown as follows, A: *Enterorhabdus*; B: Uncultured genus within the *Coriobacteriaceae*; C: *Mogibacterium*; D: *Lachnospiraceae* UCG-008; E: *Ruminiclostridium* 6.

Baseline fecal SCFA and BCFA concentrations revealed no significant differences between young adults and elderly (Table 6.2). Independent of age group, large individual differences were found for all SCFAs as indicated by the relatively high SD.

Table 6.2 - Fecal 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
Acetic acid (mean ± SD)	225.9 ± 187.6	201.6 ± 145.2	0.469
Propionic acid (mean ± SD)	71.1 ± 66.4	58.1 ± 53.2	0.281
Butyric acid (mean ± SD)	59.2 ± 45.0	56.6 ± 49.8	0.785
Valeric acid (mean ± SD)	8.4 ± 6.4	9.3 ± 6.7	0.473
Isobutyric acid (mean ± SD)	6.8 ± 3.7	7.2 ± 6.0	0.715
Isovaleric acid (mean ± SD)	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 6.3A), indicating relatively small differences in exhaled breath profiles, which is in line with fecal 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 6.3B). This is in accordance with PCA analysis performed on the complete breath profiles (Figure 6.S3).

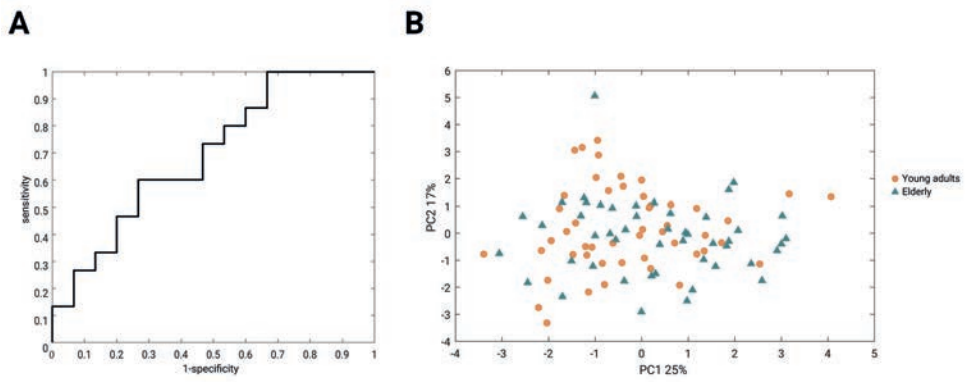

Figure 6.3 - A: Receiver operating characteristic curve performed on the validation set, with area under the curve = 0.70. B: PCA score plot, performed on a set of 15 VOCs that were found important (set of the most discriminatory VOCs selected in at least 80% of RF iterations) for classification in the 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.

Four weeks of sugar beet pectin supplementation did neither alter fecal microbiota composition, nor SCFA- and 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 6.4A-B) and in-depth microbial composition (*i.e.* detailed taxa comparison), nor impact on microbial phylogenetic diversity (Figure 6.4C) and InvSimpson diversity indices (Figure 6.4D). 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 6.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 6.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 6.S1).

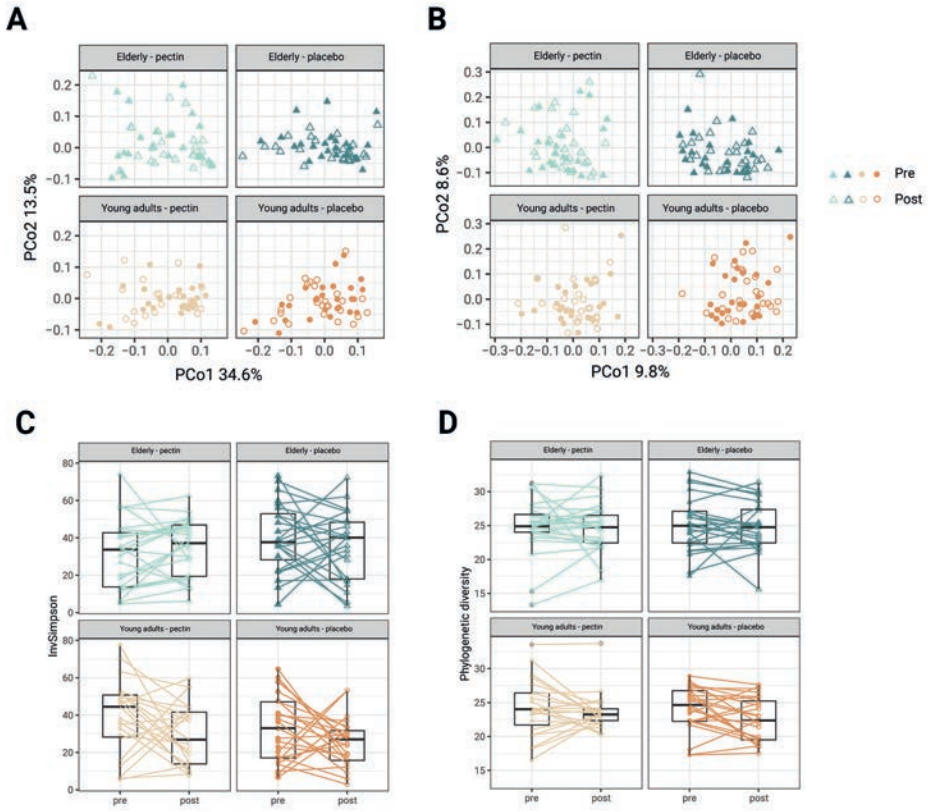

Figure 6.4 - Intervention effects on fecal microbiota composition and alpha diversity in young adults and elderly. PCoA plots at baseline and after four weeks sugar beet pectin or placebo supplementation based on A: weighted UniFrac and B: unweighted UniFrac, showed no clear groupings in microbiota profiles between pre- and post-intervention. C: Comparison of phylogenetic diversity and D: InvSimpson 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.

Four weeks of sugar beet pectin intake did also not significantly change fecal SCFA or BCFA concentrations in young adults, nor in elderly (Table 6.3). 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.

Table 6.3 - 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		
		Pre-intervention	Post-Intervention	*P*-value *	Pre-intervention	Post-Intervention	*P*-value *
Acetic acid (mean ± SD)	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 (mean ± SD)	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 (mean ± SD)	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 (mean ± SD)	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 (mean ± SD)	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 (mean ± SD)	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	

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

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 6.5A-B). No clear groupings were found between post and pre-intervention indicating similarity in breath profiles.

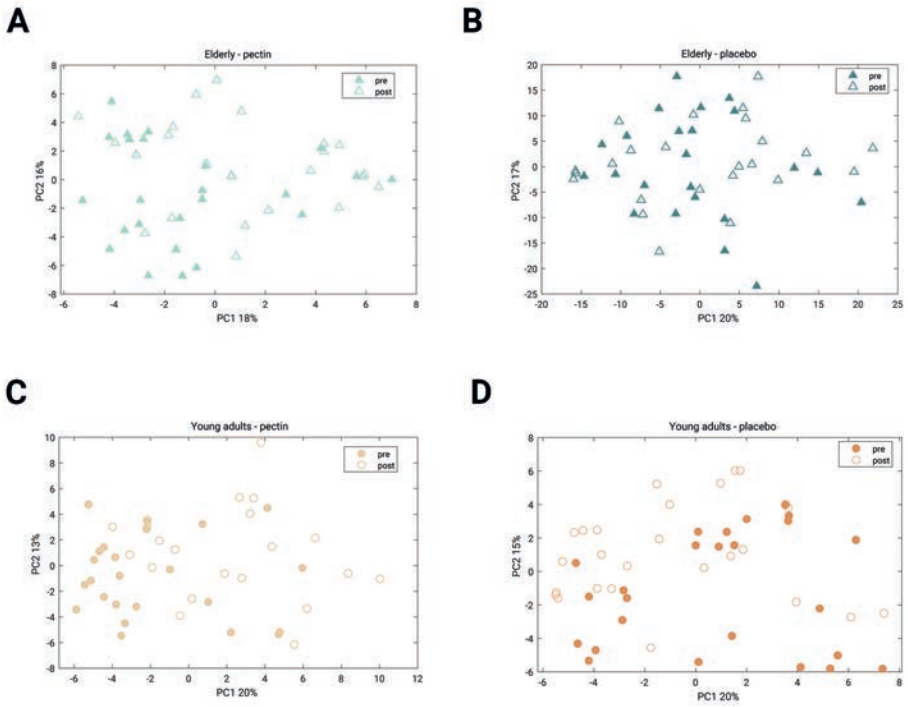

Figure 6.5 - PCA score plot based on the set of A: 11 volatile metabolites in the exhaled breath of elderly to discriminate between pre and post-pectin intervention and B: 12 volatile metabolites in the exhaled breath of elderly to discriminate between pre and post-placebo intervention. PCA score plots performed on the set of C: 14 VOCs measured in exhaled breath of young adults to discriminate between pre and post-pectin intervention and D: 16 VOCs measured in exhaled breath of young adults to discriminate between 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 6.5C-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 fecal microbiome, nor on breath metabolite profiles, neither in elderly nor in young adults.

Discussion

In the present study we compared healthy young adults versus healthy elderly and studied the effect of sugar beet pectin supplementation on fecal microbiota composition, fecal SCFA and exhaled breath VOC profiles. We hypothesized that intestinal microbiota and metabolite profiles in feces 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 fecal SCFA and exhaled VOC profiles between age groups. In addition, in neither of the two age groups, any effects of pectin supplementation on fecal 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 fecal 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 fecal 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-galactooligosaccharide mixture (B GOS) (10, 11) and a non-digestible carbohydrate mixture (of resistant starch, GOS, corn fiber, 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 B-GOS 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 fecal *Bifidobacterium* and *Lactobacillus* levels, as well as a significant decrease in *Clostridium* (22). In the present study, however, pectin supplementation did not affect fecal 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 fecal 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 fecal 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 fiber 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 fiber diet (44g/day of whole grain rye) or a low fiber 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 fibers used.

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 aging and changes are primarily due to alterations in health status and lifestyle factors (3). In addition, no effects of pectin supplementation on microbiota composition, fecal 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 fiber supplementations, are warranted.

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Supplementary material

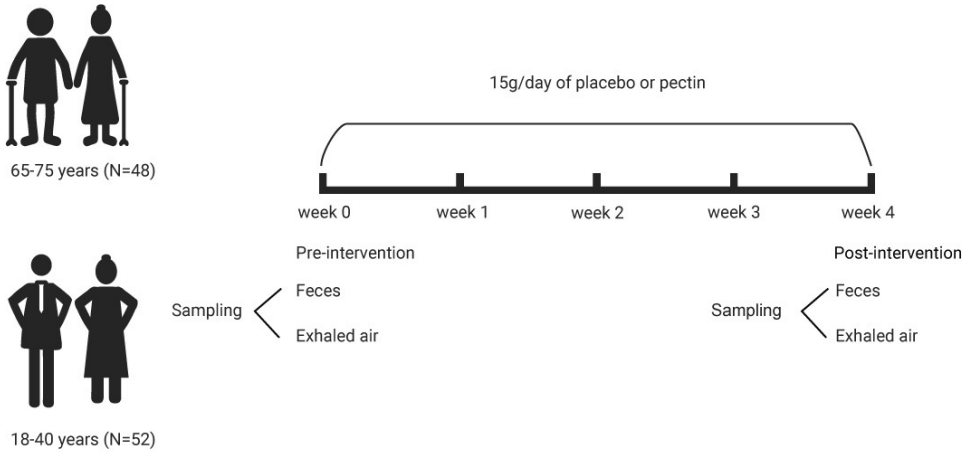

Figure 6.S1 - Schematic overview of the study design. Forty-eight healthy elderly and 52 young adults started this study. Participants consumed either 7.5g pectin or 7.5g maltodextrin (placebo) twice daily for four weeks. Feces and exhaled air were sampled before and after the intervention for analyses.

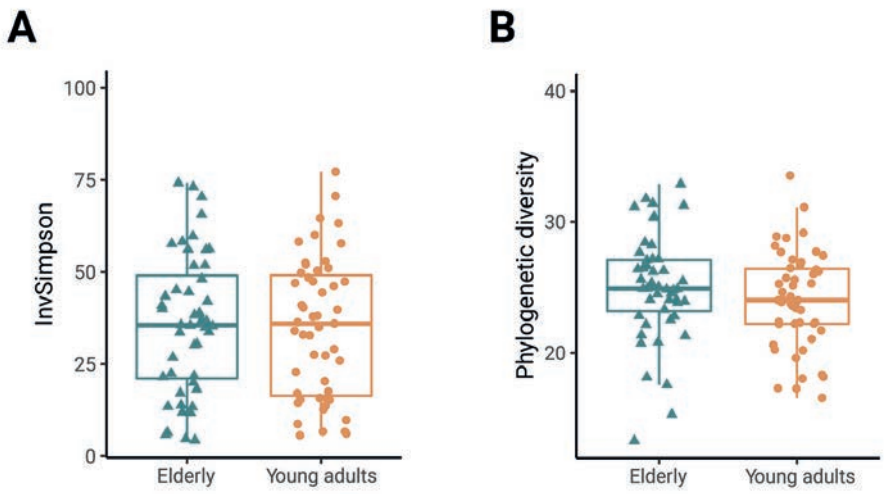

Figure 6.S2 - Fecal microbiota of young adults and elderly did not show significant differences in alpha diversity at baseline. The diversity of the microbiota was evaluated by A: Inverse Simpson's index and B: Phylogenetic diversity. Groups were compared by Wilcoxon signed rank tests.

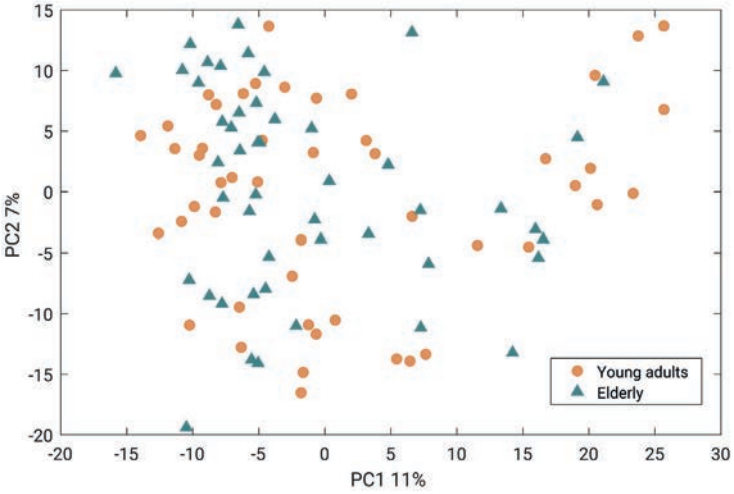

Figure 6.S3 - PCA score plot performed on the complete breath profiles of young adults and elderly. No clear groupings in exhaled breath profiles could be observed between young adults and elderly.

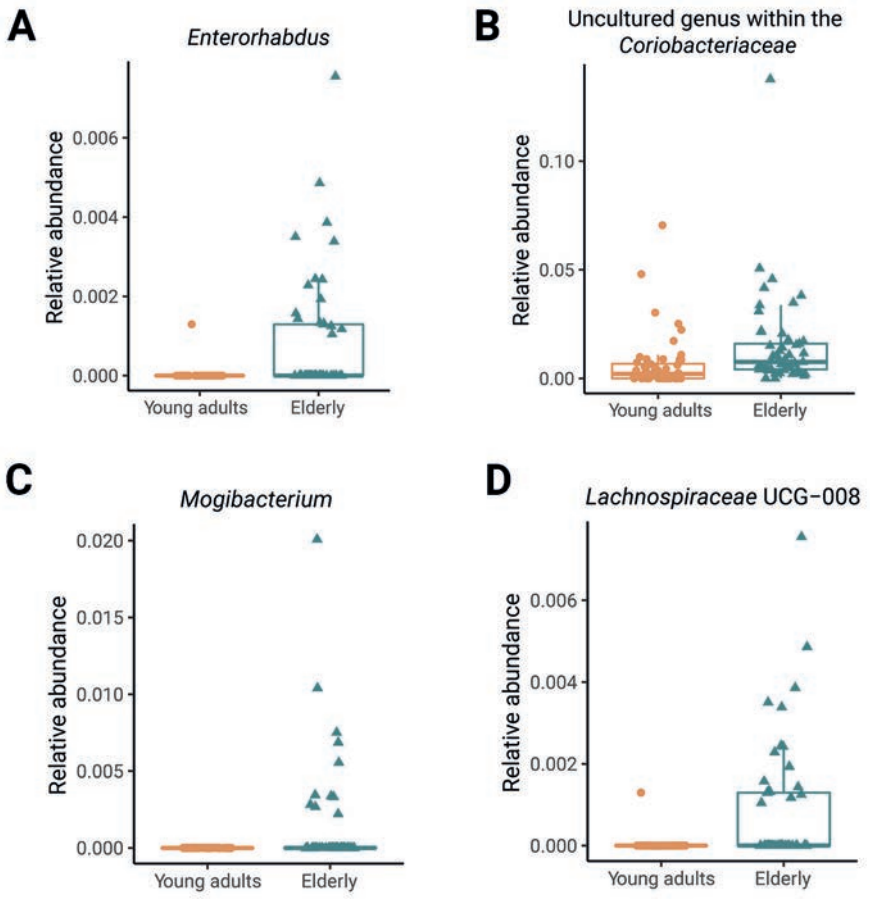

Figure 6.S4 - Significantly different genus level taxa (FDR<0.05), comparing the fecal microbiota of young adults and elderly after intervention.

Table 6.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	0.504	0.0049	0.790
BMI (kg/m^2)	0.0044	0.810	0.0009	0.959
Alcohol (units/week)	0.0264	0.275	0.0271	0.281
Sport (hours/week)	0.0023	0.890	0.0003	0.987
Sex (male/female)	0.0047	0.628	0.0094	0.402
Medication (yes/no)	0.0006	0.935	0.0029	0.759
Allergy (yes/no)	0.0035	0.696	0.0024	0.791
Vegetarian (yes/no)	0.0007	0.930	0.0240	0.092
Food supplements (yes/no)	0.0094	0.381	0.0029	0.752
Disease history (yes/no)	0.0116	0.320	0.0197	0.148

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 6.S2 - Inter- and intra-individual distances of the fecal microbiota over the intervention period.

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

Differences between inter-individual distance and intra-individual distance were tested using T-tests. Higher value in distance indicates smaller similarity.

7

Chapter 7

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

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Submitted

Abstract

Aging is accompanied with an increased risk to become frail, and is associated with intestinal microbiota perturbations as well as immunosenescence and higher oxidative stress levels. Prebiotics such as galacto-oligosaccharides (GOS) are considered as a targeted intervention to beneficially alter these aging related factors. Our aims were to compare prefrail elderly with younger adults with respect to fecal microbiota composition and -activity, immune and oxidative stress markers and to investigate the effects of GOS supplementation in both groups. In a randomized, double-blind, placebo-controlled, cross-over study, 20 prefrail elderly (70-85 years) and 24 healthy adults (25-50 years) received 21.6 g/day Vivinal® GOS Powder or placebo. Fecal and blood samples were collected at baseline and after four weeks of intervention. Fecal samples were used for microbiota profiling by 16S rRNA gene amplicon sequencing, and for analysis of short-chain fatty acids (SCFAs). Blood samples were used for analyses of cytokine production (*i.e.* after 24h stimulation by 10μg/ml LPS or PHA), C-reactive protein (CRP), malondialdehyde (MDA), trolox equivalent antioxidant capacity (TEAC), uric acid (UA) and non-UA TEAC. Jaccard and unweighted UniFrac distances showed that the overall microbiota composition was not significantly different between elderly and adults, although elderly had significantly lower relative abundance of *Bifidobacterium* ($P = 0.027$) at baseline. After four weeks of GOS supplementation, the overall microbiota profile of both age groups changed significantly, accompanied by significant increases in bifidobacteria ($P < 0.001$), and a significant decrease in microbial diversity (InvSimpson) in adults ($P = 0.002$) but not in elderly ($P = 0.101$). Fecal metabolites, stimulated cytokine levels as well as CRP, MDA, TEAC and UA did not differ between age groups ($P \geq 0.125$), nor between GOS and placebo interventions ($P \geq 0.236$). Non-UA TEAC values were higher in elderly compared with adults ($P < 0.001$), but not significantly different between interventions ($P \geq 0.455$). In conclusion, elderly showed lower fecal bifidobacteria compared with adults, but fecal metabolites and parameters of immune function and oxidative stress were not significantly different. Four weeks galacto-oligosaccharides supplementation in both prefrail elderly and healthy adults increased fecal bifidobacteria, but did not impact fecal metabolites, immune function and oxidative stress.

Introduction

In line with a continuous rising life expectancy, the aging population is growing worldwide. Aging is associated with a decline of physiological function, including *e.g.* 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 is composed of a complex community of microbes dominated by anaerobic bacteria, and plays a key role in intestinal immune and defense capacity (4), either directly by microbe-cell interactions or indirectly via bacterial metabolites. In a recent review from our group, it was concluded that lower microbial diversity, richness and alterations in abundance of several bacterial groups are found in elderly, being most pronounced in frail elderly and in association with lifestyle risk factors (5). The mechanisms through which the intestinal microbiota perturbations contribute to age-related immunosenescence is however unclear (6). Immunosenescence refers to age-related alterations in immune function, and is characterized 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 health care costs in the elderly. One such approach could be via the intake of galacto-oligosaccharides (GOS). GOS are non-digestible oligosaccharides, which have been classified as prebiotic because of its impact on the microbiota and host benefits. 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 fecal 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 are twofold: 1) to compare the fecal microbiota composition and -activity, immune markers and oxidative stress markers of prefrail elderly versus healthy adults, and 2) to study the impact of four weeks GOS supplementation in both groups. We hypothesized that the fecal microbiota, and parameters of immune function and systemic oxidative stress show perturbations in prefrail elderly when compared with healthy adults, which can be beneficially altered by four weeks GOS supplementation.

Methods

The study 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, NCT03077529). The study was performed 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.

Subjects

Men and women without gastrointestinal (GI) complaints, Body Mass Index (BMI) 20 - 30 kg/m^2 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 (13) to determine physical frailty 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 co-morbidities are more frequent in elderly, they were not general exclusion criteria, but considered to reflect (prefrail) aging. Both were checked by a medical doctor and subjects were only allowed to participate if medication and co-morbidities 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.

Study design

This study was designed as a randomized, double-blind, placebo-controlled, cross-over study with a 4-6 weeks wash-out period between the intervention periods (Figure 7.1). Per age group (*i.e.* elderly and adults), randomization 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 computerized program (http://www.randomization.com), and stratifying 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 Vivinal® GOS Powder (FrieslandCampina Ingredients, Amersfoort, the Netherlands), containing 15.0 g/day of 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.* color, taste and odor), 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 and after four weeks GOS and placebo supplementation, fecal and blood samples were collected, and three-day dietary records and stool characteristics scores were completed at home (Figure 7.1). Fecal samples were immediately after defecation stored at -20 ºC, and after arrival at the study site stored at -80 ºC for later analyses. Venous blood samples were collected after an overnight fast, and until further use stored at -80 ºC and room temperature, respectively. The GI symptom rating scale (GSRS) was completed at baseline and at weekly intervals. Supplement intake continued until all

measurements were finished.

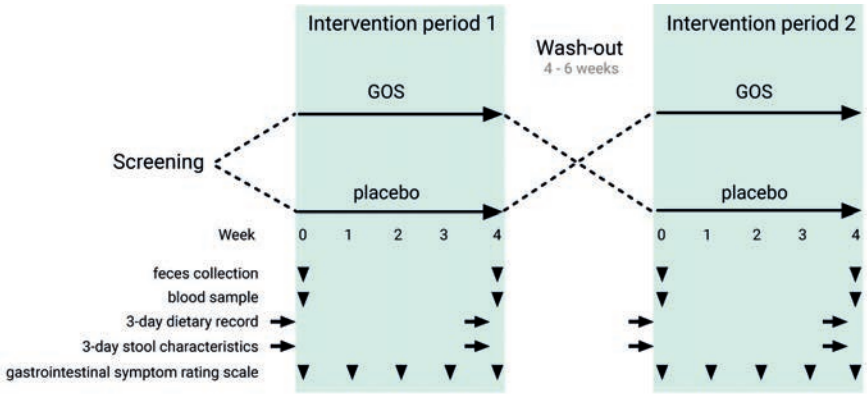

Figure 7.1 - Study set-up and timeline. Screening, GOS and placebo supplementation, feces collection, blood sampling, three day dietary records and stool characteristics, and gastrointestinal symptom rating scales were completed on the weeks as indicated by arrows. The wash-out period was four to six weeks. Intake of GOS and placebo continued until all measurements of the specific intervention period were finished.

Microbiota profiling

Total DNA was isolated from feces and subsequently purified as described previously (14). The microbiota profiling was determined by sequencing of barcoded 16S ribosome RNA (rRNA) gene amplicons using Illumina Hiseq2500 (2 x 150 bp). The V4 region of 16S rRNA gene was amplified in triplicate using 515F(15)-806R (16) primers and purified DNA as template. 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 sequencing data was processed using NG-Tax default settings (17).

qPCR analysis

Total DNA was also used for quantitative PCR (qPCR) using CFX384 Touch ™ Real-Time PCR Detection System (Bio-Rad, California, USA). The reaction mixture composed of 6.25 µl iQ™ SYBR ® Green Supermix, 0.25 µl forward primer (10mM), 0.25 µl reverse primer (10mM), 3.25ul nuclease free water and 2.5 µl DNA template. Primers targeted total bacteria (18) and *Bifidobacterium spp.* (19). 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 seconds, annealing at 52 °C for 30 seconds and elongation at 72 °C for 30 seconds. The program for total bifidobacteria was: 94°C

for 5 min, followed by 40 cycles of 94 °C for 20 seconds, 55 °C for 50 seconds, 72 °C for 50 seconds. Both programs were ended with a melt-curve from 60 °C to 95 °C with 0.5 °C per step. Quantification of total bacteria and total bifidobacteria were performed in triplicates. Data was analyzed using the CFX manager ™ (Bio-Rad). The relative abundance of *Bifidobacterium* was calculated by dividing the total 16S rRNA gene copy number of *Bifidobacterium* by total bacterial 16S rRNA gene copy number.

Fecal metabolites profiling

Organic acids in the feces were measured using High performance liquid chromatography (HPLC), equipped with 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 (RI) detector with a temperature of 40 °C. 1g feces was dissolved 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 for analysis. The chromatograms were depicted and analyzed with the 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 feces for five hours at 60 °C (Concentrator plus, Eppendorf, Hamburg, Germany).

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 Lipopolysaccharide (LPS) *E. coli* 055:B5 (L4524, Sigma-Aldrich, St. Louis, MO, USA) to stimulate monocytes and 10 µg/ml Phytohemagglutinin-M (PHA) (L8902, Sigma-Aldrich, St. Louis, MO, USA) to stimulate T-cells. After 24 hours incubation at 37°C and 5% CO_2, 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 manufacturers' instructions and samples were measured using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FACSDiva ™ Software (BD Biosciences, San Jose, CA, USA). Serum CRP concentrations were determined by immunoturbidimetric assay using Cobas 6000 analyzer (Roche, Mannheim, Germany).

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 concentrations were quantified in 100 µl plasma after derivatization with thiobarbituric acid into a fluorescent chromogen during 1 h boiling. 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 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 Trolox equivalent antioxidant capacity (TEAC), according to Fischer *et al.* (20) with some minor modifications. The 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS·+) radical solution was generated by adding approximately 2 U/ml horse radish peroxidase type II (Sigma-Aldrich, St. Louis, MO, USA) and 20 µM hydrogen peroxide to a solution of 5 mM ABTS in a 145 mM sodium phosphate buffer (pH = 7.4) until the absorption was around 0.7 at λ = 734 nm. To correct the antioxidant capacity of plasma for individual differences in the highly abundant antioxidant UA, UA plasma concentrations were quantified by HPLC (21). The UA corrected TEAC (non-UA TEAC) values can be related to the presence of other water-soluble antioxidants in plasma such dietary polyphenols and vitamins.

Gastrointestinal tolerance

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, diarrhea, and constipation (22). 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 analyzed as described previously (23).

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 analyzed using the online dietary assessment tool of The Netherlands Nutrition Centre (www.voedingcentrum.nl), which is based on the Dutch Food Composition Dataset (NEVO, National Institute for Public Health and Environment, Ministry of Health, Welfare and Sport, the Hague, the Netherlands). 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 (24, 25).

Statistical analyses

In a study investigating the effects of GOS on different bacterial groups using fluorescent in situ hybridization (FISH) in healthy elderly volunteers, significant changes in bifidobacteria (proxy parameter for saccharolytic activity) and *Bacteroides spp* (indicator of less favorable bacteria) were observed (12). The estimated effect after five weeks GOS intervention was an increase of 0.59 ± 0.44 cells/g feces for bifidobacteria and a decrease of 0.018 ± 0.022 cells/g feces 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 equaled 8 and 17 subjects, respectively. O'keefe *et al.* (26) investigated the overall microbiota profile in healthy individuals by comparing two weeks high-fiber and low-fiber diets in a cross-over setting, it was shown that 20 subjects was sufficient to find effects on the overall microbiota profile (26). Taking all above into account, we decided to include 20 subjects per age group, which should be sufficient to pick up changes in overall microbiota profiles, as well as in specific genera.

Per parameter, we described the comparison between elderly and adults, and

subsequently the effects of GOS versus placebo interventions in both age groups. For this, normality of the data was checked by histograms and was summarized 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 normalized to relative abundance and the microbial diversity indices (Faith's phylogenetic diversity (PD) and Inverse Simpson) were calculated based on amplicon sequence variant (ASVs). Based on weighted UniFrac, unweighted UniFrac, Bray-Curtis and Jaccard distance, principle coordinate analysis were used to demonstrate the microbiota variation at ASVs level. Significant differences between groups were tested by permutational multivariate analysis of variance (PERMANOVA). All statistical analysis for microbiota data were conducted in R (R-3.5.0).

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 statistically significant. *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.

Results

Study subjects, gastrointestinal tolerance and dietary intake

After evaluating 66 elderly and 33 adults for eligibility, 20 elderly (all Fried frailty score of 1) and 24 adults (all Fried frailty score of 0) were randomized in the study. Three adults dropped out and one adult was non-compliant to the intervention (Figure 7.2). As per protocol analyses led to the same conclusions, only the results of the intention to treat analyses are shown. Baseline characteristics indicate that besides age, also medication use and BMI were significantly different between age groups (Table 7.1).

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 significant different between GOS and placebo intervention in elderly nor in adults (all $P \geq 0.058$) (Supplementary Figure 7.S1), thereby indicating that both GOS and placebo products were well-tolerated. 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 $P \geq 0.170$; data not shown).

As shown in Supplementary Table 7.S1, energy, macronutrient, micronutrient as well as polyphenol intake levels did not differ significantly between elderly and adults at baseline. Moreover, these dietary intake levels were not significantly different between four weeks GOS and placebo supplementation in the elderly, nor in the adults (Supplementary Table 7.S1).

Moreover, elderly and adults returned on average 97.6% and 95.0% of the empty sachets, respectively.

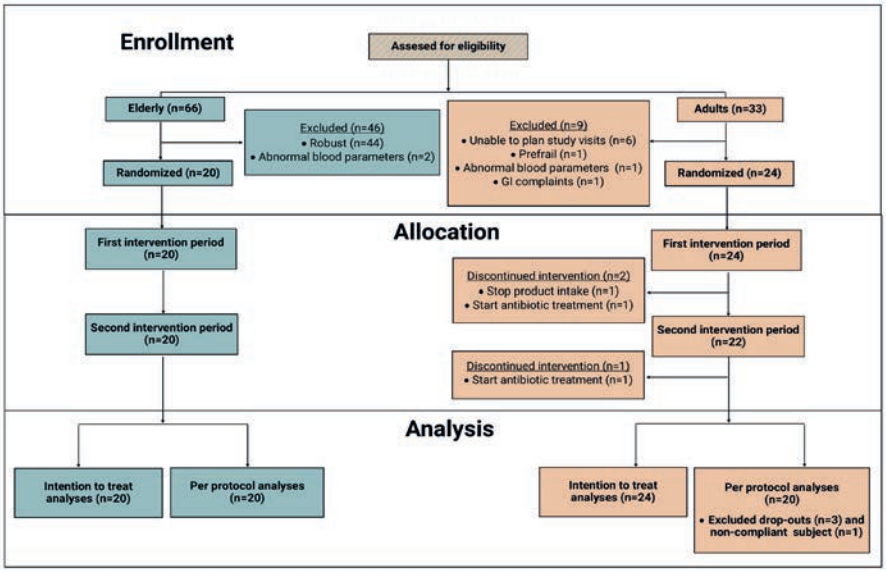

Figure 7.2 - Flow diagram of the study.

Table 7.1 - 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	0.251
1 – 7 units/wk	55.0	62.5	
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.

Fecal microbiota profile

Principle coordinate analysis (PCoA) based on Bray-Curtis (considering relative abundance of ASVs) and weighted UniFrac (considering bacteria relative abundance and their position in the phylogenetic tree) revealed significant differences between the microbiota of adults and that of elderly (Figure 7.3A and C), 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 (Figure 7.3B and D), indicating the differences in microbiota profile are mainly driven by bacteria high in relative abundance. Therefore, we investigated the differences between elderly and adults, and the effects of GOS on genera with an average relative abundance above 2.5% (Table 7.2). The relative abundance of *Bifidobacterium* was significantly lower in elderly compared with adults ($P = 0.027$, Table 7.2), and confirmed by qPCR ($P = 0.036$, Figure 7.4). The relative abundance of other genera ($P \geq 0.473$, Table 7.2) as well as microbial richness and diversity (both $P = 0.942$, Supplementary Figure 7.S2) were not significantly different between elderly and adults.

PCoA based on weighted UniFrac and Bray-Curtis distance (Figure 7.5A and C) were significantly different after four weeks of GOS supplementation, both in adults (both $P = 0.003$) as well as elderly ($P = 0.010$ and $P = 0.005$, respectively). However, no significant differences were observed based on unweighted UniFrac and Jaccard distance (Figure 7.5B and D; ($P > 0.05$)). The relative abundance of *Bifidobacterium* increased significantly after four weeks GOS supplementation in elderly and adults (both $P < 0.001$, Table 7.2), which is confirmed by qPCR for elderly ($P < 0.001$) but not for adults ($P = 0.493$) (Figure 7.4). Furthermore, after four weeks wash-out upon GOS supplementation, the relative abundance of *Bifidobacterium* decreased back to baseline in both age groups. The relative abundance of other genera was not significantly different between GOS versus placebo neither in elderly, nor in adults ($P \geq 0.180$, Table 7.2). Though, the microbial diversity decreased after four weeks GOS supplementation in both groups, being significant only in adults ($P = 0.002$), but not in elderly ($P = 0.101$) (Supplementary figure 7.2A). Microbial richness was not significantly affected by GOS supplementation in elderly and adults ($P \geq 0.113$) (Supplementary Figure 7.2B). Furthermore, the significant effects of GOS on relative abundance of *Bifidobacterium* and on microbial diversity were not significantly different between elderly and adults ($P \geq 0.337$).

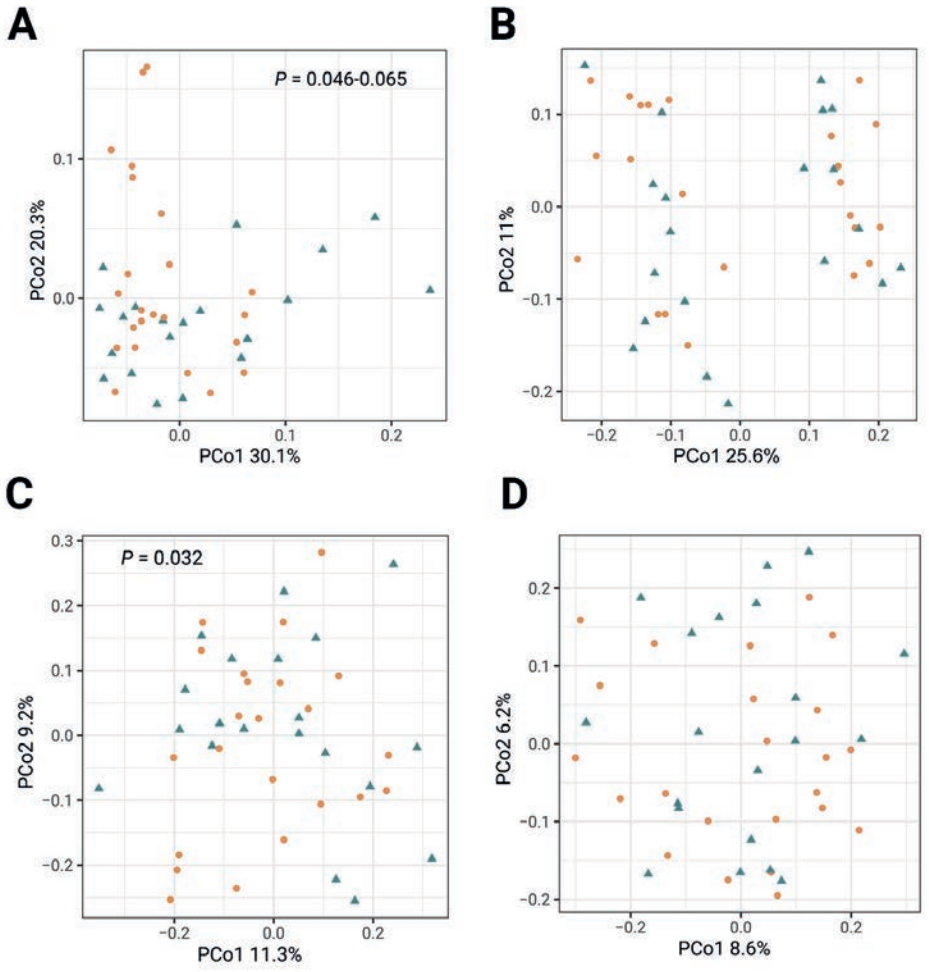

Figure 7.3 - PCoA plots of the fecal microbiota of 20 elderly (green triangles) and 23 adults (orange dots) based on A: weighted UniFrac, B: unweighted UniFrac, C: Bray-Curtis and D: Jaccard distance matrices, based on sequencing data of 16S rRNA gene. PERMANOVA was used to compare the microbiota of elderly and adults in the PCoA plots. PCoA; Principle coordinate analysis.

Table 7.2 - 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)					
	GOS		Placebo		*P*-value	GOS		Placebo		*P*-value	*P*-value (elderly vs adults at baseline)
	Pre	Post	Pre	Post		Pre	Post	Pre	Post		
Blautia	0.091 [0.060; 0.106]	0.079 [0.037; 0.108]	0.100 [0.063; 0.131]	0.079 [0.048; 0.117]	0.637	0.092 [0.067; 0.139]	0.056 [0.038; 0.081]	0.098 [0.072; 0.145]	0.088 [0.061; 0.103]	0.275	0.784
Bifidobacterium	0.024 [0.011; 0.071]	0.260 [0.022; 0.349]	0.027 [0.016; 0.047]	0.042 [0.006; 0.075]	<0.001	0.070 [0.049; 0.145]	0.304 [0.228; 0.525]	0.083 [0.025; 0.152]	0.113 [0.041; 0.196]	<0.001	0.027
Faecalibacterium	0.065 [0.033; 0.081]	0.058 [0.038; 0.083]	0.073 [0.030; 0.096]	0.061 [0.024; 0.090]	0.637	0.096 [0.069; 0.118]	0.070 [0.047; 0.091]	0.077 [0.049; 0.109]	0.075 [0.057; 0.109]	0.180	0.592
Unclassified genus within Lachnospiraeceae family	0.030 [0.014; 0.090]	0.046 [0.019; 0.076]	0.043 [0.021; 0.085]	0.044 [0.012; 0.054]	0.474	0.039 [0.026; 0.066]	0.034 [0.012; 0.065]	0.038 [0.018; 0.061]	0.062 [0.021; 0.084;	0.180*	0.698
Subdoligranulum	0.037 [0.019; 0.075]	0.022 [0.013; 0.039]	0.038 [0.017; 0.059]	0.022 [0.012; 0.053]	0.474	0.036 [0.027; 0.047]	0.016 [0.011; 0.031]	0.040 [0.021; 0.063]	0.032 [0.017; 0.057]	0.275	0.846
Prevotella 9	0.001 [0.000; 0.099]	0.002 [0.000; 0.106]	0.002 [0.000; 0.068]	0.000 [0.000; 0.118]	0.637	0.000 [0.000; 0.008]	0.000 [0.000; 0.006]	0.000 [0.000; 0.002]	0.000 [0.000; 0.003]	0.678	0.473
Ruminococcus 2	0.024 [0.002; 0.086]	0.012 [0.000; 0.074]	0.043 [0.003; 0.059]	0.027 [0.003; 0.060]	0.474	0.004 [0.000; 0.063]	0.012 [0.000; 0.030]	0.022 [0.000; 0.048]	0.026 [0.000; 0.050]	0.275	0.592
Christensenellaceae R-7 group	0.010 [0.004; 0.041]	0.011 [0.002; 0.027]	0.019 [0.003; 0.038]	0.015 [0.003; 0.046]	0.275	0.017 [0.006; 0.035]	0.013 [0.003; 0.027]	0.022 [0.005; 0.043]	0.021 [0.008; 0.034]	0.975	0.784
Bacteroides	0.014 [0.002; 0.045]	0.007 [0.002; 0.032]	0.025 [0.006; 0.055]	0.015 [0.002; 0.022]	0.378	0.023 [0.003; 0.036]	0.012 [0.004; 0.038]	0.027 [0.006; 0.041]	0.033 [0.017; 0.063]	0.275	0.700

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. *P*-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. * Significant before correction for multiple testing ($P = 0.047$).

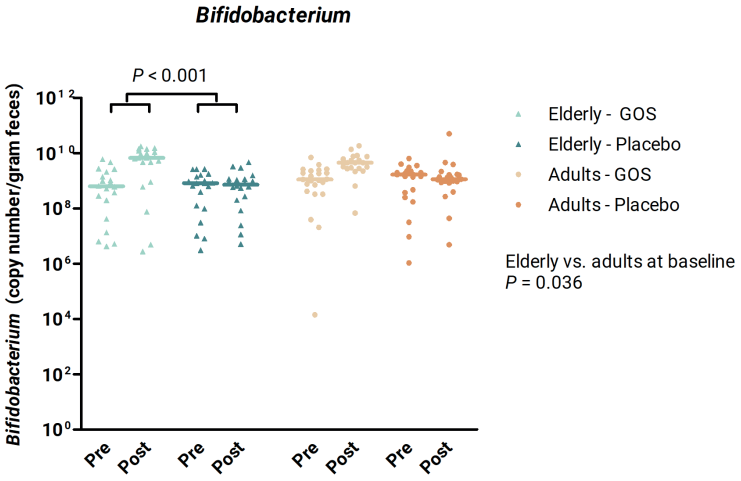

Figure 7.4 - *Bifidobacterium* (copy number/g dry feces) based on qPCR, pre and post GOS and placebo intervention periods in 20 elderly and 23 adults. Sample sizes vary due to drop-outs and technical reasons. Values are presented in scatter plots with median line. Elderly vs. adults at baseline of the 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.

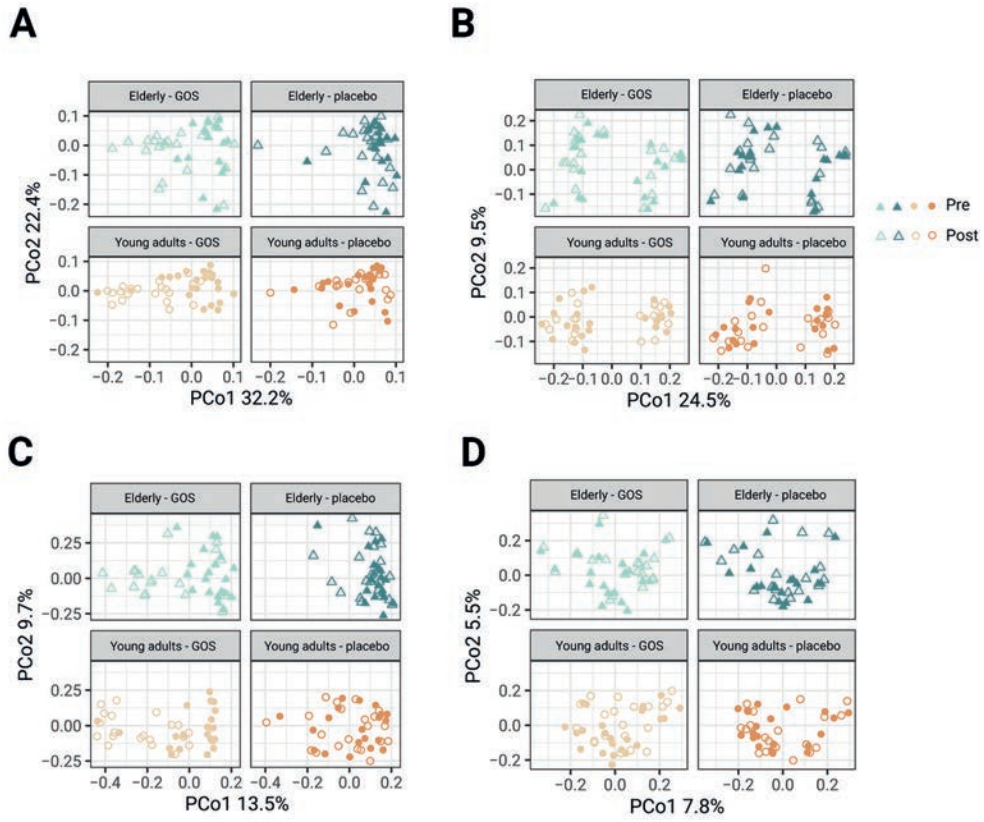

Figure 7.5 - Fecal microbiota profiles, pre (filled symbols) and post (open symbols) GOS and placebo intervention periods in 20 elderly and 23 adults. PCoA plots are based on A: weighted UniFrac, C: Bray-Curtis distance matrices demonstrated clear separation over time, but not based on B: unweighted UniFrac and D: Jaccard distance matrices. Sample sizes vary due to drop-outs and technical reasons. Interventions were compared with PERMANOVA. PCoA; Principle coordinate analysis.

Fecal metabolites

Acetate, propionate, isobutyrate and succinate concentrations did not significantly differ between elderly and adults ($P \geq 0.125$) (Table 7.3). Butyrate concentrations were significantly lower in elderly compared with adults before, but not after FDR correction for multiple testing ($P = 0.034$ and $P = 0.125$, respectively) (Table 7.3). Furthermore, metabolites concentrations did not change significantly after four weeks GOS versus placebo supplementation neither in the elderly, nor in the adults ($P \geq 0.520$) (Table 7.3).

Table 7.3 - Fecal metabolites concentrations (µmol/g dry content) of pre and post GOS and placebo intervention periods of the elderly (n=20) and adults (n=23).

	Elderly (n=20)					Adults (n=23)					
	GOS		Placebo		*P*-value	GOS		Placebo		*P*-value	*P*-value (elderly vs adults at baseline)
	Pre	Post	Pre	Post		Pre	Post	Pre	Post		
Acetate	64.83 [39.49; 92.70]	69.53 [45.22; 112.98]	81.71 [52.96; 149.39]	73.80 [38.67; 145.81]	0.926	94.81 [71.61; 135.82]	111.00 [55.63; 171.26]	77.90 [54.10; 113.59]	85.90 [44.09; 116.88]	0.520	0.188
Propionate	20.78 [13.39; 33.87]	20.91 [15.69; 36.76]	28.71 [16.90; 41.28]	28.21 [16.33; 38.70]	0.926	31.66 [18.58; 43.37]	27.64 [19.03; 39.90]	28.28 [18.44; 43.36]	25.86 [15.72; 40.71]	0.659	0.125
Butyrate	14.74 [8.65; 21.68]	15.28 [10.74; 20.62]	16.55 [10.31; 24.69]	24.40 [7.08; 34.52]	0.926	24.93 [19.12; 43.50]	22.14 [12.64; 36.14]	21.71 [10.44; 36.41]	17.78 [11.03; 41.22]	0.692	0.125*
Isobutyrate	2.32 [1.20; 3.44]	3.30 [1.83; 20.62]	3.25 [1.85; 5.75]	2.88 [1.80; 5.08]	0.926	2.64 [0.00; 4.31]	2.82 [0.20; 4.69]	3.48 [1.42; 3.87]	3.38 [0.68; 6.61]	0.575	0.487
Succinate	0.45 [0.09; 2.66]	0.35 [0.08; 1.39]	0.29 [0.10; 2.02]	0.18 [0.09; 0.62]	0.895	0.34 [0.00; 1.32]	0.35 [0.03; 0.87]	0.18 [0.01; 1.19]	0.23 [0.01; 2.05]	0.520	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) of Benjamini-Hochberg. * Significant before correction for multiple testing ($P = 0.034$).

Immune parameters

Cytokine production (IL-1β, IL-6, IL-8, IL-10, TNFα and IFN-γ) in plasma after 24 hours LPS or PHA whole blood stimulations and serum CRP concentrations were not significantly different between elderly and adults at baseline (Figure 7.6, all $P \geq 0.803$). Moreover, these parameters did not differ significantly between four weeks GOS and placebo supplementation neither in the elderly nor in the adults (Figure 7.6, all $P \geq 0.964$). Overall, these results suggest that GOS does not significantly impact parameters of immune response in elderly or adults.

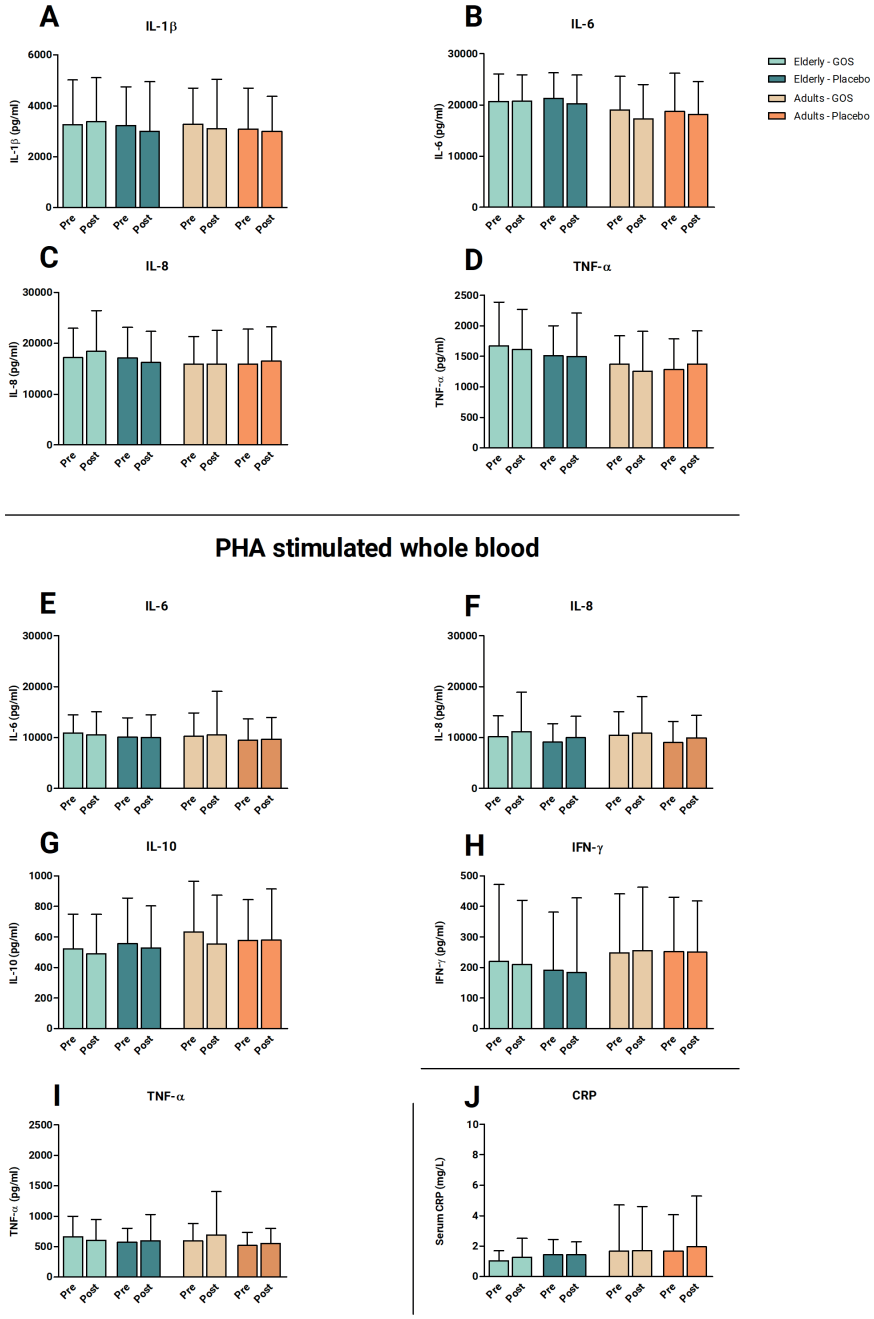

Figure 7.6 - Whole blood cytokine production after 24 hours, A-D: 10 µg/ml LPS or, E-I: 10 µg/ml PHA stimulation, and J: serum CRP concentrations, pre and post GOS and placebo intervention periods in 20 elderly and 24 adults. Values are presented in bars with mean and SD. Sample sizes vary slightly due 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.

Parameters of systemic oxidative stress

Plasma MDA, TEAC and UA concentrations did not differ significantly between elderly and adults at baseline (Figure 7.7A-C) (all $P \geq 0.128$). However, baseline non-UA TEAC values (Figure 7.7D) were significantly higher in elderly compared with adults ($P < 0.001$). None of the markers did significantly differ between four weeks GOS and placebo supplementation neither in elderly, nor in adults (Figure 7.7A-D, all $P \geq 0.236$). These observations indicate that GOS did not significantly parameters of systemic oxidative stress in elderly and in adults.

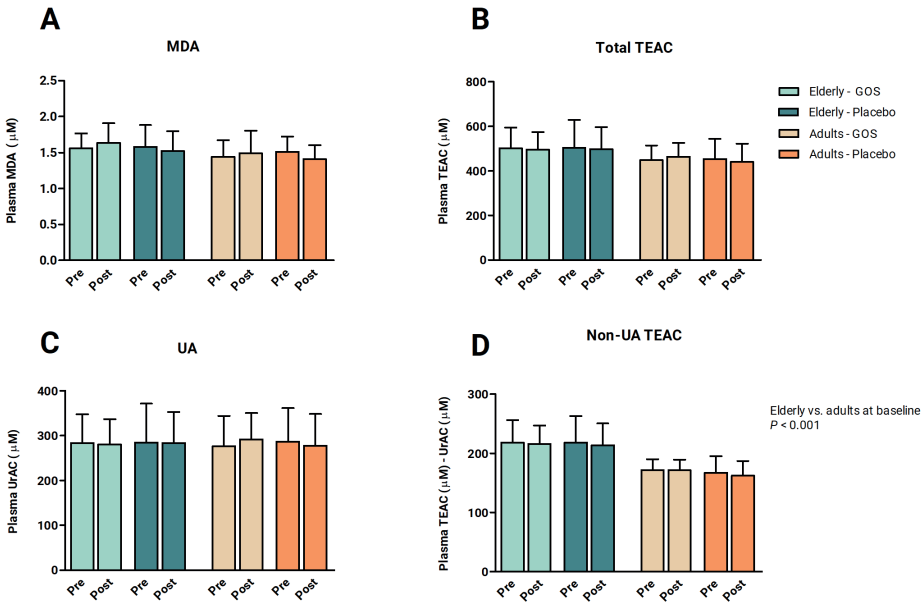

Figure 7.7 – A: MDA, B: total TEAC, C: UA and D: non-UA TEAC values, pre and post GOS and placebo intervention periods in 20 elderly and 24 adults. Values are presented in bars with mean and SD. Sample sizes vary due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention 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.

Discussion

We have shown that the overall fecal microbiota composition did not differ between elderly and adults, except for significantly lower relative abundance of *Bifidobacterium* in the elderly when compared with adults. After four weeks of GOS supplementation, the overall microbiota profile of both age groups changed significantly, accompanied by significant increase in *Bifidobacterium* which was driving a significant drop in microbial diversity (in adults), but not microbial richness. Fecal metabolites as well as parameters of immune function and systemic oxidative stress did not show significant differences neither between elderly and adults, nor between GOS and placebo intervention periods.

Based on a review from our group, we found that microbiota perturbations in elderly was more pronounced in elderly with impaired health status (*e.g.* frailty) (5). In the current study, apart from significantly lower bifidobacteria in prefrail elderly versus healthy adults, the overall microbiota profile did not differ. However, others did find differences. Claesson *et al*. (27) 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 co-morbidity 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, not only physical) or the Barthel Index (assessing performance in activities of daily living), found a negative association between microbial richness and frailty level (28, 29). The significantly lower fecal bifidobacterial abundance in prefrail elderly compared with healthy adults is in line with previous studies (30-32), and may be related to the lower microbial diversity, as in this parameter relative abundance is taken into account (in contrast to microbial richness). As found previously (12, 33-35), in the current study, four weeks GOS supplementation (15 g/day) did significantly increase bifidobacteria and alter the overall microbiota profile of adults and elderly. Fecal metabolites did not show significant differences neither between elderly and adults, nor between GOS and placebo intervention periods. Though, these findings should be interpreted with care, because fecal metabolites do not reflect metabolite production of the proximal colon (*i.e.* assumed site of GOS fermentation).

As several studies have observed that immunosenescence is common in elderly, we have evaluated cytokine responses after *ex vivo* LPS and PHA 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.* (36) who found that LPS-stimulated IL-1β and TNF-α, but not IL-6 levels, were significantly 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 not differ significantly between elderly and adults, is in line with previous findings on IL-6 production after PHA stimulation in isolated peripheral blood mononuclear cells (PBMC) (37, 38). We also evaluated serum CRP as frailty has been associated with inflammation (39). 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. Four weeks of GOS supplementation (*i.e*. 21.6 g/day) 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). Apart from some methodological differences, a clear explanation for the contrasting findings is not available.

As immune function is associated with oxidative stress (8), and elevated oxidative stress levels and lower antioxidant capacity have been reported in elderly (40-44), we also determined the concentrations of 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 any of the age groups. Interestingly, when we corrected TEAC values 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 reveal that the included prefrail elderly appeared to be relatively healthy, with no significant baseline differences between elderly and adults, and lack of a

GOS effect on fecal metabolites as well as parameters of immune function and systemic oxidative stress. 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 fiber intervention studies in comparable populations (45, 46). 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.

In conclusion, we showed that bifidobacteria were lower in prefrail elderly compared with adults, but fecal metabolites and parameters of immune function and oxidative stress were not significantly different. Further, in this well-controlled study in relatively healthy populations, four weeks GOS supplementation increased bifidobacteria and thereby decreased microbial diversity, but did not affect microbiota activity, 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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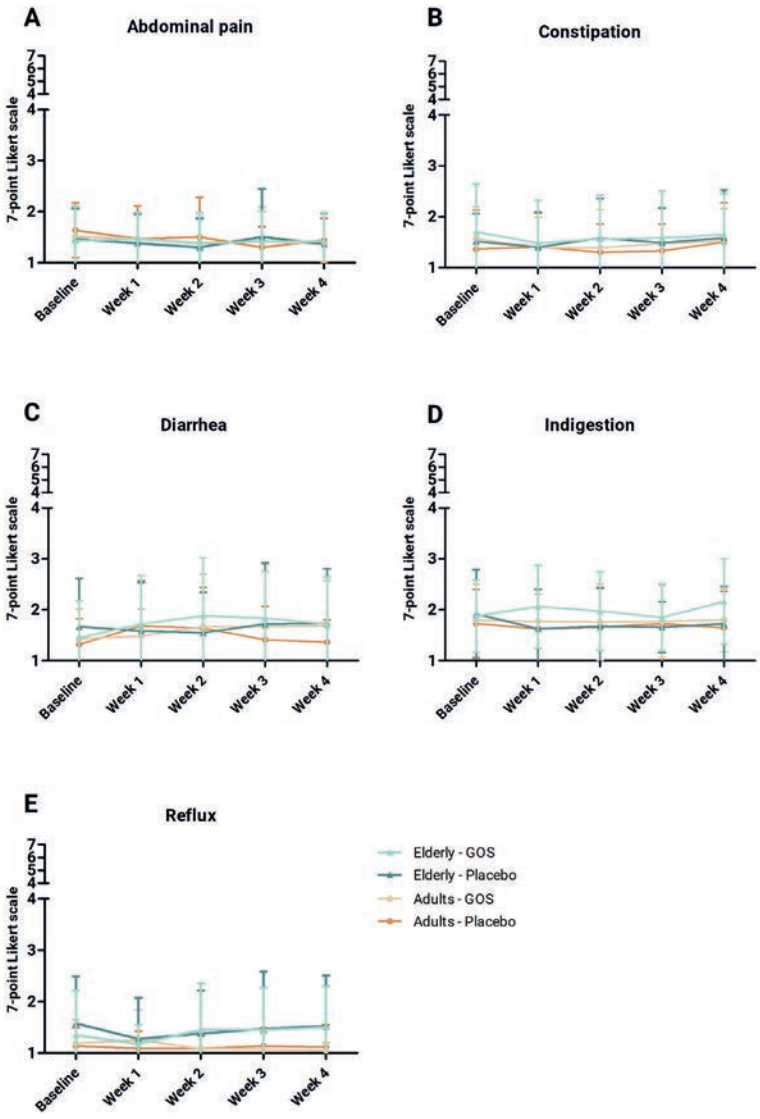
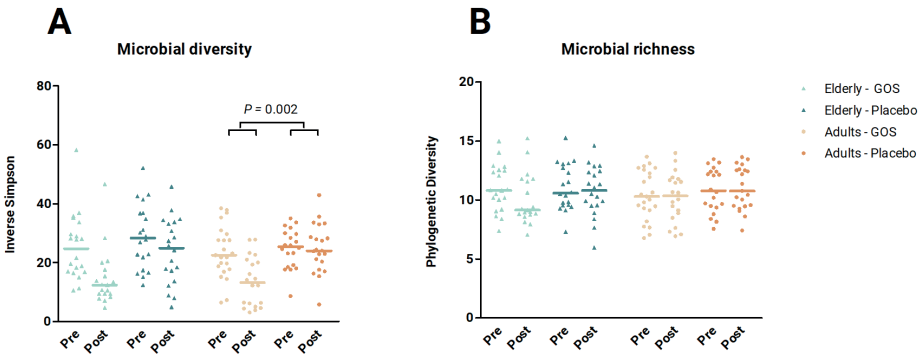
Supplementary material



Supplementary Figure 7.S1 - Gastrointestinal symptoms at baseline and every week of GOS and placebo intervention periods in elderly and adults. 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 7.S2 – A: microbial diversity and B: microbial richness in fecal samples of 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 7.S1 - Dietary intake of pre and post GOS and placebo intervention periods of the elderly (n=20) and adults (n=24).

	Elderly (n=20)					Adults (n=24)					
	GOS		Placebo		*P*-value	GOS		Placebo		*P*-value	*P*-value elderly vs adults at baseline
	Pre	Post	Pre	Post		Pre	Post	Pre	Post		
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
Fiber (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

	Elderly (n=20)					Adults (n=24)					
	GOS		Placebo		*P*-value	GOS		Placebo		*P*-value	*P*-value elderly vs adults at baseline
	Pre	Post	Pre	Post		Pre	Post	Pre	Post		
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 ($P = 0.029$).

Chapter 8

General discussion

Main findings

In this thesis, we aimed to investigate the impact of nutritional interventions on intestinal health, with a special focus on age-related changes. Adaptations in gastrointestinal (GI) physiology and in intestinal microbiota composition and activity have been reported for elderly in general, being most pronounced in association with frailty and impaired health status (**Chapter 2**). Intestinal barrier and immune function as well as the microbiome are considered key modulators of intestinal health. In **chapter 3** we showed that intestinal barrier function, determined by a combined *in vivo* and *ex vivo* approach, was found to be maintained with aging both in healthy individuals and in patients suffering from irritable bowel syndrome (IBS). Nevertheless strengthening of the intestinal barrier by dietary interventions, especially in stressed conditions, is an attractive target to reduce the risk of developing aging-related comorbidity. We showed in a randomized controlled dietary intervention trial that two weeks supplementation with the synbiotic Ecologic® 825/FOS P6 did not significantly impact on the intestinal barrier function in healthy adults, neither in stressed nor in unstressed conditions (**chapter 4**). Subsequently, the impact of (candidate) prebiotics was studied in four-week intervention periods using relatively high dosages (*i.e.* 15 g/day). We found that sugar beet pectin supplementation also did not alter intestinal barrier function (**chapter 5**), nor profiles of the fecal microbiota and exhaled volatile organic compounds in healthy elderly or younger adults (**chapter 6**). The impact of galacto-oligosaccharides (GOS) supplementation in more vulnerable elderly (*i.e.* prefrail elderly, as determined by the Fried criteria) compared with healthy adults was studied in **chapter 7**. This GOS supplementation resulted in an increase in fecal *Bifidobacterium* in both prefrail elderly and healthy adults. However, fecal metabolites, immune function and systemic oxidative stress were not affected by GOS supplementation.

Human studies

Many studies aiming to beneficially alter the intestinal microbiota, intestinal epithelial integrity, and immune activation have been performed in animals and *in vitro*. These are often the basis or rationale for human studies, although the actual number of well-designed randomized controlled trials in relevant target populations is still

limited. The human studies presented in **chapters 3, 5, 6 and 7** showed no significant impact of the synbiotics and (candidate) prebiotics studied on (intestinal) health parameters. These findings do not support the results from studies investigating similar products and outcomes in rodents (1-3). Although we can learn from those animal studies, one should realize that physiological and genetic differences exist between humans and animals (4, 5). With regard to the GI tract, the pig is generally considered the best model (6), whereas most studies are performed in mice or rats. Furthermore, animals in scientific experiments are mostly derived from inbred species with limited genetic variability. Thereby, study outcome variation in animal studies is in general smaller compared with human studies, which increases reproducibility of results and reduces variation in responses (4). Although this allows to detect small intervention effects, it limits the translation of results to the human situation. Furthermore, environmental factors such as diet and medication use are also more complex in humans and vary substantially between individuals (5). Therefore, effects of interventions in animals (such as nutritional supplements or drugs) need to be confirmed in humans, preferably in the actual target population. In the current thesis, we focused on the aging population, as there is increasing interest to obtain further insight into the function of the intestinal barrier, the role of the intestinal microbiota and immune function in elderly. In this population it is relevant to investigate the impact of environmental factors such as dietary intake and medication use, but also for example living situation, self-reliance and co-morbidities. Therefore, we studied the effects of a synbiotic and (candidate) prebiotics in semi-controlled settings. Apart from supplementation of the investigational products and standardizing the most important possible confounders, subjects, all being healthy individuals (including community dwelling pre-frail elderly), were asked to maintain their lifestyle including habitual diet, as alterations in the diet may impact the microbiota composition and activity. Furthermore, only stable use of medication was allowed throughout the study, which was not expected to impact outcomes. This approach increased the external validity (*i.e.* generalizing effects beyond the studied population). It should however be noted that in the studies performed in this thesis, subjects were mainly recruited via advertisements. This may induce a bias by including more health conscious subjects, even in the prefrail elderly group.

Dietary strategies to improve intestinal health

Probiotics and prebiotics are often proposed as dietary interventions that may help to improve intestinal health (*e.g.* intestinal barrier function and intestinal microbiota composition and -activity), as well as general health and well-being (7). To date, many research projects focus on (non-selective) use of probiotics, prebiotics, and synbiotics to induce beneficial health effects. We studied different products, including a synbiotic (*i.e.* comprising 9 different probiotic strains in combination with fructo-oligosaccharides) and two (candidate) prebiotics (pectin and GOS), which may be fermented differently by the intestinal microbiota. Aparte from GOS, which significantly increased fecal *Bifidobacterium* in prefrail elderly and adults, neither of these interventions showed a significant effect on other parameters of the microbiota composition or -activity, intestinal barrier function, immune function or systemic oxidative stress (**chapters 4, 6 and 7**). Prebiotics are fermented by intestinal bacteria, predominantly in the colon. The exact location of fermentation depends on the complexity of the biochemical structure of the prebiotic as well as host factors, such as the microbial composition and GI motility. Pectin is a more complex non-digestible carbohydrate than GOS. The sugar beet pectin used is composed of methyl esters at the C6-carboxyl group and rhamnogalacturonan, with acetylation of homogalacturonan. Pectin was assumed to require various different microbial enzymatic steps for its breakdown, and thereby may be fermented more distally in the colon. However, we did not find changes in fecal bacterial taxa or the overall microbiota structure. A clear explanation for these findings is not available. Galacto-oligosaccharides (provided as Vivinal® GOS, 95% DP 2-5) are considered to be readily easily fermentable and thereby may have more proximal effects. Although we did find changes in fecal bifidobacteria in the GOS study, this was not accompanied by altered fecal metabolites levels. We should acknowledge that fecal samples are often used as a surrogate sample for intestinal microbiota composition- and activity analyses. However, they are not necessarily representative for specific intestinal segments such as the proximal colon, as the microbiota composition differs between segments, and most metabolites (especially short-chain fatty acids) are readily absorbed and/or cross-fed. In future studies, it would be interesting to include luminal content and tissue sampling of for example the ascending, the transverse and/or the sigmoid colon, to be able to investigate the microbiota activity in specific intestinal segments.

Nutritional intervention studies can provide insight in causal relations between intestinal microbiota and potential health effects. However, in our well-controlled intervention studies we showed that in healthy and prefrail individuals, reinforcement of intestinal barrier function as well as parameters of host health (*i.e.* immune function and oxidative stress), was not achieved via addition of a synbiotic and (candidate) prebiotics to the habitual diet. This may be because these are only single products within the complex dietary intake including several meals, beverages and snacks. Major dietary changes (*i.e.* from high-fat/low-fiber diet to low-fat/high-fiber diet, or Mediterranean whole diet) have been found to affect the intestinal microbiota composition and especially the activity (8-10), while also improving for example metabolic health (*i.e.* reduce plasma cholesterol levels (11)) and reducing colon cancer risks (12). Inducing major changes in the habitual diet may therefore also be an attractive approach for future studies in aging populations. This seems to be especially interesting since it is generally considered that (subgroups of) elderly have an altered dietary intake, although further scientific evidence is needed. Other nutritional strategies to improve intestinal health include the intake of *e.g.* polyphenols that may beneficially alter the microbiota, intestinal barrier function and gastrointestinal inflammation (13). Future studies are needed to show whether a combination of nutritional supplements may increase the potential beneficial impact on intestinal health.

Measuring intestinal barrier function

Interest in the role of the intestinal barrier is increasing as this barrier is suggested to be involved in the pathophysiology of several intestinal and extra-intestinal diseases, as well as in comorbidities that are associated with aging. Evaluation of human intestinal barrier function is challenging as good non-invasive markers are largely lacking. The intestinal barrier consists of various components (*e.g.* bacteria, mucus layer, antimicrobial peptides, epithelial single cell layer and immune cells). Many studies focuses on the epithelial barrier. The neighboring epithelial cells are sealed by a highly dynamic junctional complex, comprising of various proteins that may be affected by potential stressors, leading either to temporary dislocation and/or and altered gene or protein expression or modulation. Therefore, careful evaluation of the intestinal barrier, on both functional and molecular level, *in vivo,* in humans is

crucial. Although some previous studies suggested that inulin or synbiotics intake can improve the intestinal barrier functon (14, 15), in **chapters 3, 4** and **5,** we did not observe significant effects of synbiotic- and pectin supplementation on GI segment-specific intestinal permeability *in vivo* as determined by the multi-sugar test. This may be due to a well-functioning intestinal barrier in the populations we studied, *i.e.* healthy adults and healthy elderly. Other studies also did not show significant differences in urinary sugar ratios after probiotic supplementation (*i.e.* single species and multispecies) in healthy subjects (16, 17), and arabinoxylan supplementation in obese subjects (18), whereas analyses in diseased individuals did find an increased permeability. For example, in patients with chronic obstructive pulmonary disease (19), compensated liver cirrhosis (20), diarrhea-predominant IBS (21), hemodialysis (22) and multiple sclerosis patients (23), when compared with healthy controls, the multi-sugar test has shown significantly higher urinary sugar recoveries and/ or urinary sugar recovery ratios. It remains to be determined whether nutritional interventions including probiotic and prebiotics may reinforce intestinal barrier function in these populations. Furthermore, the use of stressors as well as *ex vivo* functional and molecular analyses can be of additional value to explore potential preventive effects and mechanistic insights of barrier function. In **chapter 3,** we showed that indomethacin significantly increased urinary sucrose excretion and L/R ratio, which is in line with other studies (16, 17, 24, 25). This supports the use of indomethacin as stressor in intestinal permeability studies. Indomethacin has been shown to decrease zonula occludens-1 (ZO-1) and occludin gene expression as well as redistribution of their localization *in vitro* in gastric (26) and colonic monolayers (27). The disruption in epithelial barrier function was found to be prevented by polyphenols (27). Ideally the potential preventive or restorative intervention should target the same mechanism through which a stressor impairs intestinal barrier function. Other factors known to increase urinary L/R ratio are for example ethanol intake (28), physical exercise (29, 30), and psychosocial stress (*e.g.* public speech) (31). These factors can either be used as a stressor to check the resilience of the intestinal barrier, but should also be considered as potential confounders when studying the intestinal barrier (21).

In **chapters 3, 4 and 5**, we used the multi-sugar test to determine segment-specific intestinal permeability. As compared to single markers, ratios of urinary excretion of a larger molecule (*i.e.* considered to permeate paracellularly, its permeation rate increases in case of an impaired barrier function) with that of a smaller molecule

(*i.e.* primarily permeating transcellularly and assumed to remain stable under stressed conditions) correct for factors such as gastric emptying rate, intestinal transit time, gastrointestinal secretions and renal clearance (32). Two to five hour urinary recovery ratios of lactulose to mannitol (L/M), and of lactulose to rhamnose (L/R) are often used to assess small intestinal permeability *in vivo* (25, 33). It should be noted that studies on the impact of pro- and prebiotics and of aging on small intestinal permeability differ in the sugar dosages applied. A high dose of lactulose can induce osmotic effects and thereby impact intestinal permeability (25). In our studies, low sugar dosages (0.5-1 g) were used in order to limit potential osmotic effects. We also combined intestinal barrier function analyses with an *ex vivo* setup (**chapters 4 and 5**), *i.e.* mounting freshly obtained biopsies from the sigmoid colon in Ussing chambers under unstressed and chemically stressed conditions. No significant effect of aging or pectin intervention were observed in these experiments. The absence of an effect on intestinal barrier function was further confirmed by analyses of the expression of tight junction and adherence junction proteins. We consider these analyses to be complementary to intestinal permeability determined by the multi-sugar test, as functional differences are often small, especially given the dynamic nature of the junctional complex. Recently, interest is growing in non-invasive single biomarkers or sets of biomarkers for intestinal barrier function (34-36), which enables further investigation of the pathophysiology of diseases that relate to disturbances in GI barrier function, and may also applicable to evaluate effects of interventions, especially in larger populations. Although, some studies assessed circulating ZO-1 or claudins as non-invasive biomarkers of epithelial damage (37), these are not generally considered to be reliable candidate biomarkers, especially considering the complexity of the intercellular junctional complex. Due to this challenge, it may be more relevant to focus on the consequences of barrier dysfunction, *e.g.* permeation of bacteria or toxins. Given the recent development in molecular microbiome analyses, it would be interesting to use 16s rRNA based methods to evaluate the presence of microbes or their products in the circulation.

Future perspectives

Aging is a highly complex process which affects physiological, metabolic, immunological and cognitive processes. These are directly and/or indirectly linked to intestinal functioning and can impact the development of age-related comorbidity. We therefore consider it to be very relevant to better understand the underlying mechanisms and to investigate how they can be addressed by targeted interventions. The intervention studies presented in the current thesis, did not result in significant changes in health parameters (*i.e.* intestinal barrier function, immune function or oxidative stress). This lack of effects is likely due to the well-preserved health in the study populations, which included healthy individuals as well as prefrail elderly. Future studies aiming to improve health parameters in elderly should include more susceptible elderly, for example based on biomarkers. As studied in aging populations, single as well as clusters of biomarkers (*e.g.* inflammation, hematological, or lipid biomarkers) correlate well with for example physical functioning, susceptibility to infection, and responses to vaccination (38-40). Identification based on microbiota-targeted biomarkers may also be relevant, but because of its complexity we cannot clearly distinguish yet between a healthy and unhealthy microbiota.

Concluding remarks

The studies presented in this thesis focused on the role of nutritional interventions and aging in intestinal health. We showed that intestinal barrier and immune function, as well as the intestinal microbiome, are largely preserved with (healthy) aging. Furthermore, we provided evidence that synbiotics and (candidate) prebiotics did not affect the intestinal barrier in healthy adults, nor in healthy and prefrail elderly. In future intervention studies, it is recommended to select more vulnerable (elderly) populations, for example by using relevant biomarkers.

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A

Addendum

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Summary

Intestinal health is an important target to improve overall health and well-being. Apart from effective digestion and absorption of food, an adequate intestinal barrier and immune function, as well as a normal and stable intestinal microbiome are considered to contribute to intestinal health. Proper intestinal functioning also contributes to healthy aging, and may prevent and/or delay health impairments such as frailty and associated comorbidities. Nutritional interventions (such as intake of synbiotics and (candidate) prebiotics) may improve intestinal health in aging populations, although human scientific evidence is scarce. The studies described in this thesis investigated the impact of aging and nutritional interventions on intestinal barrier function (including the epithelial barrier as well as immune parameters) and on the intestinal microbiota composition and activity, as important domains contributing to intestinal health.

In **chapter 2** we reviewed the effects of human aging, and contributing factors such as frailty and medication use, on gastrointestinal (GI) physiology and on the intestinal microbiota. In general, the number of high quality human studies investigating GI physiology with aging was limited, and outcomes were not related to other relevant parameters of GI function. Though, age-related adaptations in GI physiology and in intestinal microbiota composition and activity were reported for elderly in general, being most pronounced in association with frailty and impaired health status. Further, we concluded that a typical intestinal microbiota of elderly is hard to define, given the large interindividual differences in intestinal microbiota composition. We suggest that future studies should shift towards investigating associations between GI physiology and intestinal microbiota in well-characterized subgroups of elderly, and how GI physiology and the intestinal microbiota can be modulated by targeted interventions.

Intestinal barrier function is considered a key modulator of intestinal health, and often reported to be impaired in elderly, although this is mostly based on animal studies. As physiological and genetic differences exist between humans and animals, we investigated the effects of aging on intestinal barrier function in humans in **chapter 3**, by combined *in vivo* and *ex vivo* experiments. Healthy subjects and irritable bowel syndrome (IBS) patients of older (65-75 years) and younger adult age (18-40 years) were compared. *In vivo,* GI site-specific permeability was assessed by a multi-sugar test, and potential confounders were taken into account. Sigmoid

biopsies were collected from subgroups of healthy individuals for *ex vivo* Ussing chamber experiments, gene transcription of barrier-related genes, and staining of junctional proteins. *In vivo* as well as *ex vivo* experiments consistently showed no significant differences between healthy young adults and elderly on intestinal barrier-related parameters. In IBS patients, gastroduodenal and colonic permeability did not differ significantly, but small intestinal and whole gut permeability were higher in elderly versus young adults, mainly driven by the IBS-diarrhea subtype. This latter observation is in line with previous findings. In general, our study revealed that the functional capacity of the intestinal barrier is maintained with aging in the populations studied.

In **chapter 4,** we studied the modifiable potential of two weeks synbiotic supplementation (*i.e.* multispecies probiotic mixture with 10 g/day fructo-oligosaccharides) on intestinal barrier function *in vivo* in healthy adults in unstressed and stressed conditions. In this randomized, double-blind, controlled, parallel study, 10 subjects ingested synbiotic products and 10 subjects ingested control products for two weeks. At baseline and after two weeks supplementation, GI site-specific permeability was assessed by the multi-sugar test in the absence and presence of an indomethacin challenge. Furthermore, plasma zonulin, as well as cytokines and chemokines levels (*i.e.* parameters of immune function) were determined. GI symptoms and stool frequency were recorded on a weekly base. We observed that two weeks synbiotic intake increased stool frequency, but did not affect intestinal permeability (neither under basal nor under indomethacin-induced stressed conditions), immune function, or GI symptoms in healthy adults.

In **chapter 5** and **chapter 6**, the effects of four weeks supplementation of the candidate prebiotic pectin (15 g/day of this complex dietary fiber) on intestinal barrier function, and on profiles of the fecal microbiota and exhaled breath, in adults and elderly were investigated. In this randomized, double-blind, placebo-controlled, parallel study, 52 healthy young adults (18-40 years) and 48 healthy elderly (65-75 years) were included. In **chapter 5**, we assessed *in vivo* GI site-specific permeability by the multi-sugar test, and parameters of defense capacity (*i.e*. salivary and fecal secretory immunoglobulin A (sIgA) and immunoglobulin (IgA) in serum) both pre- and post-intervention. Further, sigmoid biopsies were collected post-intervention from subgroups of healthy individuals for *ex vivo* Ussing chamber experiments, gene transcription of barrier-related genes, and staining of junctional proteins. We found that intestinal barrier function (*i.e.* intestinal permeability *in vivo* and *ex*

vivo and parameters of defense capacity) was not affected by four weeks pectin supplementation, neither in healthy young adults, nor in healthy elderly. In **chapter 6**, we assessed fecal microbiota composition, short-chain fatty acids (SCFAs), and exhaled volatile organic compounds (VOCs) pre- and post-intervention. Results showed that four weeks pectin supplementation did not significantly alter fecal microbiota, SCFA or exhaled VOC profiles in healthy young adults and healthy elderly. In contrast to healthy aging, frailty seems to play an important role in age-related changes in immune function and the intestinal microbiota, as also reported in chapter 2. Therefore, we studied the impact of four weeks galacto-oligosaccharides (GOS) supplementation on immune- and microbial parameters in prefrail elderly and healthy adults in **chapter 7**. In a randomized, double-blind, placebo-controlled, cross-over study, 20 prefrail elderly (70-85 years) and 24 healthy adults (25-50 years) received 21.6 g/day Vivinal® GOS and placebo. Fecal microbiota composition and SCFAs, as well as immune parameters and parameters of systemic oxidative stress determined in plasma, were determined pre- and post-intervention. Prefrail elderly showed lower fecal bifidobacteria compared with healthy adults; however, fecal metabolites, parameters of immune function, and oxidative stress were not significantly different. Four weeks GOS supplementation in both prefrail elderly and healthy adults increased fecal bifidobacteria, but did not impact fecal metabolites, immune function, or oxidative stress.

In **chapter 8**, we summarized and integrated the main findings of this thesis. Furthermore, we pointed to the relevance of human studies and the most important differences with animal studies. New insights and future directions of dietary strategies to improve intestinal health, measuring intestinal barrier function, as well as research in aging populations, are also discussed.

Samenvatting

Een goede darmgezondheid is belangrijk voor de algehele gezondheid en het welzijn. Behalve een effectieve spijsvertering en absorptie van voedsel, zijn een adequate darmbarrière en immuun functie, evenals een normale en stabiele darmmicrobiota, belangrijk voor de darmgezondheid. Een goede darmgezondheid draagt ook bij aan gezond ouder worden en kan gezondheidsproblemen zoals kwetsbaarheid en bijbehorende comorbiditeiten voorkomen en/of vertragen. Voedingsinterventies (zoals inname van synbiotica en (kandidaat) prebiotica) kunnen de darmgezondheid potentieel verbeteren bij ouderen, al is wetenschappelijk bewijs in humane studies schaars. De studies beschreven in dit proefschrift onderzoeken de impact van veroudering en van voedingsinterventies op de darmbarrière functie (inclusief de epitheliale barrière en immuun parameters), en op de samenstelling en activiteit van de darmmicrobiota, als belangrijke factoren die bijdragen aan darmgezondheid.

In **hoofdstuk 2** hebben we een overzicht gegeven van de effecten van veroudering, en daaraan gerelateerde factoren zoals kwetsbaarheid en medicatiegebruik, op de gastro-intestinale (GI) fysiologie en op de darmmicrobiota. Over het algemeen was het aantal hoogwaardige humane studies naar GI-fysiologie en veroudering beperkt, en de resultaten waren niet gerelateerd aan andere relevante parameters van GI-functie. Leeftijd-gerelateerde aanpassingen in de GI-fysiologie en in de samenstelling en activiteit van darmmicrobiota werden gerapporteerd voor ouderen in het algemeen, en bleken vooral gerelateerd aan factoren als kwetsbaarheid en een verminderde gezondheidstoestand. Verder concludeerden we dat een typische darmmicrobiota bij ouderen moeilijk te definiëren is, vanwege de grote interindividuele verschillen in de samenstelling. Toekomstige studies zouden zich kunnen richten op het onderzoeken van associaties tussen GI-fysiologie en darmmicrobiota in goed gekarakteriseerde subgroepen van ouderen, alsook op hoe de GI-fysiologie en de darmmicrobiota gemoduleerd kunnen worden door gerichte interventies.

Darmbarrière functie wordt beschouwd als een belangrijke modulator van darmgezondheid. Op basis van dierstudies werd regelmatig geconcludeerd dat de darmbarrière functie is verstoord bij ouderen. Vanwege fysiologische en genetische verschillen tussen mens en dier, onderzochten we in **hoofdstuk 3** de effecten van veroudering op de darmbarrière functie in mensen, door zowel *in vivo* als *ex vivo* experimenten uit te voeren. Gezonde proefpersonen en patiënten met prikkelbare darm syndroom (PDS) van oudere (65-75 jaar) en jongere volwassen leeftijd (18-

40 jaar) werden vergeleken. *In vivo* werd GI segment-specifieke permeabiliteit onderzocht met behulp van de zogenaamde multi-suikertest. Daarnaast werd met potentiële confounders rekening gehouden. Biopten uit het sigmoïd deel van het colon werden verzameld in subgroepen van gezonde individuen voor *ex vivo* Ussing kamer experimenten, het bepalen van gen-transcriptie van barrière-gerelateerde genen en kleuring van zogenaamde "tight junction" eiwitten. Zowel de *in vivo* als *ex vivo* experimenten lieten geen significante verschillen zien tussen gezonde jongvolwassenen en ouderen op darmbarrière-gerelateerde parameters. Bij PDS-patiënten was de gastroduodenale- en colon permeabiliteit niet significant verschillend, maar de permeabiliteit van de dunne darm en de gehele darm was hoger bij ouderen versus jongvolwassenen. Dit was voornamelijk het geval bij PDS-patiënten met het diarree subtype. Dit laatste komt overeen met eerdere studiebevindingen. Concluderend bleek uit ons onderzoek dat de functionele capaciteit van de darmbarrière behouden blijft bij veroudering in de door ons bestudeerde groepen.

In **hoofdstuk 4** hebben we de effecten bestudeerd van twee weken synbioticum inname (multispecies probioticum met 10 g/dag fructo-oligosacchariden) op de darmbarrière functie *in vivo* bij gezonde volwassenen, in niet-gestresste en gestresste omstandigheden. In deze gerandomiseerde, dubbelblinde, gecontroleerde, parallelle studie namen 10 proefpersonen synbiotische producten en 10 proefpersonen controleproducten in gedurende een periode van twee weken. Bij aanvang en na afloop van deze interventie werd GI segment-specifieke permeabiliteit onderzocht met behulp van de zogenaamde multisuikertest. Dit zowel in afwezigheid als, op een andere dag, in aanwezigheid van een stressor (inname van indomethacine). Verder werden plasma zonuline, evenals cytokines en chemokines bepaald als respectievelijke parameters van darmbarrière en immuun functie. GI symptomen en ontlastingsfrequentie werden wekelijks geregistreerd. Resultaten toonden aan dat twee weken inname van een synbioticum de frequentie van de ontlasting verhoogde, maar geen effect had op de darmpermeabiliteit (onder basale omstandigheden en na indomethacine-geïnduceerde gestresste omstandigheden), immuunfunctie of GI symptomen in gezonde volwassenen.

In **hoofdstuk 5** en **hoofdstuk 6** hebben we effecten van vier weken inname van het kandidaat-prebioticum pectine (15 g/dag van deze complexe voedingsvezel) op de barrièrefunctie van de darm, op profielen van de fecale microbiota en op metabolieten in uitademingslucht bij zowel volwassenen als bij ouderen onderzocht. In deze gerandomiseerde, dubbelblinde, placebo-gecontroleerde, parallelle studie

werden 52 gezonde jongvolwassenen (18-40 jaar) en 48 gezonde ouderen (65-75 jaar) geïncludeerd. In **hoofdstuk 5** hebben we opnieuw *in vivo* GI segment-specifieke permeabiliteit onderzocht met behulp van de zogenaamde multi-suikertest, alsook parameters van afweer (secretoir immunoglobuline A (sIgA) in speeksel en in feces, en immunoglobuline (IgA) in serum) getest voor en na de interventieperiode. Na de interventieperiode werden ook biopten uit het sigmoïd deel van het colon verzameld van subgroepen van gezonde individuen voor *ex vivo* Ussing kamer experimenten, voor het bepalen van de mate van transcriptie van barrière-gerelateerde genen, en voor kleuring van zogenaamde "tight junction" eiwitten. We vonden dat de darmbarrière functie (darmpermeabiliteit *in vivo* en *ex vivo,* alsook parameters van afweer) niet werd beïnvloed door het gedurende vier weken dagelijks innemen van pectine in gezonde jongvolwassenen en gezonde ouderen. In **hoofdstuk 6** hebben we de samenstelling van fecale microbiota en korte-keten vetzuren, alsook van metabolieten in uitademingslucht voor en na de interventie bestudeerd. De resultaten lieten zien dat het gedurende vier weken dagelijks innemen van pectine geen invloed had op de samenstelling van fecale microbiota en korte-keten vetzuren, noch op de samenstelling van metabolieten in uitademingslucht bij gezonde jongvolwassenen en gezonde ouderen.

In tegenstelling tot gezonde veroudering, lijkt kwetsbaarheid een belangrijke rol te spelen bij leeftijd-gerelateerde veranderingen in het immuunsysteem en de darmmicrobiota, zoals ook gerapporteerd werd in **hoofdstuk 2**. Daarom hebben we in **hoofdstuk 7** de impact van dagelijkse inname van galacto-oligosachariden (GOS) gedurende vier weken op het immuunsysteem en de darmmicrobiota bestudeerd bij ouderen in een voorstadium van kwetsbaarheid (pre-kwetsbaarheid) en bij gezonde volwassenen. In een gerandomiseerde, dubbelblinde, placebo-gecontroleerde, cross-over studie, ontvingen 20 pre-kwetsbare ouderen (70-85 jaar) en 24 gezonde volwassenen (25-50 jaar) Vivinal® GOS (21,6 g/dag) en placebo. Zowel fecale microbiota samenstelling en korte-keten vetzuren, alsook immuunparameters en parameters van systemische oxidatieve stress gemeten in het bloedplasma, werden bepaald voor en na de beide interventieperioden. Voor de eerste interventieperiode hadden pre-kwetsbare ouderen minder fecale bifidobacteriën vergeleken met gezonde volwassenen, maar fecale korte-keten vetzuren gehaltes en parameters van immuunfunctie en oxidatieve stress verschilden niet tussen beide groepen. Vier weken GOS inname in pre-kwetsbare ouderen en gezonde volwassenen leidde tot een verhoging van fecale bifidobacteriën, maar had geen invloed op fecale korte-

keten vetzuren, immuunfunctie en oxidatieve stress.

In **hoofdstuk 8** zijn de belangrijkste bevindingen van dit proefschrift samengevat en geïntegreerd. Verder is de relevantie van humane studies benadrukt en zijn de belangrijkste verschillen met dierexperimenteel onderzoek toegelicht. Ook werden nieuwe inzichten besproken met betrekking tot toekomstige voedingsinterventies om de darmgezondheid te verbeteren, het meten van de darmbarrière functie en onderzoek bij ouderen in het algemeen.

Valorization

With the studies presented in this thesis, we aimed to improve our understanding of the impact of nutritional interventions and aging on intestinal health. The knowledge we gained will be put into societal and economical perspective in this valorization paragraph.

The population is aging worldwide, although geographical differences exist (1). It was estimated that the number of individuals aged 65 years and older increases from 617 million (representing 8.5% of the total world population) in 2015 to 1.6 billion (16.7% of the total population) in 2050 (1). The biological aging process contributes to age-related conditions such as frailty and (co)morbidity (2, 3). Collectively, this trend imposes an increasing challenge for society in terms of the burden on health care systems and economic consequences by increasing health care costs (1). Therefore, the World Health Organization (WHO) has decided that global action is urgently needed. Hence, the current decade (*i.e.* 2020-2030) is labeled as the 'decade of healthy aging', with the opportunity to bring together governments, civil society, international agencies, healthcare professionals, academia, the media and private sectors. The goal is to improve the lives of older people, their families, and the communities in which they live by collaborative actions. At an individual level, elderly who experience these extra years of life in good health, continue to participate and are an integral part of families and communities. However, if the added years are dominated by poor health, social isolation or dependency on care, the implications for elderly overall are negative. At population level, this can be expressed as health-adjusted life years (HALY), including quality-adjusted life years (QALY) and disability-adjusted life years (DALY) (4). In the concept of healthy aging, the aim is to induce QALY gain and thereby limit DALY. Overall, this would improve quality of life of (vulnerable) elderly, and reduce health care costs.

With the research presented in this thesis, we aimed to increase our understanding on aging and intestinal health, and tried to improve intestinal health by nutritional interventions. Optimizing dietary intake (*i.e.* quantity and quality) and thereby nutritional status in specific (elderly) populations can be one strategy to achieve the goals of the 'decade of healthy aging'. However, several other aspects have to be taken into account as well. The impact of specific (nutritional) interventions differs between less developed and more developed countries, because of different standards and needs. In addition, fundamental domains of a healthy aging phenotype in lifestyle-

based intervention studies should be included: physiological and metabolic health, physical capability, cognitive function, social well-being and psychological well-being (5). Nutrition may directly or indirectly contribute to healthy aging via several of these domains. A multi-domain intervention approach should be considered with improvements in overall diet, physical activity, as well as psychological and social support. In addition, functional foods (*i.e.* foods or dietary components that may provide a health benefit beyound basic nutrition) have received increased attention in the past decades (6). Beneficial effects of functional foods have been shown *in vitro* and in animal studies, but evidence of (well-controlled) human studies is inconclusive. As dietary habits are largely personally and culturally determined, it remains challenging to induce major changes in consumers overall diet. Thereofore, functional foods are an interesting stategy to induce dietary changes and further improve healthy aging in general and/or specific domains.

Intestinal health is important as target to improve overall health and well-being in the general population as well as in elderly. An adequate intestinal barrier and immune function, as well as normal and stable intestinal microbiota are suggested to contribute to intestinal health (7-9). By definition, the intestinal microbiota can be modulated by probiotics and/or prebiotics intake. From the late 90s onwards, probiotics and prebiotics research increased rapidly, mainly driven by technological developments and commercial perspectives. To date, strong and consistent evidence is still lacking on beneficial intestinal and extra-intestinal health effects in general populations including elderly. With regard to the microbiome, reported effects are mainly limited to an increase of the administered probiotic strain, increased bifidobacterial counts and/or short-chain fatty acid levels. The latter are important for intestinal health by serving as energy substrate for the intestinal epithelium, reinforcement of the epithelial barrier, as well as having amongst others anti-inflammatory and anti-oxidative effects (10). As a result, the majority of filed health claims on probiotics and prebiotics improving intestinal health have been rejected by the European Food Safety Authority (EFSA). In this thesis, there was strong focus on the effects of probiotics and prebiotics on intestinal barrier function and the intestinal microbiota, as these were suggested to be perturbated in elderly. Our findings, however, indicated that two weeks synbiotic intake and four weeks (candidate) prebiotic intake does not translate into pronounced beneficial intestinal health effects in healthy adults and in healthy elderly. One explanation may be that

the populations we studied were too healthy to further improve the selected health parameters. Future intervention studies aiming to intestinal health should select more vulnerable subgroups of (frail) elderly, preferably by using biomarkers and/or based on the outcome parameters. Furthermore, we must acknowledge that there are still gaps in our understanding of the interactions between dietary components, the intestinal microbiota, as well as intestinal and extra-intestinal physiological functions. Depending on their chemical structure (in case of prebiotics) and/or microbes included (in case of probiotics), physiological effects may differ. Thereby chosing the best prebiotic or probiotic to target specific mechanisms is challeging.

Probiotics and prebiotics are widely available as dietary supplements and are incorporated in for example dairy products. Despite many efforts, the exact value of probiotics and prebiotics for health, for the society and for the economy remains to be determined. By increasing our understanding on a 'healthy' intestinal microbiota composition, in relation to intestinal microbiota metabolic activity, intestinal health and overall well-being, the impact of probiotics and prebiotics may expand as well. Other factors influencing the lack of evidence for potential benefit of probiotics and prebiotics include the large inter-individual variation in microbiota composition and the complexity of the overall diet (*i.e.* various different foods consumed over a given reference period) and consumer behavior (*e.g.* costs, taste, compliance).

The research projects described in this thesis were all performed in the setting of a consortium and were partly funded by the government, Top Institute for Food and Nutrition (TIFN), and CarboKinetics program within the NWO Carbohydrate Competence Center partnership. Industrial partners contributed to these consortia via funding, knowledge exchange (*e.g.* in meetings and presentations), and by providing nutritional study products. As researchers, we acted independently with respect to study design and methodology, in performing the studies and in the analysis and interpretation of results, as well as the process of manuscript writing and publication. By these collaborations, government, industry, and academia performed pre-competitive research to study the impact of nutritional interventions in different populations (*i.e.* adults and elderly). This enabled us to set up and conduct comprehensive and expensive human studies. The study objectives were twofold: 1) to investigate the effects of aging on parameters of intestinal health and 2) to investigate the effects of nutritional interventions on parameters of intestinal health. Human intestinal barrier function, including the commensal intestinal microbiota and intestinal permeability, is largely maintained with healthy

aging. This is in contrast to previous findings in animal studies. Factors such as diet, medication use and co-morbidites are generally more complex in humans compared with animals, and vary substantially between individuals as well as with aging. By taking into account such variation, we increase the knowledge on diverse (elderly) populations, and increase the scientific impact of our studies. Although the results of the nutritional intervention studies in this thesis in general are negative, most of the studies described in this thesis have been published in peer reviewed journals in the fields of gastroenterology and nutrition, and data are available for the public domain and the scientific community (11-14). We consider it important to publish negative findings to avoid publication bias. Our data contribute to a better, unbiased understanding of the implications of aging on intestinal health. This may help other groups in designing intervention studies to improve intestinal health, *e.g.* intestinal barrier function, either in diseased populations with known barrier dysfunction or in stressed healthy individuals with a stressor induced disruption in intestinal barrier. Such an approach will stimulate the development of interventional products which are better targeted towards the specific barrier related dysfunctions.

To conclude, the research presented in thesis shows that intestinal physiology, including intestinal barrier function, and intestinal microbiota composition are largely maintained with healthy aging. Perturbations have been reported, but were mainly associated with frailty and impaired health status. Therefore probiotics and prebiotics will have virtually no beneficial effects on largely preserved intestinal physiology and intestinal microbiota composition in populations of healthy adults and healthy elderly. Based on our interventions, we cannot draw conclusions on possible application of pre- and probiotics for the prevention of health problems as this was not part of the current set of investigations. Taken together, this thesis provides relevant information for researchers, for healthy elderly, for producers and consumers.

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Gut Day 2018, Wageningen, the Netherlands – Age-related changes in abdominal pain in healthy individuals and IBS patients

Wetenschapdag Interne Geneeskunde 2018, Maastricht, the Netherlands – Age-dependent changes in GI physiology and microbiota: time to reconsider?

Digestive Disease Days, spring 2017, Veldhoven, the Netherlands – The effects of four weeks pectin intake on intestinal permeability in young adults and elderly

Digestive Disease Days, spring 2017, Veldhoven, the Netherlands – Age-related changes in abdominal pain in healthy individuals and IBS patients
Digestive Disease Days, spring 2016, Veldhoven, the Netherlands – The effects of two weeks synbiotic supplementation on intestinal permeability: a randomized controlled trial

Gut Day 2014, Amsterdam, the Netherlands – The effects of two weeks synbiotic supplementation on intestinal permeability: a randomized controlled trial

Poster presentations

Gut Day 2018, Wageningen, the Netherlands – The effects of four weeks pectin intake on intestinal permeability in young adults and elderly

3rd Meeting of the Federation of Neurogastroenterology and Motility 2018, Amsterdam, the Netherlands – Age-related changes in abdominal pain in healthy individuals and IBS patients

Digestive Disease Week 2017, Chicago, USA – The effects of four weeks pectin intake on intestinal permeability in young adults and elderly – Awarded as 'poster of distinction'

Digestive Disease Week 2017, Chicago, USA – Age-related changes in abdominal pain in healthy individuals and IBS patients

Digestive Disease Week 2015, Washington, USA – The effects of two weeks synbiotic supplementation on intestinal permeability: a randomized controlled trial

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Curriculum vitae

Ellen Wilms was born on 25 January 1989, in Beringe (Helden), the Netherlands. After completing her pre-university education at Bouwens van der Boijecollege (Panningen) in 2008, she studied Health Sciences at Maastricht University (Maastricht) with the specializations 'bioregulation' and 'human movement sciences'. She obtained her BSc degree in 2011. Subsequently, she completed the two-year MSc Health Food Innovation Management at Maastricht University (Venlo). After graduating in 2013, Ellen started as PhD candidate at the department Internal Medicine, division of Gastroenterology and Hepatology within NUTRIM School of Nutrition and Translational Research in Metabolism at Maastricht University. She was supervised by prof. dr. A.A.M. Masclee, prof. dr. D.M.A.E. Jonkers, and dr. F.J. Troost. All human intervention studies were performed in consortium contexts, and partly funded by the Rijksdienst voor Ondernemend Nederland, Top Institute for Food and Nutrition (TIFN), and CarboKinetics program within the NWO Carbohydrate Competence Center (NWO-CCC) partnership.