Nutritional interventions focusing on gastrointestinal and metabolic health

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Layout: Tiny Wouters
Cover design: Kira van Landschoot | www.vankira.nl
Print: Giel Nijssen Grafisch Adviesbureau

ISBN: 978-94-6333-129-6

Printing of this thesis was financially supported by Maastricht University, BioActor BV, Nederlandse Vereniging voor Gastroenterologie, Catharina Hospital, Pentax Medical, DSM, Imagelabonline & Cardiovascular, Dr. Falk Pharma.

Nutritional interventions focusing on gastrointestinal and metabolic health

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. Rianne M. Letschert,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 17 maart 2017 om 12.00 uur

door

Bouke Nicolette Hubertus Salden

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- Big things have small beginnings -

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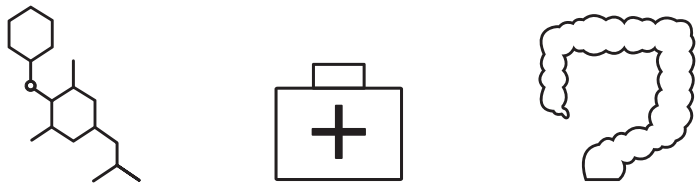

chapter 1

General introduction

General introduction

The statement that food may provide therapeutic benefits is clearly not new. The tenet, 'Let food be thy medicine and medicine be thy food' was embraced already about 2500 years ago by Hippocrates. Initially, scientific focus was given on the identification of essential elements (*e.g.* vitamins) and their role in the prevention and therapy of various dietary deficiency diseases. In recent years, the focus has shifted to explore the role of diet in diseases linked to excess food intake and 'overnutrition'. Furthermore, food is now being examined intensively to identify physiologically active components that potentially have health promoting effects, particularly in chronic diseases or premalignant and malignant disorders or in mild disturbances of organ physiology. This food category is now recognized as 'functional foods'.

In this thesis, we aim to investigate the effects of several nutritional interventions on gastrointestinal and metabolic health. To study these effects we not only focused on healthy, lean subjects but also on obese individuals.

Overweight, obesity and high-fat diet

The prevalence of overweight, obesity and related diseases (*e.g.* cardiovascular diseases (CVD) and diabetes mellitus type 2 (DM2)) is increasing worldwide. Adults with a body mass index (BMI) of >25 kg/m^2 are categorized as overweight, those with a BMI of >30 kg/m^2 as obese.[1] In the Netherlands in 2015, 50.3% of the adults aged 20 years and older were overweight and 13.7% were obese.[2] Worldwide in 2014, approximately 39% of people aged 18 years and over were overweight and 13% obese.[3] The increased prevalence of obesity and associated diseases is primarily due to an increase in caloric availability and fat consumption, and a reduction in physical activity in daily life.[4] Growing evidence supports the idea that also the gut microbiome plays an important role in the development of fat mass and energy homeostasis.[5] Common features associated with overweight and obesity are alterations in gut microbiota,[6,7] impaired gut barrier function,[8-10] (gastrointestinal) and systemic low-grade inflammation,[11-14] endothelial dysfunction,[15] hypertension,[16] glucose homeostasis disorders[4] and lipid homeostasis disorders.[17] Obesity and the associated gastrointestinal and systemic metabolic abnormalities are well-recognized risk factors for the development of DM2 and CVD.[18-20]

Obesity imposes a tremendous economic burden on society through increased total direct (treatment obesity-associated diseases) and indirect (productivity losses) medical costs. In Europe, it has been calculated that in general the direct health care costs of obesity account already for up to total 4% of national health expenditure. It is indicated that indirect health care costs could amount to twice the direct health care costs.[21] Significant reduction

in these costs could be accomplished by prevention of overweight or by a delay in the onset of overweight, obesity and associated diseases.

Gastrointestinal and systemic metabolic consequences

Gut barrier

Every day, our gastrointestinal tract is exposed to a multitude of different microorganisms and nutrient compounds for subsequent interaction. Balancing this interaction requires a complex control system that is able to prevent entry of antigens and microorganisms into the body, while it allows exchange of molecules between host and environment and absorption of nutrients from the diet.[22,23] In a healthy organism, the gut barrier is able to fulfill all these needs simultaneously. The gut barrier is a functional unit, organized as a multi-layer system comprising the mucus, epithelial cells, the innate and adaptive immune cells forming the gut-associated lymphoid tissue (GALT) and the gut microbiota.[24]

Mucus protects the epithelium from potentially harmful microorganisms and antigens, but also functions as a lubricating agent for food particles. The mucus layer is dense and only allows low amounts of bacteria to get close to or penetrate the epithelium, which would potentially induce an increase in intestinal permeability with subsequent inflammatory reaction. Furthermore, it is the habitat of the gut microbiota. In addition, the mucus layer serves as food and a binding site for the bacteria, and is probably involved in the selection of specific microbial species, essential for the maintenance of intestinal integrity, homeostasis and function.[24,25]

The gastrointestinal *epithelium* is a single cell layer which is comprised of different cell types, acting as a physical barrier by inhibiting the passage of luminal contents into the systemic circulation through the formation of complex protein-protein networks that mechanically connect adjacent cells and seal intercellular spaces.[26] Tight junctions (TJ), adherent junctions (AJ) and desmosomes are the three main junctional complexes connecting adjacent epithelial cells. TJ consist of transmembrane proteins (occludins, claudins, junctional adhesion molecule-A) connected to the actin cytoskeleton via plaque proteins (ZO-1, ZO-2 and α-catenin) and these proteins regulate paracellular permeability of the epithelium. AJ and desmosomes anchor epithelial cells to one another and confer mechanical strength to the epithelial barrier.[27-29]

Obesity is associated with changes in gut microbiota, which in turn may affect the intestinal permeability via changes in the expression, localization and distribution of TJ proteins[9,10,12,30] and via the endocannabinoid system (eCB). This system consists of endogenous bioactive lipids that are able to alter the distribution of TJ proteins by activating cannabinoid receptors, expressed throughout the gastrointestinal tract.[31] A 'leak' in the paracellular

absorption route enables antigens to pass from the intestinal milieu, challenging the immune system to produce an immune response resulting in inflammation and oxidative stress. Gut barrier dysfunction is regarded as an early event or trigger in the development of various intestinal diseases, and of metabolic endotoxemia present in obesity, DM2 and CVD.[32,33]
GALT, gut-associated lymphoid tissue, is a complex network of immune cells representing a functional barrier that protects the 'milieu interieur' from external threats. GALT drives immunological responses to pathogenic microorganisms. Proper functioning of such networks is essential for the maintenance of gut homeostasis, preventing transport of luminal contents towards and into the systemic circulation thus preventing the activation of an immune response and the induction of an inflammatory state. This underlies the pathogenesis of many gastrointestinal diseases, such as inflammatory bowel diseases (IBD) and celiac disease.[34]

Gut microbiota

The human gut microbiome refers to the entire population of microorganisms colonizing the gut. It is a complex ecosystem that includes at least 10^{14} bacteria and also other microbes, such as yeasts and viruses.[24] It is an organ by itself, which plays an important role within our body, being involved in many functional processes essential for our homeostasis, such as the metabolism of nutrients and drugs, the regulation of many metabolic pathways, the maintenance of epithelial integrity, the modulation of gastrointestinal motility, the stimulation and maturation of both systemic and mucosal immunity, and the production of vitamins and micronutrients.[35,36] The majority of the gut bacteria is non-pathogenic and co-habit with the gut mucosa in a symbiotic relationship. Intestinal microbiota convert undigested food components into various metabolites, such as short-chain fatty acids (SCFA), which can be further processed by the host and appear to be important signalling molecules. Many of the beneficial physiologic properties of the microbiota can be attributed to the production of these SCFA, particularly acetate, propionate and butyrate.[37-39]
In humans, the interaction between genetic and environmental factors (diet and physical activity) is considered as the main contributor for the development of obesity. Recently, gut microbiota have emerged as a potentially important factor influencing development of overweight. However, the exact mechanisms through which the gut microbiota contribute to obesity are still unclear. It has been suggested that the microbiome has the capacity to increase energy harvested from the diet, and that it is able to influence host energy balance and metabolism by modulating host signaling pathways.[5,40-42] Furthermore, the gut microbiome might contribute to the development of low-grade inflammation by production of lipopolysaccharide (LPS), a powerful pro-inflammatory molecule from the cell wall of Gram-negative bacteria, which is continuously produced in the host gut with the death and lysis of Gram-negative bacteria.[7,11] Today, many questions still remain to be answered: for

instance whether changes in gut microbiota composition and diversity are a cause or a consequence of the observed pathology.

Low-grade inflammation

Persistently high circulating levels of inflammatory cytokines (IFN-gamma and TNF-alpha) are often observed in obese humans, and may cause an impaired gut barrier function by affecting TJ proteins.[43,44] An increased intestinal permeability may lead to translocation of bacterial endotoxins, such as LPS, into the systemic circulation where it is able to activate inflammatory pathways.[45] Other processes contributing to these elevated LPS levels are an increase in chylomicron-driven LPS transport and a decrease in processes involved in intestinal LPS degradation (alkaline phosphatase activity).[12,30,46] Additionally, the state of insulin resistance, often observed in obese individuals, promotes inflammation by impairing the anti-inflammatory effects of insulin.[47,48]

Vascular function

The vascular endothelium is a monolayer of cells between the lumen and the vascular smooth muscle cells. It plays a crucial role in the maintenance of vascular homeostasis by keeping a refined balance between vasodilation and vasoconstriction.[49] Nitric oxide (NO), generated by endothelial nitric oxide synthase (eNOS), is of pivotal importance in regulating the arterial tone and thus in regulating endothelial function (EF).[50] It is able to dilate all types of blood vessels, is considered to be a potent inhibitor of platelet aggregation and adhesion and can also inhibit leukocyte adhesion.[51] Two important events in atherogenesis are the recruitment and differentiation of circulating monocytes to the endothelium, and the uptake of cholesterol and oxidized low-density lipoproteins (LDL) by tissue macrophages to form lipid-foam cells. Cellular adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectins participate in leukocyte-endothelial interactions and are strongly expressed on macrophages within atherosclerotic plaques.[52,53] Therefore, based on the combination of its effects, endothelial NO is considered an important anti-atherogenic defense mechanism in vasculature.

Exposing endothelial cells to obesity and cardiovascular risk factors leads to oxidative stress. As a result, endothelial dysfunction (ED) can occur due to a decreased NO bioavailability by a reduction in eNOS expression or acceleration in NO degradation.[15,51] Subsequently, it plays a key role in the development and progression of atherosclerosis. ED is often manifested by an impaired capacity of the vascular endothelium to dilate, which can be quantified by the degree of flow-mediated dilation (FMD), a non-invasive NO-dependent endothelial function assessment.[54]

Another aspect of vascular function that can be affected by obesity is blood pressure. Both animal and human studies have revealed a strong relationship between obesity and hypertension.[55] Several mechanisms have been identified as potential causes of obesity-induced hypertension. As mentioned above, in obesity-associated ED the vasodilating properties of the endothelium are impaired.[56] Also, increase in both peripheral vasoconstriction and renal tubular sodium reabsorption,[57] increase in sympathetic activity by obesity-related factors (leptin, hyperinsulinemia, circulating free fatty acids (FFA)),[58-62] overactivation of the renin-angiotensin system (RAS)[63,64] and insulin resistance[65,66] are proposed additional mechanisms mediating hypertension in obesity. A linear relationship exists between blood pressure (BP) and mortality from stroke and ischemic heart disease.[67] Hence, interventions aimed at preventing or improving obesity would greatly impact the management of ED, obesity-induced hypertension and related diseases.

Glucose and insulin metabolism

In healthy individuals, plasma glucose is tightly controlled by the balance between glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues. Insulin serves as the primary regulator of plasma glucose, by increasing glucose uptake from muscle and fat, and by inhibiting hepatic glucose production. Furthermore, it stimulates glycogen synthesis.[68]

Obese individuals are at risk for developing resistance to the cellular actions of insulin. This is characterized by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in fat and muscle.[68,69] Proposed mechanisms, explaining the relationship between increased adipose mass and systemic insulin resistance, focus on dysregulation of interconnected endocrine (FFA, adipokines and cortisol), inflammatory, neural and cell-autonomous pathways.[70] Insulin resistance is considered the key etiological factor in DM2.

Lipid metabolism

Numerous metabolic processes are involved in the uptake, transport and storage of lipids. In the intestinal lumen, triglycerides (TG) are lipolyzed into FFA and taken up by the enterocyte, where they are assembled in TG again. Cholesterol is in the enterocyte transformed into cholesterol-esters. TG, cholesterol-esters, phospholipids and apolipoprotein B together form chylomicrons, which enter the systemic circulation. After food-derived TG and FFA reach the liver, TG-rich lipoproteins called very low density lipoproteins (VLDL), are synthesized. Adequate lipolysis of chylomicrons and VLDL by lipoprotein lipase (LPL) delivers FFA to the heart, skeletal muscle and adipose tissue for energy expenditure and storage. The liberated FFA is then taken up by adipocytes and re-synthesized into TG. During the

process of lipolysis also chylomicron remnants and dense LDL are formed, these products are taken up by the liver. The intestine and liver also play an important role in the reverse cholesterol transport by the synthesis of high-density lipoproteins (HDL). HDL promotes the uptake of cholesterol from peripheral tissues and returns it to the liver.[71]

Typical dyslipidemia in obesity consists of hypertriglyceridemia in combination with an increase in small dense LDL and a low HDL.[17] In hypertriglyceridemia, increased FFA fluxes to the liver are a consequence of increased FFA release from adipose tissue and reduced FFA clearance from plasma. The increased FFA flux results in hepatic accumulation of TG followed by an increase in VLDL synthesis. Lipolysis of TG-rich lipoproteins is impaired in obesity, due to competition for lipolysis between VLDL and chylomicrons, reduced LPL expression in adipose tissue and reduced LPL activity. Also, HDL metabolism is affected by obesity. An increased number of TG-rich lipoproteins results in an increased exchange of cholesterolesters from HDL for TG from VLDL and LDL. In addition, TG-depleted small dense LDL is formed by removing TG and phospholipids from LDL.[71-73] Furthermore, also insulin plays an important role in lipid metabolism as it stimulates LPL activity, facilitates FFA uptake, inhibits hydrolysis of intracellular lipids postprandially, and regulates FFA mobilization from adipose tissue during fasting states.[68] Dyslipidemia in obesity is an established independent risk factor for the development of CVD.[74]

Nutritional interventions in healthy, overweight and obese individuals

Prevention or improvement of gastrointestinal and chronic metabolic diseases by a nutritional intervention appears to be a promising approach in health and obesity. At present, a number of dietary strategies are available.

Prolyl endoproteases

Gluten is a storage protein present in wheat, barley and rye and is exceptionally rich in proline. Proline-cleaving proteases are absent in the human gastrointestinal tract, ensuring long proline-rich gluten peptides to enter the small intestine upon ingestion, subsequently triggering an abnormal immune response in subjects suffering from wheat allergy or coeliac disease.[75-77] Subjects with non-coeliac gluten sensitivity also experience distress after gluten intake, but no allergic or autoimmune mechanisms are involved in this condition.[78-81] It has been proposed that in these subjects a gluten-induced activation of the innate immune system occurs.[79] In general, treatment of these gluten-related disorders is based on excluding gluten-containing cereals from the diet, which is often difficult to comply with as

gluten-free products may not always be labeled as such or may not always be at hand during social events or travelling.[82-84]

The *Aspergillus Niger*-derived prolyl endoprotease (AN-PEP) belongs to a family of enzymes having the ability of cleaving at internal proline residues within a peptide.[85] The use of AN-PEP seems to be a promising approach to digest unintended dietary gluten as *in vitro* studies have shown that this enzyme is capable of degrading gluten under acidic stomach conditions and is safe for human use.[86-89] However, *in vivo* data confirming these promising *in vitro* results regarding the gastric degradation of gluten are still lacking.

Prebiotics

Prebiotics are non-digestible compounds that selectively stimulate growth and/or activity of one or a limited number of microbial species in the gut with potential health benefits to the host.[90] Prebiotic fermentation leads to beneficial host effects by the following major mechanisms: (1) by increasing the relative abundance of beneficial bacteria (*i.e.* bifidobacteria, lactobacilli), (2) by increasing SCFA production and reducing the production of putrefactive compounds, and (3) by impacting on the gut's immune system.[91,92] The most common prebiotics used in gut microbiota modulation are the inulins, fructooligosaccharides, various types of galactooligosaccharides and resistant starches. A limitation of these prebiotics is their rapid fermentation in the proximal colon.[93,94] Dietary carbohydrate is the main fermentable substrate in the proximal colon and as it is degraded during bacterial fermentation, protein takes over as the dominant fermentable substrate towards the distal colon. However, bacterial protein metabolism produces toxic and potentially carcinogenic compounds, playing an important role in the development of distal intestinal chronic diseases.[95] Thus, there is currently much interest in the development of prebiotics having the ability to persist towards the distal region of the colon.

Arabinoxylans (AX), the most abundant non-digestible carbohydrates present in wheat, form an interesting novel class of potential prebiotics.[96-98] Due to their structure, AX rely on a whole spectrum of (microbial) enzymes for degradation, resulting in a more distal fermentation and activity.[97-99] Furthermore, AX exist in different forms, ranging from long-chains to enzymatically modified short-chain fractions.[100] Previous studies indicate that AX structure affects the fermentation pattern and immune modulation, with the highest activity observed for high-molecular weight AX.[99,101,102] Prior *in vitro* and animal studies have shown promising effects of high-molecular weight AX on several aspects of the gut barrier,[93,103-105] immune system[103] and metabolic markers.[103] To date, no human studies have been published exploring the effect of such AX extract on these parameters.

Probiotics

Another proposed nutritional intervention, able to change gut microbiota composition, is the use of probiotics. The internationally endorsed definition of probiotics is live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[106] In order to establish an effect, probiotic bacteria will have to survive passage through the gastrointestinal tract. The effects can be classified in three modes of action; they are able to (1) inhibit the action of pathogenic bacteria, (2) stimulate and modulate host defenses and gut barrier, and (3) produce important metabolites for the host.[107,108] Most probiotic products currently on the market contain lactic acid bacteria, such as lactobacilli and streptococci, or bifidobacteria.[109] These bacteria are all normal constituents of the human gut microbiota.

In this thesis we investigate the probiotic effects of a *Bacillus indicus* strain. Bacillus species as probiotics are attractive as they form heat-stable spores. These spores are capable of surviving the gastric barrier without loss of function, are stable to many food-processing steps and can be stored at room temperature.[110,111] No previous human study investigating the effect of this specific strain on gut microbiota composition, activity and gut barrier is available, but animal and human studies exploring the effect of other bacillus strains have shown beneficial effects.[112-117]

Phytonutrients

Phytonutrients, also known as phytochemicals, are chemicals produced by plants. These phytochemicals are used to protect the plant from UV light, insects or pests and produces color and other organoleptic characteristics.[118] Many of these phytochemicals have antioxidant properties and might have the potential to improve health. In this thesis we discuss two different phytonutrients, polyphenols and carotenoids.

Polyphenols are a large group of bioactive plant compounds and are divided into different classes based upon their chemical structure. The gut microbiota plays a critical role in transforming dietary polyphenols into absorbable biologically active species.[119] Recent animal and human studies have shown that dietary polyphenols are able to modulate the human gut microbiota.[120,121] In addition, large epidemiological studies link increased consumption of polyphenol-rich foods with reductions in CVD morbidity and mortality.[122-124] Flavonoids are the largest class of polyphenols, present in many foods of plant origin.[125] Hesperidin, a flavonoid abundantly present in the peels of citrus fruits, to a large extent reaches the colon intact where it is subsequently deglycosylated by intestinal microbiota to produce the active aglycone hesperitin.[126,127] Several human intervention studies have shown promising effects of hesperidin on different cardiovascular biomarkers, such as low-grade inflammation, endothelial function, blood pressure, glucose / insulin metabolism and

blood lipid profiles.[128-132] However, in humans hesperidin is poorly soluble, resulting in a limited bioavailability and biological activity. While natural hesperidin products contain high concentrations of the most active form of hesperidin (hesperitin-7-O-rutinoside 2S), typical hesperidin extracts on the market mainly contain the less active 2R enantiomer. In this thesis, we study the bioavailability and biological activity of a hesperidin 2S extract with a specific enantiomer configuration similar to natural hesperidin.

Carotenoids are organic pigments, synthesized mainly in plants but have also been isolated from yeasts, fungi, marine algae, micro-algae and some species of bacteria. Humans are not able to synthesize carotenoids themselves, and therefore must absorb them from their diet. The incidence of CVD is notably reduced in areas where a 'Mediterranean diet', containing large amounts of fruit and vegetables, and thus carotenoids, predominates.[133,134] In part, the protective effects of carotenoids seem to be related to their potent antioxidant activity. A link between carotenoid intake and protection from LDL oxidation, atherosclerotic progression, hypertension, low-grade inflammation and endothelial dysfunction has been shown.[135-137] The majority of commercially available carotenoids are derived from plant-based materials. However, the quality and consistency of the end product varies tremendously according to the plant growth conditions.[138,139] In addition, these carotenoids are often rapidly degraded in the human gastrointestinal tract by the action of gastric juice. As indicated before, carotenoids can also be synthesized by some species of bacteria. Of specific interest are carotenoids produced by a variety of spore-forming bacillus strains, as these strains are able to deliver gastric-stable carotenoids with equal or even higher bioavailability levels than well-established sources of dietary carotenoids in use today.[140] In addition, in an *in vitro* model these bacterial carotenoids showed to have a 10-fold higher antioxidant activity compared to lycopene, one of the most potent antioxidants known (data not published yet). However, no human data regarding the bioavailability, antioxidant activity and systemic effects of these bacterial carotenoids are available up to now.

Aim and outline of the thesis

In this thesis, we aimed to investigate the effects of nutritional interventions on gastrointestinal and metabolic health in healthy, overweight and obese individuals. The efficacy of AN-PEP in the gastric degradation of gluten was assessed in healthy (lean) individuals. We chose to study the effect of several other nutritional interventions in overweight and obese subjects. We consider overweight and obesity as "mild disease", associated with a broad spectrum of well-recognized risk factors for the development of chronic metabolic diseases such as CVD and DM2. Figure 1.1 gives an overview of these risk factors, the (inter)relationship between obesity, high-fat diet and risk factors, and the chapters in which each risk factor is measured and discussed.

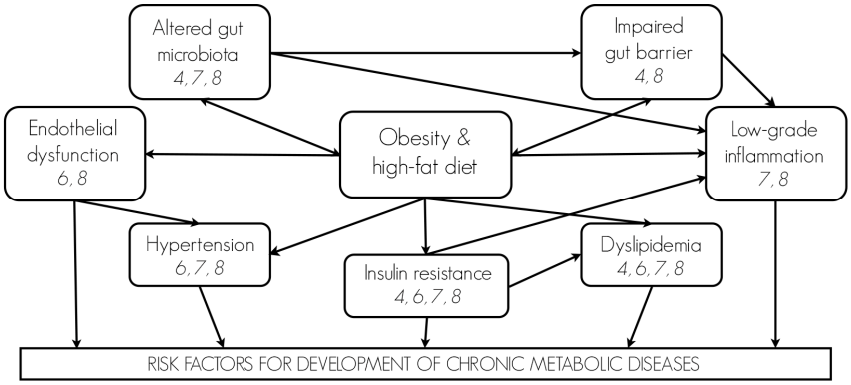

Figure 1.1 Schematic overview of (inter)relationship between obesity, high-fat diet and associated risk factors. The numbers below a risk factor demonstrate the chapters in which the respective risk factor is measured and discussed.

The first part of this thesis (**Chapters 2 & 3**) focuses on investigating the efficacy of AN-PEP on gastric gluten degradation in healthy individuals. Furthermore, also the effect of meal caloric density and gastric emptying rate on the enzyme's efficacy was assessed (**Chapter 2**). In order to display a systemic (patho)biological effect, a compound has to be absorbed from the gastrointestinal tract. The onset of systemic effects depends on the rate of absorption and on gastric emptying rate. In **Chapter 3** we critically evaluate the use of paracetamol as a non-invasive method for measuring gastric emptying rate.

The second part of this thesis (**Chapter 4-8**) explores the impact of different nutritional compounds on parameters of gut and systemic metabolism in overweight and obese, yet healthy, individuals. In **Chapter 4** we investigate the effects of 6 weeks daily supplementation of AX on the gut barrier. The study was specifically designed to integrate

both the analysis of TJ proteins structure and functioning of this barrier. The aspects of the gut barrier we studied include gene transcription and protein expression of TJ proteins in sigmoid colon mucosa, gastrointestinal permeability, microbial community composition and its metabolic activity. In addition, we also assessed whether AX are capable of modulating the immune response. Another nutritional compound we studied is hesperidin, a flavonoid found in citrus fruits. In humans, low solubility of hesperidin limits its bioavailability and biological activity. In **Chapter 5** we studied the *in vivo* bioavailability of hesperidin. First, we compared the bioavailability of a single intake of standard hesperidin with a hesperidin 2S extract, which was specifically developed for its improved bioavailability. Secondly, we aimed to study the bioavailability of this hesperidin extract in more detail by conducting a placebo controlled trial in which participants received products for 5 consecutive days. **Chapter 6** investigates the potential role of hesperidin 2S in the regulation of endothelial function and blood pressure, while **Chapter 7** reveals whether it has a role in glucose and insulin metabolism. Furthermore, local effects of hesperidin 2S on gastrointestinal environment were assessed. The last nutritional intervention we performed relates to PD01, a *Bacillus indicus* strain known to produce *in vitro* high amounts of carotenoids. Carotenoids are lipophilic antioxidants, and a potential preventive role of these antioxidants in the development of CVD has been proposed. Furthermore, *in vitro* data indicate that PD01 itself is able to modulate composition of gut microbiota. Hence, both the effects of the bacillus strain on microbiota composition and functioning and the effects of the carotenoids on CVD risk markers were explored (**Chapter 8**).

Finally, **Chapter 9** summarizes the main findings of all studies presented in this thesis, and discusses the new insights, future perspectives and potential implications for further research and future applications.

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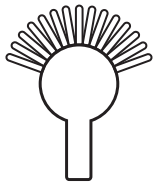

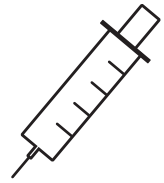

chapter 2

Randomised clinical study: Aspergillus niger-derived enzyme digests gluten in the stomach of healthy volunteers

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Alimentary Pharmacology & Therapeutics

2015 Aug;42(3):273-85

Abstract

Background

Aspergillus niger prolyl endoprotease (AN-PEP) efficiently degrades gluten molecules into non-immunogenic peptides *in vitro*.

Aim

To assess the efficacy of AN-PEP on gluten degradation in a low and high calorie meal in healthy subjects.

Methods

In this randomised, double-blind, placebo-controlled, cross-over study 12 healthy volunteers attended to four test days. A liquid low or high calorie meal (4 g gluten) with AN-PEP or placebo was administered into the stomach. Via a triple-lumen catheter gastric and duodenal aspirates were sampled, and polyethylene glycol (PEG)-3350 was continuously infused. Acetaminophen in the meals tracked gastric emptying time. Gastric and duodenal samples were used to calculate 240-min area under the curve ($AUC_{0\text{-}240min}$) of α-gliadin concentrations. Absolute α-gliadin $AUC_{0\text{-}240min}$ was calculated using duodenal PEG-3350 concentrations.

Results

AN-PEP lowered α-gliadin concentration $AUC_{0\text{-}240min}$ compared to placebo, from low and high calorie meals in stomach (low: 35 vs. 389 μg*min/ml; high: 53 vs. 386 μg*min/ml; $P < 0.001$) and duodenum (low: 7 vs. 168 μg*min/m.; high: 4 vs. 32 μg*min/ml; $P < 0.001$) and absolute α-gliadin $AUC_{0\text{-}240min}$ in duodenum from low (2,813 vs. 31,952 μg*min; $P < 0.001$) and high (2,553 vs. 13,095 μg*min; $P = 0.013$) calorie meals. In the placebo group, high compared to low calorie meal slowed gastric emptying and lowered duodenal α-gliadin concentration $AUC_{0\text{-}240min}$ (32 vs. 168 μg*min/ml; $P = 0.001$).

Conclusions

AN-PEP significantly enhanced gluten digestion in the stomach of healthy volunteers. Increasing caloric density prolonged gastric residence time of the meal. Since AN-PEP already degraded most gluten from low calorie meals, no incremental effect was observed by increasing meal caloric density.

Introduction

Gluten is a storage protein present in wheat, barley and rye and is exceptionally rich in proline, rendering gluten peptides resistant to gastrointestinal digestion. Proline-cleaving proteases are absent in the human gastrointestinal tract. Therefore, long proline-rich gluten peptides reach the small intestine intact after ingestion.[1] About 1% of the western population is suffering from celiac disease.[2-5] In these patients, exposure of duodenum and proximal small intestine to the specific amino acid sequences of such poorly digested proline-rich gluten peptides triggers an abnormal immune response. This causes inflammation with infiltration of lymphocytes in the intestinal mucosa and ultimately villous atrophy and crypt hyperplasia.[6] Adverse reactions to gluten consumption are not limited to subjects suffering from celiac disease. Presently non-celiac gluten sensitivity has been clinically recognized as a separate condition in which neither allergic nor autoimmune mechanisms are involved.[7,8] The symptoms experienced by these subjects are often identical to those seen in celiac disease.[9,10]

A lifelong gluten-free diet is the only treatment for individuals who cannot tolerate gluten. However, a gluten-free diet is hard to comply with as gluten-free products may not always be labelled, and may not always be at hand during social events or travelling.[11-13]

Prolyl endopeptidases belong to a family of enzymes with the ability to cleave at internal proline residues within a peptide.[14] Early investigations on oral protease therapy as approach to degrade gluten have focused on bacterial prolyl oligopeptidases.[15-18] However, several *in vitro* studies conducted with such enzymes revealed only low enzymatic activity at acidic stomach pH and rapid degradation of these enzymes by pepsin.[16] Moreover, these enzymes were not able to prevent passage of potentially harmful gluten fragments into the small intestine.[16,19] But, other enzymatic preparations have shown to be capable of degrading complex gluten proteins both *in vitro* and *in vivo*.[20-23]

In this respect, the *Aspergillus niger*-derived prolyl endoprotease (AN-PEP) also presents a promising option to degrade inadvertent dietary gluten. The use of the enzyme as food supplement has undergone successful evaluation by the French Agency for Food, Environmental and Occupational Health & Safety.[24] AN-PEP is active between pH 2 and 6, with optimum activity at gastric pH between 3 and 5.[19] In a dynamic, multi-compartmental gastrointestinal *in vitro* model, AN-PEP was shown to degrade almost all immunogenic gluten epitopes from gluten-containing meals into non-immunogenic fragments during passage through the stomach compartment.[25] A pilot study in celiac patients showed that a combination of AN-PEP and gluten was safe and well tolerated.[26] Before AN-PEP can be used as a future digestive aid for subjects intolerant to gluten, it is essential that the promising *in vitro* results are confirmed in a human *in vivo* study, focussing on duodenal gluten delivery after intake of gluten-containing meals with and without AN-PEP. Thereafter the efficacy and safety of AN-PEP should be evaluated in target populations.

Our aim was to investigate the efficacy of AN-PEP on gluten degradation in an intragastrically delivered gluten-containing meal in healthy volunteers. To standardize meal intake, we administrated the meal intragastrically at a fixed rate instead of by oral ingestion. Secondly, we hypothesized that increasing the caloric density of a meal enhances gluten degradation by delaying gastric emptying and thereby prolonging gastric residence time of the meal.

Materials and methods

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center (MUMC+) and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as revised in 2008 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study was performed at the MUMC+ from December 2011 to May 2012. All participants gave their written informed consent before participation. The trial has been registered in the Clinical Trials register (NCT01335503) and the Dutch trial register (NTR2780).

Subjects

Healthy volunteers aged 18-45 years were recruited by advertisement. All subjects were screened by means of a standardized general physical examination. Reasons for exclusion included: history of gastrointestinal disorders or gastrointestinal surgery interfering with gastrointestinal function; history of major disease, use of medication (except oral contraceptives) within 14 days before testing; dieting; pregnancy; lactation; excessive alcohol consumption (>20 alcoholic consumptions/week); and smoking.

Design and intervention

In this double-blind, randomized, placebo-controlled, crossover study, participants attended to four test days with at least 1 week washout period between two test days. At test days, participants were randomized in a double-blind fashion to 1 of the 24 possible orders of the four interventions; a low calorie gluten meal with AN-PEP or placebo, or a high calorie gluten meal with AN-PEP or placebo. The randomization list was generated by an independent and blinded statistician using a computerised procedure. All participants and investigators remained blinded to treatment until the analyses were completed. After an overnight fast, a triple-lumen catheter (adapted from Freka® Trelumina, Fresenius Kabi Nederland B.V., Zeist, The Netherlands) was introduced trans-nasally via the stomach into the duodenum, under fluoroscopic guidance. The proximal lumen, with an infusion port

positioned in the stomach, was used for administration of the test meal and aspiration of gastric contents. The second lumen, positioned 5 cm distal to the pylorus, was used for continuous perfusion of the inert dilution marker polyethylene glycol (PEG)-3350. The third and distal lumen (positioned at tip and located 15 cm distal to the pylorus) was used for aspiration of duodenal contents. The catheter position was secured by radiology and regularly checked during the tests by measuring pH of each aspirate. Meals, mixed with acetaminophen (Centrafarm B.V., Etten-Leur, The Netherlands), and either AN-PEP or placebo, were infused into the stomach over a 5-min period, at a rate of 80 ml/min. A PEG-3350 (Norgine B.V., Amsterdam, The Netherlands) solution (15 mg/ml in 0.9% saline) was continuously infused into the proximal part of the duodenum at 3 ml/min to calculate the gluten amount corrected for dilution of duodenal content by endogenous secretions.[27-30] Infusion started 60 min prior to meal infusion, to achieve steady state conditions in fluid secretion and absorption at the start of meal infusion, and continued until 240 min. Gastric and duodenal content was sampled at baseline (t=0 min), and after start of meal infusion at t=15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 min. Gastric content was also aspirated at t=5 and t=10 min. Mixing of the meal with AN-PEP or placebo and acetaminophen was performed immediately prior to the start of infusion. Approximately 3 ml and 2 ml were aspirated from the gastric and duodenal port, respectively, for pH and gluten epitope measurements. Also, acetaminophen concentrations in gastric samples were measured. The samples were immediately frozen in liquid nitrogen to stop enzyme activity and were subsequently stored at -80°C until analysis.

Gluten meals

Dry meals were packaged in sachets of airtight tinfoil and stored at room temperature. On test days, meals were prepared at a food-grade kitchen facility. During preparation of the test meal, 1 g acetaminophen was added. Table 2.1 shows the composition of the meals. All test meals contained 5.2 g of gluten powder, of which 4.0 g was gluten protein (Syral, Aalst, Belgium). Encapsulated refined olive oil powder (Vana Grasa 80B, FrieslandCampina, Kievit B.V., Meppel, The Netherlands) was used as fat source for the meals and together with maltodextrin as additional energy source for the high calorie meal, and sodium caseinate (DMV, Veghel, The Netherlands) was used to match both meals for protein content. The dry meal powders were dissolved in a total volume of 300 ml tap water of 40°C by stirring with a spoon and subsequently mixing with a Turrax (IKA, Ultra Turrax® T25, Staufen, Germany). Low and high caloric meals were not blinded to the investigator.

Table 2.1 Recipe and nutritional composition of the low and high calorie test meal per 300 g portion.

	Low calorie meal	High calorie meal
Vana Grasa (g)	7.0	22.0
Sucrose (g)	17.0	16.9
Maltodextrin (g)	0.0	36.3
Caseinate (g)	0.7	0.3
Gluten powder (g)	5.2	5.2
Citric acid (g)	0.1	0.1
Water (g)	270.9	245.1
Protein (g)	4.9	4.9
Fat (g)	5.7	17.7
Carbohydrate (g)	18.0	56.5
Ash (g)	0.2	0.5
Protein (kcal)	19.6	19.6
Fat (kcal)	51.6	159.6
Carbohydrate (kcal)	71.9	225.8
Total (kcal)	143.1	405.1
Caloric density (kcal/g)	0.5	1.4
Osmolarity (mOsm/kg)	194.8	373.6
pH	6.0	6.0

AN-PEP enzyme and placebo

The AN-PEP enzyme was obtained from DSM Food Specialties (Delft, The Netherlands). A total of 6.1 ml of AN-PEP corresponding with 1.600.000 Protease Picomol International (1 Protease Picomol International is the amount of enzyme that releases one picomole of p-nitroaniline per second under defined assay conditions) in a total of 100 ml water was added to the 300 mL test meals. A 6.1 ml solution consisting of 4.8 g water, 1.3 g maltodextrin, 0.01 g caramel liquid (Brenntag, Deerlijk, Belgium), 0.03 g citric acid, and 0.02 g sodium benzoate (Wuhan Youji, Wuhan, China) at pH 4.2, with a similar appearance to AN-PEP, served as placebo.

Sample pre-treatment

Upon thawing, the enzyme in the gastric and duodenal samples was immediately inactivated by increasing the pH of the sample to 11-12 using 1 M NaOH, heating at 85°C for 10 min and neutralizing the pH with 1 M HCl. For the gluten content analysis, 100 µl from each sample was frozen again at -80°C for further analysis by ELISA, or mixed with loading buffer (60% glycerol, 300 mM Tris, pH 6.8, 12 mM EDTA, pH 8.0, 12% sodium dodecylsulphate, 864 mM 2-mercaptoethanol, 0.05% bromophenol blue), boiled for 5 min for further analysis by Coomassie Blue staining and western blot.

Gluten monitoring by ELISA analyses

The sample was diluted 40-5000 times in phosphate-buffered saline and the presence of the DQ2.5-glia-α3 epitope was quantified using the Gluten-Tec ELISA assay (EuroProxima B.V., Arnhem, The Netherlands) according to the manufacturer's instructions.[31] The DQ2.5-glia-α3 epitope is directly adjacent to the 33-mer that contains the immunodominant DQ2.5-glia-α1 and DQ2.5-glia-α2 epitopes. As the α-gliadins contain only a single copy of the DQ2.5-glia-α3 epitope, the measurement of this epitope provides an accurate estimate of the actual α-gliadin content of the samples.[32]

Volume marker

PEG-3350 concentrations were determined in samples obtained from the distal aspiration port in the duodenum, using reversed-phase HPLC in combination with evaporative light scatter detection. The analysis was based on PEG analysis as described by van Wijck *et al.*[33] PEG-3350 concentrations were used to calculate the dilution of duodenal samples by endogenous secretions, using the formula described by Beglinger *et al.*[27]

$$V = (F \times [PEG]_{perfused} \times 15) / (PEG_{measured})$$

V represents the calculated duodenal volume (ml per 15 or 30 min); F the flow rate of PEG solution perfused (3 ml/min); $[PEG]_{perfused}$ the concentration of PEG in the perfusate; $PEG_{measured}$ the concentration of PEG in the duodenal juice collected for 15 or 30 min. The number '15' has to be replaced by '30' if the time interval between two samples was 30 min.
To calculate the absolute duodenal gluten amount at a certain time point, the calculated duodenal volume was multiplied with the gluten concentration at that time point.

Gluten monitoring by western blot

Measurement of gluten epitopes by ELISA is an indirect method of gluten analysis. To confirm the presence of intact gluten proteins and relatively large fragments thereof we also performed Western blot analysis. The proteins present in samples isolated from the stomach were separated on SDS-PAGE, blotted onto polyvinylidenefluoride membrane and the gliadin proteins were visualized with a monoclonal antibody specific for the immunodominant DQ2.5-glia-α1 epitope.[34]

Presence of AN-PEP in gastric and duodenal samples

To assess the presence of AN-PEP protein in gastric and duodenal samples, the protein in the samples was separated on SDS-PAGE followed by Coomassie Blue staining.

Assessment of gastric emptying

Gastric emptying rate was measured at all test days of 6 of the 12 participants who completed the study, randomly chosen. Gastric emptying rate was determined according to the changes of the acetaminophen concentration over time in the stomach, with stomach samples taken at predetermined time points. Gastric fluid samples were first deproteinated by adding a 10% solution of trichloroacetic acid. After centrifugation (800 *g*, 5 min) the supernatant was injected into an HPLC using a reversed-phase method with UV detection at 250 nm (Agilent 1100 series HPLC Value System, Waldbronn, Germany). A composition of MilliQ and acetonitrile (97: 3%v/v) was used to elute the samples. As the total gastric volume changes constantly after meal ingestion, it was difficult to calculate the concentration via a formula. A calibration curve of acetaminophen was used to calculate the acetaminophen concentration in the samples. As a pragmatic approach, the total gastric emptying time (in minutes) was derived from this acetaminophen concentration-time curve. The time point when the acetaminophen concentration in a gastric sample is zero indicates the complete passage of the test meal into the duodenum and is thus considered to represent total gastric emptying time.

Gastrointestinal symptoms questionnaire

At the end of each test day, participants were requested to complete a 'Symptoms Diary', to ensure that all gastrointestinal complaints of the test day, caused by the intervention, were reported to the investigator. This questionnaire included 8 items, each rated on a five-point Likert scale. The lowest score, 1, denotes no symptoms and 5 denotes the most pronounced symptoms. Items that were included in the 'Symptoms Diary' were: abdominal discomfort, abdominal pain, abdominal distension, constipation, diarrhoea, flatus, eructation and nausea.

Statistics

The primary outcome of this study was the effect of AN-PEP on gluten degradation, measured by the difference in the 240-min AUC ($AUC_{0\text{-}240min}$) of duodenal gluten epitope concentration between AN-PEP and placebo. Secondly, we investigated the effect of AN-PEP on gluten degradation, measured by the difference in $AUC_{0\text{-}240min}$ of absolute amounts of gluten epitopes in the duodenum between both interventions. The $AUC_{0\text{-}240min}$ was

calculated by using the trapezoidal equation. We calculated that a sample size of 12 subjects would be required based on a standardized effect-size of 1.3, a power of 90%, and *alpha* = 0.05 (one sided). Seventeen subjects were recruited taking into account a drop-out of 5 subjects. Baseline characteristics are presented as mean (SD) for numerical variables and number (%) for categorical variables. Differences in AUCs, gastric emptying rate and 'Symptom Diary' scores between combinations of treatment (AN-PEP or placebo) and meal (high or low calorie) were assessed using linear mixed models based on restricted maximum likelihood, where the natural logarithm of the AUCs was taken to account for the expected non-normality. The linear mixed model accounts for the correlation between repeated measures within a person (cross-over design) and missing data, where a likelihood approach was used assuming data missing at random. Fixed factors were treatment, meal, treatment*meal, and test-day. The best fitting covariance structure, *i.e.* structure of variances over different test-days and correlations between test-days, was based on Akaike's Information Criterion. All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 20.0, IBM Corp, Armonk, NY, USA). Two-sided $P \leq 0.05$ were considered statistically significant.

Results

Study subjects

A total of 12 healthy volunteers [67% male; age 26 ± 6 years; BMI (in kg/m^2) = 22 ± 3] were included in the study. One of these subjects did not complete the fourth test day and only the results of the two high calorie meal test days were available for inclusion in the analyses. In two other subjects the catheter progressed more distally into the small intestine at one occasion, causing administration of (part of) the meal directly into the duodenum. Data of these experiments were omitted, but data from their remaining test days were still included in the analysis as the linear mixed model accounts for missing data. Initially 17 subjects were enrolled in the study, but 5 subjects dropped out due to discomfort related to the nasoduodenal tube. Data of drop-outs were omitted from analyses.

pH of gastric samples

The mean gastric pH of gastric samples, taken during test days when low and high calorie meals combined with AN-PEP were infused, ranged between 2.3 and 5.3 and was similar to the pH range of the samples of placebo-containing meals (data not shown).

DQ2.5-glia-α3 concentrations in stomach and duodenum and absolute amount in duodenum

The mean DQ2.5-glia-α3 concentrations in stomach and duodenum samples after ingestion of low and high calorie meals with and without AN-PEP are shown in Figure 2.1 and the $AUC_{0\text{-}240min}$ in Table 2.2. The mean duodenal DQ2.5-glia-α3 concentrations per participant are shown in Figure 2.2. Over a 240-min period, AN-PEP reduced the gastric DQ2.5-glia-α3 concentrations, compared to placebo, both after ingestion of the low (35 vs 389 μg*min/ml; $P < 0.001$) and the high (53 vs 386 μg*min/ml; $P < 0.001$; Figure 2.1A and B; Table 2.2) calorie meals. This was also observed for duodenal DQ2.5-glia-α3 concentrations (low calorie: 7 vs. 168 μg*min/ml; high calorie: 4 vs. 32 μg*min/ml; $P < 0.001$; Figure 2.1C and D; Table 2.2). In the placebo intervention, the duodenal DQ2.5-glia-α3 concentrations were significantly lower after intake of a high compared to a low calorie meal (32 vs. 168 μg*min/ml; $P = 0.001$; Figure 2.1C and D; Table 2.2). In the presence of AN-PEP this difference was not present (4 vs. 7 μg*min/ml; $P > 0.05$; Figure 2.1C and D; Table 2.2) and low duodenal DQ2.5-glia-α3 concentrations were observed after intake of both a high and low calorie meal. The $AUC_{0\text{-}240min}$ of DQ2.5-glia-α3 concentrations in the duodenum of AN-PEP-receiving subjects was around or lower than the limit of detection (26.7 μg/l * 240 min= 7 μg*min/ml) and lower than the limit of quantification (89 μg/l * 240 min= 21 μg*min/l) of the ELISA assay. The pattern for the duodenal DQ2.5-glia-α3 concentrations corresponds with the data for absolute duodenal DQ2.5-glia-α3 amount, which is corrected for the dilution during the digestion process. AN-PEP lowered the calculated absolute duodenal α-gliadin compared to placebo in both low (2,813 vs. 31,952 μg*min; $P < 0.001$) and high (2,553 vs. 13,095 μg*min; $P = 0.013$; Figure 2.1E and F; Table 2.2) calorie meals.

Gluten monitoring by western blot

Western blot analysis of stomach samples indicated that gluten degradation was accelerated by the addition of AN-PEP. Compared to samples of placebo-containing meals, samples of AN-PEP-containing meals showed generally a markedly faster degradation of DQ2.5-glia-α1. In many cases, very little or even no gluten protein could be detected when AN-PEP was taken with the meal. In contrast, in gastric samples of placebo-containing meals, in some cases DQ2.5-glia-α1 was still detectable up to 2 h after meal infusion. In duodenal samples, the Western blots were unable to detect significant amounts of intact gluten proteins, neither with AN-PEP, nor with placebo (Figure 2.3). This finding

suggests that in both cases little or no intact water insoluble gluten protein reaches the duodenum.

Overall, for the majority of the meals analysed, the pattern on gluten degradation by both ELISA and Western blot showed a correlation, demonstrating the robustness of the study results.

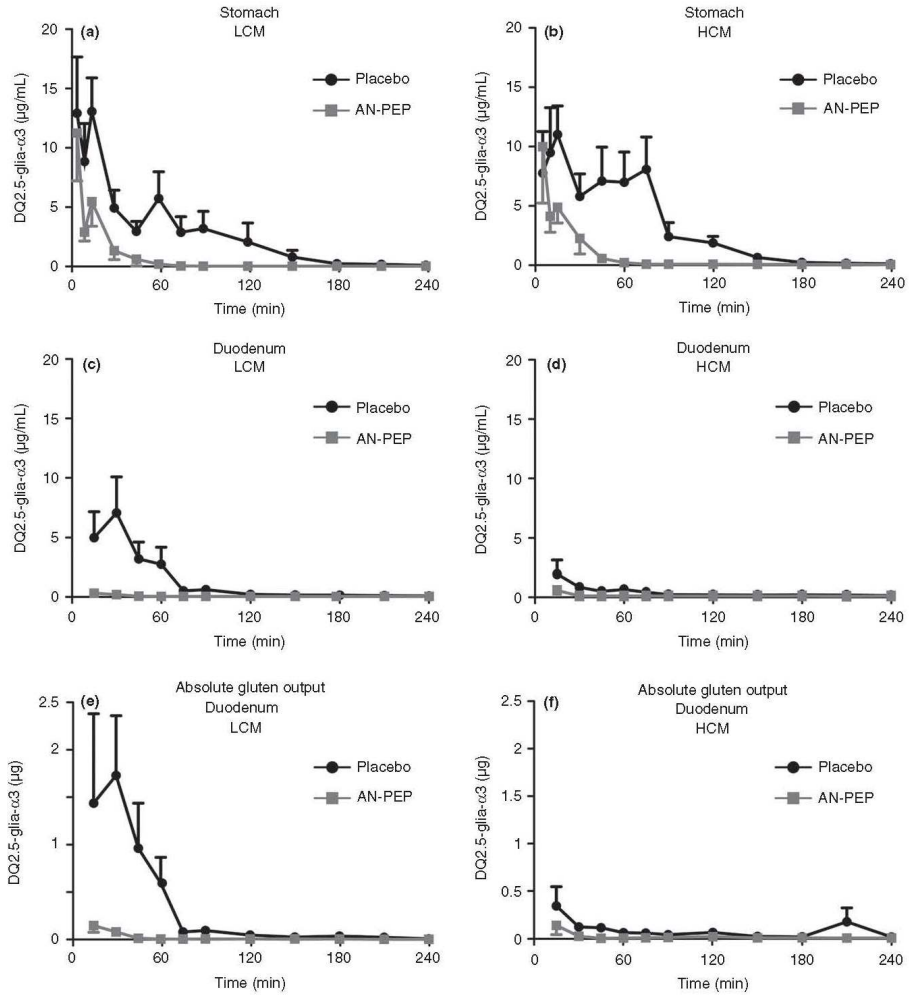
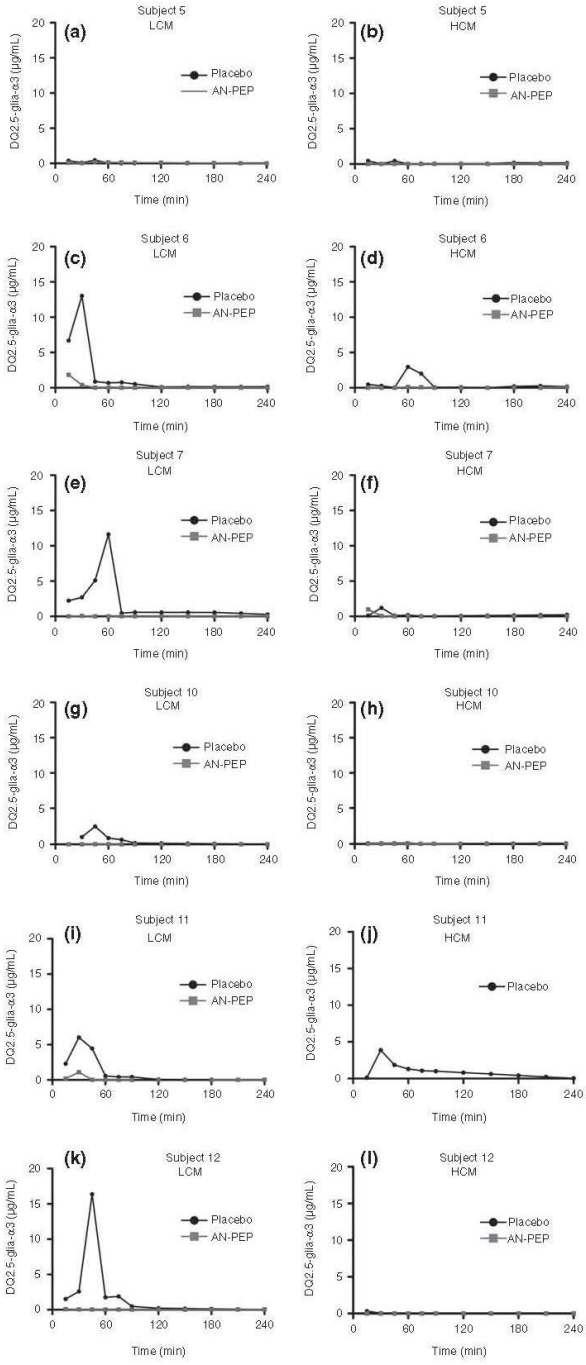


Figure 2.1 A: DQ2.5-glia-α3 concentration (mean ± SEM) over time in the stomach in low calorie meals; B: DQ2.5-glia-α3 concentration (mean ± SEM) over time in the stomach in high calorie meals; C: DQ2.5-glia-α3 concentration (mean ± SEM) over time in the duodenum in low calorie meals; D: DQ2.5-glia-α3 concentration (mean ± SEM) over time in the duodenum in high calorie meals; E: Absolute DQ2.5-glia-α3 output (mean ± SEM) over time in the duodenum in low calorie meals; F: Absolute DQ2.5-glia-α3 output (mean ± SEM) over time in the duodenum in high calorie meals.


(a)
Subject 5
LCM
(b)
Subject 5
HCM
(c)
Subject 6
LCM
(d)
Subject 6
HCM
(e)
Subject 7
LCM
(f)
Subject 7
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(g)
Subject 10
LCM
(h)
Subject 10
HCM
(i)
Subject 11
LCM
(j)
Subject 11
HCM
(k)
Subject 12
LCM
(l)
Subject 12
HCM
Placebo
AN-PEP
DQ2.5-glia-α3 (μg/mL)
Time (min)
0
5
10
15
20
0
60
120
180
240

Figure 2.2

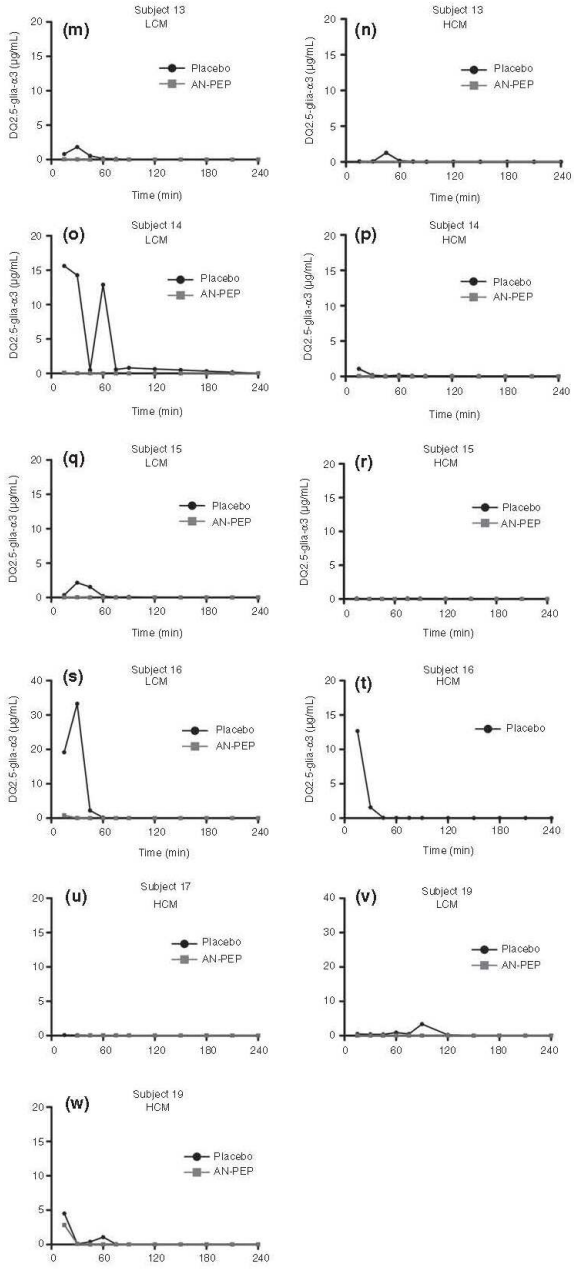

Figure 2.2 DQ2.5-glia-α3 concentration over time in the duodenum of the individual subjects in low calorie meals (LCM) and high calorie meals (HCM). (a) + (b): subject 5. (c) + (d): subject 6. (e) + (f): subject 7. (g) + (h): subject 10. (i) + (j): subject 11. (k) + (l): subject 12. (m) + (n): subject 13. (o) + (p): subject 14. (q) + (r): subject 15. (s) + (t): subject 16. (u): subject 17. (v) + (w): subject 19.

Table 2.2 $AUC_{0\text{-}240min}$ of DQ2.5-glia-α3 concentrations in stomach and duodenum and the $AUC_{0\text{-}240min}$ of absolute DQ2.5-glia-α3 amount in duodenum.

		Low calorie meal		High calorie meal	
		Placebo	AN-PEP	Placebo	AN-PEP
$AUC_{0\text{-}240min}$ *DQ2.5-glia-α3, μg*min/ml*					
Stomach	Mean	389	35 [a]	386	53 [a]
	95% CI	180-840	17-73	192-775	25-113
Duodenum	Mean	168	7 [a, d]	32 [b]	4 [a, e]
	95% CI	80-352	3-14	16-63	2-9
$AUC_{0\text{-}240min}$ *DQ2.5-glia-α3, μg*min/ml*					
Duodenum	Mean	31,952	2,813 [a]	13,095	2,553 [c]
	95% CI	12,670-80,579	1,206-6,555	5,967-28,730	884-7,369

[a] $P < 0.001$ compared to placebo; [b] $P = 0.001$ compared to low calorie placebo meal; [c] $P < 0.05$ compared to placebo; [d] Below the level of quantitation 21 μg*min/ml; [e] Below the level of detection 7 μg*min/ml.

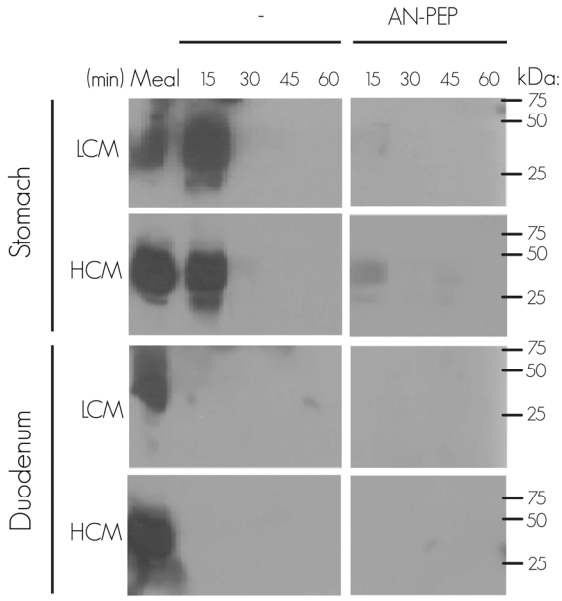

Figure 2.3 Representative Western blot showing degradation of water-insoluble DQ2.5-glia-α1 over time in the stomach and duodenum in low and high calorie meals with and without AN-PEP.

Presence of AN-PEP in gastric and duodenal samples

After administration of AN-PEP-containing meals, a band with AN-PEP's characteristic molecular weight (66 kD) was visible in the gastric samples of 14 different test days (Figure 2.4). In the other test days, the AN-PEP signal was either too weak to be detected or

masked by other proteins with a similar electrophoretic mobility. In the placebo-containing meals, a band with this particular molecular weight was always absent (data not shown). With high calorie meals, AN-PEP was detectable for a longer period than with low calorie meals. AN-PEP was not found in the duodenal samples of any test day (Figure 2.4), possibly due to degradation of the enzyme by trypsin or chymotrypsin under conditions of high pH in the duodenum.

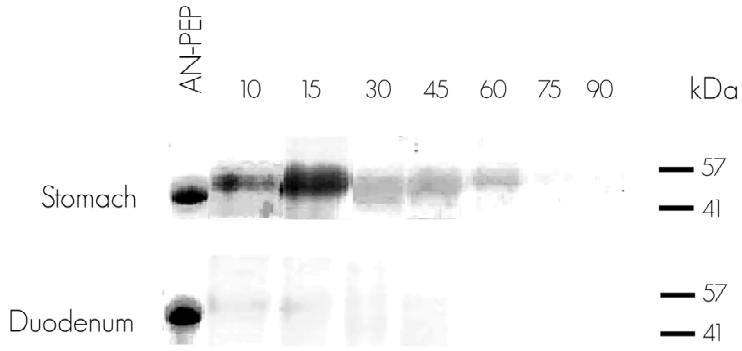

Figure 2.4 Representative SDS-PAGE gel showing the presence of AN-PEP protein in gastric and duodenal aspirates.

Gastric emptying

The mean of total gastric emptying time of the high calorie meals was approximately twice as long as compared to the low calorie meals, being significantly different in the presence of placebo (172 vs. 88 min; $P = 0.014$) but not in the presence of AN-PEP (154 vs. 100 min; $P = 0.100$).

Gastrointestinal symptoms questionnaire

Mild gastrointestinal symptoms were reported on some occasions during low and high calorie meal intervention. Overall, discomfort was low for each different intervention. No significant differences were observed in reported gastrointestinal symptoms between meal types in combination with AN-PEP or placebo (data not shown).

Discussion

In the current placebo-controlled intervention study, AN-PEP-mediated gluten digestion was studied in the stomach and duodenum of healthy volunteers. This is the first study showing that the AN-PEP enzyme efficiently degrades gluten from a meal in the stomach of human subjects.

Considering the enzyme's optimum pH range between 3 and 5, and the mean pH of the gastric samples ranged between 2.3 and 5.3 with both low and high calorie meals, this points to optimal enzyme activity during the entire digestive process in the stomach.[19] Irrespective of the caloric density of the meal, the enzyme degraded almost all gluten present in the stomach within a period of 1 hour whereas with placebo, gluten were present for 3 hours. Furthermore, the addition of AN-PEP did not result in differences in gastrointestinal-related symptoms compared to placebo, confirming that intake of the enzyme is safe and well tolerated by human subjects.[26]

We also tested whether increasing meal caloric density would improve gluten degradation by delaying gastric emptying and thus prolonging exposure time of gluten proteins to AN-PEP and endogenous gastric proteases. Whereas the low calorie meal in the stomach was emptied within about 1.5 hour, the gastric emptying of the high calorie meal took about twice as long. Delayed gastric emptying also resulted in longer gastric residence time of AN-PEP. These findings are in line with a previously reported delay in gastric emptying rate with increased meal caloric density in human subjects.[35] Within 1 hour, AN-PEP degraded gluten to concentrations around or below reliably detectable levels, irrespective of meal caloric density. Without AN-PEP enzyme, high gluten concentrations were present in the stomach when given with a low or high calorie meal, supporting the notion that pepsin exerts only minimal proteolytic action on dietary gluten.[36] Interestingly, in absence of the enzyme, less gluten reached the duodenum with high than with low calorie meals. Possibly, the fat content of the high calorie meals supported gluten digestion in the duodenum by increasing pancreatic enzyme outputs which has been described for high-fat as compared to low-fat diets.[37]

We used a marker infusion technique to correct for dilutions resulting from biliary and pancreatic secretions that might have influenced the gluten concentrations measured in the duodenum. The absolute gluten values obtained, therefore, represent a better measure of true gluten exposure than the gluten concentrations. The absolute amount of gluten reaching the duodenum was significantly reduced with AN-PEP irrespective of meal caloric density, in line with the findings for gluten concentrations. The duodenal gluten degradation pattern was comparable between concentrations and absolute amounts suggesting little influence of duodenal dilutions. Insoluble gluten measurements confirmed that AN-PEP is able to significantly reduce gluten before entering the duodenum.

A band on SDS-PAGE with AN-PEPs characteristic molecular weight was observed in gastric, but not in duodenal aspirates, indicating AN-PEP is present and active in the stomach but not in the duodenum. Possibly, under duodenal neutral pH conditions, bile and pancreatic enzymes may have degraded the enzyme.

Apart from AN-PEP, other enzymes detoxifying gluten are currently under investigation. A mixture of two proteases, namely PEP derived from *Sphingomonas capsulate* and a barley protease (EP-B2), termed ALV003 has previously been shown to be capable of degrading complex gluten proteins *in vitro*.[20,23] In a human setting, ALV003 was well tolerated and effective in detoxifying 1 g of gluten.[21] A recent study in celiac patients showed that ALV003 attenuated small intestinal mucosal injury induced by 6 weeks ingestion of 2 g gluten daily.[22] Another protease mix, STAN-1, showed effective *in vitro* gluten-degrading properties.[38] These enzymes have been investigated for their applicability as a future celiac disease drug therapy.

This study made use of a triple lumen nasogastroduodenal catheter. This enabled the simultaneous administration of a test meal, infusion of a dilution marker, and aspiration of gastric and duodenal contents. Clear benefit of this approach is that it allowed us to measure the actual gluten concentration present in duodenum samples. This information is important for safe use in subjects intolerant to gluten. Further, by infusion of the dilution marker we could calculate the absolute intraduodenal gluten appearance. In none of previous mentioned studies, investigating other gluten-detoxifying enzymes, true gluten presence in the duodenum has been measured. To standardize each meal intake, AN-PEP was added to the meal and thereafter immediately infused intragastrically at a standardized rate, to avoid differences in gluten degradation during meal consumption between interventions, caused by variable meal ingestion rates. We acknowledge that this does not represent a fully physiological meal setting, in which solid food and AN-PEP are ingested separately and undergo the normal physiologic processes of mixing in the stomach. A randomized placebo-controlled trial is underway in which the efficacy of AN-PEP in an actual meal setting will be investigated. Furthermore, this technique also has another drawback. Migration of the catheter, due to gastrointestinal peristalsis, caused erroneous infusion of the meal into the duodenum in two subjects at one occasion, as was noted based on pH profiles. These data were excluded from analysis.

The AN-PEP enzyme has been developed as a dietary supplement that in conjunction with a gluten-free diet may help subjects intolerant to gluten to digest unintended dietary gluten. Despite these promising results, the data do not prove that AN-PEP allows subjects intolerant to gluten to ingest gluten safely. Oral enzymes cannot replace a gluten-free diet, yet our observations suggest that AN-PEP may be useful as a digestive aid to help digest hidden gluten. The enzyme may protect against unintentional ingestion of gluten on a daily basis, during social events or travelling. A randomized placebo-controlled trial is underway

to address AN-PEP's efficacy in the target population which is necessary prior to AN-PEP's use to be considered safe and effective.

In conclusion, AN-PEP efficiently degrades gluten from a meal in the stomach of healthy volunteers before entering the duodenum. Increasing the caloric density of a gluten meal slowed gastric emptying rate and prolonged gastric residence time of the enzyme. Since AN-PEP with a low calorie meal already degraded almost all gluten, a high calorie meal could not further increase the efficacy of the enzyme to digest gluten.

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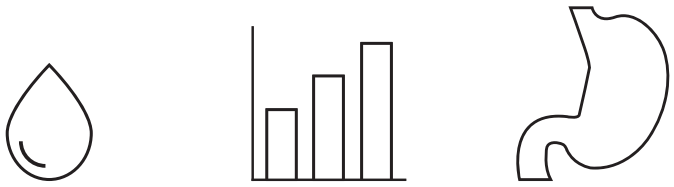

chapter 3

Paracetamol as a post prandial marker for gastric emptying, a food-drug interaction on absorption

Bartholomé R, Salden BN, Vrolijk MF, Troost FJ, Masclee AA, Bast A, Haenen GR

PLoS One 2015; 10:e0136618

Abstract

The use of paracetamol as tool to determine gastric emptying was evaluated in a cross-over study. Twelve healthy volunteers were included and each of them consumed two low and two high caloric meals. Paracetamol was mixed with a liquid meal and administered by a nasogastric feeding tube. The post prandial paracetamol plasma concentration time curve in all participants and the paracetamol concentration in the stomach content in six participants were determined. It was found that after paracetamol has left the stomach, based on analysis of the stomach content, there was still a substantial rise in the plasma paracetamol concentration time curve. Moreover, the difference in gastric emptying between high and low caloric meals was missed using the plasma paracetamol concentration time curve. The latter curves indicate that (i) part of the paracetamol may leave the stomach much quicker than the meal and (ii) part of the paracetamol may be relatively slowly absorbed in the duodenum. This can be explained by the partition of the homogenous paracetamol-meal mixture in the stomach in an aqueous phase and a solid bolus. The aqueous phase leaves the stomach quickly and the paracetamol in this phase is quickly absorbed in the duodenum, giving rise to the relatively steep increase of the paracetamol concentration in the plasma. The bolus leaves the stomach relatively slowly, and encapsulation by the bolus results in relatively slow uptake of paracetamol from the bolus in the duodenum.
These findings implicate that paracetamol is not an accurate post prandial marker for gastric emptying. The paracetamol concentration time curve rather illustrates the food-drug interaction on absorption, which is not only governed by gastric emptying.

Introduction

In order to display a systemic biological effect most compounds first have to be absorbed from the gastro-intestinal tract. The onset of the effect depends heavily on the rate of absorption. Absorption mainly takes place once the compound has reached the intestine. Gastric emptying is considered to be the bottleneck in uptake, and numerous studies have been conducted on the effect of nutrition and disease on this process.

Paracetamol is the number one tool for monitoring gastric emptying.[1] To assess gastric emptying, paracetamol is mixed through a meal. After ingestion, the meal containing paracetamol will enter the stomach, and the meal is digested. Gradually the meal-paracetamol mixture passes through the pyloric sphincter into the duodenum. The large surface area, the peristaltic movement, the relatively thin membrane and the rich blood supply of the intestine enable rapid uptake in the duodenum. It is assumed that paracetamol will be absorbed almost immediately after leaving the stomach. Moreover, it is assumed that the passage time of paracetamol through the stomach is identical to that of the meal. Based on these assumptions, the rate of paracetamol uptake into the plasma would be governed by gastric emptying and consequently gastric emptying might be deduced from the time course of the plasma concentration of paracetamol.[2-5]

This approach with paracetamol as marker molecule is regarded as an established and well validated method and is extensively applied. The wide application and acceptance of the procedure, might explain why a critical attitude is lacking. Often, only a small number of samples (sometimes even only one) is taken which will give only a rudimentary kinetic profile. Surprisingly, only a few studies addressed the validity of the method.[6,7] In the most extensive literature review on the subject is was stated that "it is a simple, noninvasive, and economical method, making it suitable for application on a larger scale but further research should be performed under standardized conditions to allow wide scale clinical use".[6] Moreover, in a recent study, we obtained data which are in conflict with the widely accepted model which prompted us to carefully evaluate the use of paracetamol as post prandial marker for gastric emptying. This revealed two major pitfalls, *i.e.* (i) part of the paracetamol may leave the stomach much quicker than the meal and (ii) part of the paracetamol may be relatively slowly absorbed after entering the duodenum.

Materials and methods

Clinical study

In the randomized cross-over study "*Aspergillus niger*-derived enzyme effectively digests gluten in the stomach of healthy volunteers"[8] (December 2011 to May 2012) gastric

emptying was assessed with the use of paracetamol. The healthy volunteers attended four test days with at least one week washout period between two test days. On two test days the volunteers were given a low caloric meal (143 kcal), administered via a nasogastric feeding tube (Freka® Trelumina, Fresenius Kabi Nederland b.v., Zeist, The Netherlands). One of the two low caloric meals given to each of the volunteers was mixed with Aspergillus niger-derived prolyl endoprotease (AN-PEP). On the other two test days a high caloric meal (405 kcal) was administered. Also one of the two high caloric meals given to each of the volunteers was mixed with AN-PEP. The AN-PEP treatment had no effect on the paracetamol data and therefore the results of the meal treated with AN-PEP were combined with that of the meal that was not treated.

Prior to administration, 1000 mg paracetamol was added to the meals and the meal was thoroughly mixed. The rate of gastric emptying was determined using two different strategies. The common strategy is based on the paracetamol plasma concentration. Blood samples were taken 0, 10, 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after administration of the meal. Further details regarding materials and methods are described earlier by Salden *et al.*.[8] The flow diagram of the study is presented in Figure 3.1.

A total of 12 (8 male and 4 female) healthy volunteers (average age 26 ± 6 years, average BMI 22 ± 3 kg/m^2) were included in the study. One of these subjects did not complete the fourth test day. In two other subjects the catheter progressed more distally into the small intestine on one occasion (based on the high pH of the sample), indicating that (part of) the meal was directly administered into the duodenum. These experiments were omitted, but the other experiments of these subjects were included in the data analysis. Initially 17 subjects were enrolled in the study, but 5 subjects dropped out due to discomfort related to the nasoduodenal tube. Data of dropouts were omitted from analyses.

The amount of paracetamol taken up on 10 and 240 minutes after intake was derived from the area under concentration-time curve in all subjects. The second strategy to determine gastric emptying was based on the paracetamol concentration in the content of the stomach. Samples were aspirated from the stomach on 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 minutes after intake. In 6 subjects a complete set of stomach samples could be obtained, and the paracetamol content of the stomach samples of these subjects was determined. Paracetamol metabolites were not determined, because the aim of the study was to evaluate the use of paracetamol as tool for gastric emptying. The paracetamol concentration-time curve derived from the HPLC analysis of the sample was used to determine gastric emptying.[9]

The fractionation of paracetamol in the stomach was mimicked by adding 100 µl HCl-solution (0.01 M) to 400 µl of the meal to obtain a pH of 2. After centrifugation the pellet was resuspended in a sodium-phosphate buffer of pH 7.4 and centrifuged again. In both supernatants the paracetamol concentration was determined using HPLC.

The study has been approved by the Institutional Review Board of Maastricht University, and all clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent has been obtained from the participants. The study has been registered as NTR2780 in the Dutch trialregister (Nederlands Trialregister).

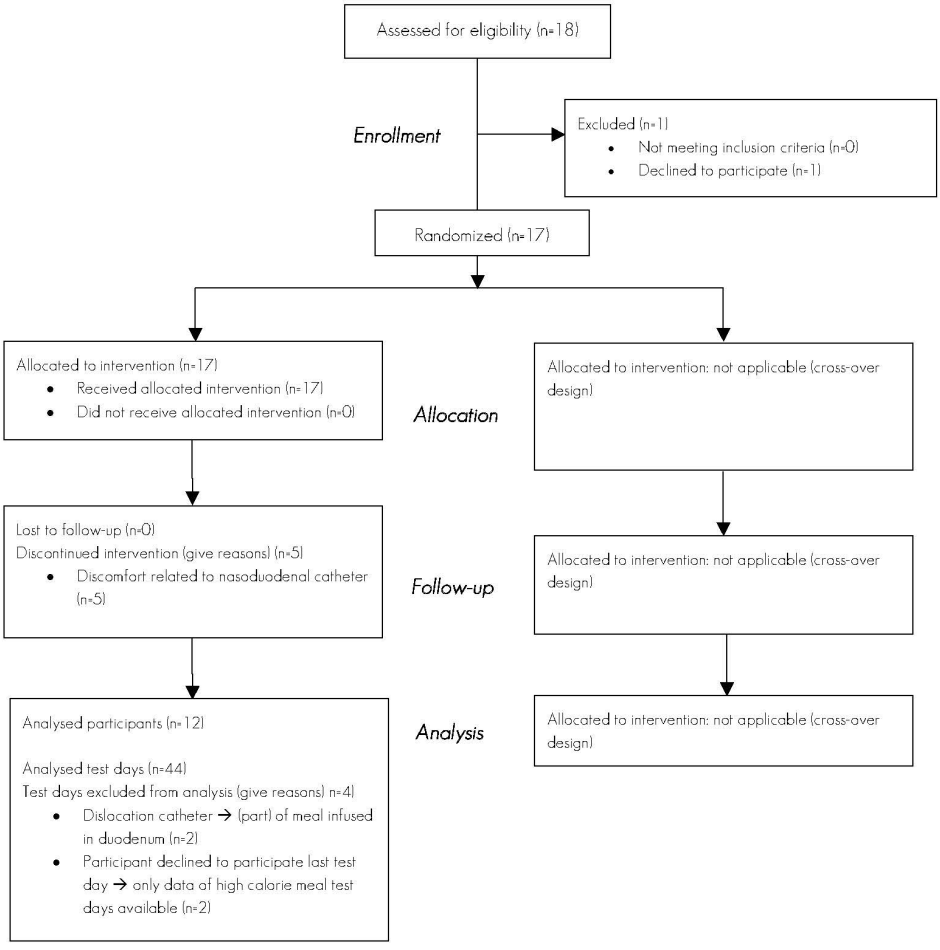

Figure 3.1 Consort Flow Diagram of the study.

Determination of paracetamol

For the determination of the paracetamol concentration, the samples were deproteinated by adding trichloroacetic acid. After centrifugation (800 x *g*, 5 minutes) the supernatant was

analyzed using reversed phase HPLC with UV detection at 250 nm. The eluent was a mixture of MilliQ and Acetonitrile (97: 3% v/v). The quality control samples as well as the calibration curves were well within the specifications for pharmacokinetic studies.[10] For each subject and each treatment, an extra control sample taken before the administration of Paracetamol was taken. In none of these control samples interfering peaks were observed. The analyses were performed blinded to the study.

Statistics

The concentration time curve was statistically evaluated in Prism 6 (GraphPad Software, La Jolla, USA). Differences in pharmacokinetic parameters between groups were analyzed using ANOVA. Post-hoc testing was performed using a paired t-test with Bonferroni correction. A $P < 0.05$ was considered to be statistically significant.

Results

A liquid meal was administered as a slurry by a nasogastric feeding tube. Paracetamol was mixed through the meal prior to administration for monitoring gastric emptying. Subsequently, the concentration of paracetamol in plasma was determined post prandial. In six volunteers, also the concentration of paracetamol in the content of the stomach was determined.

Partition of the paracetamol

The paracetamol level in plasma rose after administration of the meal, until it reached a maximum concentration (C_{max}) relatively quickly with the low caloric meals. After reaching C_{max}, the concentration time curve of paracetamol in plasma showed a biphasic exponential decay. Surprisingly, the exponential decay immediately after reaching C_{max} was higher compared to that at a later stage ($P < 0.05$). An illustration of this kinetic profile is given in Figure 3.2A.

From the plasma paracetamol data the amount of paracetamol which was taken up was calculated. It appeared that in most subjects, already within 15 minutes, an unexpected high amount of paracetamol was absorbed, even up to 70% of the total dose (Figure 3.2). The uptake in two phases indicates that paracetamol, administered mixed with the meal, is divided into two fractions. One fraction enters quickly the duodenum and is responsible for the fast uptake. The other fraction apparently stays relatively long in the stomach and is responsible for the relatively slow uptake.

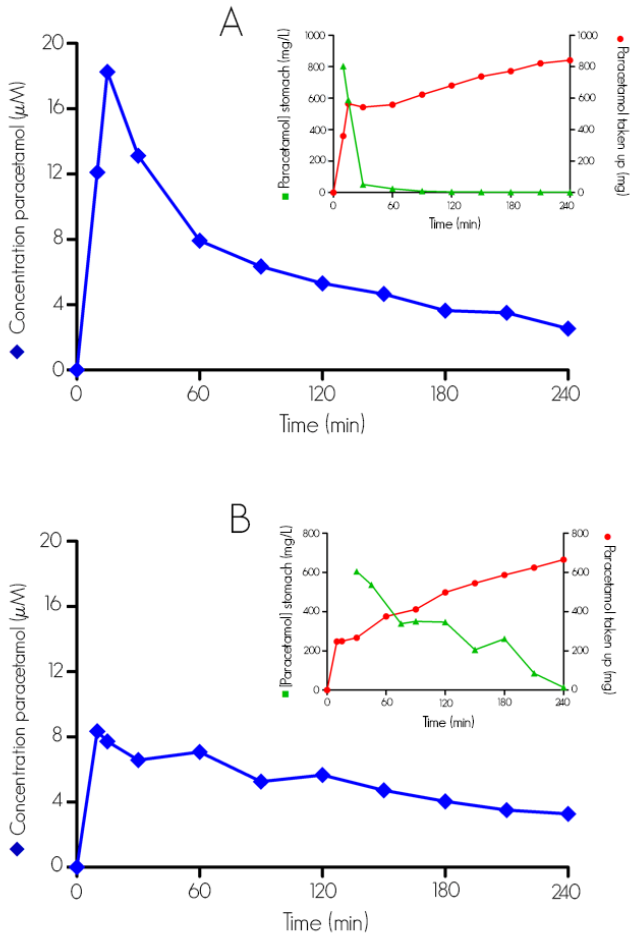

Figure 3.2 The plasma paracetamol concentration in time after administration of a low or high caloric meal containing 1 g paracetamol via a nasogastric tube. (●) (Panel A, low caloric meal; panel B, high caloric meal). A typical example is shown. The inserts show the uptake of paracetamol based on the plasma concentration curve (●) and the concentration of paracetamol detected in the stomach content (●) (panel A, low caloric meal; panel B, high caloric meal).

Encapsulation of part of the paracetamol by the meal

To further investigate the fractionation of paracetamol, a hydrochloric acid solution (to obtain a pH of 2) was added to the low caloric meal containing paracetamol in a test tube to mimic the condition in the stomach. The obtained mixture was centrifuged and two thirds of the paracetamol was retrieved in the supernatant. The pellet was mixed with buffer (150 mM sodium phosphate buffer, pH 7.4) and centrifuged again. This extract of the pellet

appeared to comprise approximately one tenth of the original amount of paracetamol (Figure 3.3). Similar results were obtained with the high caloric meal instead of the low caloric meal (data not shown). These experiments substantiate that in the stomach the paracetamol is divided into two fractions, which are (i) a fraction in which paracetamol is dissolved in the liquid content of the stomach and (ii) a fraction in which paracetamol is encapsulated by the contents of the meal that precipitate in the acid environment of the stomach.

The conclusion is corroborated when the plasma concentration in time is compared to the level of paracetamol detected in the content of the stomach. A slow, but substantial paracetamol uptake in the plasma occurs even when the paracetamol has left the stomach, as shown in Figure 3.2. This indicates that slow release in the intestine of the paracetamol that is encapsulated by a meal, is a rate-limiting process in the uptake.

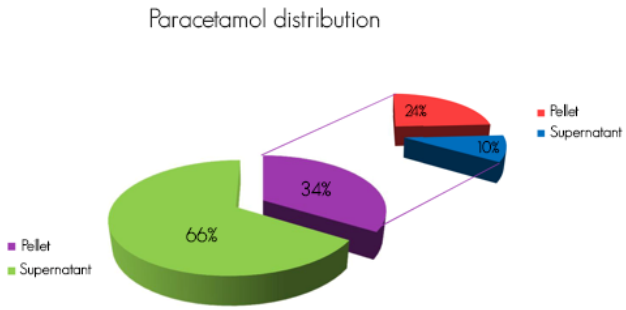

Figure 3.3 Encapsulation of paracetamol by the meal. The meal containing paracetamol was mixed with a hydrochloric acid solution (pH 2) in a test tube to mimic the conditions in the stomach. After centrifugation two thirds of the paracetamol was retrieved in the supernatant. Extraction of the pellet with buffer (150 mM sodium phosphate buffer, pH 7.4) released one tenth of the paracetamol.

High and low caloric meals

In our study, high and low caloric meals were given to the volunteers. The rate of gastric emptying of these meals was estimated from the plasma paracetamol concentration and the concentration of the first time point (10 minutes) was used for this. After 10 minutes a substantial amount of paracetamol, on average 35%, was taken up. No difference was found in the 10 minutes paracetamol concentrations between the low and high caloric meal, suggesting that gastric emptying did not differ between both types of meal (Figure 3.4A). Also after 240 minutes, the plasma paracetamol data indicate that there is no difference in gastric emptying between the low and high caloric meals (Figure 3.4B).

The time at which no paracetamol was detected in the stomach content is a direct measure for gastric emptying. These stomach data show that gastric emptying was statistically longer with the high caloric meal compared to the low caloric meal (Figure 3.4C). The lower gastric emptying of a high caloric meal compared to that of a low caloric meal is well documented, and this difference is confirmed by the longer retention of paracetamol in the stomach samples after the high caloric meal compared to that after the low caloric meal. The difference between the low and high caloric meal is not observed in the gastric emptying determined using the plasma paracetamol concentration (Figure 3.4).[11]

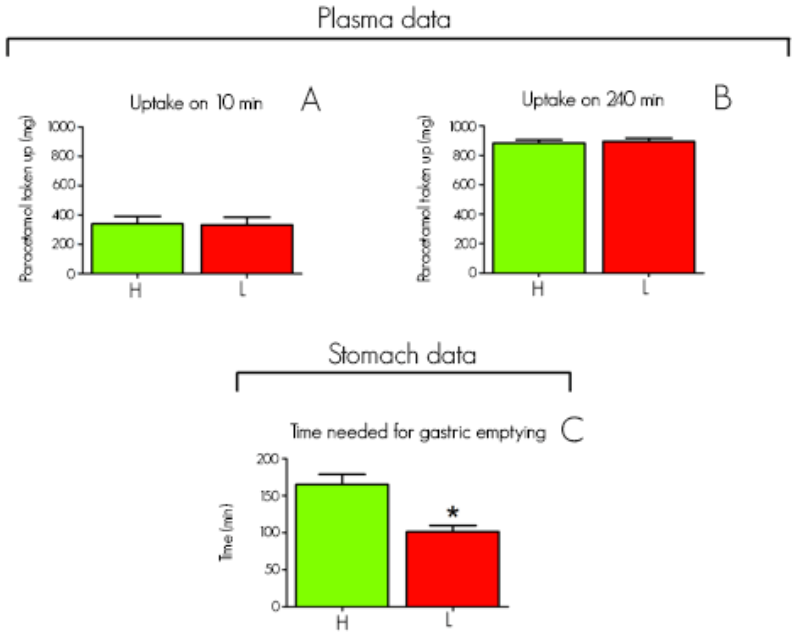

Figure 3.4 Discrepancy in gastric emptying deduced from the paracetamol plasma data and deduced from content in the stomach. The amount of paracetamol taken up after 10 min (panel A) and 240 min (panel B) calculated form the concentration time curve of paracetamol in plasma do not show a difference between the high and low caloric meals. However, the time needed for gastric emptying determinded by the paracetamol content of the stomac (panel C), show a clear difference in gastric emptying between the high and low caloric meals. * different form the high caloric value ($P < 0.05$).

Discussion

In the present study, the use of paracetamol as tool for gastric emptying is evaluated. The nasogastric feeding tube which was used in our experimental set up, assured that the complete and homogeneous meal/paracetamol mixture was in the stomach at the start of

the experiment. After administering the meal, a relatively steep initial increase in the plasma concentration of paracetamol was seen, indicative for a relatively quick uptake. After the quick initial rise, the plasma paracetamol concentration demonstrated a more prolonged, gradual uptake of paracetamol in the blood. Even when all paracetamol has left the stomach, based on analysis of the stomach content, there was still a slow but substantial rise uptake with all meals and in all volunteers. That there still is a substantial uptake of paracetamol when all of the paracetamol has left the stomach, already indicates that the plasma paracetamol levels do not only reflect gastric emptying.

The biphasic time course of the paracetamol curve can be explained by the division of paracetamol into two fractions after entering the stomach, a fraction that is encapsulated by the meal and a fraction that is dissolved in the liquid content of the stomach. The aqueous phase is expected to leave the stomach quickly and paracetamol in an aqueous phase is readily absorbed in the duodenum. Deduced from the relatively high and early peak in the plasma paracetamol time course, most paracetamol is in this aqueous phase. This is corroborated by the test tube experiment, whereby acid is added to the meal and only a small part of the paracetamol remains in the solid content of the meal that precipitates. Apparently, the steep initial increase of paracetamol plasma concentration should not be mistaken for a rapid gastric emptying which would give an overestimation of the rate of gastric emptying.

Part of the paracetamol is encapsulated by the meal and this fraction might be used to determine the gastric emptying. However, after all paracetamol has passed the stomach, still a slow uptake of paracetamol in the plasma is detectable. This indicates that the paracetamol is not instantaneously taken up once the meal containing paracetamol reaches the duodenum. Apparently, paracetamol encapsulated by the meal is only slowly released from the bolus and this slow release appears to be the bottleneck in paracetamol uptake in our experiment. This is also corroborated by the test tube experiment, paracetamol encapsulated by the meal after acid precipitation is only partial released by an extraction. The slow release of the encapsulated paracetamol is probably also the result of the relatively high viscosity of the bolus, which reduces the rate of absorption in the intestine.[3, 11-13] In fact, the bioaccessibility, *i.e.* the release of the compound from the matrix in the intestine, is the rate limiting factor for absorption. This was previously also observed for phenolic compounds in bread.[14]

It is well established that the rate of gastric emptying of high caloric meals is slower than that of low caloric meals.[15] This is confirmed by the paracetamol concentration time course in the stomach in our study that does demonstrate a slower rate of gastric emptying for the high caloric meals. However, the plasma paracetamol data fail to reflect this difference in gastric emptying of low and high caloric meals. This confirms that for the meals studied, the plasma paracetamol concentration time curve is not a valid marker for gastric emptying.

Although plasma paracetamol concentrations are an inappropriate measure of the gastric emptying, by definition the paracetamol concentration time curve does reflect the absorption of paracetamol. Actually, our study shows the effect of food on the rate of absorption of paracetamol. The absorption of a drug in the gastrointestinal tract bears major clinical relevance. Often it is advised to take medication in combination with or after a meal to avoid a peak concentration which would prevent side effects and possible toxicity. Our study shows that this is not necessarily the case. In case of enteral feeding still a relatively high peak concentration can arise. Moreover, the duration of the effect might be extended by encapsulation of the drug by the meal and the slow release of the drug from the drug-meal bolus.

Food-drug interactions are not limited to an effect of food on gastric emptying; apparently, it also includes separation of the drug into different fractions, which has an impact on both the onset and duration of the pharmacotherapeutic effect. However, in studying food-drug interactions, it is important to realize that a drug might be separated in two fractions which have a large difference in their rate of absorption. In the use of paracetamol plasma levels as a post prandial marker for gastric emptying, the food-drug interaction on absorption may lead to incorrect conclusions.

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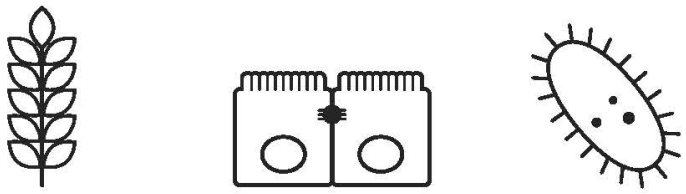

chapter 4

Reinforcement of intestinal epithelial barrier by arabinoxylans in overweight and obese subjects: a randomized controlled trial

Salden BN, Troost FJ, Wilms E, Truchado P, Vilchez-Vargas R, Pieper D, Jáuregui R, Marzorati M, van de Wiele T, Possemiers S, Masclee AA

Clinical Nutrition, article in press

Abstract

Background & aims

Obesity and metabolic diseases are associated with alterations in microbial composition and impaired gut barrier. Previous *in vitro* and animal studies have shown that arabinoxylans (AX) have the potential to modulate gut microbiota and gut barrier and therefore could have a protective role. Primary aim of the study was to investigate the effect of AX on intestinal permeability. Secondary aims included the effect of AX on gene transcription and protein expression of tight junctions (TJ), intestinal microbiota composition and activity, immune response and metabolic markers in overweight and obese individuals.

Methods

In this randomized, double-blind, placebo-controlled trial, 47 overweight subjects were randomly assigned to groups receiving 7.5 g/d AX (*n = 16*), 15 g/d AX (*n = 17*) or 15 g/d placebo (*n = 14*) for 6 wks. Intestinal permeability was investigated using a multi-sugar test. Sigmoid colon tissue was obtained from a subgroup (*n = 26*) for analyzing gene transcription and mucosal expression of TJ proteins. Fecal samples were collected to assess microbial composition and activity. Furthermore, the production of cytokines by stimulated peripheral blood mononuclear cells (PBMCs) was examined. Blood was also sampled for measuring metabolic markers.

Results

No significant changes in gastrointestinal permeability and TJ protein expression were observed after 6 wks AX supplementation compared to placebo. However, gene transcription of occludin was upregulated in the 7.5 g AX group, and transcription of claudin-3 and claudin-4 were upregulated in the 15 g AX group compared to placebo. Furthermore, fecal microbiota diversity was decreased after 6 wks 15 g AX treatment, but no change in relative abundance of dominant phyla was observed. AX intake significantly decreased fecal pH and increased fecal concentrations of total SCFAs, acetate, propionate and butyrate, compared to placebo. Additionally, a decreased TNFα production by stimulated PBMCs was observed after 15 g AX treatment. No changes in metabolic markers were detected.

Conclusions

Regular consumption of AX resulted in a more beneficial fermentation profile in overweight and obese individuals. Further studies are required to assess whether such fermentation profile will translate into improved gut barrier function and immune health.

Introduction

The gastrointestinal (GI) epithelial barrier is a complex system that includes an active local immune defense, a physical barrier comprised of a network of epithelial cells which are connected by tight junction (TJ) proteins to control permeability, a mucus layer, secretion of antimicrobial peptides, intestinal microbiota and luminal microbial metabolism.[1] Recent research has focused on intestinal microbiota and their effects on intestinal physiology, nutrient digestion, luminal metabolism and immune function.[2] Short-chain fatty acids (SCFA) are the end products of fermentation of non-digestible carbohydrates by intestinal microbiota and have an important role in maintenance of intestinal homeostasis. Changes in gut microbiota composition may affect gut barrier function via changes in expression, localization and distribution of TJ proteins, thereby influencing gut permeability.[3] Changes in gut barrier function are regarded as early events or triggers in the development of various intestinal diseases, and also in the development of systemic metabolic diseases, such as diabetes mellitus type 2 (DM2) and cardiovascular diseases (CVD).[4]

Modulation of gut microbial communities by prebiotics appears to be an interesting concept to enhance the gut barrier, to treat or even prevent the onset or aggravation of chronic diseases. Prebiotics are non-digestible food ingredients selectively stimulating growth and/or activity in the GI microflora conferring potential health benefits to the host.[5] A limitation of most prebiotics is their rapid fermentation in the proximal colon. Arabinoxylans (AX), the most abundant non-digestible carbohydrates present in wheat, form a novel class of potential prebiotics.[6] Due to their structure, AX rely on a whole spectrum of (microbial) enzymes for degradation. They are more gradually fermented along the colon, resulting in more distal fermentation.[6,7] AX exist in different forms, ranging from long-chains to enzymatically modified short-chain fractions. *In vitro* research has shown that the AX form affects the fermentation pattern and immune function, high-molecular weight AX being most active.[7,8] Promising outcomes of high-molecular weight AX on the gut barrier,[9,10] gut microbiota,[9,11,12] immune system[9] and metabolic markers[9] have been shown both in *in vitro* and in animal studies. To date, no human data are available on the combined effect of such AX on intestinal environment, gut barrier and immune system.

Therefore, the purpose of this study was to investigate the effects of a high-molecular weight AX concentrate on the gut barrier, intestinal microbiota, immune system and metabolic control in humans in overweight and obese individuals. We hypothesized that 6 wks AX supplementation ameliorates the gut barrier, improves colonic microbiota composition and its metabolic activities, improves the immune system, enhances metabolic control and is well-tolerated in overweight and obese individuals. We specifically chose to study an overweight and obese population, as overweight and obesity are associated with an impaired gut barrier function. Primary aim of this study was to investigate the effect of AX on intestinal permeability, measured by a multi-sugar test. Secondary, we aimed to

investigate the effect of AX intake on gene transcription and protein expression of TJ, on fecal microbiota composition and fecal pH, ammonium and SCFA concentrations, on cytokine production by stimulated peripheral blood mononuclear cells (PBMCs), on blood concentrations of glucose, insulin and lipids, and on GI tolerance.

Materials and methods

The Medical Ethics Committee of the Maastricht University Medical Center (MUMC+) approved the trial and the study was performed at the MUMC+ from March 2012 to December 2012 in full accordance with the principles of the Declaration of Helsinki of 1964 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). Before participation all participants provided written informed consent.

Subjects

Healthy overweight and obese volunteers aged 18-70 y with a BMI between 28-35 kg/m^2 were recruited by local advertisement. Key exclusion criteria were: any medical condition that might interfere with the study and might jeopardise the health status of the participant; smoking; abuse of alcohol (>20 consumptions/wk) and drugs; no consistently stable body weight for at least 6 months (± 2 kg); plans to lose weight or following an energy restriction diet during study period; use of medication, vitamin- or mineral supplements, consumption of pro-, pre- or synbiotics during study period; use of antibiotics in the 90 days prior to start of the study; pregnancy and lactation; history of any side effects towards the intake of pro-, pre-, or synbiotic supplements of any kind. Throughout the trial, subjects consumed their habitual diet. Before start of the study, subjects were informed about the prohibited pre-, pro- and synbiotics and the food products containing them.
To assess the difference in GI permeability between this study population and a group of age and sex matched healthy lean subjects, we used the GI permeability data obtained with exactly the same protocol in healthy controls. These data have recently been published by our group.[13]

Design and intervention

This study was set up as a randomized, placebo-controlled, double-blind, parallel group study. Each subject was tested on three occasions, while a subset of these subjects additionally provided sigmoid mucosal tissue samples on two separate occasions. Participants were randomly assigned in a double-blind fashion to one of the three

intervention arms: 7.5 g AX, 15 g AX or a placebo (15 g maltodextrin). An independent and blinded person generated the randomization list, using a computerized method. All participants and investigators remained blind to intervention until all analyses were finalized. Participants were instructed to refrain from consumption of alcohol and strenuous physical exercise on the day before each test day. After an overnight fast, subjects handed in a fecal sample on the first test day. Fecal samples were collected one day before or on a test day, and stored at -20°C until arrival at the study site. Subsequently, venous blood samples were collected from an antecubital vein in the fore-arm. Then, subjects ingested a multi-sugar drink, to assess gastrointestinal permeability, and collected full urine output for 24 h. A subset of the participants underwent a standard flexible sigmoidoscopy without bowel preparation on the second test day. Seven mucosal tissue samples of approximately 5 mg each were obtained from the sigmoid colon by using a standard forceps (diameter: 2.8 mm) and directly after collection frozen in liquid nitrogen. Two tissue samples were embedded in Tissue-tek (Sakura Finetek, Tokio, Japan) prior to freezing for immunohistochemical analyses. After completion of the baseline measurements, participants received the study product for the first 3 wks. After 3 wks of daily supplementation, the third test day was organized. Again, a fecal sample was handed in, blood samples were taken and study products for another 3 wks were provided. After 6 wks of daily administration of the study product, the fourth and fifth test days took place. Measurements were identical to the baseline measurements performed during the first and second test days, respectively. The same subgroup of subjects underwent again a sigmoidoscopy. Also, all participants had to complete a questionnaire at weekly intervals, to assess the presence of GI symptoms, stool frequency and stool consistency. To assess compliance, participants were asked to collect the empty sachets and to return these at the last visit.

AX concentrate and placebo

BioActor BV (Maastricht, The Netherlands) supplied AX with a purity of 62%, degree of substitution of 0.7, and a varying degree of polymerization, with an average above 60; the composition of the batch used for the study was 67% non-starch polysaccharides (62% AX), 18% protein, 0.5% lipids, 3.8% ash. Maltodextrin (Glucidex IT 19; Roqette Frères, Lestrem Cedex, France) served as placebo. The study products were provided to subjects as a powder in sachets. The placebo group received per day two sachets, each containing 7.5 g Maltodextrin, the 7.5 g AX group received per day two sachets, each containing 3.75 g AX combined with 3.75 g Maltodextrin and the 15 g AX group received per day two sachets, each containing 7.5 g AX. Subjects were asked to stir the content of one sachet in 200 ml liquid (*e.g.* juice, water, milk) and ingest the solution twice daily (every morning and every evening) for 6 wks.

Gastrointestinal permeability

GI permeability was investigated using a validated multi-sugar test, quantifying 24-h urinary excretion of five different ingested sugars reflecting the permeability of 4 segments of the GI tract. Firstly, fasted volunteers emptied their bladder and drank the multi-sugar drink, consisting of 1 g lactulose (Centrafarm, Etten-Leur, the Netherlands), 0.5 g L-rhamnose (Danisco, Copenhangen, Denmark), 1 g erythritol (Now Foods, Bloomindale, IL, USA), 1 g sucralose (Brenntag, Sittard, the Netherlands) and 1 g sucrose (Van Gilse, Dinteloord, the Netherlands) dissolved in 150 mL tap water. Participants were instructed to collect their 24 h urine output in two different fractions; one fraction containing the 0–5 h urine output and a second fraction containing the 5–24 h urine output. It was not allowed to ingest any foods or drinks, except for water, throughout the first urine fraction (0-5 h) collection. Gastroduodenal and small intestinal permeability were reflected by the 0-5 h urinary sucrose excretion and the lactulose and L-rhamnose (L/R) ratio, respectively. In the 5-24 h urine, the sucralose to erythritol (S/E) ratio represented colonic permeability, while in the 0-24 h urine it indicated whole gut permeability. After collection of the 24 h urine output, total volume was quantified and urine aliquots were frozen at -80°C. Urinary sugars were analysed using HPLC-MS as reported earlier.[14] The intestinal permeability test and the analysis of urine samples in the study of Mujagic *et al.*[13] was performed in exactly the same way and done by the same research group as in our study.

Gene transcription of TJ and associated proteins

Gene transcription of TJ and associated proteins (claudin-3, claudin-4, occludin, myosin light-chain kinase (MLCK) and zonula occludens-1 (ZO-1)) in sigmoid colon tissue samples was determined by quantitative real-time polymerase chain reaction, as earlier presented by Pijls *et al.*[15] Housekeeping genes included GAPDH and 18S RNA. Data are shown as normalized expression ratios.

Protein concentrations of claudin-3

Sigmoid mucosal tissue was used for the determination of claudin-3 protein concentrations. Biopsy specimens were kept in liquid nitrogen and ground in a nitrogen cold mortar. The powder was gently scraped with a spatula of the auger and dissolved in 150 µl phosphate-buffered saline (PBS) (Invitrogen 10010, pH 7.4) containing protease inhibitor cocktail (Sigma P8340) with a concentration from 10 µl PI/ml PBS and centrifuged for 20 min at 10,000 rpm at 4°C. Supernatant was stored at -80°C. The concentration of claudin-3 was determined with the sandwich ELISA kit for human claudin-3 (Cloud Clone Corp., Houston USA Kit: SEF293Hu). The microtitre plate provided in this kit was precoated with an

antibody specific to claudin-3. Standards of samples (samples were 1 to 5 diluted) were added to the suitable microtitre plate wells together with a claudin-3 specific biotin-conjugated antibody. Then, avidin conjugated to horseradish peroxidase was applied on each microplate well and incubated. Subsequently tetramethylbenzidine substrate solution was added, and only the wells containing claudin-3, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a color change. Addition of sulphuric acid solution ended the enzyme-substrate reaction. The change in color was quantified using spectrophotometry (wavelength: 450 nm ± 10 nm). The concentrations of claudin-3 in the tissue was defined by comparison of the O.D. of the tissue sample to that of the standard curve. Total protein determination in the sigmoid tissue was done using Pierce® BCA Protein Assay Kit (ThermoFisher, Waltham, MA USA). Samples were 1 to 15 diluted. Data are presented as pg claudin-3 per ug total protein.

Immunofluorescence staining of ZO-1 and occludin

Immunofluorescent staining of ZO-1 and occludin was done as presented earlier.[16] Leica TCS SPE confocal microscopy (Leica Micro systems GmbH, Mannheim, Germany) was used to analyse images. Obtained images were quantified by calculating the AUC. Briefly, to assess the fluorescent staining of both TJ proteins in the TJ region, mucosal tissue was exposed to confocal analysis of Z regions perpendicular to the apical cell surface of the epithelium. Three randomly selected areas within 3 different Z regions were analyzed. The middle part of the villi was used for analyzing the staining. Image J software created the plot profiles reflecting staining intensity and AUC was calculated using Matlab R2013a software (Natick, MA, USA).

Microbiota composition and activity

DNA extraction

To extract DNA from fecal samples (100 mg) samples were resuspended in 1 mL of lysis buffer (Tris/HCl (100 mM pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) SDS). Cells were lysed in a Fast Prep®-24 instrument (MP Biomedicals, Solon, OH, USA) for 30 s at 1800 rpm and extractions were performed using phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with ice-cold isopropanol and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The amount and quality of DNA was measured using an ND 1000 spectrophotometer (ThermoFisher, Waltham, MA, USA).

Gut microbiota composition

We used 16S targeted DNA-based Illumina with MiSeq platform to assess the gut microbiome structure in the fecal samples. Primers for the 16S rRNA gene amplified the hypervariable region V1-V2. After sequencing, quality filtering was performed as previously described and sequences were taxonomically annotated using RDP database with 80% threshold.[17] Sequencing depth was normalized to the minimum reads per sample, using the Phyloseq package from R. Rarefaction curves and biodiversity indices were calculated using the vegan package from R. Rarefaction curve analysis showed that the use of at least 10.000 reads was sufficient to sample the complete diversity of the library.
The following metrics of alpha diversity were determined: observed richness, Shannon diversity index and Simpson evenness. Beta diversity was assessed by Bray-Curtis (BC) dissimilarity. BC is an ecological diversity index representing the extent of change in microbiota composition. This index takes into consideration both the bacterial taxa detected in a specimen, and their relative presence.

Fecal pH, ammonium and SCFA determination

Fecal samples were collected by the participants and stored at -20°C until handed in at the study site. For SCFA and ammonium analyses, aliquots of approximately 1 g of fecal samples were diluted and homogenized with 6 mL demineralized water. After removal of the particulate material by centrifugation (10 min, 500 x *g*), the supernatants were stored at -20°C prior to analysis. SCFA concentrations in the supernatants were determined using gas chromatography with flame ionization detector, based on the SCFA concentration analysis described by Possemiers *et al.*.[18] The sum of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate represent total SCFA. Ammonia concentrations in the supernatants were measured using the method previously presented.[19]
To determine fecal pH, an aliquot of approximately 1 g feces was homogenized by mixing into 10% (w/w) demineralized water. Directly after homogenization pH was assessed.

Production of cytokines by stimulated peripheral blood mononuclear cells

Blood was collected in sodium heparine tubes (Becton & Dickinson, Franklin Lakes, NJ, USA) and used within 1 h for the isolation of PBMCs. To examine *ex vivo* cytokine production by PBMCs, cells were isolated from whole blood and stimulated with Phytohemagglutinin-M (PHA). PHA-stimulation is used to bind antigen-presenting cells and T-cells and thereby PHA induces T-cell proliferation. This technique is used to enlarge the current activities of the T-cells present in the culture. Lymphoprep gradient centrifugation was performed according to the manufacturer's instructions (Takeda Nederland B.V., Hoofddorp, The Netherlands). Freshly isolated cells were diluted in RPMI 1640 culture medium containing HEPES and

L-glutamine (ThermoFisher, Waltham, MA USA), with 1% added Penicillin/Streptomycin, Sodium Pyruvate, and heat inactivated human serum pool. Cells were seeded $1*10^6$ in 24-well flat bottom culture plates (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) and T-cell proliferation was stimulated with PHA (Roche Diagnostics Nederland B.V., Almere, The Netherlands). After 48 h, the culture medium was harvested and stored at -80°C until further analysis. Interferon-gamma (IFNγ) was measured using a sandwich ELISA according to the manufacturer's instructions (eBioscience, Vienna, Austria). IL-2, IL-10, IL-12p40 and TNFα were measured with a multiplex chemoluminescence assay (Meso Scale Diagnostics, Rockville, Maryland USA).

Blood lipids, glucose and insulin

Blood samples were collected at baseline, after 3 wks of supplementation and at the end of the supplementation period. Serum concentrations of glucose, total cholesterol, LDL- and HDL-cholesterol and triglycerides were measured using spectrophotometry. Plasma insulin was determined using a luminescence-enhanced immunoenzymatic assay. Insulin sensitivity was estimated by the quantitative insulin sensitivity check index (QUICKI) index as: 1 / (log insulin 0 h + log glucose 0 h).

Gastrointestinal tolerance

The gastrointestinal symptom rating scale (GSRS) was used to assess the presence of GI symptoms. This questionnaire consists of 15 seven-point graded items that can be combined into 5 symptom clusters reflecting reflux, abdominal pain, indigestion, diarrhea, and constipation. The absence of troublesome symptoms is represented by a score of 1, while a score of 7 reflects very inconvenient GI symptoms.[20] Defecation frequency and stool consistency were assessed using the Bristol stool chart. Subjects completed these questionnaires at weekly intervals during the study period.

Statistical analyses

The primary outcome of the study was the effect of 6 wks 7.5 g AX and 15 g AX supplementation on GI permeability, compared to placebo. Secondary outcomes include the effect of 6 wks AX supplementation versus placebo on gene transcription and protein expression of TJ, fecal microbiota composition and activity, cytokine production by PBMCs, metabolic markers and on GI tolerance. Sample size was determined for the primary outcome of the study, using a significance level *alpha* = 0.017 and a power of 80%. A study in diet-induced obese mice, investigating the effect of a dietary fiber on GI permeability,[21] was used for calculating the sample size. To detect a difference in intestinal permeability of

0.3 ± 0.25 μg/ml (mean ± SD) a total of 45 subjects would be required. Baseline characteristics are displayed as mean (SD) for numerical variables. Baseline values in age and BMI between overweight and obese individuals and lean controls were compared using independent t-test, and baseline numbers in sex were evaluated with chi-square test. Differences in gut barrier (function), fecal pH, relative abundance of main phyla of the fecal microbiota, alpha diversity metrics, fecal SCFA concentrations, immune response, blood parameters and digestive tolerance between placebo and 7.5 g AX and placebo and 15 g AX were assessed using linear mixed models with group (placebo, 7.5 g AX and 15 g AX), time (0, 3 and 6 wks for AX effect) and group*time as fixed factors, where an unstructured covariance structure was used for repeated measures. The linear mixed model accounts for the correlation between repeated measures and missing data, where a likelihood approach was used assuming data missing at random.

GI permeability data were not normally distributed, as assessed by Shapiro-Wilk Test. Mann-Whitney U test was used to evaluate the difference in baseline GI permeability between healthy lean and overweight and obese individuals. Statistical analysis was performed using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY, USA).

R language for statistical computing [http://www.R-project.org] was used for normalizing sequencing depth, calculating rarefaction curves and biodiversity indices. It was also used to generate exploratory heat maps, using the heatmap.2 function from the R package gplots on logarithmic transformed data. To determine the effect of intervention on the shared diversity between samples, beta diversity metrics (BC dissimilarity) were calculated for placebo versus 7.5 g AX and placebo versus 15 g AX. A Principal coordinate analysis (PCoA), based on the BC similarity distances, was used in order to visualize the microbial variation among placebo, 7.5 g AX and 15 g AX. A two-way crossed analysis of similarity (ANOSIM) was used to determine if the microbial communities were significantly different between treatment groups. These analyses were performed using PAleontological STatistics version 3.11 (PAST, Øyvind Hammer, Natural History Museum, University of Oslo). For all comparisons, two-sided p-values ≤ 0.05 were considered statistically significant.

Results

Study subjects

A total of 47 healthy volunteers were enrolled in the study; 14 subjects were assigned to the placebo group, 16 subjects to the AX 7.5 g group and 17 subjects to the AX 15 g group. Forty-five volunteers completed the entire study protocol. One participant dropped out during the intervention period because of a pneumonia and consequent antibiotic treatment. The second dropout developed gallstones during the study period and had to

undergo surgery. From these participants only baseline characteristics were available (Online Supplemental Material Figure S4.1). Sigmoidoscopy was performed in 9 participants in the placebo group, 9 participants in the 7.5 g AX group and 8 participants in the 15 g AX group. Baseline characteristics are presented in Table 4.1.
Data of a group of 33 lean age and sex matched controls, derived from Mujagic *et al.*,[13] were used to evaluate differences in GI permeability between lean and overweight/obese subjects.

Table 4.1 Baseline characteristics in the overweight and obese study population, and lean controls.

	Overall cohort *(n = 47)*	Placebo *(n = 14)*	AX 7.5 g *(n = 16)*	AX 15 g *(n = 17)*	Lean controls *(n = 33)*
Age (y)	48 ± 16	49 ± 17	49 ± 17	47 ± 15	42 ± 3
Sex (M/F)	25/22	8/6	10/6	7/10	13/20
BMI (kg/m^2)	31.0 ± 2.4	31.4 ± 3.1	30.2 ± 1.9	31.5 ± 2.2	22.0 ± 0.31
Sigmoidoscopy (n)	26	9	9	8	n/a

Differences in age and BMI between overall cohort and lean controls tested with independent t-test, differences in sex between overall cohort and lean controls tested with chi-square test. Data are given as mean ± SD. $P < 0.001$ overall cohort vs. lean controls. AX, arabinoxylans.

Gastrointestinal permeability

Baseline GI permeability was significantly increased in our healthy, but overweight population, compared to an age and sex matched healthy, lean population (all $P < 0.001$, Table 4.2).

Table 4.2 Gastrointestinal permeability, reflected by urinary sugar excretion (μmol) and ratios of excreted sugars (in 0-5, 5-24 and 0-24 h fraction) for lean, and overweight and obese subjects.

Sugar excretion	Lean *(n = 33)*	Overweight & obese *(n = 45)*	*P* value
0-5 h sucrose	2.06 [0.51; 6.91]	7.75 [4.68; 13.91]	<0.001
0-5 h L/R ratio	0.016 [0.009; 0.036]	0.050 [0.030; 0.108]	<0.001
5-24 h S/E ratio	0.009 [0.006; 0.015]	0.018 [0.014; 0.023]	<0.001
0-24 h S/E ratio	0.010 [0.005; 0.014]	0.020 [0.014; 0.024]	<0.001

Differences between lean subjects and overweight and obese subjects tested with Mann-Whitney U test. Data are given as median [IQR, *i.e.* Q1; Q3]. L/R, lactulose/l-rhamnose. S/E, sucralose/erythritol.

Six weeks effect

Table 4.3 presents the effect of 6 wks intervention on GI permeability. Gastroduodenal permeability, as indicated by the sucrose excretion in the 0-5 h urine fraction, did not significantly change after both 7.5 g AX and 15 g AX supplementation compared to placebo (all $P \geq 0.224$). Neither was the 0-5 h urinary L/R ratio, reflecting small intestine permeability, altered after the AX interventions compared to placebo (all $P \geq 0.219$).

Colonic and whole gut permeability, respectively reflected by the S/E ratio in 5-24 h and the 0-24 h urine fractions, did not show significant differences among AX and placebo groups after 6 wks supplementation (all $P \geq 0.257$).

Table 4.3 Gastrointestinal permeability, reflected by urinary sugar excretion (μmol) and ratios of excreted sugars (in 0-5, 5-24 and 0-24 h fraction) at baseline and after 6 wks supplementation.

Sugar excretion	Intervention	Baseline	End	*P* value
0-5 h sucrose	Placebo	5.43 [3.97;8.74]	7.10 [4.99; 11.75]	
	7.5 g AX	10.76 [8.39; 21.89]	12.52 [8.38; 17.55]	0.256
	15 g AX	7.05 [3.38; 13.24]	7.88 [4.99; 9.29]	0.224
0-5 h L/R ratio	Placebo	0.045 [0.030; 0.073]	0.065 [0.038; 0.170]	
	7.5 g AX	0.070 [0.040; 0.120]	0.060 [0.048; 0.135]	0.464
	15 g AX	0.050 [0.030; 0.115]	0.065 [0.040; 0.095]	0.219
5-24 h S/E ratio	Placebo	0.020 [0.014; 0.025]	0.018 [0.016; 0.023]	
	7.5 g AX	0.016 [0.012; 0.020]	0.020 [0.013;0.024]	0.766
	15 g AX	0.020 [0.014; 0.026]	0.017 [0.013; 0.024]	0.810
0-24 h S/E ratio	Placebo	0.020 [0.015; 0.025]	0.017 [0.015; 0.020]	
	7.5 g AX	0.018 [0.014; 0.022]	0.018 [0.013; 0.025]	0.257
	15 g AX	0.020 [0.014; 0.024]	0.016 [0.014; 0.024]	0.986

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model with correction for baseline values. Data are given as median [IQR, *i.e.* Q1; Q3]. AX, arabinoxylans. L/R, lactulose/l-rhamnose. S/E, sucralose/erythritol.

Gene transcription of TJ and associated proteins

Results regarding the effects of AX administration on gene transcription of TJ and associated proteins are given in Table 4.4. Transcription of claudin-3 and claudin-4 was significantly upregulated in participants receiving daily 15 g AX for 6 wks, compared to participants receiving placebo ($P = 0.012$ and $P = 0.046$ respectively). Furthermore, a trend towards upregulation of the TJ protein occludin was seen after 15 g AX intake compared to placebo ($P = 0.057$). In the 7.5 g AX group we observed a significant upregulation of occludin compared to placebo ($P = 0.031$). No significant changes in gene transcription for MLCK and ZO-1 between both AX groups and the placebo group were observed (all $P \geq 0.087$).

Protein concentration of claudin-3, ZO-1 and occludin

At baseline, protein concentrations of claudin-3 were 6.10 ± 0.61 (mean ± SEM), 4.92 ± 0.41 and 7.02 ±1.49 pg claudin-3 per ug protein for placebo, 7.5 g AX and 15 g AX respectively. Protein concentration of ZO-1 and occludin in biopsies were quantified by calculating an AUC. At baseline, AUCs of ZO-1 were 159 ± 34 (mean ± SEM), 126 ± 33 and 177 ± 41 and of occludin 112 ± 19, 142 ± 35 and 140 ± 29 for placebo, 7.5 g AX and 15 g AX respectively. No significant differences were observed in sigmoid protein

concentrations of claudin-3, ZO-1 and occludin between placebo and AX interventions after 6 wks supplementation (all $P \geq 0.144$, data not shown).

Table 4.4 Gene transcription of TJ and associated proteins (expressed as normalised expression ratios) at baseline and after 6 wks supplementation.

Gene transcription	Intervention	Baseline		End		Difference in means	95% CI	*P* value
		Mean	SD	Mean	SD			
Claudin-3	Placebo	1.13	0.06	1.12	0.04			
	7.5 g AX	1.09	0.04	1.13	0.03	0.02	-0.01, 0.05	0.225
	15 g AX	1.12	0.05	1.16	0.04	0.05	0.01, 0.08	0.012
Claudin-4	Placebo	1.13	0.04	1.11	0.03			
	7.5 g AX	1.10	0.04	1.12	0.03	0.02	-0.01, 0.05	0.152
	15 g AX	1.10	0.05	1.13	0.04	0.03	0.00, 0.06	0.046
MLCK	Placebo	1.08	0.06	1.08	0.03			
	7.5 g AX	1.06	0.03	1.10	0.03	0.03	0.00, 0.06	0.087
	15 g AX	1.06	0.06	1.09	0.05	0.02	-0.01, 0.05	0.236
OCLN	Placebo	1.14	0.05	1.14	0.02			
	7.5 g AX	1.13	0.03	1.17	0.03	0.04	0.00, 0.07	0.031
	15 g AX	1.15	0.07	1.17	0.05	0.03	-0.01, 0.07	0.057
ZO-1	Placebo	1.17	0.05	1.17	0.04			
	7.5 g AX	1.15	0.04	1.19	0.04	0.02	-0.01, 0.05	0.197
	15 g AX	1.14	0.05	1.17	0.04	0.01	-0.02, 0.05	0.516

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model. The presented difference in means is corrected for baseline differences. AX, arabinoxylans. MLCK, myosin light-chain kinase. OCLN, occludin. ZO-1, zonula occludens.

Gut microbiota composition

From 37 study participants (placebo: *n*=12; 7.5 g AX: *n* = 9; 15 g AX: *n* = 15) gut microbiota composition data were available. Data of 8 participants were not available because there was no amplification in sequencing or data were removed due to quality control issues.

Phylum relative abundance

16S targeted DNA-based Illumina with MiSeq platform allowed to obtain at least 30.000 usable reads per sample. The dominant phyla across all baseline fecal samples were firmicutes (mean 52.5%, SD 10.5%) and bacteroidetes (mean 36.0%, SD 10.1%), followed by proteobacteria (mean 7.7%, SD 7.6%) and actinobacteria (mean 3.0%, SD 2.9%). The relative abundance of these four dominant phyla did not change significantly after 6 weeks intake of 7.5 g AX or 15 g AX, compared to placebo (all $P \geq 0.061$; Figure 4.1).

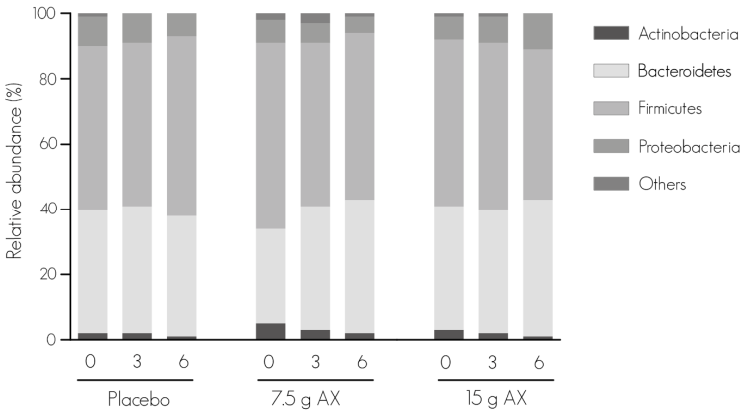

Figure 4.1 Relative abundance of the main phyla according the 16S-targeted Illumina data for placebo, 7.5 g AX and 15 g AX at baseline (0), after 3 wks supplementation (3) and after 6 wks supplementation (6). No significant differences in relative abundance between placebo, 7.5 g AX and 15 g AX were during study period observed, as tested with linear mixed model with correction for baseline values. AX, arabinoxylans.

Fecal samples alpha diversity

Six wks treatment with the two dosages AX did not change the Simpson evenness index, compared to placebo (all $P \geq 0.063$, Table 4.5). However, 6 wks intake of 15 g AX but not 7.5 g AX significantly reduced the observed richness and lowered Shannon diversity, compared to placebo ($P = 0.029$ and $P = 0.036$ respectively, Table 4.5). Six wks treatment with 7.5 g AX or 15 g AX versus placebo did not change the Simpson evenness index.

Microbiota composition and taxon distribution

The PCoA analyses, based on the BC (dis)similarity matrix of the bacterial community sequence data, per test day are shown as Online Supplemental Material Figure S4.2, S4.3 and S4.4. In order to see if treatment during study period significantly altered fecal microbial composition, a two-way ANOSIM was performed. Two-way ANOSIM applying the BC coefficient showed no significant differences for treatment ($P = 0.746$) using time as factor. The heat map analysis showed that variations observed were not linked to a specific dosage of the test product and were masked by the interindividual variability among subjects. The cluster dendrogram showed that, with a few exceptions mainly related to the 15 g AX, samples from the same donor cluster together at baseline, after 3 wks intervention and end of the study, irrespective of the treatment received (data not shown).

Table 4.5 Observed richness, Shannon diversity and Simpson evenness indices at baseline and after 6 wks supplementation.

Indices	Intervention	Baseline		End		Difference	95% CI	*P* value
		Mean	SD	Mean	SD	in means		
Observed richness	Placebo	295.3	21.4	285.2	44.9			
	7.5 g AX	301.4	49.3	287.8	33.1	-1.9	-39.1, 35.3	0.918
	15 g AX	272.4	40.3	235.8	62.5	-36.7	-69.3, -4.0	0.029
Shannon diversity	Placebo	4.13	0.28	4.05	0.33			
	7.5 g AX	4.28	0.37	4.03	0.31	-0.11	-0.41, 0.20	0.473
	15 g AX	4.08	0.25	3.75	0.43	-0.29	-0.55 -0.02	0.036
Simpson evenness	Placebo	0.96	0.02	0.96	0.02			
	7.5 g AX	0.97	0.02	0.94	0.03	-0.01	-0.04, 0.01	0.278
	15 g AX	0.96	0.01	0.94	0.03	-0.02	-0.04, 0.00	0.063

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model. The presented difference in means is corrected for baseline differences. AX, arabinoxylans.

Fecal pH, ammonium and SCFA concentrations

Data regarding fecal pH, ammonium and SCFA concentrations are depicted in Table 4.6. Fecal pH was significantly reduced after 6 wks 7.5 g AX supplementation, compared to placebo (P = 0.012). Six wks daily intake of both 7.5 g and 15 g AX increased the concentration of total SCFA, compared to a decrease in the placebo group, the difference being statistically significant (7.5 g AX: P = 0.012; 15 g AX: P = 0.006). The same was observed for fecal concentrations of acetate (7.5 g AX: P = 0.038; 15 g AX: P = 0.016) and butyrate (7.5 g AX: P = 0.001; 15 g AX: P = 0.030). Fecal propionate concentrations significantly increased in the 15 g AX group, compared to placebo (P = 0.026). AX intake versus placebo did not affect fecal ammonium concentrations (7.5 g AX: P = 0.111; 15 g AX: P = 0.370). No significant differences between interventions were seen in fecal pH, ammonium and SCFA concentrations after 3 wks supplementation (all $P \geq 0.082$, data not shown).

Production of cytokines by stimulated peripheral blood mononuclear cells

Production of the pro-inflammatory cytokine TNFα by stimulated PBMCs was significantly reduced after 6 weeks 15 g AX intake (P = 0.035; Table 4.7) compared to placebo. No significant differences were found regarding the production of the other measured cytokines (all $P \geq 0.176$; Table 4.7).

Table 4.6 Fecal pH, ammonium (mg/l) and short-chain fatty acid concentrations (μmol/g feces) at baseline and after 6 wks supplementation.

	Intervention	Baseline	End	*P* value
pH	Placebo	7.3 [7.0; 7.6]	7.7 [7.3; 8.1]	
	7.5 g AX	7.1 [6.8; 7.5]	7.0 [6.9; 7.4]	0.013
	15 g AX	7.2 [6.9; 7.9]	7.4 [6.9; 7.7]	0.086
Ammonium	Placebo	58.5 [46.1; 74.5]	44.8 [34.7; 59.9]	
	7.5 g AX	53.8 [45.8; 69.7]	55.4 [41.9; 91.4]	0.111
	15 g AX	43.4 [28.2; 60.8]	51.8 [31.5; 88.1]	0.370
SCFA: total	Placebo	67.4 [46.4; 97.0]	59.1 [31.7; 63.8]	
	7.5 g AX	63.6 [50.0; 86.2]	71.6 [54.7; 88.3]	0.012
	15 g AX	49.3 [33.7; 72.5]	59.2 [41.8; 80.3]	0.006
SCFA: acetate	Placebo	34.8 [29.2; 49.9]	31.2 [17.4; 35.4]	
	7.5 g AX	35.6 [28.6; 44.7]	35.0 [25.4; 53.3]	0.038
	15 g AX	27.8 [17.9; 40.1]	34.0 [23.0; 44.5]	0.016
SCFA: propionate	Placebo	11.1 [7.7; 18.4]	9.8 [6.1; 12.2]	
	7.5 g AX	11.5 [8.7; 17.0]	12.5 [9.0; 15.5]	0.229
	15 g AX	10.2 [6.4; 17.0]	12.7 [8.4; 17.6]	0.026
SCFA: butyrate	Placebo	9.0 [6.1; 27.4]	6.7 [3.7; 9.9]	
	7.5 g AX	10.9 [8.7; 14.3]	11.9 [9.4; 17.0]	0.001
	15 g AX	6.9 [4.5; 15.2]	7.4 [5.2; 14.6]	0.030

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model with correction for baseline values. Data are given as median [IQR, *i.e.* Q1; Q3]. AX, arabinoxylans. SCFA, short-chain fatty acids.

Table 4.7 Cytokine production by stimulated PBMCs (ng/mL) at baseline and after 6 wks supplementation.

Cytokine production	Intervention	Baseline	End	*P* value
IFNγ	Placebo	29.8 [5.0; 75.5]	48.8 [12.6; 419.2]	
	7.5 g AX	14.2 [9.3; 59.3]	38.7 [2.5; 104.5]	0.176
	15 g AX	54.9 [26.5; 85.3]	49.5 [10.7; 87.7]	0.211
Il-2	Placebo	15.2 [0.9; 38.2]	7.5 [1.2; 23.6]	
	7.5 g AX	0.3 [0.1; 7.3]	5.3 [0.2; 41.1]	0.427
	15 g AX	10.7 [0.4; 53.4]	1.5 [0.2; 49.9]	0.489
Il-10	Placebo	6.7 [2.6; 18.6]	12.8 [11.4; 27.1]	
	7.5 g AX	3.8 [1.5; 10.1]	5.3 [3.2; 18.4]	0.205
	15 g AX	8.3 [5.0; 18.3]	11.5 [5.6; 17.2]	0.402
Il-12p40	Placebo	0.02 [0.01; 0.16]	0.05 [0.02; 0.17]	
	7.5 g AX	0.02 [0.01; 0.03]	0.04 [0.02; 0.06]	0.210
	15 g AX	0.02 [0.01; 0.07]	0.02 [0.00; 0.06]	0.861
TNFα	Placebo	72.9 [44.5; 147.6]	97.2 [52.4; 119.3]	
	7.5 g AX	56.9 [28.6; 103.4]	61.9 [34.0; 138.7]	0.708
	15 g AX	83.0 [50.5; 96.0]	60.9 [27.4; 77.6]	0.035

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model with correction for baseline values. Data are given as median [IQR, *i.e.* Q1; Q3]. AX, arabinoxylans. IFN, interferon. TNF, tumor necrosis factor.

Blood lipids, glucose and insulin

At baseline, total cholesterol, LDL- and HDL-cholesterol, triglycerides, free fatty acids, glucose and insulin lied within normal blood value ranges. These parameters were not

significantly altered by either 3 wks or 6 wks supplementation with 7.5 g or 15 g AX compared to placebo (all $P \geq 0.083$; Table 4.8). Subsequently, no change between intervention groups was observed regarding insulin sensitivity (all $P \geq 0.768$, Table 4.8).

Table 4.8 Blood lipids (mmol/l), glucose (mmol/l), insulin (pmol/l) and QUICKI at baseline and after 6 wks supplementation.

Metabolic markers	Intervention	Baseline		End		Difference	95% CI	*P* value
		Mean	SD	Mean	SD	in means		
Total cholesterol	Placebo	5.39	1.00	5.37	1.24			
	7.5 g AX	5.83	1.00	5.71	1.05	-0.11	-0.49, 0.27	0.563
	15 g AX	5.59	0.80	5.46	0.99	-0.12	-0.49, 0.25	0.524
LDL-C	Placebo	3.26	0.77	3.29	0.95			
	7.5 g AX	3.67	0.83	3.56	0.99	-0.14	-0.52, 0.23	0.442
	15 g AX	3.39	0.75	3.16	0.90	-0.26	-0.62, 0.10	0.148
HDL-C	Placebo	1.42	0.31	1.46	0.35			
	7.5 g AX	1.44	0.41	1.41	0.41	-0.06	-0.20, 0.08	0.357
	15 g AX	1.60	0.36	1.64	0.45	0.00	-0.14, 0.13	0.972
Triglycerides	Placebo	1.57	0.58	1.37	0.51			
	7.5 g AX	1.79	1.32	2.06	2.11	0.41	-0.08, 0.90	0.102
	15 g AX	1.32	0.64	1.47	0.82	0.42	-0.06, 0.89	0.083
Glucose	Placebo	5.38	0.49	5.39	0.66			
	7.5 g AX	5.54	0.90	5.36	0.50	-0.08	-0.45, 0.28	0.659
	15 g AX	5.26	0.54	5.12	0.38	-0.24	-0.59, 0.12	0.186
Insulin	Placebo	75.8	29.8	62.4	26.3			
	7.5 g AX	124.8	250.5	68.3	33.9	3.74	-25.4, 32.9	0.797
	15 g AX	70.7	35.9	68.4	48.7	6.26	-22.4, 34.9	0.661
QUICKI	Placebo	0.26	0.01	0.27	0.02			
	7.5 g AX	0.26	0.02	0.27	0.02	-0.0018	-0.01, 0.01	0.768
	15 g AX	0.27	0.02	0.27	0.02	0.0003	-0.01, 0.01	0.963

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model. The presented difference in means is corrected for baseline differences. AX, arabinoxylans. QUICKI, quantitative insulin sensitivity check index.

Gastrointestinal tolerance

No significant differences between intervention groups regarding reported GI symptoms were observed during the study (all $P \geq 0.118$, data not shown). Mean scores remained under 2 during all treatments, which means that the participants during intervention experienced no troublesome symptoms. Neither 7.5 g nor 15 g AX significantly affected stool frequency or consistency throughout the entire study period (all $P \geq 0.271$, data not shown).

Discussion

In this study, we have shown that GI permeability is compromised in overweight and obese individuals when compared to lean controls. Six wks intake of 7.5 g or 15 g AX per day did not affect GI permeability, as assessed by the multi-sugar test. Although no differences were found in the relative abundance of the dominant phyla, intake of 15 g AX resulted in reduced microbial richness and diversity, when compared to placebo. AX supplementation increased fecal SCFA concentrations. Furthermore, 7.5 g AX intake significantly lowered fecal pH. This modulation of the intestinal luminal environment may positively affect the gut barrier, as reflected by upregulation in the transcription of different TJ proteins in sigmoid biopsies, although we did not detect significant changes in TJ protein expression. The decreased TNFα production by stimulated PBMCs in the high dose (15 g) AX group points to downstream positive anti-inflammatory effects.

Evidence indicating that obesity is associated with an impaired gut barrier is mainly derived from animal models.[3] Human studies on gut barrier and obesity are scarce, and results contradictory.[22,23] Here, we showed that gut permeability is increased in obese compared to lean healthy volunteers. AX treatment did not affect gastroduodenal, small intestinal, colonic or whole gut permeability, as assessed with a multi-sugar test. However, interpretation of these results must be done with caution as permeability testing with sugars might be influenced by individual differences in gastric emptying and intestinal transit time, parameters we did not measure in these participants. Furthermore, although we aimed to examine a homogenous study population, possible confounders (*i.e.* lifestyle, sex) could have affected permeability results. Prebiotics, such as inulin, were shown to improve GI permeability in obese subjects after dietary intervention for 9 wks.[24] The discrepancy in results between our and above study might be explained by various factors, such as differences in method of measuring GI permeability, sample size and duration of study product intake. Furthermore, we assessed the effects of AX intake during weight maintenance, while they investigated the effect during a weight loss intervention.[24] Some animal studies have shown beneficial effects of AX on an impaired gut barrier.[9,10] This is the first human study analysing the effects of a (putative) prebiotic on the expression of TJ proteins in sigmoid mucosa in overweight subjects. From a subset of our study population we obtained mucosal samples from sigmoid colon and despite the small sample size, 7.5 g AX showed to significantly upregulate gene transcription of the TJ protein occludin, while 15 g AX significantly upregulated claudin-3 and claudin-4. These proteins are structural transmembrane components, which organize complex TJ protein systems, determining paracellular permeability. These results are in line with the preclinical data in obese mice, where AX significantly increased mRNA concentrations of TJ proteins expression.[9] No significant changes in sigmoid TJ protein concentrations were observed after AX treatment.

Modulating gut microbiota composition by prebiotic compounds, and thereby influencing its effect on intestinal homeostasis, appears to be a promising strategy to treat and prevent chronic diseases. This is the first human study investigating the effect of a high molecular weight AX on the microbial community composition in overweight and obese individuals. *In vitro* and mice studies have shown that long-chain AX can be efficiently fermented, leading to specific stimulation of bifidobacterial species and beneficial fermentation profiles.[9,11] While in previous studies the effect of this particular AX concentrate on the microbiota was assessed by selective qPCR protocols,[10-12] in the current trial we made use of a metagenomic approach. Here, we observed a decrease in microbial richness and diversity after 15 g AX supplementation, but no change in overall microbiota composition was seen. Intestinal microbiota converts indigestible food components into metabolites, such as SCFA, that can be processed by the host. SCFA are able to exert antioxidant, anti-inflammatory and immunomodulating effects.[25] Furthermore, the SCFA butyrate functions as the principal energy source for the proliferation and differentiation of colonic epithelial cells and is essential in establishing and maintaining the gut barrier by regulating the expression of TJ proteins.[25] We demonstrated that the fecal concentration of the individual and total SCFA were increased upon intake of AX compared to a decrease in the placebo group. Our findings confirm previous data from *in vitro* and *in vivo* studies. Two different *in vitro* models, often used to assess modulation of the gut microbiome (SHIME, TIM-2), demonstrated that long-chain AX specifically increase propionate concentrations.[11] In germ-free rats, inoculated with human feces, long-chain AX increased the concentrations of acetate, propionate, butyrate and total amount of SCFA.[12] In a human study, medium-chain AX significantly increased fecal concentrations of both propionic and butyric acid after 4 wks intake.[26] Other prebiotics, such as inulin and fructo-oligosaccharides, showed less pronounced effects on SCFA production in humans.[27,28] Concomitantly, we found a significant decrease in fecal pH in the 7.5 g AX group. A more acidic luminal environment may contribute to the reduction of pathogenic bacteria and creates a more favourable environment for the growth of beneficial bacteria.

To assess the effect of AX on immune response we applied an *ex vivo* model using PHA-stimulated whole blood to evaluate the capacity to produce cytokines. We found a significant decrease in production of the pro-inflammatory cytokine TNFα after supplementation with 15 g AX, suggesting a capacity to reduce the intensity of an acute pro-inflammatory reaction. In obese mice AX showed to have an anti-inflammatory effect.[9] Human data regarding the effect of prebiotics on immune modulation, assessed via the current *ex vivo* model, are limited. One study in healthy subjects showed that a prebiotic mixture had the capacity to reduce the pro-inflammatory pathway and to stimulate anti-inflammatory pathways.[26] Pro-inflammatory cytokines are associated with the development of systemic IR, ß-cell dysfunction and CVD.[29] One important function of SCFA is suppressing the production of such cytokines.[25] In the present human trial we observed an increase in

fecal SCFA concentrations after AX treatment, providing a possible explanation for the decrease in TNFα production by PBMC.

Obesity is typically associated with a wide cluster of metabolic alterations, including glucose homeostasis disorders and dyslipidemia. AX showed to improve glycemic control and blood triglycerides concentrations in subjects with an impaired glucose tolerance or DM2.[29] In the present study, supplementation with AX in healthy overweight and obese subjects did not affect systemic glucose, insulin and lipid concentrations. This can be explained by the fact that the participants had normal values from beginning of the study.

The daily ingestion of AX did not influence bowel habits, confirming previous observations in prebiotics intervention studies.[28] The low prevalence of GI complaints during AX supplementation provides evidence that AX is well-tolerated by humans.

This study followed a unique approach to assess the gut barrier, by combining functional analyses (multi-sugar test) with TJ analyses in mucosal tissue specimens. Some potential shortcomings should be considered. Firstly, we measured fecal SCFA concentrations to assess the microbiota fermentation capacity. SCFA are efficiently absorbed from the gut, with only 5% to 10% being excreted via the feces. Fecal SCFA concentrations hence do not accurately reflect the exact intestinal SCFA concentration. However, previous studies showed that the ratio between the respective SCFA, measured in feces and colon, is comparable to each other.[31] Thus, changes in fecal SCFA concentration reflect relative changes in intestinal concentrations of the separate SCFA. Furthermore, as we choose to focus on the effects of AX on gut barrier function and gut microbiota, we did not obtain information regarding hepatic health of our study subjects. Prebiotics target multiple metabolic impairments associated with obesity-related non-alcohol fatty liver disease, and thus the effects of AX on hepatic health in this study population may be another interesting objective to assess. We did not control the diet and exercise pattern of our participants, as we aimed to evaluate the effects of AX when supplementing the habitual diet, which is in line with future applications. Furthermore, in this study we observed potentially advantageous findings regarding gut barrier and immune function, however these findings are still theoretical and were not translated into clinically positive outcomes. Future research is required to assess whether these findings can improve gut barrier function and immune health.

In conclusion, we have shown that 6 wks AX supplementation resulted in a more beneficial fermentation (SCFA) profile in overweight and obese subjects. In addition, an increased gene transcription of several transmembrane proteins was observed, although no changes in TJ proteins expressions or GI permeability were detected. Furthermore, we showed that 15 g AX might have an anti-inflammatory potential and that the intake of AX was well-tolerated.

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Supplemental material

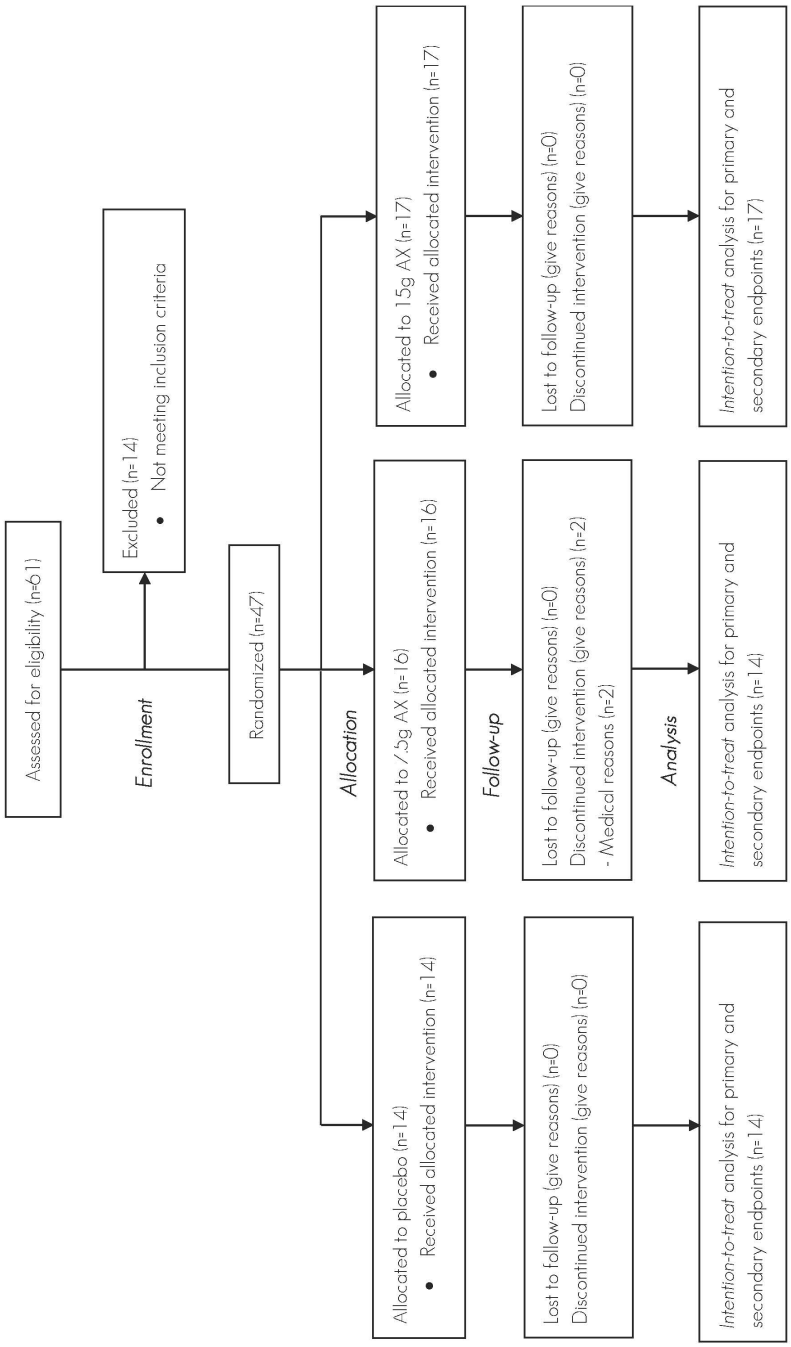


Figure S4.1 CONSORT flow diagram.

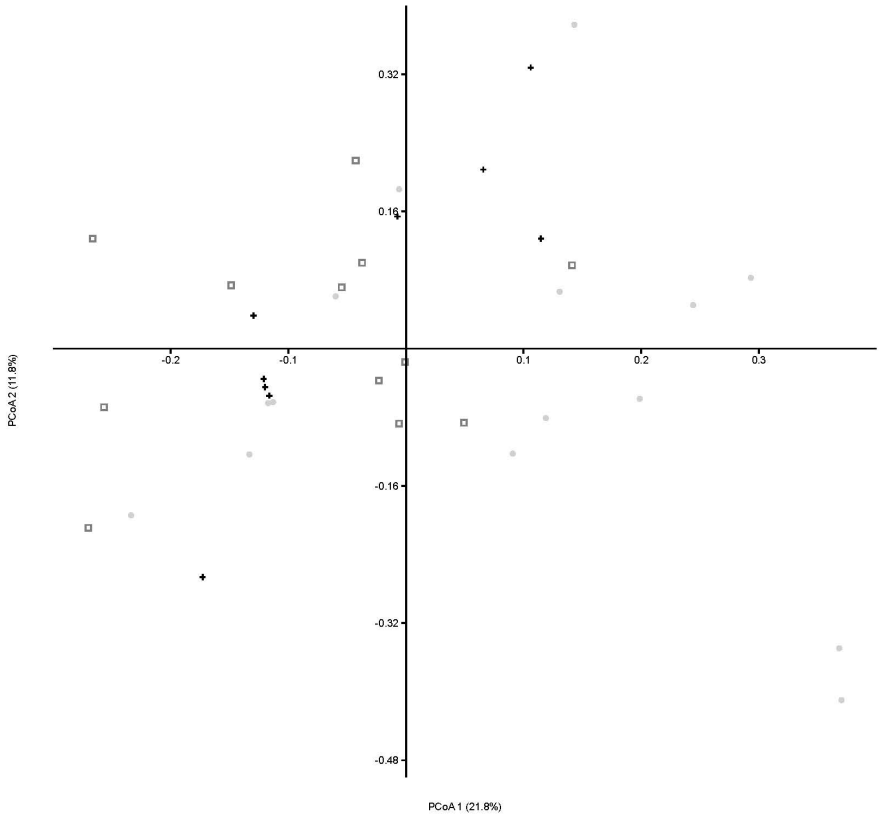


Figure S4.2 Clustering of samples due to intervention by PCoA, based on BC similarity distance, at baseline. Fecal samples collected from different treatment groups are represented by gray squares for placebo, black plusses for 7.5 g AX and lightgray dots for 15 g AX. No significant differences in microbial community composition between placebo, 7.5 g AX and 15 g AX during study period were observed, as assessed using two-way ANOSIM. ANOSIM, analysis of similarity. AX, arabinoxylans. BC, Bray-Curtis. PCoA, principle coordinate analysis.

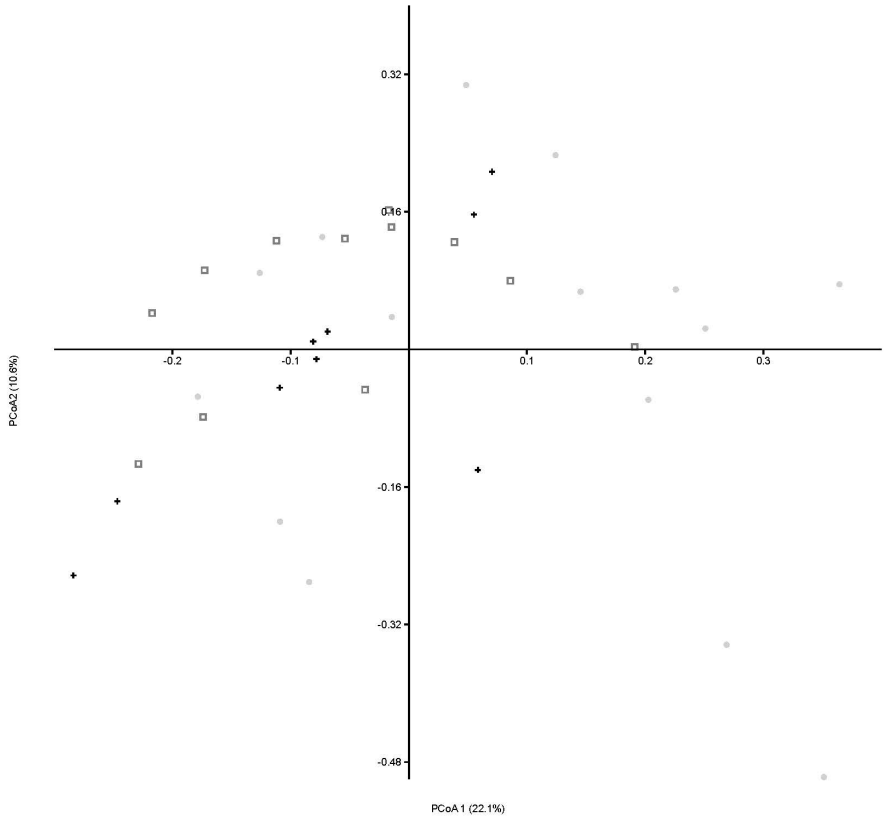


Figure S4.3 Clustering of samples due to intervention by PCoA, based on BC similarity distance, after 3 wks intervention. Fecal samples collected from different treatment groups are represented by gray squares for placebo, black plusses for 7.5 g AX and lightgray dots for 15 g AX. No significant differences in microbial community composition between placebo, 7.5 g AX and 15 g AX during study period were observed, as assessed using two-way ANOSIM. ANOSIM, analysis of similarity. AX, arabinoxylans. BC, Bray-Curtis. PCoA, principle coordinate analysis.

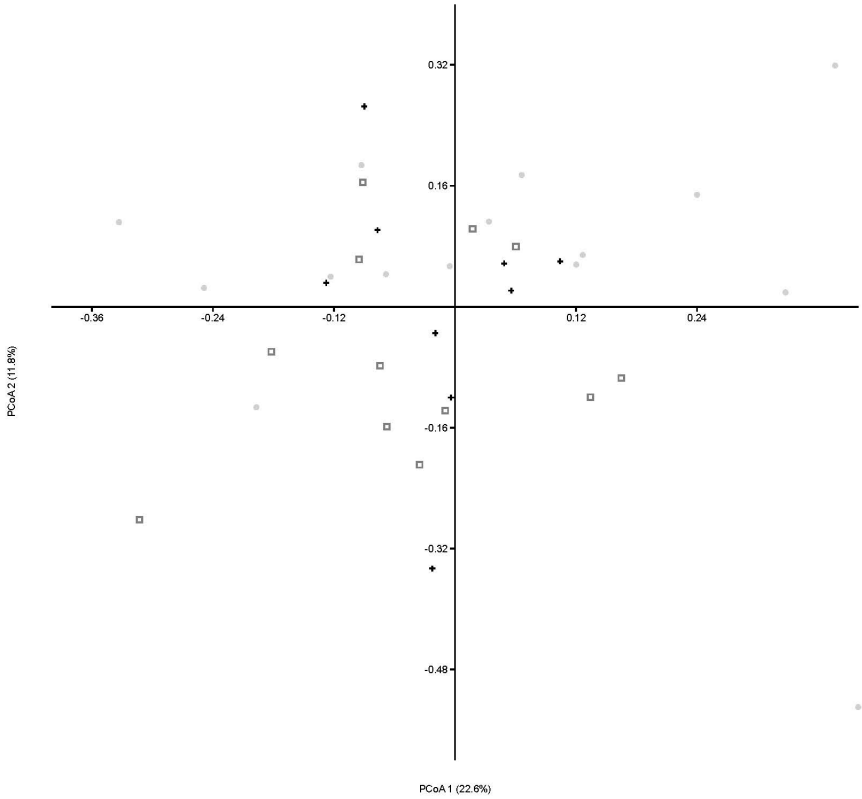
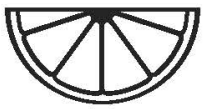
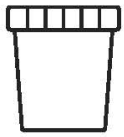


Figure S4.4 Clustering of samples due to intervention by PCoA, based on BC similarity distance, after 6 wks intervention. Fecal samples collected from different treatment groups are represented by gray squares for placebo, black plusses for 7.5 g AX and light gray dots for 15 g AX. No significant differences in microbial community composition between placebo, 7.5 g AX and 15 g AX during study period were observed, as assessed using two-way ANOSIM. ANOSIM, analysis of similarity. AX, arabinoxylans. BC, Bray-Curtis. PCoA, principle coordinate analysis.





chapter 5

In vivo bioavailability of hesperidin 2S is improved by micronization: a randomized, double-blind cross-over study

Salden BN, Troost FJ, Possemiers S, Bast A, Masclee AA

Abstract

Background
The bioavailability of hesperidin in humans is low, partly due to its poor water solubility. Micronization is known to decrease particle size of a compound, thereby improving its solubility and thus absorption.

Objective
To investigate the bioavailability of a single dosage micronized hesperidin 2S compared to an identical dosage standard non-micronized hesperidin in healthy individuals.

Design
In this double-blind, randomized, crossover exploratory study 11 healthy individuals attended to two test days. Subjects randomly consumed one dosage standard non-micronized hesperidin and one dosage micronized hesperidin 2S. Urine was collected for 24 h. Blood was sampled at 8 different time points over a 10 h period.

Results
LC-MS/MS analysis of urine and plasma samples revealed the metabolites hesperetin-3-O-glucuronide, hesperetin-7-O-glucuronide and hesperetin-7-O-sulphate. The cumulative and relative excretion of total hesperetin (P = 0.032) and individual metabolites (all $P \leq 0.044$) in 24 h was significantly higher after micronized hesperidin 2S intake, compared to standard hesperidin. Also the plasma hesperetin $AUC_{0\text{-}4h}$ was higher after intake of the micronized product, compared to non-micronized product (P = 0.032).

Conclusions
The bioavailability of micronized hesperidin 2S was higher than standard non-micronized hesperidin. This finding may beneficially influence the biological activity and systemic health effects of this formulation.

Introduction

Flavonoids comprise a large group of polyphenolic compounds present in fruits and vegetables. Several large studies have shown that the intake of flavonoid-rich foods has positive effects on cardiovascular diseases (CVD) and their related risk factors.[1,2] Hesperidin (HE; hesperetin-7-O-rutinose 2S), a naturally extracted and purified flavanone glycoside from sweet orange peels, has drawn attention due to its positive effects on metabolic and cardiovascular health.[3-6] In order to exert health effects *in vivo*, it is essential that flavonoids are bioavailable, absorbed from the gastrointestinal (GI) tract and reach the systemic circulation. Following oral administration, hesperidin is absorbed across the GI tract, but its bioavailability is low in humans.[7,8] Several factors limit the bioavailability of hesperidin after oral intake, one of them is its poor water solubility.[9] Furthermore, for many flavonoid glycosides, after conversion to its aglycone, the small intestine is the major site of absorption.[10] However, hesperidin is a flavonoid consisting of a flavonoid backbone (hesperetin) bound to rutinose. To release and enable the absorption of the hesperidin aglycone, hydrolysis of rutinose by β-glucosidase derived from gut microbiota is required.[11] This process mainly occurs in the large intestine, where the aglycone hesperetin is released and further conjugated into glucuronidated and sulphated metabolites by enterocyte cells or by the liver, these metabolites may then be absorbed into the blood.[8,12] As a result, circulating levels of hesperetin are very low: hesperetin sulfates (3'- and 7-O-sulfate) and glucuronides (3'- and 7-O-glucuronide) are the main circulating metabolites upon hesperidin consumption.[7,8,13] An additional complex factor is related to the specific enantiomer configuration of hesperidin (*i.e.* S versus R). Hesperidin consists of a mixture of S- and R-enantiomers, it has a S:R ratio between 1:1 and 5:1, depending on its source.[14,15] While in nature the 2S-enantiomer of hesperidin, and subsequently the S-hesperetin enantiomer, is dominant, commercially available hesperidin and hesperetin are a mixture of both stereo-isomers. The two enantiomers may display distinct kinetic and dynamic properties, with stronger biological effects elicited by the S-enantiomer compared to the R-enantiomer.[16,17]
Several attempts have been made to improve the bioavailability and subsequent bioactivity of hesperidin.[18-20] Here, we developed a micronized hesperidin 2S formulation with a S:R ratio of 4:1 (Cordiart). Micronization has previously been used to increase the bioavailability of other polyphenols.[21] The particle size of the micronized hesperidin 2S is approximately 10 times smaller than the standard hesperidin extract.
Primary aim of this study was to investigate the bioavailability of a single dose of micronized hesperidin 2S, as measured by 24 h urinary excretion and plasma AUC of hesperidin metabolites, compared to a standard non-micronized hesperidin formulation (S:R ratio of 1.5:1). We hypothesized that micronized hesperidin 2S has an improved bioavailability compared to a standard non-micronized hesperidin product, as shown by a higher 24 h urinary excretion and plasma AUC of hesperidin metabolites.

Subjects and methods

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center + (MUMC+) and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study was performed at the MUMC+ in June and July 2013. All participants gave written informed consent before participation.

Subjects

Healthy volunteers aged 18-75 years with a BMI between 18-30 kg/m^2 and normal laboratory values were recruited by advertising in the local media. Laboratory values included haemoglobin (Hb, defined as: M 8.2-11.0 mmol/l, F 7.3-9.7 mmol/l); haematocrit (Ht, defined as: M 0.41-0.52 l/l, F 0.36-0.48 l/l); serum alanine transaminase (ALT, defined as: M <45 U/l, F <34 U/l); serum aspartate transaminase (AST, defined as: M <35 U/l, F <31 U/l); gamma-glutamyl transferase (GGT, defined as: M <55 U/l, F <40 U/l); bilirubin total (defined as: <20 μmol/l); serum creatinine (defined as: M 60-115 μmol/l, F 50-100 μmol/l). Exclusion criteria were: any medical condition that might interfere with the study and might jeopardize the health status of the participant; smoking; use of medication / vitamin-, mineral-, or antioxidant supplements; pregnancy and lactation; history of any side effects towards the intake of flavonoids or citrus fruits; failure to comply with prohibited intake of hesperidin rich food products.

Design and intervention

In this double-blind, randomized, crossover exploratory study each subject attended to two test days with at least 1 week washout period in between. All participants and investigators remained blind to treatment until all analyses were completed. Participants were requested to abstain from strenuous physical exercise and consumption of alcohol the day prior to each test day. Furthermore, we instructed them to maintain their habitual diet during the study period, but to abstain from intake of hesperidin-rich foods starting 3 days prior to start of the study period. After an overnight fast, subjects arrived at the study site and an intravenous catheter was inserted into the antecubital vein of the arm. Blood sampling was performed at baseline and 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 10 h after intake of the study product. Furthermore, after intake of the study product, subjects collected full urine output for 24 h. One hour after intake of the study product, the participants received a standard breakfast. During a test day they also received a standard snack, lunch and dinner. At the end of the test day the intravenous catheter was removed.

Micronized hesperidin 2S and standard hesperidin

Micronized hesperidin 2S (450 mg, supplied as 500 mg Cordiart, BioActor BV, Maastricht, The Netherlands) was extracted from the *Citrus sinesis* immature fruits, containing both the S and R enantiomers in the natural 4:1 S:R ratio of hesperidin. Standard hesperidin (450 mg) was provided by Ferrer HealthTech (Spain) and had a S:R molar ratio of 1.5:1. A total of 450 mg hesperidin corresponds to 222.8 mg hesperetin. The study products were formulated into capsules, each containing 250 mg study product. Subjects were asked to ingest two capsules within 1 min with 200 ml water.

Urine collection

Twenty-four hour urine was collected in 2L bottles with added dibutylhydroxytoluene (anti-oxidant) to preserve the metabolites during the collection period. The total urine volume was weighed before samples were stored at -80°C until further analysis.

Blood collection

During a test day blood samples were taken for the determination of hesperidin, hesperidin 2S and its metabolites. These samples were collected in sodium heparin tubes and immediately centrifuged at 1300 *g* for 10 min at 4°C. Thirty-six μl/ml 4 mM hydrochloric acid (HCl) was added to the plasma in support of the LC-MS measurements of the metabolites. All plasma samples were stored at -80°C until further analysis.

LC-MS/MS analysis of metabolites

LC-MS/MS analysis of the samples revealed the following metabolites: hesperetin-3-O-glucuronide (Hp3G), hesperetin-7-O-glucuronide (Hp7G) and hesperetin-7-O-sulphate (Hp7S). The optimum mass spectrometer parameters for the detection of the hesperetin metabolites were optimized, connecting the column inlet directly to a special ionization source called 'Jet Stream'. The source parameters were the following: capillary voltage -3500 V, charging potential -500 V, nebulizer pressure 40 (psi), auxiliary gas heated to 275°C and introduced at a flow rate of pure nitrogen 9 L/min. The multiple reaction monitoring (MRM) was the method of choice due to the high selectivity and sensitivity in LC-MS/MS, monitoring 4 transitions for each analysis: hesperetin, m/z 301→164; hesperetin glucuronide, m/z 477→301; hesperetin diglucuronide, m/z 653→(477)→301; hesperetin sulfoglucuronide, m/z 557→(477)→301; hesperetin sulfate, m/z 381→301; with a dwell time for each transition of 8 ms. Concentrations of hesperetin diglucuronide and hesperetin sulfoglucuronide metabolites were estimated by synthesized hesperetin

glucuronide calibration curves (10, 5, 1, 0.5, 0.1 μM). The cumulative excretion of total hesperetin in 24 h urine was calculated by adding the concentrations of the 3 different metabolites per test day.

Statistical analyses

Primary outcome of the study was the bioavailability of micronized hesperidin 2S, measured by the difference in cumulative excretion of hesperetin in 24 h urine and by the difference in the 240-min and 600-min area under the plasma concentration-time curves ($AUC_{0\text{-}4h}$ and $AUC_{0\text{-}10h}$) of plasma total metabolite concentration between micronized hesperidin 2S and standard hesperidin. As this is an exploratory study, no formal sample size calculation was made. Based on other studies investigating the bioavailability of polyphenols by measuring the urinary excretion and the plasma concentrations of the metabolites, we estimated that at least 8 participants would be sufficient for evaluation of the primary aim.[19,22] Baseline characteristics are presented as mean (SD) for numerical variables. The AUCs were calculated using the trapezoidal method. Differences in urinary excretion and plasma AUCs of Hp3G, Hp7G and Hp7S and total hesperetin between intervention groups (micronized hesperidin 2S or standard hesperidin) were assessed using linear mixed models with group (micronized hesperidin 2S or standard hesperidin) as fixed factor, where an unstructured covariance structure was used for repeated measures. The linear mixed model accounts for the correlation between repeated measures and missing data, where a likelihood approach was used assuming data missing at random. The estimated means and SEM obtained from this model were presented for each group. Two-sided *P*-values ≤ 0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY, USA).

Results

Study subjects

Initially, 11 healthy volunteers were enrolled in the study [38% male; age 27 ± 8 yrs; BMI (in kg/m^2) 22 ± 2], of which 10 completed the entire study protocol. From the drop-out only baseline characteristics were available.

Urinary metabolites

The cumulative and relative excretion of hesperetin in 24 h urine is shown in Table 5.1 and Figure 5.1. The urinary excretion of the individual metabolites Hp3G, Hp7G, Hp7S and of

total hesperetin was significantly higher after micronized hesperidin 2S intake compared to standard hesperidin intake (P = 0.044, P = 0.024, P = 0.018, P = 0.032 respectively). Relative urinary excretion of total hesperetin as a percentage of intake was 3.73% for the standard hesperidin formulation compared to 5.62% for the micronized hesperidin 2S formulation.

Table 5.1 Ingested dose, plasma AUCs and 24 h urinary excretion of hesperetin after intake of standard hesperidin and micronized 2S HE[1]

	Standard HE	Micronized HE 2S	*P* value
Ingested dose (mg)	222.79	222.79	-
AUC_{0-4h} (μmol h/l)	0.4 ± 0.1[2]	1.1 ± 0.3	0.032
AUC_{0-10h} (μmol h/l)	4.9 ± 1.1	7.1 ± 1.3	0.225
Total urinary excretion (24 h)			
μmol	27.52 ± 4.53	41.48 ± 7.35	0.032
mg	8.31 ± 1.37	12.53 ± 2.22	
Relative urinary excretion percentage of intake	3.73 ± 0.61	5.62 ± 1.00	0.032

[1]Differences between standard hesperidin and micronized hesperidin 2S tested with linear mixed model. HE, hesperidin. [2]Estimated mean ± SEM (all such values)

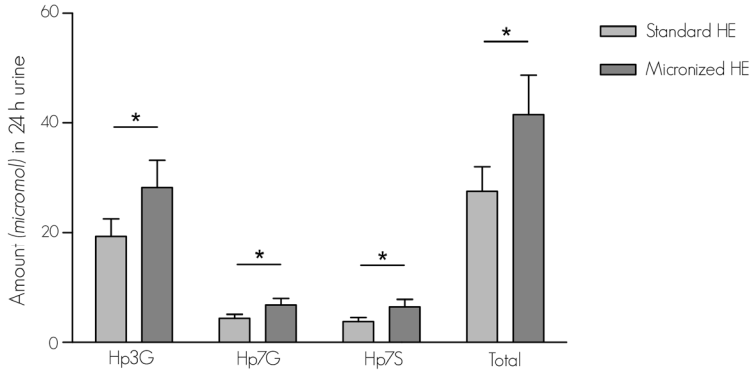


Figure 5.1 The cumulative excretion of Hp3G, Hp7G, Hp7S and total hesperetin in 24 h urine after intake of standard hesperidin and micronized hesperidin 2S. Data presented as: mean ± SEM. Data of standard hesperidin are represented by light gray, data of micronized hesperidin 2S by dark gray. Differences between standard hesperidin and micronized hesperidin 2S tested with linear mixed model. * significant ($P \leq 0.050$) difference between standard hesperidin and micronized hesperidin 2S.

Plasma metabolites

The AUC_{0-4h} and AUC_{0-10h} of plasma hesperetin are shown in Table 5.1 and Figure 5.2. The mean and individual time-vs.-plasma concentration curves for hesperetin are shown in Figure 5.3, Figure 5.4A and 5.4B. The AUC_{0-4h} of plasma hesperetin was significantly higher after micronized hesperidin 2S intake compared to standard hesperidin (P = 0.032). However, the

$AUC_{0\text{-}10h}$ was not significantly different between interventions (P = 0.225). Also, there were no significant between-group differences at each measured time point (all $P \geq 0.194$).

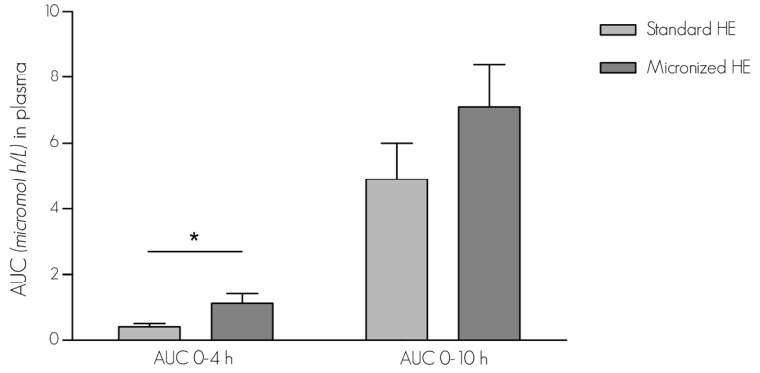


Figure 5.2 The $AUC_{0\text{-}4h}$ and $AUC_{0\text{-}10h}$ of plasma total hesperetin after intake of standard HE and micronized 2S HE. Data presented as: mean ± SEM. Data of standard HE are represented by light gray, data of micronized 2S HE by dark gray. Differences between standard hesperidin and micronized hesperidin 2S tested with linear mixed model. * significant ($P \leq 0.050$) difference between standard hesperidin and micronized hesperidin 2S.

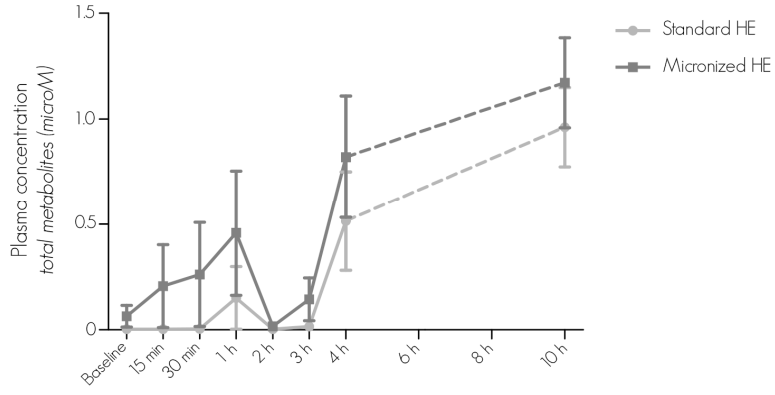


Figure 5.3 Change in plasma concentration of total hesperetin (μM) from baseline to 10 h after intake of standard HE and micronized 2S HE. Data presented as: mean ± SEM. Data of standard HE are represented by light gray dots, data of micronized 2S HE by dark gray squares. Differences between standard hesperidin and micronized hesperidin 2S tested with linear mixed model. No significant differences in plasma total hesperetin per time point between interventions were observed (all $P \geq 0.194$).

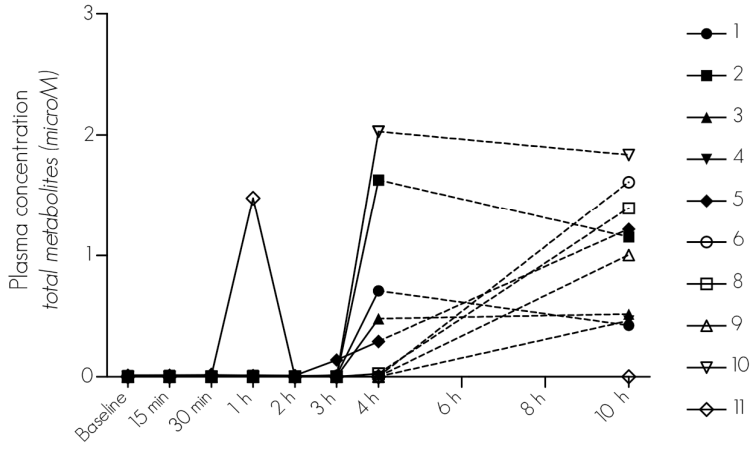


Figure 5.4A Individual plasma concentration curves for total hesperetin (μM) from baseline to 10 h after intake of standard hesperidin. Subject numbers are shown.

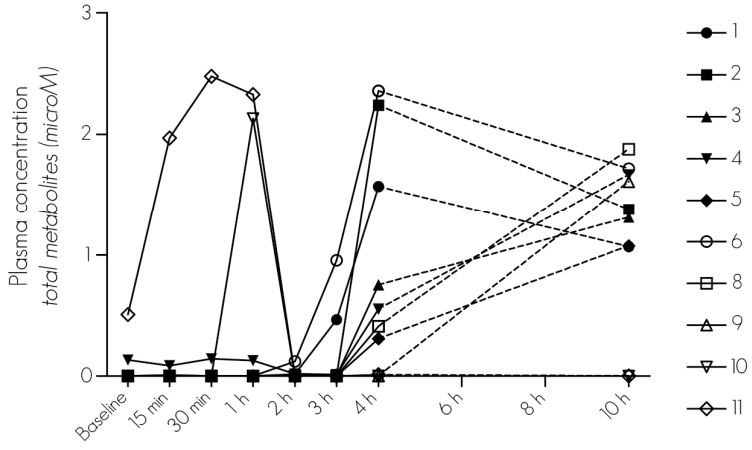


Figure 5.4B Individual plasma concentration curves for total hesperetin (μM) from baseline to 10 h after intake of micronized hesperidin 2S. Subject numbers are shown.

Discussion

The present study demonstrates that micronized hesperidin 2S is more bioavailable than standard hesperidin, as shown by a significant increase in the cumulative excretion of the individual metabolites Hp3G, Hp7G and Hp7S and of total hesperetin in 24 h urine, after

ingestion of one dose micronized hesperidin 2S, compared to standard hesperidin. Additionally, plasma hesperetin $AUC_{0\text{-}4h}$ and $AUC_{0\text{-}10h}$ increased after intake of micronized hesperidin 2S, compared to standard hesperidin, being significant for the $AUC_{0\text{-}4h}$.

It has been shown that the oral bioavailability of hesperidin is low in humans, due to a combination of factors.[7,8] Hesperidin is highly water insoluble and it requires colonic microbiota to release and enable the absorption of its aglycone hesperetin.[8,9,11,12] A way to improve the dissolution process of a product is to reduce the size of the product particles by micronization.[23] Here, we compared the bioavailability of a micronized hesperidin 2S formulation to that of a non-micronized hesperidin formulation. The micronized 2S product showed to be more bioavailable than the non-micronized standard product, by excreting significantly higher amounts of the 3 main hesperidin metabolites in 24 h urine. Human studies investigating the bioavailability of hesperidin have reported lower relative urinary excretion over 24 h.[7,8,19,24,25] To assess plasma bioavailability we calculated the $AUC_{0\text{-}10h}$ of the individual metabolites and of total hesperetin. Higher AUCs were observed for the individual metabolites and for total hesperetin after micronized hesperidin 2S intake, compared to placebo, but it did not reach significance. Blood samples were taken at baseline and 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 10 h after study product intake. We particularly focused on the first 4 h after study product intake as we expected metabolites to be detected more rapidly in the plasma. We had two reasons to assume this: 1) we expected the micronized hesperidin 2S to be absorbed more rapidly, and 2) we administered the study product while the participant was fasted. Indeed, the individual plasma concentration curves for hesperetin show that in a number of cases detection of metabolites occurs earlier after intake of the micronized hesperidin 2S formulation, compared to the non-micronized hesperidin formulation. However, we have not determined plasma metabolite concentrations between 4 h and 10 h after study product intake. Based on literature data, a peak in plasma hesperetin is expected to occur between 5 to 7 h after study product intake.[7,8,19,24] Therefore, the plasma $AUC_{0\text{-}10h}$ is probably not an accurate reflection of the real bioavailability. As hesperetin concentrations are higher at each measured time point after intake of micronized hesperidin 2S, it can be assumed that this is also the case between 4 h and 10 h. Very likely, the plasma $AUC_{0\text{-}10h}$ is an underestimation of the true bioavailability of the micronized product. As we measured plasma metabolites regularly during the first 4 h after study product intake, we are able to calculate an accurate plasma hesperetin $AUC_{0\text{-}4h}$. Indeed, bioavailability of micronized hesperidin 2S, compared to standard hesperidin, showed to be significantly improved already during the first 4 h after study product intake.

In the current study we observed interindividual variation regarding plasma metabolite excretions. In most subjects the plasma concentration peak lies between 4 and 10 h. The latter indicates that the compound is indeed absorbed from the distal parts of the small intestine or the colon where enzymes, capable of releasing the aglycone hesperetin, are

present.[8,11,12] In some subjects, a peak in plasma metabolite concentration was observed within or at 1 h after study product intake. This might seem to be too short a time for a compound to reach the ileum or colon. However, subjects ingested the study product with water in a fasting state; this might have accelerated transit of the compound through the GI tract. Also, micronizing the compound could have affected the rate of absorption. Interindividual variation in polyphenol bioavailability has also been found in other human intervention studies.[7,26-28] Most likely, the observed variations are caused by interindividual differences in intestinal microbiota composition and activity.[28,29] Also, the colonic conversion rate of polyphenols to metabolites can vary greatly between individuals due to differences in transit time and substrate availability.[30]

The present data show that the main circulating metabolites, formed after oral intake of hesperidin, were hesperetin glucuronides and sulphates. This is in line with other trials assessing the bioavailability of hesperidin in humans.[3,18] Studies investigating the biological effects of these specific metabolites are limited. It has been reported that they beneficially contribute to cardiovascular health.[31-33] An *in vitro* study demonstrated that hesperetin glucuronide increased nitric oxide (NO) release from endothelial cells, indicating that it is able to improve vascular dilatation.[3] A study in hypertensive rats showed that hesperetin glucuronide has blood pressure lowering effects, endothelium-dependent vasodilatory activity and anti-inflammatory properties.[31] Furthermore, glucuronated and sulphated metabolites of hesperidin showed to reduce the adhesion of monocytes to endothelial cells and to modulate the expression of genes related to inflammation, two important aspects involved in atherogenesis.[32] Finally, Giménez-Bastida *et al.* found a reduction of PAI-1, a thrombogenic protein involved in a wide range of CVD as well as in cell migration, by hesperitin and its metabolites.[33] It should be noted that the above mentioned results regarding the beneficial effects of hesperidin metabolites in cardiovascular health have been obtained from *in vitro* or animal studies.

A limitation of the present study is the lack of available blood samples between 4 h and 10 h after study product intake for measuring hesperidin metabolites. In line with this, we were not able to calculate an accurate $AUC_{0\text{-}10h}$. However, as we regularly obtained blood samples between 0 h and 4 h after study product intake and collected full urine output over 24 h, we could still reliably measure and interpret the bioavailability of hesperidin 2S in this study.

In conclusion, the results of this study demonstrate that the bioavailability of micronized hesperidin 2S is higher compared to standard non-micronized hesperidin in healthy individuals. The enhanced bioavailability may positively affect its biological activity. Owing to a great interindividual diversity in intestinal environment, the bioavailability, biological activity and systemic health promoting effects of a polyphenolic compound can be highly variable within the human population.

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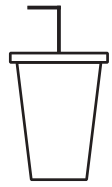
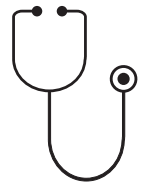

chapter 6

Randomized clinical trial on the efficacy of hesperidin 2S on validated cardiovascular biomarkers in healthy overweight individuals

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American Journal of Clinical Nutrition 2016; 104: 1523-33

Abstract

Background

Endothelial dysfunction (ED) is involved in the development of atherosclerosis. Hesperidin, a citrus flavonoid with antioxidant and other biological properties, potentially exerts beneficial effects on endothelial function (EF).

Objective

We investigated the effect of hesperidin 2S supplementation on EF in overweight individuals.

Design

This was a randomized, double-blind, placebo-controlled study in which 68 individuals were randomly assigned to receive hesperidin 2S (450 mg/d) or a placebo for 6 wk. At baseline and after 6 wk of intervention, flow-mediated dilation (FMD), soluble vascular adhesion molecule-1 (sVCAM-1), soluble intracellular adhesion molecule-1 (sICAM-1), soluble P-selectin (sP-selectin), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were assessed. Acute, reversible ED was induced by intake of a high-fat meal (HFM). A second FMD scan was performed 2 h postprandially, and adhesion molecules were assessed 2 and 4 h postprandially. An additional exploratory analysis was performed in subjects with baseline FMD $\geq$ 3%.

Results

No significant change in fasting or postprandial FMD was observed after 6 wk of hesperidin intake compared with placebo intake. However, there was a trend for a reduction of sVCAM-1, sICAM-1, sP-selectin, SBP, and DBP after 6 wk of hesperidin treatment. In the FMD $\geq$ 3% group, hesperidin protected individuals from postprandial ED (P = 0.050) and significantly downregulated sVCAM-1 and sICAM-1 (all $P \leq 0.030$). The results reported in the current article were not adjusted for multiplicity.

Conclusions

Six weeks of consumption of hesperidin 2S did not improve basal or postprandial FMD in our total study population. There was a tendency toward a reduction of adhesion molecules and a decrease in SBP and DBP. Further exploratory analyses revealed that, in subjects with baseline FMD $\geq$ 3%, hesperidin 2S improved ED after an HFM and reduced adhesion molecules. These results indicate the cardiovascular health benefits of hesperidin 2S in overweight and obese individuals with a relatively healthy endothelium.

Introduction

The vascular endothelium is a monolayer of cells between the lumen and vascular smooth muscle cells. The vascular endothelium plays a crucial role in the maintenance of vascular homeostasis by keeping a refined balance between vasodilation and vasoconstriction.[1] Nitric oxide (NO) is of pivotal importance in the regulation of the arterial tone and, thus, endothelial function (EF).[2] Chronic exposure to cardiovascular disease (CVD) risk factors [*i.e.*, obesity,[3] hypertension,[4] diabetes mellitus [5,6]] and to oxidative stress impairs the endothelium[7] and may lead to endothelial dysfunction (ED). ED is often manifested by an impaired capacity of the vascular endothelium to dilate as result of insufficient NO bioavailability.[8] ED has a key role in the development and progression of atherosclerosis, in which circulating leukocytes are recruited to the vascular endothelium and further migrate into subendothelial spaces, which are mediated by cellular adhesion molecules [*i.e.*, soluble vascular adhesion molecule-1 (sVCAM-1), soluble intracellular adhesion molecule-1 (sICAM-1), soluble E-selectin (sE-selectin), and soluble P-selectin (sP-selectin)].[9,10] Hence, interventions aimed at improving ED may provide an attractive approach in the prevention of atherosclerosis and CVD.

Hesperidin, which is a flavonoid that is abundantly present in the peels of citrus fruit, may have such potential. Hesperidin (hesperetin-7-O-rutinoside), to a large extent, reaches the colon intact where it is subsequently deglycosylated by intestinal microbiota to produce the active aglycone hesperitin.[11,12] Hesperitin has previously been shown to upregulate endothelial NO-synthase activity and, therefore, has intrinsic potential to protectively affect EF.[13] However, low solubility limits the bioavailability and resulting biological activity in humans. Furthermore, although natural products typically contain high concentrations of the most-active form of hesperidin (hesperitin-7-O-rutinoside 2S), typical hesperidin extracts mainly contain the less-active 2R enantiomer.[14,15] To overcome both the limited bioavailability and the typically suboptimal enantiomer composition, we studied a hesperidin 2S extract (Cordiart; BioActor BV) that has a specific enantiomer configuration that is similar to natural hesperidin and was specifically developed for having improved bioavailability profiles.

The primary aim of our study was to investigate the efficacy of 6 wk of supplementation of the specific hesperidin 2S formulation on EF as measured with the use of flow-mediated dilation (FMD) in a population of healthy but overweight individuals. We selected overweight individuals because this condition is associated with chronic low-grade inflammation, and in these subjects, mild ED regularly occurs.[16] Because we recruited from the general population rather than from a selection of a population with proven ED, we induced temporary, reversible ED by providing participants with a high-fat meal (HFM) during test days.[17,18] This method allowed us to further assess the effect of a single dosage of hesperidin 2S on HFM-stressed EF. Our secondary aim was to investigate the 6-wk

effects of this formulation on circulating adhesion molecules, blood pressure (BP), and metabolic markers. We hypothesized that 6 wk of supplementation with this specific hesperidin 2S extract would improve EF, downregulate adhesion molecules, lower systolic blood pressure (SBP) and diastolic blood pressure (DBP), and enhance metabolic control.

Methods

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center+ and was conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (1998). The study was performed at the Maastricht University Medical Center+ from March 2014 to October 2014. All participants gave written informed consent before participation. This trial was registered at clinicaltrials.gov as NCT02228291.

Subjects

Healthy volunteers who were aged 18–65 y and had BMI (in kg/m^2) between 25 and 35 were recruited through advertisements in the local media. Key exclusion criteria were as follows: type 2 diabetes mellitus (defined as fasting plasma glucose concentration ≥ 7 mmol/L); any medical condition that might have interfered with the study and/or jeopardized the health status of the participant; smoking; abuse of alcohol (>20 alcoholic U/wk) and recreational drugs; no consistently stable body weight for ≥ 3 mo (±3 kg); plans to lose weight or follow an energy-restriction diet during the study period; any medication, vitamin supplements, mineral supplements, or antioxidant supplements during the study or in the 90 d before start of the study; use of antibiotics in the 90 d before the start of the study; pregnancy and lactation; history of any side effects toward intake of flavonoids or citrus fruit; and failure to comply with prohibited intake of hesperidin-rich food products and food products that influence EF. For the exploratory subgroup analysis, the following additional exclusion criterion was applied: baseline FMD <3%. The cutoff of 3% was based on data from a large meta-analysis on EF.[19] In this study, it was illustrated that FMD values <3% were associated with a Framingham Risk Score of ~40%, thereby making improvements of a short-term intervention, as in our study, on a chronically impaired endothelium rather unlikely. In addition, the repeatability of low FMD values is decreased, thereby making results of statistical tests that evaluate FMD differences less robust.[20,21] During the study period, subjects consumed their habitual diets. At the time of inclusion, all subjects were informed about the prohibited hesperidin-rich food products and food products that might potentially influence EF.

Design and intervention

This study was designed as a randomized, placebo-controlled, double-blind, parallel-group study (Supplemental Figure S6.1). Each subject underwent 2 test days. Participants were randomly assigned in a double-blind fashion to one of the following 2 intervention arms: hesperidin 2S or placebo (cellulose). An independent and blinded person generated the list of random assignment with the use of a computerized procedure. All participants and investigators remained blind to the treatment until all analyses were completed. Participants were requested to abstain from strenuous physical exercise, consumption of alcohol and caffeine, and intake of vitamin C on the day before each test day.[22] Assessments took place in a quiet, temperature-controlled (20–24°C) room.

After an overnight fast, subjects arrived at the study site, and anthropometric measurements (height, body weight, and waist-to-hip circumference) were performed. After 30 min of rest in the supine position, subjects underwent BP measurements. Next, we assessed the first ultrasound FMD scan of the right brachial arterial lumen to test EF. After completion of the ultrasound scan, an intravenous catheter was inserted into the antecubital vein of the left arm, and blood samples were collected. Subjects consumed a standardized HFM to induce acute, mild, reversible ED. Synchronously with the HFM, one dosage of hesperidin 2S or placebo was ingested. Exactly 2 h after meal ingestion, a second FMD scan was performed. Blood sampling was performed at 2 and 4 h after the meal. At the end of the test day, the intravenous catheter was removed, and participants received the study products for the entire study period. After 6 wk of daily supplementation, test day 2 was organized. Measurements were identical to the measurements performed on test day 1. Figure 6.1 shows the timeline of test days 1 and 2. To assess FMD variability and intrasonographer reproducibility in this specific study, a subset of 10% of participants underwent repeat FMD scans at study baseline and after 6 wk of supplementation. To assess compliance, participants were asked to save the empty and (partly) full blister packs and to return them at the last visit. Also, in the first week and last week of the study period, participants were asked to fill out a 3-d food record to assess whether they maintained their dietary habits throughout the study period.

The primary aim of our study was to investigate the 6-wk effects of the specific hesperidin 2S formulation on fasted and postprandial EF as measured with the use of FMD in healthy but overweight individuals. The secondary aim was to investigate the 6-wk effects of this formulation on circulating adhesion molecules, BP, and metabolic markers. In addition, an exploratory analysis was performed to assess the efficacy of this hesperidin 2S extract on FMD with the exclusion of subjects with strongly decreased baseline FMD values <3%. Because plasma concentrations of adhesion molecules are closely related to FMD values[23], and factors that influence BP have been proposed to operate via mechanisms that are

linked to the release of substances from the endothelium[24], the efficacy of hesperidin 2S regarding these variables was assessed in the FMD ≥3% group.

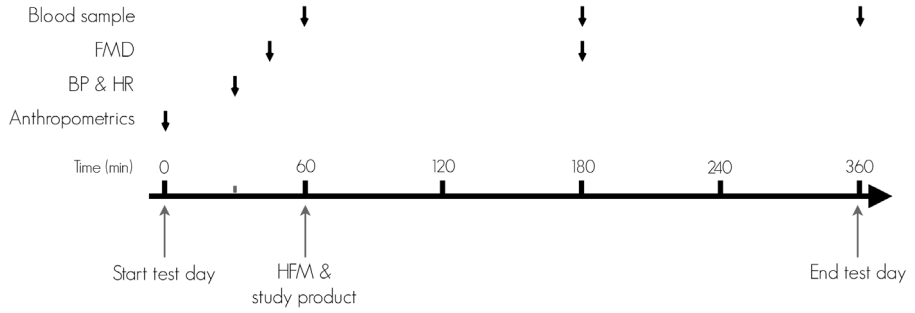

Figure 6.1 Timeline of test days 1 and 2. Blood samples, FMD, BP and HR values, and anthropometric measures were collected at different time points as indicated. BP, blood pressure; FMD, flow-mediated dilation; HFM, high-fat meal; HR, heart rate.

Hesperidin and placebo

Hesperidin 2S (450 mg supplied as 500 mg Cordiart) was extracted from the Citrus sinesis peel, which contained both the S and R enantiomers in the natural 4:1 S:R ratio of hesperidin. Cellulose (500 mg microcrystalline cellulose; Aminolabs) was administered as the placebo. The study products were formulated into capsules, each of which contained 250 mg of study product or placebo. Subjects were asked to ingest 2 capsules each morning with 200 mL H_2O just before the consumption of breakfast for 6 wk.

HFM

The meal provided during test days was a standardized HFM (2.6 MJ; energy divided as 61% fat, 33% carbohydrates, and 6% protein). The meal consisted of 125 g whole milk (Friesche Vlag; FrieslandCampina), 15 g sucrose (Van Gilse), 50 g whipped cream (Friesche Vlag; FrieslandCampina), and 150 g vanilla ice cream (Hertog; Unilever Nederland).

FMD

EF was assessed with the use of B-mode ultrasound brachial artery FMD as described previously.[25] Two trained and certified sonographers performed the strictly standardized scan protocol. The effect of the intervention on FMD was assessed both without and with an HFM challenge. For quality-control (QC) purposes, on top of FMD measurements that were performed during both study visits, in ~30% of participants, repeat-fasting QC scans

were performed at both visits (*e.g.*, 6 scans in total). In a given participant, all FMD measurements were done by the same sonographer. Thus, the sonographer performed both FMD measurements on test days 1 and 2 and, if applicable, the QC FMD measurements of the participant. B-mode ultrasound scans of the diameter of the right brachial artery lumen were obtained with the sue of a Sonix Touch ultrasound machine (Ultrasonix) that was equipped with a 7.5-MHz linear array probe as well as a study-specific, ultrasound-instrument scan protocol and settings as applied by the imaging core laboratory (Imagelabonline & Cardiovascular). A special probe-holder arm rest was used to optimize the stability and standardization of the position of the ultrasound probe. A BP cuff was placed around the forearm ~1 cm below the antecubital fossa. At rest, measurements were obtained during 1 min after which the BP cuff was inflated to 250 mm Hg to occlude the brachial artery. After 5 min of forearm ischemia, the cuff was released, and brachial diameter measurements were continuously recorded for 3 min after cuff release. Image acquisition was electrocardiogram gated on the R wave, and the ultrasound images were saved in a Digital Imaging and Communications in Medicine clip. For QC purposes and to allow for repeat scans in the case of a methodologic failure, all study scans, together with their completed web-based scan forms, were transferred securely to the core laboratory after finishing a scan. For all scans, the imaging technicians of the core laboratory instantly provided an evaluation and feedback of the sonographer scan quality. To be accepted, a precuff occlusion difference diameter of the brachial artery between follow-up study scans had to be visualized in the ≤ 0.2-mm range.

Image analysis

Accepted scans were securely stored locally on the hard-disk drive of the ultrasound instrument and automatically backed-up on the Imagelabonline & Cardiovascular core laboratory server. Available scans were batched in a per-subject fashion. The image-analysis technician was dedicated to analyze all scans of a subject and was blind to the clinical information, date of the scan, and intervention. Validated brachial FMD software (Brachial Analyzer version 6.2.3; Medical Imaging Applications) was used. In summary, the technician selected a region of interest in the longitudinal image of the brachial artery walls and lumen. The Brachial Analyzer software performed an automated tracing of the lumen-wall interfaces,[26] quantifying the brachial artery lumen diameter before cuff occlusion and after cuff release. Mean preocclusion diameters and the maximum after-cuff-release diameters were used to calculate[22] the absolute (peak diameter minus baseline diameter) and FMD [(peak diameter - baseline diameter)/baseline diameter] * 100%. After the first batched series of 30 subjects, the remaining scans of subjects were analyzed.

Adhesion molecules

During a test day, blood samples were collected in K2EDTA-coated evacuated tubes for the measurement of adhesion molecules when subjects were fasted and at 2 and 4 h after HFM intake, respectively. These samples were centrifuged at 1100 x *g* for 15 min at 4°C to obtain the plasma, which was divided into aliquots and kept frozen at −80°C until further analysis. Circulating sVCAM-1, sICAM-1, and sE-selectin in plasma were determined by commercially available ELISA kits and standards, according to the manufacturer's protocol (R&D System Europe Ltd.). Circulating plasma sP-selectin was measured by a quantitative sandwich immunoassay technique (R&D System Europe Ltd.).

Blood pressure measurements

BP was monitored with the use of a semicontinuous BP-monitoring device (Omron) on the upper left arm. At each occasion, in total, 4 BP measurements were performed. The first measurement was discarded, and the remaining 3 measurements were averaged. Measurements took place at baseline and at the end of the study period. During a test day, BP was measured before HFM consumption.

Blood lipids, glucose and insulin analysis

During a test day, blood samples were taken for the assessment of metabolic variables when subjects had fasted. Serum concentrations of glucose, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides were measured with the use of spectrophotometry (Cobas 6000 analyzer series; Roche Diagnostics). Plasma insulin was determined with the use of a luminescence-enhanced immune-enzymatic assay (Immulite 2000 immunoassay system; Siemens Healthcare). Insulin sensitivity was estimated with the use of a quantitative insulin-sensitivity check index[27] as: 1 / (log insulin 0 h + log glucose 0 h).

Statistical analyses

The primary outcome of the study was the effect of 6 wk of hesperidin 2S supplementation on FMD compared with that of a placebo. As our secondary outcome, we investigated the effect of 6 wk of hesperidin 2S supplementation compared with that of placebo on adhesion molecules, BP, and metabolic variables. In addition, the postprandial effect of hesperidin 2S on EF was investigated. The sample size was determined for the primary outcome of the study with a significance-level $\alpha = 0.05$ and a power of 80%. On the basis of previous work,[28] we calculated that a sample size of 62 subjects would be required to detect a mean ± SD difference in FMD of 1.45% ± 2.0%. Baseline characteristics are

presented as means ± SDs for numerical variables and numbers (percentages) for categorical variables. Differences in FMD, BP, and blood variables between intervention groups (hesperidin 2S or placebo) were assessed with the use of linear mixed models with the group (placebo and hesperidin 2S), time (0 and 6 wk for the 6-wk hesperidin effect; 0, 2, and 4 h for the postprandial hesperidin effect), and group x time as fixed factors, whereby a marginal model with an unstructured covariance structure was used for repeated measures (no random effects). The linear mixed model accounts for the correlation between repeated measures and missing data for which a likelihood approach was used with the assumption of data missing at random. The estimated means ± SEMs that were obtained from this model were presented for each group and each time point. A 2-sided $P \leq 0.05$ was considered statistically significant. The statistical analysis was performed with the use of IBM SPSS Statistics for Windows software (version 21.0; IBM).

Results

Study subjects

Initially, 68 healthy volunteers were enrolled in the study of whom 63 subjects completed the entire study protocol. Three participants were included and randomly assigned to a treatment but never started participation because of personal reasons. From these participants, only baseline characteristics were available. Another participant terminated the study prematurely because of an occurrence of a skin rash, which disappeared after stopping the study product. We excluded 1 participant during the study period because of overt noncompliance. The FMD ≥ 3% group entailed 48 subjects with baseline FMD ≥ 3%. Baseline characteristics in the total study population and in the FMD ≥ 3% group are presented in Table 6.1. Dietary habits of the participants were maintained throughout the entire study period.

Table 6.1 Baseline characteristics in the total study population and FMD ≥ 3% group.[1]

	Total population *(n = 68)*	Placebo *(n = 34)*	Hesperidin *(n = 34)*	FMD ≥ 3% group *(n = 48)*	Placebo *(n = 24)*	Hesperidin *(n = 24)*
Age, y	53 ± 14[2]	53 ± 14	54 ± 15	50 ± 15	50 ± 14	50 ± 16
Sex, M/F, *n*	29/39	12/22	17/17	17/31	5/19	12/12
WHR	0.94 ± 0.06	0.95 ± 0.06	0.94 ± 0.06	0.94 ± 0.06	0.94 ± 0.06	0.95 ± 0.05
BMI, kg/m^2	29.0 ± 2.6	29.7 ± 2.8	28.2 ± 2.2	29.1 ± 2.8	29.8 ± 3.0	28.5 ± 2.4

[1]FMD, flow-mediated dilation; WHR, waist-to-hip-ratio. [2]Mean ± SD (all such values)

FMD

Effect of HFM

As shown in Table 6.2, a nonsignificant decrease in FMD was observed after the ingestion of an HFM in both the placebo and the hesperidin group. The size of the decrease in postprandial FMD did not significantly differ between the 2 intervention groups (*P* = 0.670).

Effects of HFM in subjects with FMD ≥ 3%

In the FMD ≥ 3% group, the ingestion of an HFM reduced FMD in both the placebo and hesperidin groups. The size of the decrease in postprandial FMD did not significantly differ between the 2 intervention groups (*P* = 0.618) (Table 6.2).

Table 6.2 Postprandial FMD after a single dosage in the total study population (placebo: *n = 32;* hesperidin: *n = 33*) and in the FMD ≥ 3% group (placebo: *n = 24;* hesperidin: *n = 24)* at baseline (test day 1).[1]

	Placebo		Hesperidin		*P* value
	Baseline fasting	2h after HFM	Baseline fasting	2h after HFM	
FMD (%)					
Total study population	5.57 ± 0.51	5.08 ± 0.53	4.50 ± 0.51	4.23 ± 0.51	0.670
≥ 3% group	7.03 ± 0.48	6.37 ± 0.55	5.37 ± 0.48	5.04 ± 0.54	0.618

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values. There was no significant FMD decrease over time within intervention groups. FMD, flow-mediated dilation; HFM, high-fat meal.

Six-week effect: fasted

Fasted FMD did not change after 6 wk of hesperidin 2S supplementation compared with placebo intake (*P* = 0.881) (Table 6.3).

Six-week effect: fasted, in subjects with FMD ≥ 3%

No significant difference was observed in fasted brachial FMD between both interventions after 6 wk of supplementation (*P* = 0.872) (Table 6.3).

Table 6.3 Fasted FMD at baseline and after 6 wk of supplementation in total study population (placebo: *n = 32;* hesperidin: *n = 33*) and in the FMD ≥ 3% group (placebo: *n = 24;* hesperidin: *n = 24*).[1]

	Placebo		Hesperidin		*P* value
	Baseline	End	Baseline	End	
FMD (%)					
Total study population	5.57 ± 0.51	5.43 ± 0.47	4.50 ± 0.51	4.29 ± 0.47	0.881
FMD ≥ 3% group	7.03 ± 0.48	6.47 ± 0.49	5.37 ± 0.48	4.89 ± 0.50	0.872

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values.. FMD, flow-mediated dilation.

Six-week effect: postprandial

Postprandial FMD at 6 wk also did not significantly differ compared with the placebo (*P* = 0.207) (Table 6.4).

Six-week effect: postprandial, in subjects with FMD ≥ 3%

After inducing ED by an HFM, hesperidin 2S significantly protected postprandial FMD from impairment compared with the effect of the placebo (*P* = 0.050) (Table 6.4). However, this effect was nonsignificant after any correction for multiple testing (*e.g.*, Bonferroni correction for 8 tests performed as shown in Table 6.4).

Variability assessment

In total, 34 QC FMD scans were available of 19 study participants. The overall (mean) absolute difference between study FMD measurements and QC FMD measurements of complete FMD measurement sets was 0.87%.

Table 6.4 Postprandial FMD after 6 wk of supplementation (test day 2) in the total study population (placebo: *n = 32*; hesperidin: *n = 33*) and in the FMD ≥ 3% group (placebo: *n = 24*; hesperidin: *n = 24*).[1]

	Placebo		Hesperidin		*P* value
	Baseline fasting	2h after HFM	Baseline fasting	2h after HFM	
FMD %					
Total study population	5.57 ± 0.51	5.08 ± 0.53	4.21 ± 0.48	4.38 ± 0.51	0.207
FMD ≥ 3% group	6.47 ± 0.49	5.81 ± 0.56	4.85 ± 0.51	5.28 ± 0.59	0.050

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values.. FMD, flow-mediated dilation; HFM, high-fat meal.

Adhesion molecules

Six-week effect: fasted

Changes in basal circulating adhesion molecules after 6 wk of intervention are shown in Table 6.5. A borderline significant downregulation of the adhesion molecules sVCAM-1 (*P* = 0.052) and sICAM-1 (*P* = 0.056) was observed after 6 wk of supplementation with hesperidin 2S compared with the placebo. Furthermore, a downregulation of sP-selectin was seen in the hesperidin group, but this effect was NS (*P* = 0.086). No significant differences in sE-selectin concentrations were observed between the 2 treatments (*P* = 0.246).

Six-week effect: fasted, in subjects with FMD ≥ 3%

Although a significant downregulation of basal circulating sVCAM-1 (P = 0.030) and sICAM-1 (P = 0.017) was observed after 6 wk of hesperidin 2S supplementation compared with placebo intake, these effects were no longer significant after any multiple-testing correction (*e.g.,* Bonferroni correction with 5 tests performed as shown in Table 6.5). Concentrations of sE-selectin and sP-selectin were not significantly different between the 2 intervention groups (P = 0.211 and P = 0.936, respectively).

Table 6.5 Basal circulating adhesion molecules at baseline and after 6 wk of supplementation in the total study population (placebo: *n = 32*; hesperidin: *n = 33*) and in the FMD ≥ 3% group (placebo: *n = 24*; hesperidin: *n = 24*).[1]

	Placebo		Hesperidin		*P* value
	Baseline	End	Baseline	End	
Total study population, ng/mL					
sVCAM-1	214 ± 10	215 ± 10	210 ± 10	190 ± 10	0.052
sICAM-1	107 ± 5	107 ± 5	110 ± 5	100 ± 5	0.056
sE-selectin	12 ± 1	11 ± 2	11 ± 1	12 ± 2	0.246
sP-selectin	78 ± 5	83 ± 5	94 ± 5	83 ± 5	0.086
FMD ≥ 3% group, ng/mL					
sVCAM-1	210 ± 11	215 ± 12	208 ± 11	184 ± 12	0.030
sICAM-1	104 ± 5	107 ± 5	114 ± 5	103 ± 5	0.017
sE-selectin	12 ± 2	12 ± 2	11 ± 2	11 ± 2	0.936
sP-selectin	76 ± 5	84 ± 6	94 ± 5	87 ± 6	0.211

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values.. FMD, flow-mediated dilation; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sP-selectin, soluble P-selectin; sVCAM-1, soluble vascular cell adhesion molecule-1.

Six-week effect: postprandial

The effect on postprandial adhesion molecule concentrations at 6 wk was not significantly different between both interventions (all $P \geq 0.154$) (Table 6.6).

Six-week effect: postprandial, in subjects with FMD ≥ 3%

In the subgroup analysis, no significant differences in postprandial adhesion molecule concentrations were observed between both interventions (all $P \geq 0.123$) (Table 6.6).

Table 6.6 Postprandial circulating adhesion molecules after 6 wk of supplementation (test day 2) in the total study population (placebo: *n = 32*; hesperidin: *n = 33*) and in the FMD ≥ 3% group (placebo: *n = 24*; hesperidin: *n = 24*).[1]

	Placebo			Hesperidin			P_1	P_2
	Baseline fasting	2h after HFM	4h after HFM	Baseline fasting	2h after HFM	4h after HFM		
Total study population, ng/mL								
sVCAM-1[2]	215 ± 10	208 ± 9	200 ± 10	190 ± 10	185 ± 9	180 ± 10	0.686	0.462
sICAM-1[2]	107 ± 5	105 ± 5	102 ± 5	100 ± 5	98 ± 5	96 ± 5	0.857	0.976
sE-selectin[2]	11 ± 2	9 ± 2	10 ± 2	12 ± 2	11 ± 2	11 ± 2	0.154	0.358
sP-selectin	83 ± 5	-	94 ± 5	83 ± 5	-	89 ± 5	-	0.479
FMD ≥ 3% group, ng/mL								
sVCAM-1[2]	216 ± 12	211 ± 10	203 ± 11	184 ± 12	179 ± 10	171 ± 11	0.999	0.988
sICAM-1[2]	107 ± 5	106 ± 6	103 ± 5	103 ± 5	101 ± 6	97 ± 5	0.841	0.756
sE-selectin[2]	12 ± 2	10 ± 2	10 ± 2	11 ± 2	11 ± 2	11 ± 2	0.182	0.123
sP-selectin	84 ± 6	-	96 ± 6	87 ± 6	-	95 ± 6	-	0.555

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values.. *P*1 represents *P* values for the analysis of baseline compared with 2h after the HFM between placebo and hesperidin. *P*2 represents *P* values for the analysis of baseline compared 4h after the HFM between placebo and hesperidin. FMD, flow-mediated dilation; HFM, high-fat meal; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sP-selectin, soluble P-selectin; sVCAM-1, soluble vascular cell adhesion molecule-1. [2]Time by treatment interaction, *P* > 0.050.

Acute effect

After intake of the HFM with one dosage of hesperidin 2S, there was a trend (*P* between 0.05 and 0.10) that was observed for a downregulation of the adhesion molecule sICAM-1 at both 2 h (*P* = 0.088) (Table 6.7) and 4 h (*P* = 0.092) (Table 6.7). No significant postprandial differences were observed regarding sVCAM-1, sE-selectin, and sP-selectin between both study groups (all *P* ≥ 0.193) (Table 6.7).

Acute effect in subjects with FMD ≥ 3%

At baseline, after intake of an HFM with one dosage of hesperidin 2S (Table 6.7), we observed a significant downregulation of sICAM-1 after both 2 h (*P* = 0.035) and 4 h (*P* = 0.050) and a trend for a downregulation of sE-selectin after 4 h (*P* = 0.062). Again, after any multiple testing correction (*e.g.*, Bonferroni correction with 8 tests performed as shown in Table 6.7), these effects were no longer significant. No significant postprandial differences were observed regarding sVCAM-1 and sP-selectin between both study groups (all *P* ≥ 0.111).

Table 6.7 Postprandial adhesion molecules after a single dosage in the total study population (placebo: *n = 32*; hesperidin: *n = 33*) and in the FMD ≥ 3% group (placebo: *n = 24*; hesperidin: *n = 24*) at baseline (test day 1).[1]

	Placebo			Hesperidin			P_1	P_2
	Baseline fasting	2h after HFM	4h after HFM	Baseline fasting	2h after HFM	4h after HFM		
Total study population, ng/mL								
sVCAM-1[2]	214 ± 10	213 ± 9	206 ± 10	210 ± 10	207 ± 9	209 ± 10	0.818	0.489
sICAM-1[2]	107 ± 5	105 ± 4	109 ± 5	110 ± 5	100 ± 4	104 ± 5	0.088	0.092
sE-selectin[2]	12 ± 1	12 ± 2	12 ± 2	11 ± 1	10 ± 2	11 ± 2	0.356	0.998
sP-selectin	78 ± 5	-	83 ± 4	94 ± 5	-	90 ± 4	-	0.193
FMD ≥ 3% group, ng/mL								
sVCAM-1[2]	210 ± 11	215 ± 10	206 ± 11	208 ± 11	199 ± 10	202 ± 11	0.139	0.863
sICAM-1[2]	104 ± 5	103 ± 5	108 ± 6	114 ± 5	102 ± 5	107 ± 6	0.035	0.050
sE-selectin[2]	12 ± 2	12 ± 2	12 ± 2	11 ± 2	10 ± 2	10 ± 2	0.111	0.062
sP-selectin	76 ± 5	-	83 ± 5	94 ± 5	-	91 ± 5	-	0.263

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values.. *P*1 represents *P* values for the analysis of baseline compared with 2h after the HFM between placebo and hesperidin. *P*2 represents *P* values for the analysis of baseline compared 4h after the HFM between placebo and hesperidin. FMD, flow-mediated dilation; HFM, high-fat meal; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sP-selectin, soluble P-selectin; sVCAM-1, soluble vascular cell adhesion molecule-1. [2]Time by treatment interaction, *P* > 0.050.

Blood pressure

Six-week effect: fasted

Results regarding the effect of hesperidin 2S administration on BP are given in Table 6.8. In the hesperidin 2S group, we observed a mean reduction of 5 mm Hg in SBP after 6 wk of intake, whereas in the placebo group, we observed a mean reduction of 2 mm Hg in SBP. DBP was reduced by 2 mm Hg after hesperidin 2S supplementation, whereas DBP increased by 1 mm Hg after placebo supplementation. A trend (*P* between 0.05 and 0.10) toward a lower SBP (*P* = 0.095) and DBP (*P* = 0.095) was observed after 6 wk of hesperidin 2S supplementation compared with placebo intake.

Six-week effect: fasted, in subjects with FMD ≥ 3%

After 6 wk of hesperidin 2S intake, a trend (*P* between 0.05 and 0.10) toward a reduction in both SBP (*P* = 0.051) and DBP (*P* = 0.069) in the FMD ≥ 3% group was observed compared with in the placebo group.

Table 6.8 BP and HR at baseline and after 6 wk of supplementation in the total study population (placebo: *n = 32*; hesperidin: *n = 33*) and in the FMD ≥ 3% group (placebo: *n = 24*; hesperidin: *n = 24*).[1]

	Placebo		Hesperidin		*P* value
	Baseline	End	Baseline	End	
Total study population					
Systolic BP, mmHg	131 ± 3	129 ± 2	135 ± 2	130 ± 2	0.095
Diastolic BP, mmHg	80 ± 2	81 ± 2	83 ± 1	81 ± 2	0.095
HR, beats/min	62 ± 1	61 ± 1	61 ± 1	62 ± 1	0.220
FMD ≥ 3% group					
Systolic BP, mmHg	128 ± 2	127 ± 2	135 ± 2	129 ± 2	0.051
Diastolic BP, mmHg	79 ± 2	79 ± 2	84 ± 2	81 ± 2	0.069
HR, beats/min	62 ± 2	61 ± 2	62 ± 2	63 ± 2	0.256

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values.. BP, blood pressure; FMD, flow-mediated dilation; HR, heart rate.

Blood lipids, glucose and insulin *(six-week effect: fasted)*

Baseline total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, glucose, and insulin were all within normal blood value ranges. These variables were not significantly altered by 6 wk of supplementation with hesperidin 2S compared with the placebo (all $P \geq 0.097$) (Table 6.9). Also, no significant change in insulin sensitivity was observed between intervention groups ($P = 0.225$) (Table 6.9).

Table 6.9 Blood lipids, glucose, insulin, and QUICKI at baseline and after 6 wk of supplementation in the total study population (placebo: *n = 32*; hesperidin: *n = 33*)[1]

	Placebo		Hesperidin		*P* value
	Baseline	End	Baseline	End	
Total cholesterol, mmol/L	5.7 ± 0.2	5.6 ± 0.2	5.5 ± 0.2	5.4 ± 0.2	0.752
LDL cholesterol, mmol/L	3.6 ± 0.2	3.6 ± 0.2	3.5 ± 0.2	3.4 ± 0.2	0.585
HDL cholesterol, mmol/L	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	0.884
Triglyceride, mmol/L	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	0.620
Glucose, mmol/L	5.0 ± 0.1	5.0 ± 0.1	4.9 ± 0.1	5.0 ± 0.1	0.348
Insulin, pmol/L	58 ± 21	53 ± 20	74 ± 21	81 ± 20	0.097
QUICKI	0.42 ± 0.01	0.23 ± 0.01	0.34 ± 0.01	0.42 ± 0.01	0.225

[1]All values are estimated means ± SEMs. Differences between placebo and hesperidin were tested with the use of a linear mixed model with correction for baseline values.. QUICKI, quantitative insulin sensitivity check index.

Discussion

In this trial, the 6-wk effect of hesperidin 2S on EF and CVD risk markers were studied in overweight and obese but healthy individuals. To investigate the effect of treatment on EF in

this healthy population, we performed a stress test and induced reversible ED through the consumption of an HFM. After 6 wk of daily intake of hesperidin 2S, no significant changes in fasted or postprandial FMD were observed. However, a nonsignificant (but nearly significant) downregulation of adhesion molecules sVCAM-1, sICAM-1, and sP-selectin was observed after 6 wk of hesperidin 2S supplementation compared with after placebo intake. Furthermore, hesperidin 2S tended to reduce both SBP and DBP. The deterioration in FMD after consumption of an HFM was attenuated by hesperidin 2S in a group of subjects with baseline FMD ≥ 3%. In addition, in this subgroup, the downregulation in sVCAM-1 and sICAM-1 was significant. However, all significant results were no longer significant after correction for multiple testing.

Atherosclerosis is a complex process involving a number of factors and inflammatory cells interacting throughout different stages of development. This process is initiated by circulating plasma LDL entering the subendothelial space in the blood vessel. LDL is oxidized by reactive oxygen species and upregulates adhesion molecules (*e.g.*, sVCAM-1, sICAM-1, sE-selectin and sP-selectin) on the endothelium and induces the expression of chemotactic agents in endothelial cells.[29] In addition, optimal functioning of the endothelium itself is essential for undisturbed functioning of the cardiovascular system. To study this endpoint, FMD is the gold-standard method for noninvasive EF assessment.[30] FMD is considered a direct and reliable measure of the vascular reactivity of the macrocirculatory system.[31,32] In the current study, we aimed to assess the effect of a specific hesperidin 2S formulation in a generally healthy population. Because this population was not screened for impaired FMD variables, it would have been unlikely that major effects on FMD could have been observed in an unchallenged condition. This unlikelihood was confirmed by the fact that no significant effect was observed on basal FMD after 6 wk of daily hesperidin 2S intake. However, after inducing temporary ED through the consumption of an HFM, hesperidin 2S attenuated the postprandial impairment of FMD in a subgroup of subjects with baseline FMD ≥ 3%. The current data support a promising preventive role for hesperidin in mitigating fed-state oxidative stressors that are known to contribute to early, preclinical atherogenesis in subjects with a relatively healthy endothelium. In the literature, one other human intervention trial, to our knowledge, investigated the effect of hesperidin on FMD and showed a significant increase in basal FMD after 3 wk of hesperidin supplementation. The study was conducted in individuals with metabolic syndrome who were specifically included for moderately impaired FMD.[13] Results from human trials that have examined the effect of other polyphenols on basal FMD have been contradicting, with some trials showing an improvement,[28,33,34] whereas other trials did not observe any effect[35,36] after several weeks of supplementation. Only a few studies investigated the effect on postprandial FMD in humans. Results of some studies[37-41] were in line with ours with some investigators indicating that a change in FMD over time may even be more closely related to a benefit of the intervention and prognosis than of a single FMD measurement.[42]

Plasma adhesion molecule concentrations reflect the degree of EF.[25] In the current study, a borderline significant downregulation of sVCAM-1 and sICAM-1 and a nonsignificant (but nearly significant) downregulation of sP-selectin was observed in the total study population

after 6 wk of hesperidin 2S intake. In the FMD ≥ 3% group, the reduction in plasma sVCAM-1 and sICAM-1 was significant. However, after correction for multiple testing, these results were no longer significant. A reduction of sVCAM-1 and sICAM-1 may reduce plaque formation, whereas the downregulation of sP-selectin may result in a more-balanced control of platelet aggregation. The combination of both effects may result in a synergistic protection of the endothelium against atherosclerosis. Other human trials that have investigated the effect of hesperidin on adhesion molecules have been scarce. Rizza *et al.*[13] showed a downregulation of the adhesion molecule sE-selectin after 3 wk of hesperidin supplementation in metabolic syndrome individuals. However, another study in overweight men did not observe significant differences in circulating adhesion molecules after 4 wk of supplementation with hesperidin.[43] Human trials that have investigated the effect of other polyphenols on adhesion molecules have shown conflicting results.[34,44-48] We anticipated seeing the effects of repeated intake of hesperidin for a certain period. The study design allowed us to also assess the effect of a single dosage of hesperidin 2S. In the FMD ≥ 3% group, the postprandial downregulation of sICAM-1 was observed after 1 dose of hesperidin 2S in response to a fat load. This observation is in line with other literature.[47,49-51]

The BP-lowering effect of hesperidin, and of polyphenols more generally, has been shown in several human-intervention studies[28,34,43,52-54] although the effect has not been confirmed in all studies.[13,33,45] Possible mechanisms by which polyphenol-rich food products lower BP involve an increase in the NO-synthase concentration[55,56] or the inhibition of the angiotensin-converting enzyme.[57,58] As previously discussed, the mitigation of fed-state oxidative stressors and the downregulation of molecules by 6 wk of hesperidin 2S use might reflect an improved functionality of the endothelium, which is capable of producing NO, with a consequent vasodilatation and decrease in BP. In this study, a nonsignificant (but nearly significant) reduction in both SBP and DBP was observed in the total study population and in the subgroup. There is a linear relation between BP and mortality from stroke and ischemic heart disease that underscores the importance of BP control.[59] The hesperidin-induced lowering of SBP (5 mm Hg) may be of clinical relevance because reductions of this magnitude are associated with a decrease in mortality of 14% from stroke and 9% from coronary heart disease.[60] In addition, we observed a hesperidin-induced reduction of 2 mm Hg in DBP, which has been calculated to result in a 17% decrease in the prevalence of hypertension, a 15% reduction in risks of stroke and transient ischemic attacks, and a 6% reduction in risk of coronary heart disease in a general population.[61]

Obesity is typically associated with a wide cluster of metabolic alterations including dyslipidemia and glucose homeostasis disorders. Both in vitro and in vivo studies have shown beneficial effects of hesperidin treatment on cholesterol[62-65] and glucose metabolism.[63,66,67] In the current study, supplementation with hesperidin 2S in overweight but otherwise healthy volunteers did not affect systemic glucose, insulin, and lipid concentrations. This finding was not unexpected, because the participants had completely normal values at the start of the trial.

Because we aimed to work with a broad, representative population rather than screening for a specific suboptimal population, we applied a unique test design to evaluate both the acute and 6-wk effects of hesperidin 2S. Some potential shortcomings should be considered. First, brachial FMD was used for measuring EF; an extrapolation of our findings to the coronary circulation should be made with caution. However, there has been extensive

evidence on the correlation of EF in the 2 vascular beds.[68,69] Second, to assess the postprandial effect of hesperidin 2S, we chose to perform the postprandial FMD 2 h after intake of the HFM. It is possible that we missed an effect between the time of intake and 2 h after intake. Furthermore, we did not fully control the diets of our participants. We instructed the subjects to maintain their habitual diets during the study period but to abstain from intakes of hesperidin-rich foods. We aimed to assess the effect of hesperidin 2S when supplementing the habitual diet, which is in line with future applications.

In conclusion, 6 wk of hesperidin 2S supplementation in healthy, overweight individuals did not improve basal or postprandial FMD. However, the supplementation appeared to reduce circulating adhesion molecules and tended to lower both SBP and DBP. In a subset of subjects with baseline FMD ≥ 3%, 6 wk of hesperidin 2S intake protected them from a deterioration in FMD after intake of an HFM. In addition, in this subgroup, the reduction in adhesion molecules was significant. Further research to confirm these exploratory findings is warranted. However, these results reflect the potential preventive therapeutic properties of hesperidin 2S. These properties were not observed when looking at the population as a whole, probably because a number of participants had such a deteriorated endothelium that could not be affected by an intervention. Our exploratory findings suggest that hesperidin 2S might be beneficial in individuals with a relatively healthy and, therefore, favorably modifiable endothelium. This possibility underlines the need for early diagnosis and reveals the opportunities for the cardiovascular health benefits of hesperidin 2S in preventive medicine.

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Supplemental material

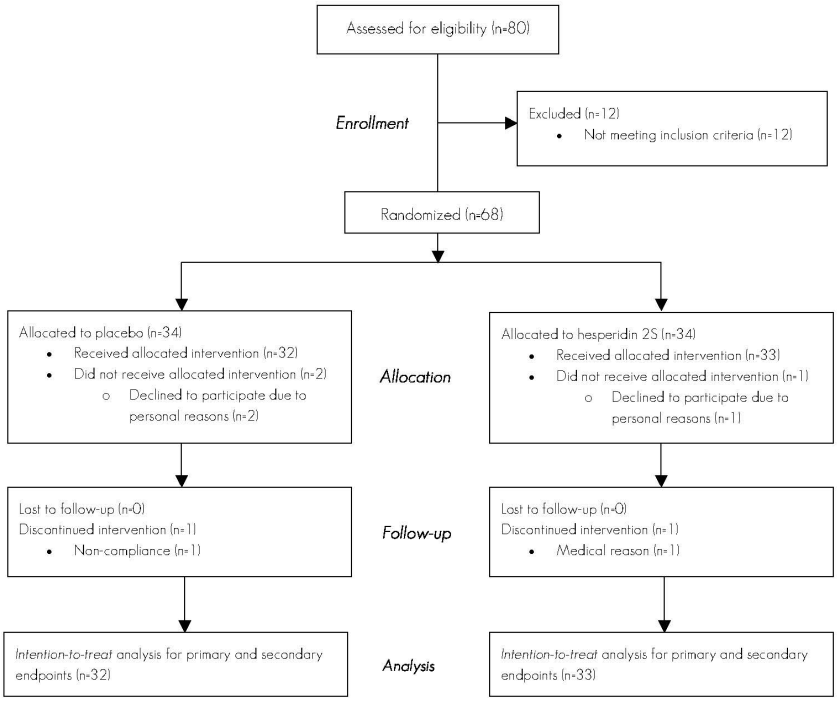
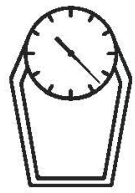


Figure S6.1 CONSORT flow diagram.



chapter 7

Effect of hesperidin 2S supplementation on systemic and intestinal metabolic parameters in subjects at risk for metabolic syndrome: a randomized controlled trial

Salden BN, Troost FJ, Possemiers S, Stevens YR, Masclee AA

Abstract

Background

The intestinal ecosystem plays an important role in host homeostasis. Obesity-related changes in gut microbiota composition and microbial activity may promote the development of metabolic disturbances, such as insulin resistance. Hesperidin, a citrus polyphenol with antioxidant and other biological properties, is poorly absorbed in the small intestine and therefore reaches the colon, where it interacts with the colonic microbiota. In line with this, it may therefore exert beneficial effects on gastrointestinal (GI) and systemic metabolic disturbances.

Objective

Primary aim of the study was to investigate the effect of 12 wks hesperidin 2S supplementation on glucose regulation. Secondly, we aimed to investigate the effect of hesperidin 2S on modulation of the intestinal environment and on systemic metabolic parameters in subjects with features of metabolic syndrome (MetS).

Design

In this randomized, double-blind, placebo-controlled study, 50 individuals were randomly assigned to groups receiving hesperidin 2S (*Citrus sinensis* extract; 450 mg/d) or placebo for 12 wks. Measurements at baseline and after 12 wks intervention included oral glucose tolerance test (OGTT), blood lipids, blood pressure (BP), fecal short-chain fatty acids (SCFA) and fecal calprotectin. Additionally, after 6 wks intervention fasting glucose, insulin, blood lipids and BP were measured.

Results

No significant changes in fasting glucose and insulin and in $AUC_{0\text{-}120min}$ glucose and $AUC_{0\text{-}120min}$ insulin were observed after 12 wks hesperidin intake, compared to placebo. While hesperidin 2S did not quantitatively affect total fecal SCFA concentrations, it beneficially altered the SCFA profile by inducing a shift from acetate to butyrate ($P = 0.020$). Fecal calprotectin showed a near-significant reduction after 12 wks hesperidin supplementation, compared to placebo ($P = 0.058$). No significant changes in blood lipids and BP were observed after hesperidin intake, compared to placebo.

Conclusions

Twelve weeks consumption of hesperidin 2S beneficially altered the fecal SCFA profile and calprotectin concentrations, without affecting glucose regulation, blood lipids or BP in subjects with features of MetS.

Introduction

The rapidly growing prevalence of obesity, and its metabolic and gastrointestinal (GI) consequences, is a major public health concern.[1] Hence, developing new preventive and therapeutic strategies is important. In this respect, the use of polyphenols appears to be an attractive approach as these compounds are potent antioxidants exerting anti-inflammatory, anti-diabetic, anti-hypertensive and antihyperlipidemic effects.[2] Hesperidin (hesperetin-7-O-rutinoside), a polyphenol abundantly present in the peels of citrus fruits, is such a promising compound. It showed to decrease the gene expression of cyclooxygenase-2, demonstrating its anti-inflammatory potential.[3] Furthermore, it significantly improved hyperglycemia by increasing glucose utilization in a diabetic mice model.[4] In humans, three weeks daily supplementation of hesperidin caused a trend towards improving insulin resistance in MetS patients.[5] Additionally, it showed to exert lipid lowering activity in hyperlipidemic rats[6] and hyperlipidemic humans.[7] Also blood pressure (BP) was reduced after hesperidin treatment, both in spontaneously hypertensive rats[8] and in humans.[9] Obesity and metabolic syndrome (MetS) have also been associated with changes in gut health, mainly caused by alterations in the composition of gut microbiota, microbial and metabolic activity.[10,11] Interestingly, recent data suggest that different polyphenolic compounds, including hesperidin, are able to modulate the gut microbiome structure and function, thereby beneficially affecting gut health.[12,13] As hesperidin is poorly absorbed in the small intestine, a major fraction reaches the colon where it indeed can interact with the intestinal microbiota.[14] However, low solubility of hesperidin limits its bioavailability and biological activity *in vivo*, including local effects in the intestine.[15] Furthermore, while natural products contain high concentrations of the most active form of hesperidin (hesperitin-7-O-rutinoside 2S), typical hesperidin extracts on the market mainly contain the less active 2R enantiomer.[16]

This study was conducted to investigate the effect of 12 wks supplementation of a hesperidin 2S extract (Cordiart), a hesperidin enantiomer which has an improved bioavailability and biological activity, on GI and on systemic metabolic parameters in individuals with features of MetS. We hypothesized that 12 wks hesperidin 2S supplementation will exert a) gastrointestinal effects with changes in metabolic activity of gut microbiota and intestinal inflammation, and b) systemic effects consisting of improvement in glucose regulation, lowering of systolic and diastolic BP, and improvement of blood lipid profile in individuals with features of MetS. Primary aim of our study was to investigate the effect of 12 wks supplementation of hesperidin 2S on glucose regulation, assessed via an oral glucose tolerance test (OGTT). Secondary, we aimed to investigate: 1) gastrointestinal effects, by measuring fecal short-chain fatty acids (SCFA) concentrations and fecal calprotectin and 2) systemic effects, by measuring BP and blood lipids.

Materials and methods

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center (MUMC+) and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study was performed at the MUMC+ from February 2015 to November 2015. All participants gave written informed consent before participation. The trial has been registered in the Clinical Trials register (NCT02610491).

Subjects

Volunteers were recruited by advertising in the local media. Inclusion criteria were age between 18 and 66 yrs, and two of the following: waist circumference >102 cm (males) or >88 cm (females), abnormal fasting serum triglycerides ≥1.7 mmol/l, abnormal fasting serum HDL-cholesterol ≤1.0 mmol/l (males) or ≤1.3 mmol/l (females), systolic BP (SBP) ≥130 mmHg and/or diastolic BP (DBP) ≥85 mmHg, elevated fasting plasma glucose ≥6.1 mmol/l.[17] Exclusion criteria were: diabetes mellitus (defined as fasting plasma glucose of ≥7 mmol/l); any medical condition that might interfere with the study and might jeopardise the health status of the participant; smoking; abuse of alcohol (>20 alcoholic units / week) and drugs; no consistently stable body weight for at least 3 months (±3 kg); plans to lose weight or following energy restriction diet during study period; use of medication / vitamin-, mineral- or antioxidant supplements; use of antibiotics in the 90 days prior to start of the study; pregnancy and lactation; history of any side effects towards the intake of flavonoids or citrus fruits; failure to comply with prohibited intake of hesperidin rich food products. During the study period, subjects consumed their habitual diet. During the trial intake of hesperidin-rich food products was prohibited.

Design and intervention

This study was designed as a randomized, placebo-controlled, double-blind, parallel group study (Online Supplemental Material Figure S7.1). Each subject underwent 3 test days. Participants were randomly assigned in a double-blind fashion to one of the two intervention arms: hesperidin 2S or placebo (cellulose). An independent person generated the randomization list, using a computerized procedure. All participants and investigators remained blind to treatment until all analyses had been completed. From 4 days prior to start of the study until the last test day, participants were instructed to refrain from consuming any hesperidin rich food products. Furthermore, participants were requested to

abstain from strenuous physical exercise and consumption of alcohol on the day before each test day. Assessments took place in a quiet, temperature-controlled (20-24°C) room. After an overnight fast, subjects collected a fecal sample on the first test day. Subsequently, anthropometric measurements (height, body weight, waist-to-hip circumference) were performed and BP was measured. Subsequently, an intravenous catheter was inserted into the antecubital vein of the arm and blood samples were collected. Next, the participant was instructed to ingest 75 g glucose (OranGluc75, Novolab, Geraardsbergen, Belgium) within 5 minutes. Blood samples for glucose and insulin concentrations were collected before and at 30, 60, 90 and 120 minutes after ingestion of the glucose load, respectively. At the end of the test day the intravenous catheter was removed and participants received the study products for the first 6 wks. After 6 wks of daily supplementation, test day 2 was organized. After an overnight fast, BP was measured and blood samples were taken. Again, study products for another 6 wks were provided. Test day 3 was conducted at the end of the supplementation period of 12 wks. Measurements were identical to the measurements performed on test day 1. To assess compliance, participants were asked to save the empty and (partly) full blister packs and to return these at the last visit. Also, in the first and last week of the study period participants were asked to fill out a 3-day food record to assess whether they maintained their dietary habits throughout the study period.

Hesperidin 2S and placebo

Hesperidin 2S (450 mg, supplied as 500 mg Cordiart, BioActor BV, Maastricht, The Netherlands) was extracted from the *Citrus sinesis* immature fruit, containing both the S and R enantiomers in the natural 4:1 S:R ratio of hesperidin. Cellulose (500 mg microcrystalline cellulose; Aminolabs; Hasselt; Belgium) served as placebo. The study products were formulated into capsules, each containing 250 mg study product or placebo. Subjects were asked to ingest two capsules each morning with 200 ml water, just before consuming breakfast, for 12 wks.

Fecal SCFA determination

Fecal samples were collected by the participants and stored at -20°C until handed in at the study site. For SCFA analysis, aliquots of approximately 1 g of fecal samples were diluted and homogenized with 6 ml demineralized water. After removal of the particulate material by centrifugation (10 min, 500 x *g*), the supernatants were stored at -20°C prior to analysis.[18] SCFA concentrations in the supernatants were determined using gas chromatography with flame ionization detector. Total SCFA was defined as the sum of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate. The analysis was identical to the SCFA analysis described by Possemiers *et al.*[19]

Fecal calprotectin

Fecal calprotectin concentration levels were determined using an ELISA for human calprotectin (Hycult Biotechnology, Uden, Netherlands), according to the manufacturer's protocol. Briefly, 5 ml of extraction buffer (0.1 M Tris, 0.15 M NaCl, 1.0 M Urea, 10 mM $CaCl_2H_2O$, 0.1 M Citric Acid and 0.5% BSA, pH 8) was added to 100 mg of feces. The suspension was vortexed and shaken for 30 minutes with a rotating shaker at 4°C. After shaking, the suspension was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was stored at -20°C until analysis.

Glucose / insulin metabolism

Oral glucose tolerance test (OGTT)

Blood samples for measuring glucose and insulin concentrations were collected before and at 30, 60, 90 and 120 minutes after ingestion of the glucose load, respectively. Serum concentrations of glucose were measured by spectrophotometry (cobas 6000 analyzer series, Roche Diagnostics, Indianapolis, USA). Plasma insulin was determined using a luminescence-enhanced immune-enzymatic assay (Immulite 2000 immunoassay system, Siemens Healthcare, Erlangen, Germany). Results are presented as glucose and insulin 120-min AUC ($AUC_{0\text{-}120\ min}$). The $AUC_{0\text{-}120min}$ was calculated by the trapezoidal method.

Homeostasis model assessment of insulin resistance (HOMA-IR)

OGTT values were used for calculating HOMA-IR index, an index of insulin resistance.[20] It was calculated as the product of fasting plasma glucose (mmol/l) and insulin (mU/l) concentrations divided by 22.5.

Quantitative insulin sensitivity check index (QUICKI)

OGTT values were also used for calculating the QUICKI, an index of insulin sensitivity.[21] It was calculated as: 1 / (log insulin 0 h + log glucose 0 h).

Insulin sensitivity index (ISI) – Matsuda[22]

OGTT values were used for calculation the composite ISI, an index of insulin sensitivity. It was calculated as: 10,000 / (√(glucose 0 h * insulin 0 h) * √(glucose mean * insulin mean)).

Blood lipids

Blood samples were collected at baseline, after 6 wks supplementation and at completion of the study at 12 wks. Serum concentrations of total cholesterol, LDL- and HDL-cholesterol and triglycerides were measured by spectrophotometry (cobas 6000 analyzer series, Roche Diagnostics, Indianapolis, USA).

Blood pressure measurements

BP was monitored using a semi-continuous BP monitoring device (Omron, Hoofddorp, The Netherlands) at the upper left arm. On each occasion, after 30 minutes of rest in the supine position, 4 consecutive BP measurements were performed. The first measurement was discarded and the remaining 3 measurements were averaged. Measurements took place at baseline, after 6 wks supplementation and at the end of the study period.

Statistics

The primary outcome of the study was the effect of 12 wks hesperidin 2S supplementation on glucose regulation, assessed via an OGTT, compared to placebo. Secondly, we investigated the effects of 12 wks hesperidin 2S supplementation on local GI parameters: fecal SCFA concentrations and fecal calprotectin, and on systemic metabolic parameters: blood lipids and BP. Sample size was determined for the primary outcome of the study, using a significance level *alpha* = 0.05 and a power of 80%. Based on a study investigating the effect of polyphenols on insulin sensitivity in overweight individuals,[23] we calculated that a sample size of 50 subjects would be required to detect a difference in ISI index of 0.98 ± 1.2% (mean ± SD). Baseline characteristics are presented as mean (SD) for numerical variables and number (%) for categorical variables. Differences in fecal SCFA, fecal calprotectin, glucose regulation, blood lipids and BP between intervention groups (hesperidin 2S and placebo) were assessed using mixed models with group (placebo and hesperidin 2S), time (0, 6 wks and 12 wks) and group*time as fixed factors, where an unstructured covariance structure was used for repeated measures. The linear mixed model accounts for the correlation between repeated measures and missing data, where a likelihood approach was used assuming data missing at random. The estimated means and SEM obtained from this model were presented for each group and each time point. Two-sided *P*-values ≤ 0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY, USA).

Results

Participants

Initially, 53 healthy volunteers were enrolled in the study, of which 50 completed the entire study protocol. Three participants were included and randomized to a treatment but never started participation due to private reasons. Baseline characteristics of the study participants are presented in Table 7.1.

Table 7.1 Baseline characteristics of study participants.[1]

	Total population *(n = 50)*	Placebo *(n = 27)*	Hesperidin 2S *(n = 23)*
Age, yr	51 ± 13[2]	50 ± 13	52 ± 11
Sex, M/F	18/32	9/18	9/14
WHR	0.93 ± 0.07	0.91 ± 0.07	0.94 ± 0.07
BMI, kg/m^2	30.8 ± 3.80	31.4 ± 4.20	30.0 ± 3.20

[1]WHR, waist-to-hip-ratio. BMI, body mass index. [2]Mean ± SD (all such values).

Fecal SCFA

Data on fecal SCFA concentrations are shown in Table 7.2. Twelve wks daily intake of hesperidin 2S did not significantly change fecal concentrations of total SCFA, branched SCFA, and of acetate, propionate and butyrate, compared to placebo. However, the butyrate proportion of the total SCFA significantly increased after 12 wks hesperidin 2S intake (P = 0.031). Furthermore, the butyrate over acetate ratio significantly increased in the hesperidin 2S group, compared to placebo (P = 0.020).

Fecal calprotectin

Fecal calprotectin showed a trend towards a reduction after 12 wks hesperidin 2S supplementation, compared to placebo (P = 0.058, Table 7.2).

Glucose / insulin metabolism

Baseline glucose and insulin concentrations were all within normal blood value ranges. No significant differences were observed in $AUC_{0\text{-}120min}$ for both glucose and insulin between intervention groups (Table 7.3). Neither were significant effects of the intervention found in glucose and insulin concentrations at baseline and at 30 min, 60 min, 90 min and 120 min after glucose load, respectively (data not shown). Consistent with these findings, hesperidin

2S supplementation did not improve insulin sensitivity or reduce insulin resistance after 6 or 12 wks supplementation, compared to placebo.

Table 7.2 Gastrointestinal parameters at baseline and after 12 wks supplementation with placebo or hesperidin 2S.[1]

	Placebo		Hesperidin 2S		*P* value
	Baseline	12 wks	Baseline	12 wks	
Proteins					
Calprotectin (ng/ml)	32.9 ± 7.8[2]	42.5 ± 7.6	47.5 ± 8.7	37.1 ± 8.4	0.058
Short-chain fatty acids					
Total (μmol/g feces)	59.8 ± 5.1	68.2 ± 5.9	72.9 ± 5.5	79.4 ± 6.4	0.806
Branched (μmol/g feces)	3.1 ± 0.3	3.2 ± 0.4	3.6 ± 0.4	3.9 ± 0.4	0.807
Acetate (μmol/g feces)	32.9 ± 3.0	38.4 ± 3.4	39.8 ± 3.3	42.9 ± 3.6	0.625
Propionate (μmol/g feces)	11.1 ± 1.3	12.9 ± 1.6	13.9 ± 1.4	14.1 ± 1.7	0.465
Butyrate (μmol/g feces)	10.4 ± 1.1	10.9 ± 1.3	12.4 ± 1.2	15.0 ± 1.4	0.249
Acetate / total SCFA ratio	0.61 ± 0.01	0.62 ± 0.01	0.61 ± 0.01	0.60 ± 0.02	0.079
Propionate / total SCFA ratio	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.812
Butyrate / total SCFA ratio	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.20 ± 0.01	0.031
Butyrate / acetate ratio	0.32 ± 0.02	0.29 ± 0.02	0.31 ± 0.03	0.35 ± 0.02	0.020

[1] Differences between placebo and hesperidin tested with linear mixed model with correction for baseline values. *P* value represents the p-value for the analysis of baseline versus 12 wks intervention between placebo and hesperidin 2S. [2]Estimated mean ± SEM (all such values).

Blood lipids

Baseline total cholesterol, LDL- and HDL-cholesterol and triglycerides were all within normal blood value ranges. These parameters were not significantly altered by 6 or 12 wks supplementation with hesperidin 2S, compared to placebo (Table 7.3).

Blood pressure

Results regarding the effect of hesperidin 2S administration on BP are given in Table 7.3. No significant changes in SBP, DBP and HR were observed after 6 or after 12 wks supplementation between intervention groups.

Table 7.3 Cardiometabolic parameters at baseline, after 6 wks and after 12 wks supplementation with placebo or hesperidin 2S.[1]

	Placebo			Hesperidin 2S			P_1	P_2
	Baseline	6 wks	12 wks	Baseline	6 wks	12 wks		
Glucose homeostasis								
Fasting glucose (mmol/l)	5.2 ± 0.1[2]	5.2 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	0.784	0.242
Fasting insulin (mU/l)	10.5 ± 1.2	11.9 ± 1.2	11.4 ± 1.3	9.3 ± 1.2	9.8 ± 1.3	10.0 ± 1.3	0.403	0.809
$AUC_{0\text{-}120min}$ glucose	790 ± 34		784 ± 33	802 ± 36		817 ± 35		0.525
$AUC_{0\text{-}120min}$ insulin	6784 ± 668		6773 ± 601	6116 ± 695		6628 ± 626		0.252
HOMA-IR	2.45 ± 0.30	2.77 ± 0.30	2.49 ± 0.32	2.14 ± 0.30	2.32 ± 0.31	2.24 ± 0.34	0.559	0.834
QUICKI	0.35 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	0.953	0.529
ISI	5.40 ± 0.62		5.62 ± 0.62	5.82 ± 0.63		5.24 ± 0.64	0.264	
Plasma lipids								
Total cholesterol (mmol/l)	5.7 ± 0.2	5.7 ± 0.2	5.7 ± 0.2	5.5 ± 0.2	5.6 ± 0.2	5.4 ± 0.2	0.828	0.802
LDL-C (mmol/l)	3.5 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	3.4 ± 0.2	3.5 ± 0.2	3.4 ± 0.2	0.496	0.970
HDL-C (mmol/l)	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	0.787	0.705
Triglycerides (mmol/l)	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.221	0.060
Blood pressure								
Systolic BP (mmHg)	131 ± 3	132 ± 3	131 ± 2	131 ± 3	135 ± 3	130 ± 3	0.105	0.913
Diastolic BP (mmHg)	82 ± 2	80 ± 2	81 ± 2	83 ± 2	83 ± 2	84 ± 2	0.269	0.287
HR (bpm)	73 ± 2	70 ± 2	73 ± 2	68 ± 3	67 ± 3	70 ± 3	0.381	0.572

[1]Differences between placebo and hesperidin tested with linear mixed model with correction for baseline values. P_1 represents the p-value for the analysis of baseline versus 6 wks intervention between placebo and hesperidin 2S. P_2 represents the p-value for the analysis of baseline versus 12 wks intervention between placebo and hesperidin 2S AUC, area under the curve. BP, blood pressure. HOMA-IR, homeostasis model assessment of insulin resistance. HR, heart rate. ISI, insulin sensitivity index. QUICKI, quantitative insulin sensitivity check index. [2]Estimated mean ± SEM (all such values).

Discussion

The effects of hesperidin 2S on glucose regulation, local GI parameters and systemic metabolic parameters were assessed in subjects with features of MetS. After 12 wks daily intake of hesperidin 2S, no significant changes were observed in glucose regulation, compared to placebo. Interestingly, although the total SCFA production did not change, hesperidin 2S significantly altered the SCFA profile by inducing a shift from acetate to butyrate. Furthermore, hesperidin 2S supplementation resulted in a reduction in fecal calprotectin that just failed to reach statistical significance. No differences in blood lipids or in BP were observed between the intervention groups.

It has been suggested that dietary polyphenols lower the risk of developing DM2. Several mechanisms of action have been described explaining through which the blood glucose lowering effects of hesperidin are mediated. First, polyphenols were shown to inhibit the digestion of dietary carbohydrates by inhibition of key enzymes involved in carbohydrate digestion.[24] Second, absorption of glucose in the intestine can be impeded by polyphenols.[25] Third, enhanced insulin-mediated tissue uptake of glucose has been observed with different polyphenols.[26] Fourth, another mechanism includes the potential of polyphenols to protect against cytokines induced pancreatic β-cell damage, thereby maintaining insulin secretion.[27] Additionally, by increasing hepatic glucokinase activity, which augments glucose utilization, and by suppressing hepatic glucose production (*i.e.* gluconeogenesis) antidiabetic effects can be exerted by several polyphenols.[28] Finally, polyphenol-mediated modulation of inflammation was shown to improve insulin sensitivity in obese mice and rats.[29]

Another mechanism through which polyphenols were shown to induce hypoglycemic effects is by modulating gut microbiota composition and their metabolic activity.[13,30] It has become evident that the gut microbiota fulfill an important role in host homeostasis, being involved in many processes such as metabolism of nutrients and drugs, regulation of several metabolic pathways, maintaining epithelial integrity, modulating GI motility, stimulation and maturation of both systemic and mucosal immunity, and production of vitamins and micronutrients.[31] SCFA are produced by bacterial fermentation of mainly undigested dietary carbohydrates. Many of the beneficial physiological properties of the gut microbiota can be attributed to the production of SCFA as these metabolites showed to exert antioxidant, anti-inflammatory and immune modulating effects.[32] SCFA are able to bind and activate G-protein-coupled receptors in gut epithelial cells. Activation of these receptors induces gut hormone secretion, such as glucagon-like peptide (GLP)-1, GLP-2 and peptide YY (PYY), which increase insulin sensitivity and decrease inflammation.[33] It has also been suggested that SCFA are able to modulate insulin sensitivity by altering fatty acid flux. Lowering postprandial free fatty acids (FFA) appeared to improve insulin sensitivity and insulin secretion.[34] The SCFA butyrate beneficially affects oxidative stress in the colonic mucosa, by increasing expression levels of the antioxidant glutathione and decreasing expression levels of uric acid, a predictor in the

development of insulin resistance.[35] In humans, increasing intestinal butyrate-producing microbes concomitantly improved insulin sensitivity.[36]

In this intervention study, we measured fecal SCFA concentrations to assess microbial activity. We did not observe differences in the quantity of acetate, propionate or butyrate production between the intervention groups. However, we did observe a hesperidin-induced alteration in SCFA profile. A significant shift from acetate towards the health-promoting SCFA butyrate was identified, possibly via an intestinal stimulation of butyrate-producing bacteria. This finding is in line with recent evidence, suggesting that a number of polyphenols and their metabolites are able to influence microbiota composition and microbial activity.[12,13] Butyrate not only improves insulin sensitivity, but also serves as an energy source for epithelial cells, exerts anticarcinogenic, anti-inflammatory and antioxidant effects, and is essential in maintaining the colonic defence barrier.[37] We did not observe any change in glucose regulation (both circulating concentrations of glucose and insulin) in the present study. It should be noted that virtually all participants had normal fasting blood glucose values and OGTT values at start of the trial. Results might have been different when glucose-intolerant subjects had been studied. However, even in glucose-intolerant subjects controversial results have been reported. Dark chocolate polyphenols showed to improve insulin sensitivity,[38] while epigallocatechin-3-gallate supplementation did not.[39]

Calprotectin, a protein secreted by neutrophils infiltrating the intestinal mucosa, is considered a marker of whole gut inflammation. Elevated concentrations of fecal calprotectin are found in patients with chronic intestinal inflammation such as inflammatory bowel disease, but also in obese individuals.[40] We found fecal calprotectin levels within the normal range, but higher than those reported in healthy controls. After the intervention, fecal calprotectin was reduced in the hesperidin 2S treated group vs. placebo, although this difference just failed to reach statistical significance. It is tempting to speculate on the mechanism of this reduction: whether it is related to changes in gut microbiota and gut metabolism.[41] It has previously been shown that pre- and probiotics can reduce fecal calprotectin levels, probably through alterations in the gut microbiome.[42,43]

The presence of dyslipidemia is common in obesity.[44] Hesperidin is known to lower blood lipids *in vitro* and *in vivo*, possibly by affecting enzymes involved in lipogenesis, fatty acid biosynthesis, triglyceride synthesis, cholesterol synthesis and cholesterol absorption, and by facilitating fecal triglyceride and cholesterol excretion.[28,45-47] Here, we did not observe alterations in systemic lipid concentrations after 6 or 12 wks hesperidin 2S intake. This finding was not unexpected as participants had completely normal values at start of the trial.

In the present study, hesperidin 2S did not improve systolic or diastolic BP. Most of the studies reporting a polyphenol-induced BP reduction have included hypertensive subjects, while in our study BP values at baseline were within normal ranges. Putative methods by which polyphenols lower BP include increasing nitric oxide synthase concentrations[48] or

inhibiting angiotensin-converting enzyme.[49] Hesperidin and other polyphenols showed to have the potential to reduce BP *in vivo*,[9,38] although other studies have not observed this.[5,50]
A few limitations of our study should be considered. First, we used the OGTT, a surrogate marker for insulin sensitivity instead of the euglycemic hyperinsulinemic clamp, which is considered the gold standard method. However, that method is an expensive, time consuming, labor intensive method requiring an experienced operator. Second, to assess the microbiota fermentation capacity we measured fecal SCFA concentrations. As these metabolites are efficiently absorbed from the gut - with only 5% to 10% being excreted via the feces - they may not accurately reflect the exact intraluminal SCFA concentrations. However, previous studies have shown that the ratio between the respective SCFA concentrations, measured in feces and colon, is comparable.[51] Thus, changes in fecal SCFA concentration reflect relative changes in intestinal concentrations of the separate SCFA. Furthermore, although we included subjects at risk for MetS, most baseline parameters were already within normal ranges at start of the study. Inducing a systemic beneficial effect in a generally healthy population through a dietary intervention is challenging. Finally, we did not fully control the diet of our participants. We instructed them to maintain their habitual diet during the study period, but to abstain from intake of hesperidin-rich foods. We aimed to assess the effect of hesperidin 2S when supplementing the habitual diet, which is in line with future applications.
In conclusion, 12 weeks oral supplementation with hesperidin 2S did not significantly influence glucose regulation in subjects with features of MetS. Interestingly, it modulated the GI environment as reflected by a shift in fermentation (SCFA) profile from acetate to butyrate, and a near-significant reduction in fecal calprotectin levels. These changes were not accompanied with changes in blood lipids or BP. These results reinforce the hypothesis that polyphenolic compounds are able to modulate gut microbiota composition and function, thereby promoting gut and host health.

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Supplemental material

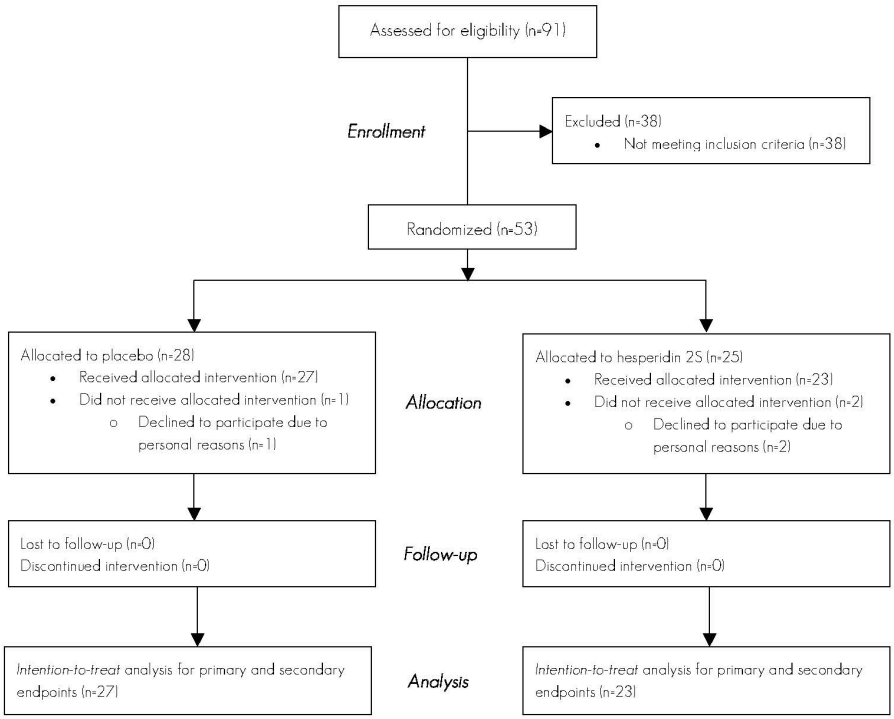
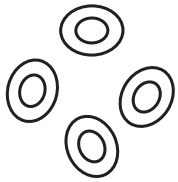
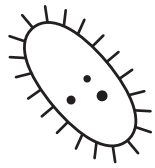


Figure S7.1 CONSORT flow diagram.

chapter 8

Randomised controlled trial on the intestinal fate and effects of the carotenoid-producing Bacillus strain PD01 on gastrointestinal and systemic parameters in overweight individuals

Salden BN, Stevens YR, Troost FJ, Possemiers S, Masclee AA

Abstract

Background

Bacillus indicus PD01 is a spore-forming bacterium, which naturally produces high levels of gastric-stable carotenoids with superior bioavailability and antioxidant properties as compared to plant carotenoids. In combination with potential direct probiotic properties of PD01 itself, the intestinal release of carotenoids may both have local effects in the intestine and systemic effects on cardiovascular or metabolic parameters. Until now no reports have been described confirming these properties in humans.

Objective

In this explorative study we aimed to confirm the intestinal fate of PD01 and the bioavailability of its carotenoids and to investigate the effect of 6 wks PD01 supplementation on both gastrointestinal (GI) and systemic parameters related to oxidative stress in overweight individuals.

Design

In this randomized, double-blind, placebo-controlled study, 68 overweight yet healthy individuals were randomly assigned to groups receiving PD01 or placebo for 6 wks. On each test day, blood was sampled to measure bacterial carotenoids, antioxidant activity, oxidative stress biomarkers, adhesion molecules and metabolic markers. Fecal samples were obtained to assess the intestinal fate of PD01 and its effect on gut microbiota composition, microbial activity and calprotectin levels. Urine was collected to assess oxidative stress and GI permeability markers. Also blood pressure (BP) was measured, and questionnaires were filled out.

Results

PD01 was able to germinate efficiently (73% vegetative cells in feces) and to release its carotenoids as shown by the detection of PD01 carotenoids in blood. In this explorative study no significant effects on intestinal barrier function nor microbiota composition nor intestinal or systemic effects could be confirmed. No adverse events were reported in relation to PD01 intake, confirming that repeated intake of PD01 was well tolerated. Additionally, PD01 intake was associated with a significant reduction in symptoms of indigestion.

Conclusions

This study provides the first evidence in humans that PD01 is able to germinate and release bacterial carotenoids, which are absorbed and detected in human blood. No intestinal or systemic effects were observed but GI symptoms improved significantly. These encouraging results warrant further large-scale research in specific target populations to investigate the specific bioactivities of PD01 at intestinal and systemic level.

Introduction

The incidence of cardiovascular diseases (CVD) varies worldwide, but is notably lower in countries where a 'Mediterranean diet' predominates. This diet contains relatively large amounts of fruit, vegetables and olive oil.[1] Carotenoids, the pigments responsible for the yellow to red color of some fruits and vegetables, have been indicated as one of the beneficial components of the Mediterranean Diet.[2] Associations between high carotenoid intake and protection against oxidative stress, inflammation, endothelial dysfunction, hypertension and atherosclerotic progression have been demonstrated.[3,4] The protective effects of carotenoids are related, at least in part, to their potent antioxidant capacity, which has been well demonstrated in *in vitro* assays.[5] However, the relevance of their antioxidant properties *in vivo* is still debated. It is likely that other biological effects of carotenoids also contribute to their health protective effects.[6]

Carotenoids are lipophilic antioxidants, synthesized mainly in plants. Humans are not able to synthesize carotenoids and therefore are dependent on dietary sources. The majority of commercially available natural carotenoids are derived from plant-based materials. However, the quality and consistency of these carotenoids varies considerably. Interestingly, carotenoid-like molecules are also synthesized in nature by a number of bacterial species. In this respect, carotenoids produced by a variety of spore-forming bacillus strains are of specific interest.[7,8] *In vitro* studies have shown that these bacterial carotenoids are stable in the stomach, are equally or even more bioaccessible and bioavailable than dietary carotenoids currently on the market, and exert a tenfold higher antioxidant activity compared to lycopene, one of the most potent antioxidants known. Furthermore, the carotenoid-producing bacillus strain itself may also possess probiotic properties.[9]

Previous *in vitro* and animal studies have provided evidence that a specific *Bacillus indicus* strain, named PD01, has excellent intestinal survival and can germinate and replicate under intestinal conditions (unpublished data). Furthermore, its carotenoids are released in the intestine and they were shown to have a high bioavailability and a promising biological activity profile with strong antioxidant properties and effects both on CVD risk markers and on the intestinal environment (unpublished data). As such effects have not yet been confirmed in humans, the present human intervention study was undertaken to investigate whether PD01 is able to survive transit through the gastrointestinal (GI) tract and thereby is able to release systemically absorbable carotenoids in an *in vivo* setting. Additionally, the GI and systemic effects of the bacillus strain and its bacterial carotenoids were examined. We specifically chose to study a healthy, yet overweight-obese population, as this condition has previously been associated with higher risk of a wide range of GI and systemic metabolic alterations including gut microbiota,[10] intestinal barrier,[11] GI and systemic low-grade inflammation,[12,13] endothelial function,[14] hypertension[15] and glucose and lipid homeostasis.[16,17] We hypothesized that PD01 is able to survive transit through the human GI

tract and that it is able to release bacterial carotenoids that can be absorbed and detected in human plasma. Furthermore, we hypothesized that 6 weeks daily PD01 supplementation beneficially affects 1) GI microbiota, barrier and metabolic function and 2) systemic antioxidant activity and metabolic function and 3) is well-tolerated in overweight individuals. Primary aim of our study was to investigate the efficacy of 6 wks daily supplementation of PD01 on oxidative stress, as measured by 24 h urinary excretion of F_2-isoprostanes, in healthy overweight individuals. Secondary, we aimed to assess the plasma bioavailability of the bacterial carotenoids during 6 wks daily PD01 supplementation, and to investigate: 1) GI effects, by assessing gut microbiota composition, fecal short-chain fatty acids (SCFA) concentrations, GI permeability, fecal calprotectin, digestive tolerance and 2) systemic effects, by measuring total antioxidant capacity, plasma malondialdehyde (MDA), plasma adhesion molecules, fasting blood glucose, insulin and lipids markers and blood pressure.

Subjects and methods

This study was approved by the Medical Ethics Committee of the Maastricht University Medical Center + (MUMC+) and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study was performed at the MUMC+ from August 2015 to December 2015. All participants gave written informed consent before participation. The trial has been registered in the Clinical Trials register (NCT02622425).

Subjects

Healthy, overweight or obese volunteers aged 18-70 years with a BMI between 25-35 kg/m^2 were recruited by advertisement. Exclusion criteria were: any medical condition that might interfere with the study and might jeopardize the health status of the participant; smoking; high intake of fruits and vegetables (> 75th percentile of dietary intake of fruits and vegetables in general Dutch population); abuse of alcohol (> 20 alcoholic units / wk) and drugs; no consistently stable body weight for at least 3 months (± 3 kg); plans to lose weight or following hypocaloric diet during study period; use of medication / vitamin-, mineral- or antioxidant supplements; consumption of pro-, pre- or synbiotics during study period and in 30 days prior to start of the study; use of antibiotics in the 90 days prior to start of the study; pregnancy and lactation; history of any side effects towards the intake of pro-, pre-, synbiotic supplements or carotenoids; failure to comply with prohibited intake of carotenoid-rich food products. During the study, the subjects consumed their habitual diet. At

the time of inclusion, all subjects were informed about the prohibited carotenoid-rich food products, pro-, pre- and synbiotics.

Design and intervention

This study was designed as a randomized, placebo-controlled, double-blind, parallel-group study. Each subject underwent three test days. Participants were randomly assigned in a double-blind fashion to one of the two intervention arms: PD01 or placebo (maltodextrin). An independent and blinded person generated the randomization list, using a computerized procedure. All participants and investigators remained blind to treatment until all analyses were completed. Participants were instructed to abstain from strenuous physical exercise, consumption of alcohol and carotenoid-rich food products on the day prior to each test day. Assessments were performed in a quiet, temperature-controlled (20-24°C) room. Subjects handed in a fecal sample on the first test day. Then, anthropometric measurements (height, body weight, waist-to-hip circumference) and BP measurements were performed. Subsequently, venous blood samples were collected from an antecubital vein in the fore-arm. Then, subjects ingested a multi-sugar drink and collected full urine output for 24 h for measurement. Finally, the subject completed a questionnaire to assess the presence of GI symptoms, stool frequency and stool consistency. After completion of the baseline measurements, participants received the study product for the following 6 wks. After 3 wks of daily supplementation, the second test day was organized. Measurements were identical to the baseline measurements performed during the first test day, with the exception of the multi-sugar drink with 24 h urine collection, which was not performed after 3 wks of intervention. After 6 wks of daily administration of the study product, the third test day was organized, which was identical to the first test day. Furthermore, a 3-day food record was completed prior to start of the study (during screening) and in the last week of the intervention period, to assess whether the participants maintained their dietary habits throughout the study period. To assess compliance, participants were asked to collect the empty sachets and to return these at the last visit.

PD01 and placebo

PD01 (ProDigest, Ghent, Belgium) is a food-grade bacterial formulation containing carotenoid-producing *Bacillus indicus* spores, which were grown and lyophilized on a maltodextrin (Pineflow; Matsutani Chemical Industry, Hyogo, Japan) carrier by Biopolis (Paterna, Valencia, Spain). The study products were provided as a powder in sachets. The PD01 group received per day one sachet containing each $5*10^9$ CFU PD01 with maltodextrin (3 g) as carrier material. The PD01 carotenoid content of the clinical batch was quantified as 2.81 μg per 10^9 CFU, thus participants received in total 14.05 μg PD01

carotenoids per day. The placebo group received one sachet containing 3 g maltodextrin per day. Subjects were asked to stir the content of one sachet in 150 ml whole fat milk and ingest the solution each morning, just before consuming breakfast, for 6 wks.

Fecal PD01 quantification

Plate counting

Prior to analysis, fecal samples were thawed for 20 minutes and homogenized in sterile distilled water. Homogenized fecal samples were serially diluted in PBS buffer. Part of the dilutions was pasteurized (30 min at 65°C) to discriminate spores and viable cells. Both pasteurized and non-pasteurized samples were plated on agar plates, and incubated overnight at 37°C. Yellow to orange colonies were counted as these represent PD01.

Blood sample pretreatment

Plasma and serum isolation

After blood collection, plasma was directly isolated by centrifugation (10 min, 1400 *g*, 4°C) and stored at -80°C. For serum isolation, blood samples were first kept at room temperature for 30 minutes. Then, serum was isolated by centrifugation (10 min, 1400 *g*, 21°C) and stored at -80°C.

Bioavailability bacterial carotenoids

After blood collection, blood samples were kept in an ice-water bath and light exposure was avoided. Plasma was isolated by centrifugation (10 min, 1400 *g*, 4°C) within 2 h following collection and stored at -80°C.

Bioavailability of bacterial carotenoids in plasma

Carotenoid extraction

In a glass tube, 1 ml of methanol containing internal standard (echinenone at 0.5 mg/l) was added to 1 ml of plasma sample. The mixture was vortexed for 10 minutes and 1 ml of dichloromethane was added. After 10 additional minutes of vortexing, the mixture was centrifuged for 10 minutes at 10°C at 800 *g*. The organic phase was collected and evaporated under argon gas.

Lipolysis of carotenoid esters

The dry extract was dissolved in 500 µl of 0.1 M phosphate buffer at pH 7.5 containing 30 mg/ml of bile salts. 250 µl of lipase from *Candida sp.* and 250 µl of lipase from *Thermomyces lanuginosis* were added, and the solution was incubated for 1 hour at 37°C while stirring. After incubation, 500 µl of methanol was added, the mixture was vortexed for 10 minutes, and 2 ml of methyl *tert*-butyl ether (MTBE) was added. After 10 additional minutes of vortexing, the mixture was centrifuged for 10 minutes at 10°C at 800 *g*. The organic phase was collected and evaporated under argon gas.

HPLC analysis

After evaporation to dryness, all extracts were dissolved in 200 µl of methanol:MTBE (1:1, v:v) and carotenoids were quantified by reverse-phase HPLC using an HP1100 Agilent system equipped with a YMC C30 column as described by Gleize *et al.*[18] The non-esterified carotenoids were identified based on retention time and UV-Visible spectra (Figure 8.1).

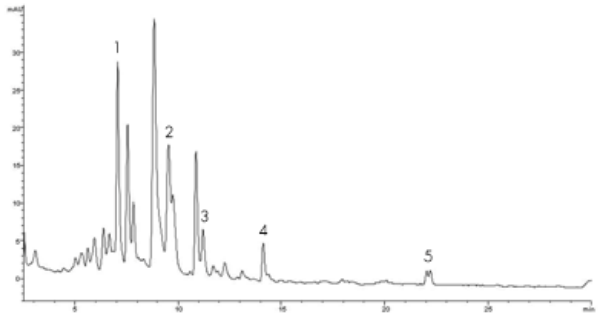


Figure 8.1 HPLC-UV VIS chromatogram of a lipophilic human plasma extract spiked with PD01 carotenoids to validate the method (1: Lutein, 2: Methyl-glycosyl-apo-8'-lycopenoate (non-esterified form of orange carotenoid produced by PD01 strain), 3: Glycosyl-apo-8'-lycopene (non-esterified form of yellow carotenoid produced by PD01 strain), 4: β-Carotene, 5: Lycopene).

Fecal microbiota composition and microbial activity

DNA extraction

Total genomic DNA suitable for PCR analysis was extracted from frozen human fecal samples using the QIAamp® DNA Stool Mini Kit (QIAGEN) following manufacturer's

instructions. The PCR products were purified, a metagenomics library prepared, sequenced and analyzed, as previously described by Hevia *et al.*[19]

Gut microbiota composition

Partial 16S rRNA gene sequences (V3 and V4 region) were amplified and sequenced as previously reported by Milani *et al.*[20] The obtained individual sequence reads were filtered to remove low quality and polyclonal sequences, trimmed, and exported as .fastq files. These .fastq files were processed using a QIIME-based custom script.[21] Quality control retained reads with a length between 140 and 400 bp, mean sequence quality scores > 20, and allowed truncation at the first base if a low- quality moving 10 bp window was found. Presence of homopolymers > 7 bp, and sequences with mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 97% sequence homology using uclust.[22] All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the SILVA database.[23] Similarities between samples were calculated by unweighted uniFrac.[24] The range of similarities was calculated between 0 and 1.

Fecal pH, SCFA, lactate and β-glucuronidase activity

Prior to analysis, fecal samples were thawed for 20 min and homogenized in sterile distilled water. Stool pH was determined for each sample upon tenfold dilution in sterile distilled water. pH was measured with a standard pH meter (Senseline F40; ProSense, Oosterhout, The Netherlands) with a conventional pH electrode (QP2111T; ProSense).
SCFA concentrations were determined as described previously by Van de Wiele *et al.*[25]
Homogenized stool samples were centrifuged at 7600 *g* for 5 minutes. Cell free supernatant (50 µl) was used for lactate measurement using a commercially available enzymatic assay kit (R-Biopharm, Darmstadt, Germany). Furthermore, cell free supernatant was diluted 1:1 (v:v) in sterile distilled water and transferred (50 µl) into a 96-well plate containing 50 µl/well of a 5 mM solution of 4-nitrophenyl β-D-glucuronide (Sigma-Aldrich, St-Louis, Missouri, USA). After 30 minutes of incubation at 37°C, the release of p-nitrophenol was quantified based on the absorbance at 405 nm using a Synergy HT multiplate reader (BioTek Instruments, Winooski, Vermont, USA). The results were expressed in µmol p-nitrophenol released per minute and per gram feces.

Metabolomic analysis on fecal samples

Metabolomic analysis was applied as previously described in detail by Vanden Bussche *et al.*[26]

Gastrointestinal permeability

Gastrointestinal permeability was assessed by using a validated multi-sugar test, measuring the 24 h urinary excretion of ingested sugar probes reflecting permeability of 4 segments of the GI tract. The method of measurement has been described previously by Mujagic *et al.*[27] Urinary sugar probes were determined by HPLC-MS as previously described.[28]

Fecal calprotectin

Fecal calprotectin concentration levels were determined using an ELISA for human calprotectin (Hycult Biotechnology, Uden, Netherlands), according to the manufacturer's protocol.

Antioxidant activity and biomarkers of oxidative stress

Total antioxidant capacity (TAC)

Plasma TAC was measured by a commercially available quantitative colorimetric assay kit, according to the manufacturer´s instructions (BioAssay Systems, Hayward, USA). Briefly, Cu^{2+} was reduced by the available antioxidants in the sample to Cu+. The resulting Cu+ formed a colored complex with a dye reagent, which was quantified colorimetrically (Bio-Tek, Synergy HT, Winooski, USA). This is proportional to the TAC in the sample. The total antioxidant capacity was expressed in mM Trolox equivalents.

Urinary F2-isoprostanes

Urinary F_2-isoprostane concentration was measured by a commercially available enzyme immune assay (EIA) kit, according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI). Briefly, 500 µl urine was acidified to pH 4 and samples were applied to prewashed C-18 SPE cartridges (Cayman Chemical Co, Ann Arbor, MI), after which the eluate was added. The ethyl acetate/methanol eluate from the SPE cartridges was subsequently dried using vacuum centrifugation and reconstituted in EIA buffer. The samples were assayed in duplicate with standards (0–3000 pg/ml of 8-iso-PGF2a), and blanks. After an incubation period of 1 h the plate was washed and *p*NPP reagent was added. The resultant color reaction was read after approximately 1 h using a Bio-Rad plate reader at 420 nm, the color development being inversely proportional to the concentration of 8-iso-PGF2a measured. To optimize the assay, it was necessary to use ultrapure water for all aqueous reagents and to manually wash the plates.

Malondialdehyde (MDA)

Plasma MDA was quantified by a commercially available microplate assay kit, according to the manufacturer's protocol (Cohesion Biosciences Limited, London, United Kingdom). Briefly, the MDA in the sample reacted with Thiobarbituric Acid (TBA) to generate the MDA-TBA adduct. The MDA-TBA adduct was quantified colorimetrically (Bio-Tek, Synergy HT, Winooski, USA).

Adhesion molecules

Circulating soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble E-selectin (sE-selectin) and soluble P-selectin (sP-selectin) were determined by commercially available ELISA and standards, according to the manufacturer´s protocol (Bender MedSystems GmbH, Vienna, Austria).

Blood glucose, insulin and lipids

Serum concentrations of glucose, total cholesterol, low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-cholesterol and triglycerides were measured by spectrophotometry (cobas 6000 analyzer series, Roche Diagnostics, Indianapolis, USA). Plasma insulin was determined using a luminescence-enhanced immune-enzymatic assay (Immulite 2000 immunoassay system, Siemens Healthcare, Erlangen, Germany). Insulin resistance (IR) was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) index. It was calculated as the product of fasting plasma glucose (mmol/L) and insulin (mU/l) concentrations divided by 22.5. Insulin sensitivity (IS) was assessed using the quantitative insulin sensitivity index (QUICKI), calculated as: 1 / (log insulin 0 h + log glucose 0 h).

Blood pressure and heart rate measurements

BP was monitored using a semi-continuous BP monitoring device (Omron, Hoofddorp, The Netherlands) at the upper right arm. At each occasion, after 30 minutes of rest in the supine position, 4 consecutive BP measurements were performed. The first measurement was discarded and the remaining 3 measurements were averaged.

Digestive tolerance

The occurrence of GI symptoms was assessed using the validated gastrointestinal symptom rating scale (GSRS), consisting of 15 items combined into five symptom clusters describing reflux, abdominal pain, indigestion, diarrhea, and constipation.[29] In the seven-point graded

Likert-type GSRS, a score of 1 represents absence of troublesome symptoms and a score of 7 represents very troublesome symptoms. Defecation frequency and stool consistency were assessed using the Bristol Stool Form Scale Chart.[30]

Statistical analysis

The primary outcome of the study was the effect of 6 wks PD01 supplementation on 24 h urinary excretion of F_2-isoprostanes, compared to placebo. Sample size was determined for the primary outcome of the study, using a significance level *alpha* = 0.05 and a power of 90%. Based on previous research,[31] we calculated that a sample size of 60 subjects would be required to detect a difference in 24 h urinary F_2-isoprostanes of 104 ± 85 pg/ml (mean ± SD). Baseline characteristics are presented as mean (SD) for numerical variables and number (%) for categorical variables. Differences in gut microbiota composition, microbial activity, GI permeability, GI inflammation, antioxidant activity, oxidative stress, adhesion molecules, metabolic markers, BP, and questionnaires between intervention groups (PD01 or placebo) were assessed using linear mixed models with group (placebo and PD01), time (1, 3 and 6 wks for 3 and 6 wks PD01 effect) and group*time as fixed factors, where an unstructured covariance structure was used for repeated measures. The linear mixed model accounts for the correlation between repeated measures and missing data, where a likelihood approach was used assuming data missing at random. The estimated means and SEM obtained from this model were presented for each group and each time point. In case of asymmetrical distribution of data, medians with interquartile ranges were displayed. The Friedman test with post-hoc Wilcoxon Signed Rank test was applied to compare the presence of PD01 in feces and bacterial carotenoids in plasma between baseline samples and samples obtained after 3 and 6 wks PD01 supplementation. The Wilcoxon Singed Rank test was used to compare the fecal microbiota composition at genus level between baseline samples and samples obtained after 6 wks supplementation in a treatment group. These statistical analyses were performed using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY, USA).

For the metabolomics analysis, chemometric data analysis was applied. First, extensive data pre-processing was conducted on the obtained full scan HRMS data files with Sieve™ 2.2 software (Thermo Fischer Scientific). Next, normalization of the dataset was performed to correct for instrumental drift. Next, a logarithmic transformation and a Pareto scaling (1/√SD, where SD is the standard deviation) were performed for inducing normality and standardizing the range of independent X-variables, respectively. Finally, multivariate regression techniques (Simca 13.5.0, Umetrics, Sweden) were used to differentiate between the obtained fingerprints. Principal component analysis (PCA) was used to reveal outliers, groups and trends, whereas (orthogonal) partial least square-discriminant analysis ((O)PLS-DA) was used to construct a prediction model that could predict the treatment from the

fecal metabolic fingerprint to explore possible suppressed metabolic effects. For all analyses, two-sided *P* values ≤ 0.05 were considered statistically significant.

Results

Subjects

In total 67 healthy volunteers were enrolled in the study, of which 62 completed the entire study protocol. One participant never started participation for a personal reason. Four participants dropped out during the intervention period; one due to the need for antibiotic treatment, another for unspecified, private reasons, while two participants experienced mild GI complaints. These complaints were present already at the start of the study and not associated to PD01 intake. From these participants only baseline characteristics were available. Baseline characteristics are presented in Table 8.1.

Table 8.1 Baseline characteristics of study participants.[1]

	Total population *(n = 67)*	Placebo *(n = 34)*	PD01 *(n = 33)*
Age, yr	53 ± 13[2]	50 ± 15	56 ± 10
Sex, M/F	29/38	12/22	17/16
WHR	0.91 ± 0.08	0.90 ± 0.08	0.93 ± 0.08
BMI, kg/m^2	29.9 ± 3.4	30.3 ± 3.6	29.5 ± 3.2

[1]BMI, body mass index. WHR, waist-to-hip-ratio. [2]Mean ± SD (all such values).

Fecal PD01 quantification

PD01 was not detected in any of the fecal samples of subjects receiving placebo. In the PD01 group, PD01 was not detected in any of the fecal samples of any of the subjects at baseline, but was present in fecal samples of all subjects after 3 wks of supplementation and in 27 of the 29 subjects after 6 wks of supplementation. There was a statistically significant difference in PD01 total cells (χ^2 (2) = 39.38, *P* = 0.000) and spores (χ^2 (2) = 42.74, *P* = 0.000) in feces after PD01 supplementation. Post hoc analysis with Wilcoxon signed-rank tests was conducted with a Bonferroni correction applied, resulting in a significance level set at *P* < 0.017. As shown in Table 8.2, significantly higher amounts of PD01 total cells were found in feces both after 3 wks and 6 wks supplementation, compared to baseline (all *P* < 0.001). No significant differences could be observed between 3 and 6 wks supplementation both for total cells and spores (both *P* ≥ 0.039), pointing to a steady state after 3 wks of PD01 supplementation. The total amount of viable PD01 cells in human feces after 6 wks of PD01 supplementation was quantified as $2.26*10^7$ CFU/g, of which $3.43*10^6$

CFU/g are PD01 spores. While administered as spores, PD01 was therefore mainly present in feces as vegetative cells (1.99*10^7 CFU/g; 73% of total counts), indicating effective germination under intestinal conditions.

Table 8.2 PD01 total cells and spores (CFU/g) in fecal samples at baseline, after 3 wks and after 6 wks supplementation with PD01 (*n = 29*).[1]

	Baseline	3 wks	6 wks
Total cells[3,4]	0 [0; 0][2]	2.50E7 [1.11E7; 3.98E7]	2.26E7 [1.06E7; 4.19E7]
Spores[3,4]	0 [0; 0]	5.77E6 [1.28E6; 1.56E7]	3.43E6 [1.85E6; 1.06E7]
Percentage of total	0	30.2 [11.1; 52.9]	26.6 [6.7; 39.5]
Vegetative cells[3,4]	0 [0; 0]	1.70E7 [4.20E6; 3.95E7]	1.99E7 [5.65E6; 2.93E7]
Percentage of total	0	69.8 [47.1; 88.9]	73.4 [60.5; 93.3]

[1]Differences between baseline and 3 wks and 6 wks supplementation tested with Friedman test with post hoc Wilcoxon signed rank test. [2]Medians [Q1; Q3] (all such values). [3]Significant difference between baseline vs. 3 wks intervention. [4] Significant difference between baseline vs. 6 wks intervention.

Bioavailability of bacterial carotenoids in plasma

PD01 carotenoids were detected in fasted plasma samples of all subjects after 3 wks and after 6 wks of PD01 supplementation. The plasma concentration of PD01 carotenoids significantly increased during the 6 wks of daily supplementation (0.000 μM at baseline, 0.044 μM at 3 wks, 0.076 μM at 6 wks, respectively; all $P \leq 0.009$, Table 8.3). The presence of (bacterial) carotenoids in plasma samples of subjects of the placebo group was not assessed. PD01 supplementation did not induce significant changes in the plasma content of lutein, β-carotene or lycopene during the study period (all $P \geq 0.121$, Table 8.3).

Table 8.3 Carotenoid concentrations (μM) in fasted plasma at baseline and after 3 wks and 6 wks supplementation with PD01 (*n = 29*).[1]

	Baseline	3 wks	6 wks
PD01 carotenoids[3,4,5]	0 [0; 0][2]	0.044 [0.002-0.167][1]	0.076 [0.019-0.233]
Lutein	0.126 [0.038-0.267]	0.124 [0.034-0.283]	0.121 [0.035-0.237]
β-Carotene	0.279 [0.029-0.887]	0.316 [0.036-0.786]	0.317 [0.031-0.786]
Lycopene	0.181 [0.018-0.461]	0.182 [0.026-0.445]	0.177 [0.018-0.392]

[1]Differences between baseline and 3 wks and 6 wks supplementation tested with Friedman test with post hoc Wilcoxon signed rank test. [2]Medians [Q1; Q3] (all such values). [3]Significant difference between baseline vs. 3 wks intervention. [4]Significant difference between baseline vs. 6 wks intervention. [5]Significant difference between 3 wks and 6 wks intervention.

Fecal microbiota and metabolic activity

Relative abundance at phylum and genus level

The dominant phyla across all baseline fecal samples were firmicutes (mean 80.3%, SD 10.9%) and bacteroidetes (mean 10.3%, SD 10.0%), followed by actinobacteria (mean 6.3%, SD 5.4%) and proteobacteria (mean 0.9%, SD 3.6%). The relative abundance of these 4 dominant phyla did not change significantly during 6 wks intake of PD01, compared to placebo (Figure 8.2). The obtained sequence reads were analyzed to obtain information on the bacterial composition of each sample at genus level. No significant changes in bacterial composition at genus level between the two treatments were observed during intervention. When analyzing the bacterial composition at genus level within the PD01 group, we observed significant increases in the fecal abundance of *Alloprevotella* ($P = 0.044$), *Blautio* ($P = 0.020$), *Butyrivibrio* ($P = 0.017$), *Dorea* ($P = 0.033$), *Flavonifractor* ($P = 0.037$), and a species belonging to the ruminococcaceae family ($P = 0.010$) after 6 wks intervention, while in the placebo group the fecal abundance of none of these bacteria significantly altered (all $P \geq 0.163$). These effects are, however, no longer significant after any multiple testing correction (*e.g.* Bonferroni).

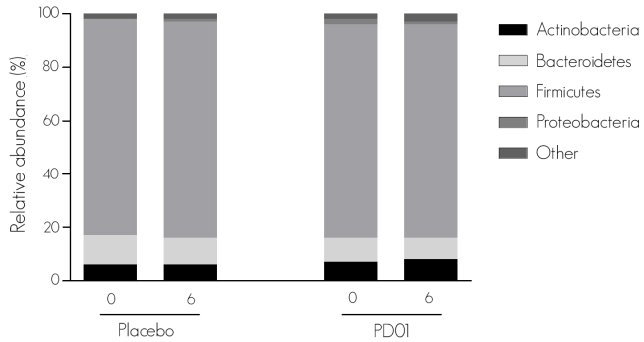


Figure 8.2 Relative abundance of the main phyla according the 16S-targeted Illumina data for placebo and PD01 at baseline (0) and after 6 wks supplementation (6). No significant differences in relative abundance between placebo and PD01 were observed during study period, as tested with linear mixed model with correction for baseline values.

Fecal samples alpha diversity

Alpha diversity was calculated through the alpha_diversity.py script using different metrics (Chao, Shannon diversity index) to take into account the species evenness and richness in each sample (Figure 8.3A and Figure 8.3B). These alpha diversity plots show that the

microbial diversity was very similar between the two interventions at baseline and after 6 wks of supplementation and, therefore, that intervention did not have major effects on the overall microbial diversity of the subjects.

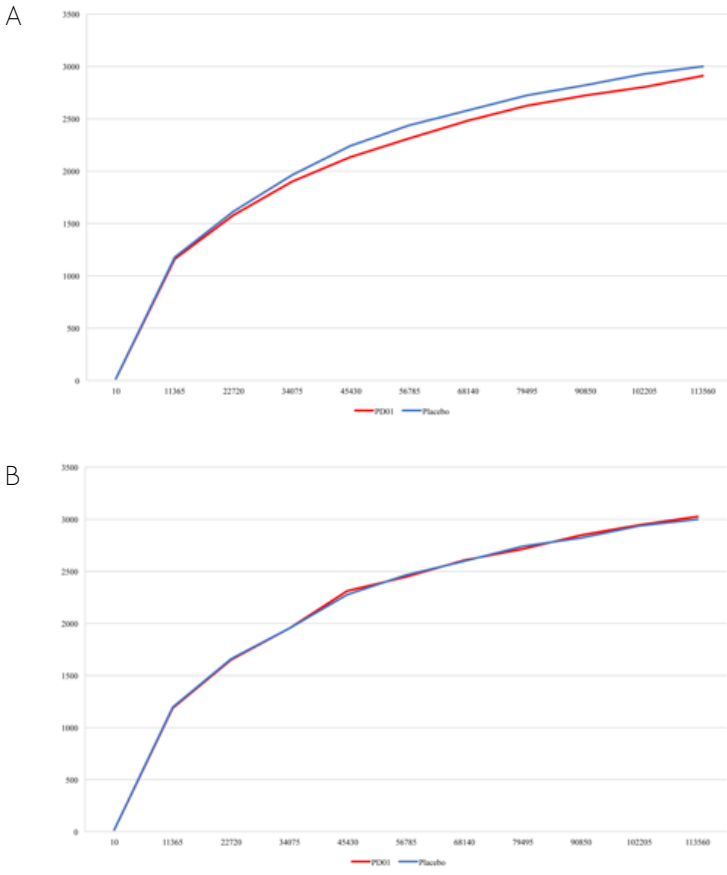


Figure 8.3 Alpha-diversity plots obtained using the Chao1 index at baseline (A) and after 6 wks of treatment (B). (red: PD01; blue: placebo).

Fecal samples beta diversity

The beta diversity index was evaluated in order to analyze the microbial diversity between samples. As shown in Figure 8.4, the sequences from the various samples were not organized into clusters but were evenly distributed, indicating that the treatment did not have major effects on the overall microbial diversity of the human gut.

Table 8.4 Metabolic activity at baseline, after 3 wks and after 6 wks supplementation with placebo or PD01.[1]

	Placebo			PD01			P_1	P_2
	Baseline	3 wks	6 wks	Baseline	3 wks	6 wks		
pH	7.3 ± 0.1[2]	7.2 ± 0.1	7.2 ± 0.1	7.1 ± 0.1	7.0 ± 0.1	7.2 ± 0.1	0.880	0.112
Lactate (μmol/g)	31.8 ± 2.4	31.4 ± 2.4	32.8 ± 2.3	35.1 ± 2.6	35.0 ± 2.5	33.7 ± 2.5	0.915	0.567
β-glucuronidase (U/g)	0.63 ± 0.16	1.02 ± 0.28	0.65 ± 0.38	0.62 ± 0.17	0.83 ± 0.30	1.68 ± 0.42	0.661	0.106
SCFA: total (μmol/g)	52.5 ± 4.0	55.8 ± 4.8	52.8 ± 4.1	56.5 ± 4.2	56.8 ± 5.2	50.6 ± 4.5	0.564	0.267
SCFA: branched (μmol/g)	2.9 ± 0.3	3.0 ± 0.4	3.1 ± 0.3	2.8 ± 0.3	3.1 ± 0.4	2.7 ± 0.4	0.719	0.517
SCFA: acetate (μmol/g)	27.4 ± 2.2	29.2 ± 2.5	28.1 ± 2.2	29.0 ± 2.4	28.4 ± 2.6	26.0 ± 2.4	0.356	0.214
SCFA: propionate (μmol/g)	9.8 ± 1.0	10.5 ± 1.1	9.7 ± 0.9	11.7 ± 1.0	10.9 ± 1.2	10.2 ± 1.0	0.175	0.214
SCFA: butyrate (μmol/g)	10.4 ± 1.0	11.0 ± 1.2	9.7 ± 0.9	10.4 ± 1.1	11.6 ± 1.2	9.2 ± 1.0	0.742	0.723

[1]Differences between placebo and PD01 tested with linear mixed model with correction for baseline values. P_1 represents the p-value for the analysis of baseline versus 3 wks intervention between placebo and PD01. P_2 represents the p-value for the analysis of baseline versus 6 wks intervention between placebo and PD01. SCFA, short-chain fatty acids. [2]Estimated mean ± SEM (all such values)

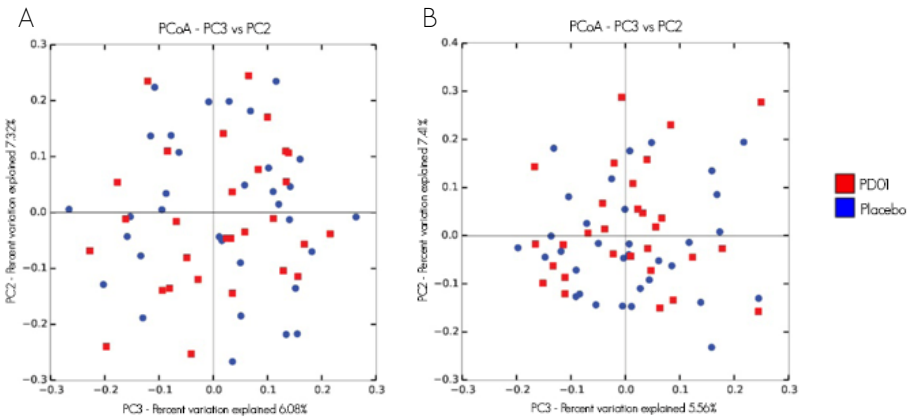


Figure 8.4 Example of beta-diversity plots comparing samples from participants receiving placebo (blue symbols) and PD01 (red symbols), collected at baseline (A) and after 6 wks supplementation (B).

Fecal pH, SCFA, lactate and β-glucuronidase activity

Results on gut metabolic activity are shown in Table 8.4. Stool pH and lactate did not significantly alter during PD01 supplementation, compared to placebo. Intake of PD01 did not significantly change the total level of SCFA, branched SCFA and the levels of the individual SCFA, compared to placebo. Neither were differences in β-glucuronidase activity after 3 wks and after 6 wks of treatment between the two intervention groups observed.

Metabolomics analysis of fecal samples

Figure 8.5 represents the PCA score plot for samples obtained at baseline and after 6 wks PD01 supplementation. No effect of intervention was observed. To investigate more in depth the metabolic effects induced by PD01, supervised OPLS-DA models were constructed per ionization mode. For the datasets, the OPLS-DA score plot (Figure 8.6) also showed no significant effect of PD01 intervention. Individuals tend to cluster as other variables (*e.g.* genetic variation, lifestyle) induced more pronounced metabolic changes than the type of intervention. To reduce the inter-individual effect, the datasets were categorized according to the combination of treatment and time of sampling, resulting in 6 categories. In this way, it was possible to compare the different time points within the PD01 treated individuals. No significant changes in metabolomics between interventions were observed throughout the study. The PCA score plots displayed a good clustering of all quality control samples, but no clear trend could be observed.

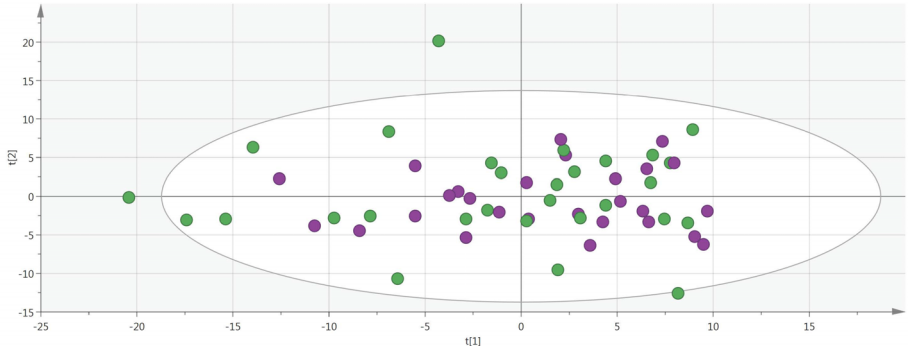


Figure 8.5 PCA score plot for the dataset categorized according to a combination of PD01 and time of sampling, where comparison is made between the human fecal metabolome at baseline (green) and after 6 wks of PD01 supplementation (purple).

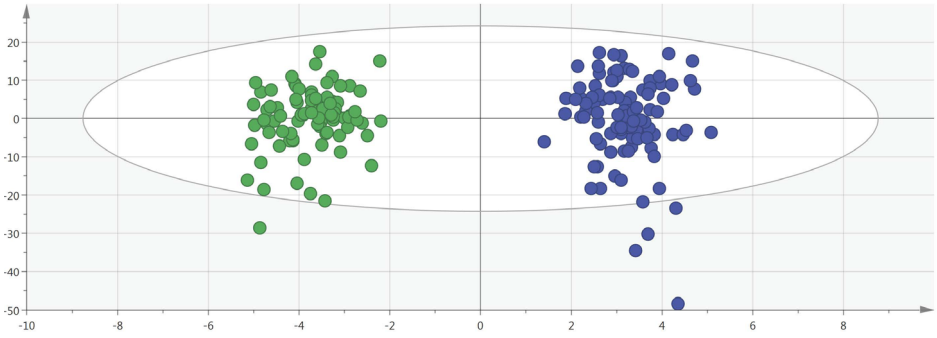


Figure 8.6 OPLS-DA score plot for the positively charged ions detected in the human feces by means of UHPLC-HRMS. (green: PD01 treatment including test day 1, 2 and 3; blue: Placebo including test day 1, 2 and 3).

Gastrointestinal permeability

Table 8.5 presents the GI permeability data. No statistically significant changes in gastroduodenal, small intestinal or colonic and whole gut permeability were observed after 6 wks PD01 supplementation compared to placebo. When analyzing the results in more detail it is apparent that a 37% decrease in gastroduodenal permeability was observed in response to PD01 compared to a 5% decrease in response to placebo ($P = 0.131$). In addition, a 33% decrease in colonic permeability was observed in the PD01 group compared to no decrease in the placebo group ($P = 0.104$).

Table 8.5 Permeability test: sugar excretion (μmol) and ratios of excreted sugars as measured in urine (in 0-2, 0-5, 5-24 and 0-24 h fraction).[1]

	Placebo		PD01		*P* value
	Baseline	6 wks	Baseline	6 wks	
0-5 h sucrose	5.290 [2.930; 11.625][2]	5.030 [3.460; 12.520]	6.975 [2.140; 19.248]	4.370 [2.540; 9.575]	0.131
0-5 h L/R ratio	0.030 [0.020; 0.040]	0.030 [0.020; 0.040]	0.035 [0.020; 0.050]	0.030 [0.020; 0.040]	0.842
5-24 h S/E ratio	0.010 [0.010; 0.020]	0.010 [0.010; 0.015]	0.015 [0.010; 0.020]	0.010 [0.010; 0.020]	0.104
0-24 h S/E ratio	0.010 [0.010; 0.020]	0.010 [0.010; 0.010]	0.010 [0.010; 0.020]	0.010 [0.010; 0.020]	0.266

[1]Differences between placebo and PD01 tested with linear mixed model. L/R, lactulose/l-rhamnose. S/E, sucralose/erythritol. [2]Median [IQR, *i.e.* Q1; Q3] (all such values).

Fecal calprotectin

Fecal calprotectin did not significantly change after 6 wks PD01 supplementation, compared to placebo (placebo: 32.6 ng/ml to 36.2 ng/ml; PD01: 46.6 ng/ml to 42.9 ng/ml; $P = 0.341$).

Antioxidant activity and biomarkers of oxidative stress

Plasma TAC and MDA, and 24 h urinary excretion of F_2-isoprostanes were not significantly changed during 6 wks PD01 supplementation, compared to placebo (Table 8.6).

Table 8.6 Antioxidant activity and biomarkers of oxidative stress at baseline and after 6 wks supplementation with placebo or PD01.[1]

	Placebo		PD01		*P* value
	Baseline	6 wks	Baseline	6 wks	
Total antioxidant capacity (μM)	366 ± 10[2]	351 ± 9	375 ± 10	363 ± 9	0.777
F_2-isoprostanes (pg/mL)	169 [100; 242][3]	142 [82; 290]	171 [108; 312]	157 [99; 289]	0.323
Malondialdehyde (nmol/mL)	0.21 [0.15; 0.28]	0.21 [0.15; 0.26]	0.21 [0.15; 0.26]	0.21 [0.15; 0.26]	0.435

[1]Differences between placebo and PD01 tested with linear mixed model with correction for baseline values. [2]Estimated mean ± SEM (all such values). [3]Median [IQR, *i.e.* Q1; Q3] (all such values).

Adhesion molecules

Basal circulating adhesion molecules at baseline and after 6 wks intervention for both the placebo and the PD01 groups are presented in Table 8.7. No significant differences in sVCAM, sICAM, sE-selectin and sP-selectin concentrations were observed between the two interventions.

Blood glucose, insulin and lipids

Baseline total cholesterol, LDL- and HDL-cholesterol, triglycerides, glucose and insulin were all within normal blood value ranges. These parameters were not significantly altered by 3 wks or 6 wks supplementation with PD01 compared to placebo (Table 8.7). Also, no significant changes in insulin resistance and insulin sensitivity, expressed by the HOMA-IR and QUICKI indices, were observed between intervention groups (Table 8.7).

Blood pressure and heart rate

Baseline systolic BP, diastolic BP and HR were all within normal value ranges at baseline. No significantly changes regarding these parameters were observed between intervention groups both after 3 wks and 6 wks supplementation (data not shown).

Digestive tolerance

Participants receiving daily PD01 scored lower on the GSRS subdimension for indigestion syndrome after 3 wks supplementation (P = 0.061, Figure 8.7), becoming significant at the end of the study period (P = 0.045, Figure 8.7). Neither PD01 nor placebo did affect stool frequency or consistency throughout the study period (data not shown).

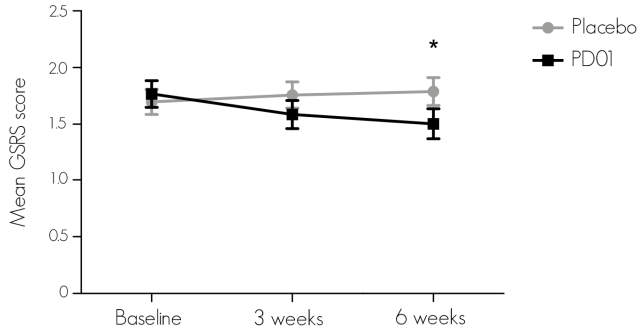


Figure 8.7 Change in GSRS score for indigestion syndrome from baseline to end of the study period. Values are means with their standard errors represented by vertical bars. * $P \leq 0.050$.

Table 8.7 Metabolic outcomes at baseline, after 3 wks and after 6 wks supplementation with placebo or PD01.[1]

	Placebo			PD01			P_1	P_2
	Baseline	3 wks	6 wks	Baseline	3 wks	6 wks		
Adhesion molecules								
sVCAM (ng/ml)	710 ± 35		682 ± 31	715 ± 38		689 ± 34		0.940
sICAM (ng/ml)	318 ± 13		320 ± 17	354 ± 14		350 ± 19		0.757
sE-selectin (ng/ml)	25.8 ± 2.1		30.1 ± 2.5	25.5 ± 2.2		29.3 ± 2.7		0.771
sP-selectin (ng/ml)	117.5 ± 6.8		128.8 ± 6.4	114.0 ± 7.3		127.7 ± 7.0		0.769
Glucose homeostasis								
Fasting glucose (mmol/l)	5.2 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	0.993	0.497
Fasting insulin (mU/l)	9.2 ± 1.1	9.5 ± 1.3	9.9 ± 1.1	9.6 ± 1.2	10.4 ± 1.3	10.0 ± 1.2	0.586	0.811
HOMA-IR	2.2 ± 0.3	2.3 ± 0.3	2.4 ± 0.3	2.3 ± 0.3	2.4 ± 0.3	2.4 ± 0.3	0.790	0.801
QUICKI	0.35 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.477	0.211
Plasma lipids								
Total cholesterol (mmol/l)	5.5 ± 0.2	5.3 ± 0.1	5.2 ± 0.1	5.6 ± 0.2	5.7 ± 0.1	5.6 ± 0.2	0.302	0.113
LDL-C (mmol/l)	3.3 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3.5 ± 0.2	3.5 ± 0.1	3.5 ± 0.1	0.438	0.192
HDL-C (mmol/l)	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	0.161	0.089
Triglycerides (mmol/l)	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	0.770	0.961

[1]Differences between placebo and PD01 tested with linear mixed model with correction for baseline values. P_1 represents the p-value for the analysis of baseline versus 3 wks intervention between placebo and PD01. P_2 represents the p-value for the analysis of baseline versus 6 wks intervention between placebo and PD01. HOMA-IR, homeostasis model assessment of insulin resistance. QUICKI, quantitative insulin sensitivity check index. sE-selectin, soluble E-selectin. sICAM-1, soluble intercellular adhesion molecule-1. sP-selectin, soluble P-selectin. sVCAM-1, soluble vascular cell adhesion molecule-1. [2]Estimated mean ± SEM (all such values).

Discussion

This is the first human study evaluating the intestinal fate of a carotenoid-producing bacillus strain as well as its potential GI and systemic effects. We have shown that PD01 survived transit through the GI tract and was able to germinate into vegetative cells under intestinal conditions and release carotenoids *in situ*. The latter were shown to have excellent bioavailability, with levels in blood at the end of the study in similar ranges as plant carotenoids. Repeated intake of PD01 was well tolerated and was even associated with a significant improvement of indigestion symptoms. In this first explorative study with obese, yet healthy volunteers, no significant effects on gut microbiota and barrier function, nor systemic effects on antioxidant activity, oxidative stress, adhesion molecules, glucose and insulin homeostasis, blood lipids and BP could be observed.

PD01 survived passage through the human GI tract and daily PD01 supplementation over a period of 6 wks led to a significant accumulation of PD01 carotenoids in plasma. PD01 carotenoids were detected in all subjects supplemented with PD01, demonstrating absorption and transport to the systemic circulation. Plasma concentrations of PD01 carotenoids were lower in comparison with those of some of the dietary carotenoids, but it should be taken into account that intake of these dietary carotenoids is of a much higher magnitude. On the other hand, intestinal absorption of bacterial carotenoids appears to be more efficient than that of dietary carotenoids. More specifically, the average lutein intake is estimated at 1-3 mg/day,[32] with plasma lutein concentrations of 0.12-0.13 μM. The calculated intake of PD01 carotenoids was only 0.014 mg/day with plasma concentrations of 0.04-0.08 μM. Despite the almost 100-fold lower intake of PD01 carotenoids, plasma levels were therefore only 2-fold lower as compared to lutein. No changes in plasma concentrations of dietary carotenoids were observed, indicating that these novel PD01 carotenoids do not compete with other carotenoids for absorption and transport.

In vitro, PD01 was shown to modulate the microbial intestinal environment by increasing the presence of two bacterial groups, namely the firmicutes and *Clostridium coccoides/Eubacterium rectale* groups. In this human intervention study we did not observe a significant effect of PD01, compared to placebo, on gut microbiota composition. No significant changes in the relative abundance of the dominant phyla or on the microbial composition at genus level were observed between groups. However, when analyzing the microbial composition over time separately in the PD01 group, significant increases in the fecal presence of several bacteria belonging to the lachnospiraceae and ruminococcaceae (part of the firmicutes) were observed, while in the placebo group no alterations in these species were detected. The lachnospiraceae and ruminococcaceae groups include many butyrate-producers, and an increase might be beneficial as butyrate plays a key role in maintaining gut homeostasis and epithelial integrity.[33]

In vitro, PD01 also altered SCFA profiles by inducing a shift from acetate to butyrate and by increasing lactate production. Here, no significant alterations were observed after PD01 vs. placebo supplementation. In both treatment groups, baseline stool pH was within normal ranges and did not significantly change after treatment. Additionally, SCFA production, monitored as a marker of saccharolytic fermentation, was stable throughout the study period and between groups. We also determined β-glucuronidase activity in the fecal samples, but found no between-group differences after 6 wks supplementation. High microbial β-glucuronidase activity has been associated with development of colon cancer as it may transform pro-carcinogenic substances carcinogenic.[34] On the other hand, high microbial β-glucuronidase activity is considered beneficial as it is associated with an increase in butyrate-producing microorganisms from the firmicutes phylum, particularly within clostridial clusters XIVa and IV.[34]

With respect to PD01-induced effects on the gut metabolome: PCA score plots revealed that interindividual variation caused by genomic variation and exposome influence (*e.g.* lifestyle factors[35]) were more dominant than potential bacillus-induced metabolomic effects, as analyzed by hierarchical clustering analysis. The best option to visualize the bacillus-induced changes would be to correct for the confounding factors (genetics, dietary factors, etc.) and to create a plot per individual. One could question the coverage of the detected metabolome but this proved more than adequate, as over 30% of the detected metabolites proved significantly altered when implementing the metabolomics fingerprinting approach on digestive fluid samples derived from *in vitro* gut simulations (unpublished data).

The above-discussed results of PD01 supplementation suggest that the current human study lacked sufficient resolution to confirm previously obtained *in vitro* and *in vivo* data. On the other hand, the obtained data should be interpreted as positive because a metabolic effect has been established (carotenoid production) without significantly disturbing microbiota composition or intestinal homeostasis of gut metabolomics. Indeed, the low incidence of GI complaints underpins the excellent tolerability of PD01 in our healthy obese individuals.

Furthermore, in addition to being well tolerated, the daily intake of PD01 for 6 wks even resulted in a significant decrease in GI symptoms of indigestion, such as borborygmi, abdominal distension, eructation and increased flatulation. This is in line with literature reports on (potential) pre-, pro- or sybiotics in healthy individuals or individuals with irritable bowel syndrome (IBS).[36-38] A possible explanation for a decrease in such symptoms is a GI decrease in bacterial groups with gas-producing abilities.[39,40]

Probiotics are suggested to improve GI permeability by stimulation of the intestinal mucosal defence.[41] The only available human intervention trial that evaluated the effect of a probiotic on GI permeability in similar subjects did not provide evidence for an improvement.[42] Interestingly, our findings suggest that GI effects of PD01 extend to improvement of GI permeability. While between group effects in GI permeability did not

reach significance, an indication towards improvement in gastroduodenal and colonic gut permeability was noted, as assessed with a validated multi-sugar test.

As PD01 produces carotenoids that have high structural similarity to lycopene, our study findings related to CVD risk markers should be compared with studies on lycopene.

Human intervention studies have shown that daily consumption of lycopene (20-30 mg/day; a dose 1000-fold higher than PD01 in this study) exerts beneficial effects on biomarkers of oxidative stress in healthy volunteers.[43] In our study, the bacterial carotenoids did not induce significant changes in TAC, urinary F_2-isoprostanes excretion or MDA. Baseline TAC values of our study population were however found to be higher than expected for overweight and obese subjects,[44] potentially masking beneficial effects of PD01.

The presence of PD01 carotenoids did not influence circulating adhesion molecules concentrations. These proteins, expressed on the vascular endothelium and on circulating leukocytes, mediate inflammatory processes and play an important role in the development and stability of atherosclerotic plaques.[45] Plasma concentrations reflect the degree of endothelial function. In our study, baseline plasma adhesion molecules values were comparable to those reported for similar study populations using the same analysis.[46] Human data among the effects of lycopene on plasma adhesion molecules are scarce and contradictory.[47,48]

Obesity may be associated with a wide cluster of metabolic alterations, including glucose homeostasis disorders and dyslipidemia. In animal studies, lycopene proved to decrease serum glucose, reduce total and LDL-cholesterol and to increase HDL-cholesterol.[49,50] In humans, results regarding the beneficial effects of lycopene on these parameters are not uniform.[51,52] Supplementing the carotenoid-producing bacillus strain PD01 to overweight and obese, but otherwise healthy volunteers, did not affect systemic glucose, insulin and lipid levels. In retrospect, since the participants had completely normal values at baseline, no significant improvement through dietary intervention was to be anticipated.

The BP lowering effect of lycopene, and of carotenoids in general, is supported by several,[53,54] yet not all,[55] human intervention trials. Carotenoids have been demonstrated to scavenge free radicals and to lower levels of reactive oxidative species, thus lowering oxidative stress. This may prevent oxidation of LDL-cholesterol, which in turn can trigger a cascade of events associated with atherogenesis.[56] Furthermore, oxidized LDL particles impair normal endothelial function by inhibiting nitric oxide release, an important vasodilator, and thus affect BP.[57] In our intervention study, no effects of PD01 treatment on systolic and diastolic BP were observed compared to placebo.

Some potential shortcomings of our study design should be mentioned. Firstly, with most baseline parameters within normal ranges, the induction of systemic beneficial changes in generally healthy subjects through dietary supplementation is challenging and when present often only marginal. Another possible limitation is the lack of a well-controlled diet. We instructed participants to maintain their habitual dietary intake throughout the study period,

as we aimed to assess the effects of PD01 as supplement to their habitual diet, which is in line with future applications. Based on the unchanged plasma concentrations of lutein, lycopene, and β-carotene it can be assumed that the habitual diet was indeed maintained. We cannot exclude that the presence of dietary carotenoids may have masked the additive effect of the bacterial carotenoids.

In conclusion, this study provides the first evidence in humans that PD01 survives transit through the GI tract and is able to germinate and release bacterial carotenoids, which are absorbed and detected in human blood plasma. Furthermore, even though systemic effects of the carotenoids could not be confirmed in this explorative study, repeated intake improved local GI parameters. These promising results warrant further research in specific target populations to investigate the specific bioactivities of PD01 in the intestine and on systemic parameters.

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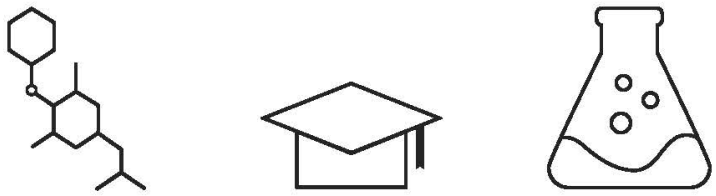

chapter 9

General discussion

Background

The relationship between food and health is complex, being multifactorial and multidimensional. Food contributes to human health and enhances the health potential, but can also be a key actor negatively influencing health and initiating or contributing to illness. The primary role of food is to provide sufficient nutrients to meet the nutritional requirements of an individual. Furthermore, food supplies the body with materials that can be used for the construction and repair of body tissues. This allows an individual to grow and thrive. Recently, there has been a growing interest in the health-enhancing role of specific foods in addition to their nutritional value. Efforts to identify functional and bioactive components from many natural sources have been intensified and have eventually led to a new group of products, the so-called functional foods. A food can be classified as "functional" when it contains a component (whether a nutrient or not) that affects one or more functions in the body in a targeted way so that positive effects on health[1] are to be expected, or when the component has a physiological or psychological effect beyond the traditional nutritional effect.[2] Functional foods may originate from plant and animal sources, from microorganisms or marine organisms.

In many low- and middle income countries access to food, availability and quality of food/nutrition are major concerns. To date, worldwide, approximately 800 million individuals are plagued by undernourishment and food insecurity.[3] These individuals, often children, consume insufficient protein and energy, and the adverse health effects of this are often compounded by deficiencies of vitamins and minerals. In these countries, easy-to-remedy nutritional deficiencies prevent 1 of 38 newborns from reaching an age of 5 years.[4] In contrast, the prevalence of overweight and obesity, linked to excess food intake and overnutrition, has more than doubled since 1980. In 2014, worldwide, more than 1.9 billion adults aged 18 years and older were overweight, and of these, over 600 million were obese.[5] Overweight and obesity are associated with alterations in gut microbiota composition and activity,[6,7] impaired gut barrier function,[8-10] gastrointestinal and systemic low-grade inflammation,[11-14] impaired vascular functions,[15,16] and glucose and lipid homeostasis disorders.[17,18] These gastrointestinal and systemic metabolic abnormalities are major risk factors for the development of diabetes mellitus type 2 (DM2) and cardiovascular diseases (CVD).[19-21] Worldwide, overweight and obesity now cause more deaths than underweight. Consequently, obesity and related illnesses have an enormous health and economic impact on society.[22]

Nutritional interventions in healthy, overweight and obese individuals

The aim of this thesis was to investigate the effects of several nutritional interventions on gastrointestinal and metabolic health in healthy, overweight and obese individuals. This thesis presents the results of six randomized-controlled trials. The nutritional interventions we studied included a prolyl endoprotease, a putative prebiotic supplement, a putative probiotic species, and two different phytonutrients.

Gastrointestinal gluten degradation by a prolyl endoprotease

Ingestion of gluten - a storage protein present in wheat, barley and rye - can induce a wide range of gluten related disorders with different pathogenic pathways, globally affecting a large proportion of the population.[23,24] In subjects suffering from celiac disease and wheat allergy, the presence of gluten peptides in the small intestine triggers an abnormal immune response resulting in intestinal inflammation and ultimately villous atrophy and crypt hyperplasia.[25] The mechanisms by which gluten induce symptoms in subjects with non-celiac gluten sensitivity remain largely unknown, but involvement of the innate immune system has been well established.[26,27] For all conditions, strict adherence to a gluten-free diet is the only effective treatment. However, the high cost, poor availability and poor palatability of gluten-free products makes it difficult to strictly adhere to such diet on the longer term.[28] In addition, gluten-free food products available on the market often contain trace amounts of gluten.[29] When maintained appropriately, a gluten-free diet is not nutritionally optimal; it may predispose to an undesirable high intake of fat and carbohydrates and lead to poor vitamin status and detrimental weight gain or weight loss.[30] Due to the consequences of gluten-free dieting, an unmet need for alternative therapies exists. Several options have been investigated. One approach is to use compounds that do not have the immunogenic proteins found in wheat derived gluten. Several wheat substitutes are available, including sorghum, almonds, rice, corn and legumes.[25,31] Another option is selecting and breeding wheat products that only contain low amounts of T-cell stimulatory sequences, thereby reducing the immunotoxic effects.[32] Furthermore, developing genetically modified gluten with reduced immunogenicity might be a potential future option for celiac disease patients. By blocking glutamine residues, the affinity of gluten peptides to bind to DQ2 and DQ8 molecules is reduced, resulting in a reduced T-cell-mediated inflammatory response.[33] Wheat fermentation with sourdough lactobacilli and fungal proteases has also been shown to reduce wheat toxicity.[34-36] However, as gluten protein is important in baking due to its strengthening and binding properties, it will be difficult to alter or select a variety of wheat completely devoid of gluten without compromising its mechanical properties. Binding glutens to polymers in the gastrointestinal tract may also prevent degradation, absorption and

triggering of an immunologic response.[37] Another interesting alternative treatment is oral enzyme supplementation to degrade gluten peptides. Food-derived gluten contains considerable amounts of proline and glutamine amino acids, which are highly resistant to enzymatic proteolysis within the human digestive tract.[38] Prolyl endoproteases are enzymes that are able to target and destroy proline and glutamine residues into small nontoxic gluten peptides before they enter the intestinal epithelium.[39] In **Chapter 2** we studied the *in vivo* gluten-degrading potential of *Aspergillus Niger*-derived prolyl endoprotease (AN-PEP). AN-PEP showed to efficiently degrade gluten from a meal in the stomach of healthy volunteers, within the time period a meal normally resides in the stomach. These *in vivo* results confirmed previously obtained *in vitro* observations.[40,41] In addition, we hypothesized that increasing the caloric density of a meal enhances gluten degradation by delaying gastric emptying rate, thereby prolonging exposure time of gluten proteins to AN-PEP, endogenous proteases and gastric acid. Indeed, increasing meal caloric density delayed gastric emptying and prolonged the gastric residence time of AN-PEP, but it did not affect its efficiency in degrading gluten. To standardize each meal intake we intragastrically administered the test meal via a triple lumen nasogastroduodenal tube, this obviously does not represent a fully physiological meal setting. Thus, data on the gluten-degrading potential of AN-PEP in an actual meal setting in gluten-intolerant subjects are needed. Also microbial prolyl endopeptidases from other species, as well as germinating cereal enzymes, have shown to possess the capacity to hydrolyze gluten peptides *in vitro*[42-44] and *in vivo*.[45-47] A combination of both, known as ALV003, already proved that it is able to attenuate gluten-induced mucosal injury in celiac disease patients.[46]

In summary, enzyme supplementation therapy targeted at degradation of immunogenic gluten peptides appears to be a promising approach. Development of such a supplement as alternative for a gluten-free diet is still in its early phase of validations and subsequent implementation. It is anticipated that enzymes first will be marketed as a supplement 1) when ingested in combination with a gluten-free diet, 2) when ingested with a normal gluten containing meal, for social reasons or 3) in conditions where persons report gluten hypersensitivity in absence of celiac disease.

Food-drug interaction

To exert a systemic effect, a compound has to be absorbed from the gastrointestinal tract. The onset of a systemic effect heavily depends on the rate of absorption, which is in part determined by the gastric emptying rate. In **Chapter 3** we critically evaluated a well-established method of measuring gastric emptying, namely by measuring plasma concentrations of paracetamol. It is assumed that paracetamol is absorbed almost immediately after reaching the small intestine and that the transit time of paracetamol through the stomach is identical to that of the water phase of a meal. Thus, gastric emptying

rate might be derived from the time course of plasma paracetamol appearance.[48,49] However, in Chapter 3, we demonstrated that paracetamol when added to a meal and mixed, was divided in two fractions. One fraction was dissolved in the liquid content of the meal which quickly left the stomach and was absorbed in the duodenum, reflected by an early, high plasma peak. A second paracetamol fraction was encapsulated by the meal, leaving the stomach more slowly and was even not taken up instantaneously after reaching the intestine. Both fractions did not accurately reflect gastric emptying rate. Additionally, this showed us that the rate-limiting factor for absorption was actually the bioaccessibility, *i.e.* release of paracetamol from the food matrix. The food matrix and structure can have both positive and negative effects on bioaccessibility.[50] Thus, it is important to realize that food-drug interactions are not limited to the effect of food on gastric emptying alone.

The human gut microbiome

The human gut microbiome harbors several trillion microbes residing in the gut.[51] It serves a wide array of functions, including regulating normal functioning of the gastrointestinal tract, harvesting energy from indigestible parts of the diet, maintaining intestinal epithelial integrity, metabolizing indigestible polysaccharides, absorbing short-chain fatty acids (SCFA) produced by bacterial fermentation and regulating intestinal transit.[10,52-55] The colonization of the human gut by micro-organisms starts at gestation, during birth and immediately afterwards. Bacteria from the mother and surrounding environment colonize the infants gut. It is affected by several factors such as genetic background, gestational age, mode of delivery, type of feeding, exposure to antimicrobials and, very important, the diet throughout life.[56] Long-term perturbations to the intestinal ecosystem can induce changes in the microbiota, contributing to the development and progression of a number of gastrointestinal disorders[57] such as irritable bowel syndrome, celiac disease, inflammatory bowel disease and colorectal cancer, as well as systemic diseases such as allergic diseases,[58] non-alcoholic steatohepatitis,[59] obesity[54,60,61] and diabetes.[62] Thus, targeting the microbiome seems to offer a promising approach to potentially ameliorate above mentioned gastrointestinal and metabolic diseases. A number of dietary strategies are available for modulating the composition and function of the intestinal microbiota; pre- and probiotics are among the most well established.

Gut microbiota, gut barrier and immune modulation by prebiotics

Prebiotics are non-digestible compounds that selectively stimulate growth and/or activity of one or a limited number of microbial species in the gut with potential health benefits to the host.[63] Many animal studies showed positive results of prebiotics in the prevention or treatment of gastrointestinal and chronic metabolic diseases, but human data remain

ambiguous.[64] SCFA are produced by bacterial fermentation of non-digestible compounds. These metabolites display several important functions; they maintain energy homeostasis, are able to affect cell proliferation and differentiation, have anti-inflammatory and immunomodulating effects, serve as an energy source for colonocytes and are essential in establishing and maintaining the gut barrier by regulating expression of tight junction proteins.[65-67] Furthermore, high amounts of intestinal SCFA lower intestinal pH, affect microbiota composition by inhibiting the growth of pathogenic bacteria, and creating a more favourable environment for the growth of beneficial bacteria.[68,69] Most studied prebiotics are fructooligosaccharides, galactooligosaccharides, inulin and resistant starch. These prebiotics are predominantly fermented in the more proximal colon and are limited in reaching the distal colon.[70,71] However, several chronic colonic diseases originate from the distal colon; consequently, there is a great interest in finding prebiotics that are able to reach, are fermented, and act in the more distal regions of the colon. Arabinoxylans (AX) form an interesting novel class of such type prebiotics, as they rely on a whole spectrum of microbial enzymes for their degradation, resulting in a more distal fermentation and action.[72-74] In **Chapter 4** we have described the results of a human study supplementing an AX extract to overweight and obese individuals for several weeks. Previous *in vitro* and animal studies already had shown that this specific extract was able to modulate gut microbiota composition and microbial activity and, as a consequence, gut barrier function.[70,75-77] In our *in vivo* study the overall microbiota composition of participants did not significantly change following AX treatment, but increases in fecal SCFA concentrations and a decrease in fecal pH were observed.

Modulation of gut microbiota by prebiotics has been associated with improvements in gut barrier function. The exact underlying mechanisms are poorly understood. An increased villus height and crypt depth, a thickened mucosal layer, increased transcription and consequently increased synthesis of tight junction proteins, and a change in the distribution and localization of tight junction proteins may contribute.[9,78-81] The integrity of the gastrointestinal epithelium depends on formation of adhesive contacts between neighbouring epithelial cells. This is mediated by the apical junctional complex, which is composed of the tight junction and subjacent adherence junction. These multi-protein complexes are considered critical regulators of the gastrointestinal barrier function. Adherence junctions are required for assembly of the tight junction, which seals the paracellular space. Tight junction proteins consist of transmembrane proteins, peripheral membrane (scaffolding) proteins and regulatory molecules. The two major tight junction transmembrane proteins are the claudins and occludins. Peripheral membrane proteins, such as members of the zonula occludens family, function as scaffolding proteins between transmembrane and cytoplasmic proteins. In addition, it has been proposed that these proteins link adherence junctions to tight junctions through protein linkages.[82,83] In **Chapter 4**, AX showed to affect gut barrier function; as upregulation in the transcription of the tight

junction proteins claudin-3, claudin-4 and occludin in sigmoid biopsies was observed. The changes in tight junction gene expression were not accompanied with changes in mucosal tight junction protein expression or in gastrointestinal permeability as assessed with a functional test.

Another important function of the colonic microbiota is its role in the development and maturation of the immune system. The gut contains an important component of the body's immune system, called gut-associated lymphoid tissue. The immune system must establish an appropriate balance between tolerance to the gut commensal microbiota and vigilance to guard against infectious agents and opportunistic pathogens. The cross-talk between the intestinal microbiota and host involves both the innate and adaptive immune system. Imbalances in gut microbiota and consequently in immune homeostasis may lead to a number of pathologies, such as obesity, DM2, inflammatory bowel disease and colorectal cancer.[84] Previous studies indicate that prebiotics can exert direct immunomodulatory effects[85,86] and indirect immunomodulatory effects through changing the gut microbiome.[87] Microbiota-dependent changes affecting the immune system include stimulation of the growth and metabolism of protective commensal intestinal bacteria. An increase in the presence of beneficial bacteria, such as bifidobacteria and lactobacilli, will provide antimicrobial effects by direct competition with pathogenic bacteria for available binding sites on the intestinal epithelium and for nutrients.[88,89] Furthermore, some bacterial species are able to produce antibacterial substances that can inhibit the growth and survival of pathogens.[90] In addition, SCFA might have direct immunomodulatory properties.[91-93] To assess the effects of AX on immune response, we applied an *ex vivo* model using PHA-stimulated whole blood to evaluate the capacity to produce cytokines (**Chapter 4**). With AX treatment we observed a significantly decreased production of the pro-inflammatory cytokine TNFα, suggesting a capacity to reduce the intensity of an acute pro-inflammatory reaction. As SCFA are able to suppress the production of such cytokines, the decrease in TNFα production might be linked to the observed increase in fecal SCFA.

Altogether, the data presented in **Chapter 4** suggest that AX modulate gut microbial activity and thereby may positively affect gut barrier and immune function.

Gut microbiota and gut barrier modulation by probiotics

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[94] Most commercial probiotic products contain lactic acid bacteria, such as lactobacilli, streptococci and bifidobacteria.[95] For a probiotic it is important that it survives the acidic intestinal environment, not being degraded before reaching the site where it is presumed to be active. As with prebiotics, despite convincing and reproducible results from animal studies showing beneficial effects of different probiotic strains on gastrointestinal and metabolic health, the data in humans remain equivocal.[64,96] In

Chapter 8 we studied the effects of a *Bacillus indicus* strain on the gut microbiota composition and activity in overweight and obese individuals. Bacillus species are gram-positive bacteria able to form endospores, *i.e.* uniquely robust entities that are able to survive extremes of temperature, irradiation and long-term storage.[97] While some spore-forming species play a role in food poisoning, several species of bacillus are consumed as food ingredients and regarded safe for human consumption.[98] Bacillus spores are able to pass the gastric barrier without loss of function, are stable to many food processing steps and can be stored at room temperature, making them attractive as a probiotic. Previous (unpublished) *in vitro* research showed that this carotenoid-producing *Bacillus indicus* strain was able to survive and germinate in the gastrointestinal tract and to modulate the intestinal environment by increasing the presence of two bacterial groups, namely the firmicutes and *Clostridium coccoides/Eubacterium rectale* group. Furthermore, it was shown to alter SCFA profiles by inducing a shift from acetate to butyrate, and it increased the lactate production. This shift may provide health benefits for the host. Indeed, in Chapter 8 we demonstrated that *in vivo* the *Bacillus indicus* strain was able to survive passage throughout the digestive tract as viable PD01 cells and spores were found in the feces of all individuals supplemented with the strain. However, no significant treatment effects regarding intestinal microbiota composition and microbial activity were observed. We also did not observe modulation of gut barrier function by the *Bacillus indicus* treatment. The only significant improvement with this *Bacillus indicus* strain we observed was a decrease in reported gastrointestinal symptoms such as borborygmi, abdominal distension, eructation and increased flatulation. The occurrence of gastrointestinal symptoms was already low at baseline of the study, but significantly reduced after 6 weeks PD01 supplementation compared to placebo. In IBS patients, it has been shown that a difference in gut microbiota composition and activity causes more intestinal gas production, impairs the transit and tolerance of gas, resulting in gastrointestinal symptoms such as bloating. It was also shown that modulation of the gut microbiome by probiotics caused a relief in these symptoms, suggesting that these products are able to induce a quantitative and qualitative gastrointestinal decrease of bacterial groups with gas-producing abilities, such as clostridium species, in the colonic mucosa.[99-101] Our findings are in line with previous literature reports on effects of (potential) pre-, pro- or synbiotics on symptoms or gastrointestinal function in healthy individuals or individuals with irritable bowel syndrome.[102-104]

Metabolic consequences of gut microbiota modulation

Alterations in gut microbiota composition have been shown to underlie or influence the development of metabolic disorders.[7,105,106] Both in Chapter 4 and Chapter 8 we explored the effect of putative pre- and probiotic products on metabolic markers. Data with respect to beneficial effects on glucose homeostasis and lipid metabolism in animals are widely

available,[75,107-110] while human studies investigating these parameters report conflicting results.[111-119] Both the AX extract and the *Bacillus indicus* strain did not affect fasting glucose, insulin and lipid concentrations in our study population. However, baseline values were already within the normal ranges, making it difficult to improve these values. Long-term intervention studies in people with pre-diabetes, metabolic syndrome or hypercholesterolemia are required to determine whether pre- or probiotics exert beneficial effects in high-risk individuals.

In vivo bioavailability of hesperidin

Large epidemiological studies have shown that the intake of fruits and vegetables exerts positive effects on metabolic and cardiovascular health.[120-123] These effects are partly due to the large amount of polyphenols that these products contain. Hesperidin is such a type polyphenol. It is abundantly present in the peels of citrus fruits.[124-129] In order to exert systemic health effects, it is essential that a compound is bioavailable and absorbed from the gastrointestinal tract into the systemic circulation. It has been demonstrated that the bioavailability of hesperidin is low in humans,[130,131] due to a combination of factors. Hesperidin is poorly water soluble[132] and it requires to be fermented by colonic microbiota to release and enable the absorption of its aglycone.[131,133,134] It has been hypothesized that glycosyl hydrolases from the genus bifidobacterium are involved in the hydrolysis of glycol-conjugate forms of phytochemicals.[135] Commercially available hesperidin supplements often elicit less biological effects than natural hesperidin due to a different enantiomer configuration. As a result, several attempts have been made to improve the bioavailability of hesperidin.[136-138] One way to improve bioavailability is by micronization of this poorly soluble compound. Micronization decreases the particle size resulting in an improved rate of dissolution and thus absorption from the gastrointestinal tract into the systemic circulation.[139] In **Chapter 5** we demonstrated that the bioavailability of a micronized hesperidin 2S extract, which has a specific enantiomer configuration similar to natural hesperidin, was improved over that of a non-micronized hesperidin extract with an enantiomer configuration similar to commercially available hesperidin extracts. Improving the bioavailability of hesperidin is important to fully exploit its beneficial properties concerning vascular and metabolic health, as studied in the paragraphs below. Furthermore, we observed considerable inter-individual differences regarding plasma metabolite concentrations. This finding is supported by other human trials investigating the bioavailability of polyphenols.[130,140,141] As differences in gut microbiota composition between subjects are common, significant inter-individual differences in the microbial transformation of polyphenols will exist.[142-144] This may serve as an explanation for inter-individual differences with respect to biological activity and systemic health effects of a polyphenolic compound.

The role of hesperidin in vascular health

Based on the results of Chapter 5, we have chosen the micronized hesperidin 2S formulation to study hesperidin-induced effects on cardiovascular and metabolic parameters in overweight and obese individuals (Chapter 6 and Chapter 7). Hesperidin is considered a strong antioxidant.[145,146] It has been demonstrated that it increases the production of nitric oxide (NO) in the vascular endothelium by phosphorylation and activation of endothelial NO synthase (eNOS).[126] NO participates in highly active metabolic and regulatory functions including the regulation of endothelial function and blood pressure, and plays a protective role in the development of atherosclerosis.[147-150] Both *in vitro* and animal studies have reported consistent, beneficial effects of hesperidin on endothelial function and blood pressure,[126,151,152] while human data is limited and contradicting.[125,126] As demonstrated in Chapter 6, six weeks daily hesperidin supplementation significantly reduced the levels of circulating adhesion molecules, reflecting an improvement in endothelial function.[153] Basal flow-mediated dilation (FMD), the gold standard method for measuring endothelial function,[154] was not improved by hesperidin. However, after inducing temporary endothelial dysfunction, hesperidin protected from postprandial deterioration in FMD in a subgroup of subjects. We excluded subjects with a very low FMD value as it is unlikely that short-term dietary interventions are able to improve such chronically impaired endothelial function[155] and additionally, the FMD measurement becomes inaccurate.[156,157] In addition, hesperidin treatment appeared to reduce both systolic and diastolic blood pressure, but this was only significant in the subgroup. As shown in Chapter 7, we did not find any effect of the same hesperidin product on blood pressure after six or twelve weeks daily intake. These contradictory findings reflect the current situation of discrepant results of hesperidin, but also of other polyphenols, on vascular function in humans.[142,158]

Impact of hesperidin on metabolic parameters

Hesperidin is able to induce glucose lowering effects through altering the transcription of genes encoding regulatory enzymes of glycolysis, gluconeogenesis and glucose utilization in the liver. In addition, it is able to change the expressions of hepatic glucose transporter 2 and adipocyte glucose transporter 4, both playing a critical role in glucose and insulin homeostasis. Furthermore, hesperidin has blood lipid lowering properties, possibly by affecting enzymes involved in lipogenesis, fatty acid biosynthesis, triglyceride synthesis, cholesterol synthesis and cholesterol absorption, and by facilitating fecal triglyceride and cholesterol excretion.[159] Both *in vitro* and animal studies have indeed shown beneficial effects of hesperidin on lipids[159-162] and glucose metabolism.[159,162-164] Positive human results also exist, but only in (pre)diabetic and hyperlipidemic subjects.[126,128,165] Both in Chapter 6

and Chapter 7 no blood glucose- or lipid lowering effects of hesperidin treatment were observed. This can be explained by the fact that the participants had normal values from start of the trial. Long-term intervention studies in subjects with (pre)diabetes, metabolic syndrome or hypercholesterolemia are needed to establish and confirm beneficial hesperidin-induced effects on systemic metabolic health markers.

Gut-microbiota dependent effects of polyphenols

As noted above, the gut microbiome plays a critical role in transforming dietary polyphenols into absorbable biologically active species. Recent data suggests that different types of polyphenolic compounds, including hesperidin, are also able to modulate the gut microbiota composition and function towards a more 'health promoting profile'.[166-171] In Chapter 7 we demonstrated that hesperidin did not affect the quantity of SCFA production, but was able to alter the SCFA profile. Hesperidin induced a significant shift from acetate to the health-promoting SCFA butyrate, possibly via intestinal stimulation of butyrate-producing bacteria. Butyrate functions as an important energy source for colonocytes, exerts anticarcinogenic, anti-inflammatory and antioxidant effects, reinforces the colonic defence barrier, and is able to modulate gut motility and satiety.[172] These results strengthen the hypothesis that polyphenols are able to modulate gut microbiota composition and function, in turn promoting gut and host health. However, more human intervention studies with a large number of subjects are needed to establish the potential prebiotic-like effects of hesperidin and other polyphenols.

Antioxidant activity of carotenoids

The incidence of CVD is notably lower in countries with a 'Mediterranean diet', containing a large amount of fruit, vegetables and olive oil.[173,174] These foods are rich in carotenoids, and a link between carotenoid intake and protection against oxidative stress, atherosclerotic progression, hypertension, inflammation and endothelial dysfunction has been shown.[175-177] The protection by carotenoids is mainly through their antioxidant activity. They act as antioxidants via several mechanisms. Firstly, they are able to quench highly reactive oxygen species, that have the capacity to oxidize nucleic acids, unsaturated fatty acids or amino acids.[178] Furthermore, they are able to inhibit free radical reactions, impeding the process of lipid peroxidation, with repair of vitamin E and C radicals.[179,180] Besides intervening in radical reactions, carotenoids have also been shown to upregulate the antioxidant response element, resulting in another way of protecting cells against highly reactive oxygen species.[181] The antioxidant capacity of carotenoids is well demonstrated *in vitro*,[178,182] but in humans evidence is still lacking that carotenoids act as antioxidants.[183] Other mechanisms explaining the beneficial effects of carotenoids include their influence on

gap-junction communication, gene function regulation, hormone and immune modulation, and modulation of drugs metabolism.[184-186]

In vivo bioavailability of bacterial carotenoids

Humans lack the capacity to synthesize carotenoids by themselves, and therefore depend on dietary intake and subsequent absorption. The majority of commercially available natural carotenoids are derived from plant-based materials, but also some bacterial species are able to synthesize these compounds. The quality and consistency of plant-based carotenoids are dependent on plant growth conditions, which vary tremendously.[187,188] Furthermore, these carotenoids are degraded in the acidic environment of the stomach.[187,188] Bacterial carotenoids, produced by spore-forming bacillus strains, deliver gastric-stable carotenoids which are equally or more bioaccessible and bioavailable than plant-based carotenoids. In Chapter 8 we studied the *in vivo* bioavailability of bacterial carotenoids in overweight and obese subjects. After supplementing the participants with a carotenoid-producing bacillus strain for six weeks, we were able to confirm that bacterial carotenoids were present in the plasma and levels increased throughout the study period. This indicates that the bacillus strain produces *in vivo* bacterial carotenoids, which are absorbed and transported to the systemic circulation. Absorption of plant-based carotenoids occurs once carotenoids are released from the food matrix. Dissolution of released carotenoids in bulk lipid droplets is followed by the formation of micelles and uptake of these micelles by duodenal mucosal cells occurs through passive diffusion. The micellular carotenoids are then incorporated into chylomicrons and via the lymphatics released into the circulation.[189] The mechanism of absorption of these bacterial carotenoids has not been elucidated up to now. Possibly, the mechanism is the same as for lycopene due to the high structural similarity of the bacterial carotenoids with lycopene.[190] Furthermore, uptake of the bacterial carotenoids did not impede the presence of lutein, β-carotene and lycopene in plasma. It has been suggested that carotenoids may compete with each other or with other lipid-soluble nutrients for their absorption and metabolism, but there is discrepancy in results from the various studies in humans.[191-195]

Carotenoids in vascular and metabolic health

Compared to plant-based carotenoids, the bacterial carotenoids were shown to have a ten-fold higher *in vitro* antioxidant activity. As noted before, the relevance of the antioxidant properties of plant-based carotenoids *in vivo* remains a matter of debate. The effects of carotenoids on oxidative stress,[196-199] endothelial function,[196,197,200] glucose homeostasis,[201] lipid metabolism[197-199,201,202] and blood pressure[199,201,203] in humans also remain contradictory. Most of the beneficial effects were obtained in individuals at risk for

developing CVD or DM2. In Chapter 8 we also explored the antioxidant effects, and effects on cardiovascular and metabolic parameters of the bacterial carotenoids. No cardiovascular or metabolic effects were observed, probably due to the fact that participants had normal values at start of the supplementation period for most parameters. Furthermore, other dietary carotenoids may have masked the effects of the bacterial carotenoids, or the production and/or absorption of the bacterial carotenoids was not high enough to induce systemic health effects.

In summary, the use of bacterial carotenoids over plant-based carotenoids may be preferred for several reasons. *In vitro* studies have shown that these bacterial carotenoids deliver gastric-stable carotenoids, which have equal or larger bioaccessibility and bioavailability levels than other, well-established sources of dietary carotenoids, and exert a higher antioxidant capacity. Furthermore, the carotenoid-producing bacillus strain also possesses the ability to act as a probiotic product.[204] In addition, a microbial fermentation using standard optimized conditions allows for producing a high-quality product with a consistent purity. However, more human *in vivo* studies are required to confirm the improved bioavailability, antioxidant capacity and systemic health effects of bacterial carotenoids over plant-based carotenoids.

Implications, suggestions for future research

Food provides taste, aroma, nutritive value and the elements for an individual to grow and live. Recently, functional and bioactive components from foods, inducing positive health effects, have been identified, the so-called functional foods. In this thesis, we have described the results of six randomized controlled trials investigating different nutritional interventions in healthy, overweight and obese individuals. All of the studied nutritional interventions may affect one or more functions in the body in a positive way and thus may be considered a functional food.

In summary, we examined food-drug interactions on the intestinal absorption of a compound, using paracetamol. In addition, the *in vivo* bioavailability of a polyphenol and of bacterial carotenoids was explored. The efficacy of a prolyl endoprotease in gastrointestinal gluten degradation was investigated in healthy humans. In overweight and obese individuals, the gut microbiota and gut barrier modulating capacities of a potential pre- and probiotic product were assessed. Furthermore, the effects of a polyphenol (hesperidin) and of bacterial carotenoids on cardiovascular and metabolic health parameters have been studied.

The potential of the gluten-degrading enzyme AN-PEP is obvious; it efficiently degrades gluten from a meal, within the time period a meal usually resides in the stomach. From a clinical perspective, additional data are needed from randomized controlled trials,

exploring the efficacy of AN-PEP in an actual meal setting in subjects intolerant to gluten. Targeting the gut microbiota by pre- and probiotics seems to be a promising approach to prevent the development or to ameliorate many gastrointestinal and metabolic diseases. *In vitro* and animal studies are widely available, showing convincing and reproducible data of pre- and probiotic products on gastrointestinal and metabolic health. However, human data are scarce. In this thesis we found clear AX-related effects on gut microbial activity, limited AX-related effects on the gut barrier and immune system, and no effects regarding gut microbiota composition. The putative probiotic, which produces carotenoids, did not induce any effects on the gut microbiota composition and function or on gut barrier, which might be considered positive: stimulating a metabolic effect (carotenoid production) without further affecting microbiota composition or intestinal homeostasis. In this thesis we also studied the effects of two different phytochemicals in overweight and obese individuals. Both hesperidin and carotenoids act as antioxidants, and many *in vitro* and animal studies have reported consistent, beneficial effects of these compounds on cardiovascular and metabolic parameters. Again, data in humans are contradictory. In our human studies, hesperidin showed to have beneficial influences endothelial function and blood pressure, while the carotenoids did not induce significant improvements. In order to exert systemic health effects it is essential that a compound is bioavailable and absorbed from the gastrointestinal tract into the systemic circulation. We have observed that several factors affect bioavailability and absorption of a compound including structure, solubility and particle size of the compound, gut microbiota composition and activity, and bioaccessibility. The different compounds, studied in this thesis, all showed to have beneficial effects in *in vitro* and in animal studies, but their efficacy in humans remains equivocal. Several explanations can be given for this.

In most studies we investigated the effects of the nutritional intervention in overweight and obese individuals. We consider overweight and obesity as "mild disease", associated with a broad spectrum of well-recognized risk factors for the development of chronic metabolic diseases such as cardiovascular diseases and diabetes mellitus type 2. However, usually these individuals were healthy at baseline. Therefore, an improvement of an already 'stable and excellent' onset condition is not to be anticipated after giving an intervention. Furthermore, a great variation exists between individuals with regard to general health, genetic make-up and expression, gut microbiome, environmental factors, lifestyle and diet. Also, large inter-individual variability exists regarding metabolite excretion of the compounds that have been described.[140,205] In addition, components of the habitual diet can impact on the bioavailability of a compound. We could not control these factors in our studies. This leads to large inter-individual differences regarding the bioavailability, absorption and biological activity of a compound.

The development of nutritional therapies that target on either prevention or on improvement of an already existing derangement of chronic gastrointestinal, cardiovascular and

metabolic diseases is important and is considered an attractive alternative for medication use. First, we need large, well-designed, placebo-controlled randomized trials investigating the safety and efficacy in well-defined or phenotyped high-risk populations. Another approach is to apply a stressor in order to induce a disturbance in homeostasis and evaluate the nutritional intervention either before or during application of the stressor. It is important that the international scientific community reaches consensus on standardized study designs for nutritional interventions in human beings. In addition, it is mandatory to study nutritional interventions in well-defined study populations to clarify in which people the intervention is actually effective.

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addendum

Summary

The primary role of food is to provide sufficient quantitative and qualitative supply of nutrients to meet the nutritional requirements of an individual. In addition food may also have health promoting effects. Initially, scientific focus was given on the identification of essential elements and their role in the treatment and prevention of various deficiencies and of diseases associated with specific deficiencies. In recent years, the role of diet in diseases linked to excess food intake and to 'overnutrition' has become topic of clinical interest and of research. The discovery and further development of functional foods is a fast growing segment of the food industry. Functional foods are defined as foods or food ingredients that affect one or more functions in the body in a targeted way so that positive effects on health are to be expected, or when the component has a physiological or psychological effect beyond the traditional nutritional effect. The role of functional foods in disease prevention and health promotion is nowadays a research topic.
In this thesis, we aimed to investigate the effects of several nutritional interventions on gastrointestinal and metabolic health in healthy, overweight and obese individuals. The nutritional interventions we studied include a prolyl endoprotease, a putative prebiotic product, a putative probiotic species and two different phytonutriets.

Gluten, a protein rich in proline, is poorly degraded in the gastrointestinal tract due to the gastrointestinal absence of proline-cleaving proteases. The presence of long proline-rich gluten peptides in the small intestine can induce a wide range of gluten related disorders with different pathogenic pathways. Prolyl endoproteases are enzymes that possess the ability to cleave at internal proline residues within the gluten. In **Chapter 2** we investigated the *in vivo* efficacy of an *Aspergillus Niger*-derived prolyl endoprotease (AN-PEP) in degrading gluten. AN-PEP efficiently degraded gluten from a meal in the stomach of healthy volunteers before entering the duodenum. Furthermore, increasing meal caloric density did delay gastric emptying time, but did not affect gluten degradation efficiency. In **Chapter 3** of this thesis we critically evaluated the use of paracetamol as postprandial marker for gastric empyting. We showed that paracetamol, when added to a meal and mixed, was divided into two fractions; one fraction encapsulated by the meal and one fraction dissolved in the liquid content of the meal. The rate of absorption of these fractions was different, and both did not accurately reflect gastric emptying rate.

Modulation of the gut microbiota by prebiotics appears to be an interesting approach to enhance gut barrier function, and to treat or even prevent the onset or deterioration of chronic diseases. A limitation of most prebiotics is their rapid fermentation in the proximal colon. Arabinoxylans (AX), the most abundant non-digestible carbohydrates present in wheat, represent an interesting novel class of potential prebiotics. Due to their structure,

they rely on a whole spectrum of (microbial) enzymes for degradation, resulting in a more distal fermentation and activity. Furthermore, AX structure affects the fermentation pattern and immune modulation, with highest acitivity observed for long-chain AX. In Chapter 4 we aimed to investigate the 6 weeks effect of two different doses high-molecular weight AX on the gut barrier, gut microbiota structure and activity, immune system and metabolic markers in overweight and obese individuals. Compared to placebo, AX intake did not significantly change the relative abundance of the dominant phyla, but a decrease in microbial richness and diversity was observed but only after 15 g AX intake, not with the lower dose of 7.5 g AX. Furthermore, the fecal concentrations of the individual short-chain fatty acids (SCFA) acetate, butyrate and propionate, and of the total SCFA were increased upon intake of AX, compared to the placebo group. Six weeks 7.5 g AX also significantly lowered fecal pH compared to placebo. This modulation in gut microbial activity may affect the gut barrier and the gut immune system. An upregulation in the transcription of the junction (TJ) proteins occludin, claudin-3 and claudin-4 in sigmoid biopsies, either after 7.5 g AX or 15 g AX supplementation, was observed. However, no significant changes in TJ protein expression nor in gastrointestinal permeability were found in the AX groups, compared to placebo. In addition, we observed a significant decrease in the production of the pro-inflammatory cytokine TNFα by stimulated peripheral blood mononuclear cells after 6 weeks 15 g AX intake.

Large scale studies have shown that intake of flavonoid-rich food products has positive effects on cardiovascular diseases (CVD) and their related risk factors. To exert health effects *in vivo* it is essential that flavonoids are bioavailable, absorbed from the gastrointestinal tract and reach the systemic circulation. Hesperidin, a flavanone glycoside from sweet orange peels, is able to exert positive effects on metabolic and cardiovascular health, but has poor bioavailability in humans. An option to improve the bioavailability of a compound is to improve the dissolution process by micronization. Furthermore, hesperidin has a specific enantiomer configuration; it consists of a mixture of S- and R-enantiomers in a S:R ratio between 1:1 and 5:1 depending on its source. Stronger biological effects are elicited by the S-enantiomer compared to the R-enantiomer. While in nature the 2S-enantiomer is dominant, commercially available hesperidin often contains a mixture of both stereo-isomers. In Chapter 5 we compared the bioavailability of standard hesperidin with that of micronized hesperidin 2S (S:R ratio of 4:1) in a cross-over study in healthy subjects. After ingestion of one dose, micronized hesperidin 2S proved to be more bioavailable than standard hesperidin. This was shown by a significant increase in the cumulative excretion of total hesperitin and of the individual metabolites in 24 hour urine, and by a significant increase in the $AUC_{0\text{-}4h}$ of plasma hesperetin after intake of micronized hesperidin 2S, compared to standard hesperidin. The improved bioavailability of micronized hesperidin 2S may positively affect its biological activity.

Stimulated by these favourable results, in **Chapter 5**, in **Chapter 6** we studied the effects of 6 weeks micronized hesperidin 2S supplementation on endothelial function, as measured by flow-mediated dilation (FMD), in overweight and obese subjects. Furthermore, also the effects on plasma adhesion molecules, blood pressure and metabolic markers were examined. Besides studying the effects on basal (fasting) endothelial function, we studied the effects of hesperidin 2S after inducing temporary, reversible endothelial dysfunction by providing participants a high-fat meal. In this randomized, placebo-controlled, double-blind, parallel-group study we observed no significant changes in fasted nor in postprandial FMD. However, trends towards downregulation of the adhesion molecules sVCAM-1, sICAM-1 and sP-selectin and towards reduction of both the systolic and diastolic blood pressure were observed after 6 weeks hesperidin 2S intake, compared to placebo. Interestingly, in an exploratory subgroup analysis, excluding those subjects with a highly impaired basal flow-mediated dilation (exclusion of subjects with basal FMD < 3%), hesperidin 2S protected from postprandial endothelial dysfunction and significantly downregulated sVCAM-1 and sICAM-1, as compared to placebo.
In another randomized, placebo-controlled, double-blind, parallel-group study we aimed to assess the 12-week effects of the same hesperidin 2S formulation on gastrointestinal and on systemic metabolic parameters in subjects with features of metabolic syndrome (**Chapter 7**). In this study, no significant hesperidin-induced effects were observed regarding glucose regulation, blood pressure and blood lipids. However, although fecal total SCFA concentrations did not change, hesperidin 2S influenced the gastrointestinal environment as reflected by a significant shift in fermentation (SCFA) profile from acetate to butyrate, and a marked but non-significant reduction in fecal calprotectin concentrations.

It has also been shown that high intake of carotenoids, lipophilic antioxidants abundant in fruits and vegetables, protects against the development of CVD and CVD related risk factors. The majority of commercially available natural carotenoids are derived from plant-based materials. However, the quality and consistency of these carotenoids varies considerably. Carotenoid-like molecules can also be produced by bacterial species. Those produced by spore-forming bacillus strains are of specific interest. Previous *in vitro* and animal studies have shown that a specific bacillus strain, named PD01, is able to produce gastric-stable carotenoids with superior bioavailability and high antioxidant activity as compared to plant carotenoids. In addition, PD01 itself may also possess probiotic properties. In **Chapter 8** we investigated whether PD01 is also able to survive transit through the gastroinestinal tract and to release systemically absorbable carotenoids in an *in vivo* setting. In addition, the gastrointestinal and systemic effects of PD01 and its bacterial carotenoids were studied in a overweight-obese study population. PD01 was shown to survive transit through the gastrointestinal tract and was able to germinate into vegetative cells under physiological intestinal conditions and to release bioavailable carotenoids *in*

vivo. No significant gastrointestinal nor systemic effects were observed after 6 weeks PD01 intake, compared to placebo.

In **Chapter 9**, we presented an overview of the main findings of this thesis, discuss new insights and future perspectives, and provide potential implications for further research.

Nederlandse samenvatting

De primaire rol van voeding is kwantitatief en kwalitatief voldoende voedingsstoffen verschaffen zodat er aan de voedingsbehoeften van een individu kan worden voldaan. Daarnaast kan voeding ook gezondheidsbevorderende effecten hebben. Aanvankelijk richtte de voedingswetenschap zich op het identificeren van essentiële elementen uit de voeding en hun rol in de preventie en behandeling van diverse deficiëntieziekten. In de afgelopen jaren ligt de wetenschappelijke en klinische focus meer op de rol van voeding in ziekten geassocieerd met overmatige voedselinname. De ontdekking en ontwikkeling van functionele voedingsmiddelen heeft "functional foods" tot een snelgroeiend onderdeel van de voedselindustrie gemaakt. Functionele voedingsmiddelen worden gedefinieerd als voedingsmiddelen of voedselingrediënten die één of meer lichamelijk functies doelgericht beïnvloeden met een gezondheidsbevorderend effect, of wanneer het product een fysiologisch effect heeft of effect op psychisch functioneren anders dan het traditionele nutritionele effect. De rol van functionele voeding in ziektepreventie en gezondheidsbevordering is tegenwoordig een belangrijk onderwerp van onderzoek.

In dit proefschrift onderzochten we de effecten van verschillende voedingsinterventies op de gastro-intestinale en metabole gezondheid van gezonde individuen en van individuen met overgewicht en obesitas. We bestudeerden de effecten van een prolyl endoprotease, een potentieel prebiotisch en probiotisch product, en van twee verschillende fytonutriënten.

Gluten, een eiwit rijk aan proline, wordt slecht afgebroken in het maag-darmstelsel door het ontbreken van proline splitsende proteasen in het maag-darmkanaal. De aanwezigheid van lange prolinerijke glutenpeptiden in de dunne darm kan meerdere gluten-gerelateerde aandoeningen via verschillende mechanismen induceren. Prolyl endoproteasen zijn enzymen die het vermogen hebben om de prolinerijke glutenpeptiden te splitsen. In **Hoofdstuk 2** onderzochten we de *in vivo* werkzaamheid van *Aspergillus Niger* prolyl endoprotease (AN-PEP) in het afbreken van gluten. AN-PEP was in staat om gluten, verwerkt in een maaltijd, efficiënt af te breken in de maag van gezonde vrijwilligers voordat deze gluten de dunne darm bereikten. Daarnaast zorgde het verhogen van de calorische dichtheid van de maaltijd tot een vertraging in maagontledigingssnelheid, maar had het geen invloed op de efficiëntie van glutenafbraak. In **Hoofdstuk 3** van dit proefschrift evalueerden we het gebruik van paracetamol als postprandiale marker voor maagontleding. We toonden aan dat paracetamol, wanneer toegevoegd en vermengd met een maaltijd, wordt onderverdeeld in twee fracties; één paracetamolfractie ingekapseld door het vaste deel van de maaltijd en één fractie opgelost in de vloeibare inhoud van de maaltijd. De snelheid van absorptie van deze fracties is verschillend, en geen van beiden geeft nauwkeurig de maagledigingssnelheid weer.

Het veranderen van de samenstelling van darmmicrobiota door prebiotische producten lijkt een interessante manier om de darmbarrièrefunctie te verbeteren en om het ontstaan of de progressie van chronische ziekten te behandelen of zelfs te voorkomen. Een beperking van de meeste prebiotica is hun snelle fermentatie in het proximale colon. Arabinoxylanen (AX), de meest voorkomende niet-verteerbare koolhydraten in tarwe, vormen een interessante nieuwe klasse van potentiële prebiotica. Door hun structuur hebben ze een breed spectrum van (bacteriële) enzymen nodig voor hun afbraak, hetgeen resulteert in een meer distale fermentatie en activiteit. Bovendien beïnvloedt hun structuur het fermentatiepatroon en bepaalt het de mate van immuunmodulatie, met de hoogste activiteit waargenomen voor lange-keten AX. In **Hoofdstuk 4** onderzochten we de effecten van twee verschillende doseringen AX, na 6 weken toediening, in gezonde personen met overgewicht of obesitas. Uitkomstmaten waren darmbarrièrefunctie, samenstelling en activiteit van de darm-microbiota, het immuunsysteem en metabole parameters. In vergelijking met placebo bleek AX de relatieve aanwezigheid van de dominante bacteriefyla in feces niet significant te beïnvloeden. Wel werd er een afname in microbiële rijkdom en diversiteit waargenomen na 6 weken 15 g AX inname, maar niet na 6 weken 7,5 g AX inname. Daarnaast waren de fecale concentraties van de verschillende korteketenvetzuren acetaat, butyraat en propionaat en van de totale hoeveelheid korteketenvetzuren verhoogd na inname van 6 weken AX, tegenover een daling in de placebogroep. Zes weken inname van 7,5 g AX zorgde voor een significante verlaging van de fecale pH, in vergelijking met placebo. Deze modulatie van microbiële activiteit in de darm zou de darmbarrièrefunctie en het immuunsysteem kunnen beïnvloeden. Ook werd er een toename van transcriptie van de tight junction (TJ) eiwitten occludine, claudine-3 en claudine-4 in sigmoid biopten waargenomen, zowel na 7,5 g AX als na 15 g AX suppletie. Echter, er werden geen significante veranderingen in TJ eiwitexpressie of in gastro-intestinale permeabiliteit gezien na AX suppletie, in vergelijking met placebo. Tenslotte zagen we in deze studie een significante daling in de productie van het pro-inflammatoire cytokine TNF-alfa door gestimuleerde perifere mononucleaire bloedcellen na 6 weken inname van 15 g AX vergeleken met inname van placebo.

Studies van grote omvang hebben aangetoond dat flavonoïde-rijke producten gunstige effecten hebben op hart- en vaatziekten en de bijbehorende risicofactoren. Om *in vivo* gezondheidseffecten te bewerkstelligen is het essentieel dat flavonoïden goed biobeschikbaar zijn, met andere woorden dat ze worden opgenomen vanuit het maag-darmstelsel en dat ze de systemische circulatie bereiken. Hesperidine, een flavonoïde glycoside uit sinaasappelschillen, kan wel degelijk positieve effecten uitoefenen op metabole en cardiovasculaire gezondheid, maar is in mensen maar in geringe mate biologisch beschikbaar. Een manier om de biologische beschikbaarheid te verbeteren is door het proces van oplossen te verbeteren middels micronisatie. Daarnaast heeft hesperidine een specifieke enantiomeer configuratie; het bestaat uit een mengsel van S- en

R-enantiomeren in een S:R-verhouding tussen 1:1 en 5:1, afhankelijk van de bron. De S-enantiomeer heeft een sterker biologisch effect dan de R-enantiomeer. Terwijl in de natuur de 2S-enantiomeer overheerst, bevatten commercieel verkrijgbare hesperidine producten vaak een mengsel van beide stereo-isomeren. In **Hoofdstuk 5** vergeleken we de biologische beschikbaarheid van standaard hesperidine met die van gemicroniseerde hesperidine 2S (S:R-verhouding van 4:1) in een cross-over studie met gezonde proefpersonen. Na inname van één dosis bleek de gemicroniseerde hesperidine 2S beter biologisch beschikbaar te zijn dan de standaard hesperidine. Dit werd aangetoond middels een significante toename van de cumulatieve uitscheiding van totaal hesperetine en van de individuele hesperidinemetabolieten in 24-uurs urine. Daarnaast was er een significante toename van de $AUC_{0\text{-}4h}$ van plasma hesperetine na inname van de gemicroniseerde hesperidine 2S, in vergelijking met standaard hesperidine. De verbeterde biologische beschikbaarheid van gemicroniseerd hesperidine 2S zou tevens de biologische activiteit gunstig kunnen beïnvloeden.

Naar aanleiding van bovengenoemde gunstige resultaten, bestudeerden wij in **Hoofdstuk 6** de effecten van 6 weken gemicroniseerde hesperidine 2S inname op endotheelfunctie, gemeten met flow-gemedieerde dilatatie, in personen met overgewicht en obesitas. Daarnaast onderzochten we ook de effecten op plasma adhesiemoleculen, bloeddruk en metabole parameters. Naast het bestuderen van de effecten op basale (nuchtere) endotheelfunctie, bestudeerden wij ook de effecten van hesperidine 2S na inductie van tijdelijke, reversibele endotheeldysfunctie door deelnemers een vetrijke maaltijd te geven. In deze gerandomiseerde, placebo-gecontroleerde, dubbelblinde, parallelle studie zagen we geen significante veranderingen in nuchtere noch in postprandiale flow-gemedieerde dilatatie. Wel zagen we een dalende trend in de adhesiemoleculen sVCAM-1, sICAM-1 en sP-selectine en in de systolische en diastolische bloeddruk na 6 weken hesperidine 2S inname, vergeleken met placebo. Daarnaast zagen we in een subgroep analyse dat hesperidine 2S een beschermend effect had ten aanzien van postprandiale endotheeldysfunctie, en dat het de adhesiemoleculen sVCAM-1 en sICAM-1 significant verlaagde, in vergelijking met de placebo. In deze subgroep analyse waren personen met een sterk verminderde basale flow-gemedieerde dilatatie (<3%) geëxcludeerd omdat verbetering van endotheelfunctie na een dergelijke korte termijn voedingsinterventie niet te verwachten is in personen met zo'n chronisch slecht functionerend endotheel.

In een volgend gerandomiseerd, placebo-gecontroleerde, dubbelblinde, parallelle studie onderzochten we de effecten van hetzelfde hesperidine 2S supplement op gastro-intestinale en systemische metabole parameters bij patiënten met kenmerken van het metabool syndroom na 12 weken inname (**Hoofdstuk 7**). In deze studie werden geen significante hesperidine-geïnduceerde effecten waargenomen ten aanzien van de glucoseregulatie, bloeddruk en lipidenconcentraties. Hoewel de totale korteketenvetzurenconcentratie in feces niet veranderde, beïnvloedde hesperidine 2S het gastro-

intestinale milieu, weerspiegeld door een significante verschuiving in het fermentatie (korteketenvetzuren) profiel van acetaat naar butyraat. Daarnaast werd er een duidelijke maar niet-significante daling gezien in fecale calprotectine concentraties.
Carotenoïden zijn lipofiele antioxidanten overvloedig aanwezig in fruit en groenten. Het is aangetoond dat een hoge inname van carotenoïden beschermt tegen de ontwikkeling van hart- en vaatziekten en gerelateerde risicofactoren. De meeste commercieel verkrijgbare natuurlijke carotenoïden zijn afkomstig van plantaardige materialen. Echter, de kwaliteit en consistentie van deze carotenoïden varieert aanzienlijk. Carotenoïden kunnen ook worden geproduceerd door bacteriën, met name carotenoïden die geproduceerd worden door sporenvormende bacillus-stammen zijn van belang. Eerdere *in vitro* en dierstudies hebben aangetoond dat een specifieke bacillus-stam, genaamd PD01, in staat is maagsapresistente carotenoïden met superieure biobeschikbaarheid en hoge antioxidant werking te produceren in vergelijking met carotenoïden afkomstig van planten. Daarnaast bezit PD01 zelf mogelijk ook probiotische eigenschappen. In **Hoofdstuk 8** onderzochten wij of PD01 in staat is om *in vivo* te overleven in het maagdarmstelsel en in staat is tot productie van systemisch opneembare carotenoïden. Daarnaast bestudeerden wij de gastro-intestinale en systemische effecten van PD01 en van de geproduceerde bacteriële carotenoïden in individuen met overgewicht en obesitas. PD01 bleek *in vivo* in staat te overleven in het menselijke maagdarmstelsel en te ontkiemen tot vegetatieve cellen met afgifte van biobeschikbare carotenoïden. Echter, er werden geen significante gastro-intestinale noch systemische effecten waargenomen na 6 weken PD01 inname, in vergelijking met placebo.
In **Hoofdstuk 9** gaven we een overzicht van de belangrijkste bevindingen van dit proefschrift, bediscussieerden we nieuwe inzichten en toekomstperspectieven, en droegen we mogelijke implicaties voor verder onderzoek aan.

Valorisation

Valorisation is the process of value-creation out of knowledge, by making this knowledge suitable and available for economic or societal utilization and to translate it into high-potential products, services, processes and industrial activity.[1] With this thesis, we aim to improve our understanding of the effects of several nutritional interventions on gastrointestinal and metabolic health, and to make the acquired knowledge and expertise available for society.

The relationship between food and health is complex, being multifactorial and multidimensional. Food provides sufficient quantitative and qualitative supply of nutrients and supplies the human body with materials that can be used for the construction and repair of structures, tissues and organs. Furthermore, the role of functional foods in disease prevention and health promotion is emerging. Functional foods are defined as foods or food ingredients that affect one or more functions in the body in a targeted way so that positive effects on health are to be expected, or when the component has a physiological or psychological effect beyond the traditional nutritional effect.[2,3] On the other hand, food can also negatively influence health and initiate or contribute to illness. To date, in many low- and middle income countries access to food is still limited and the availability and quality of nutrition is poor, leading to severe undernourishment and food insecurity.[4] In contrast, the prevalence of overweight and obesity, linked to excess food intake and 'overnutrition', has worldwide more than doubled since 1980. In 2014, approximately 39% of people aged 18 years and over were overweight and 13% obese.[5] Also in the Netherlands, the number of individuals with overweight and obesity is tremendous; in 2015, 50.3% of the adults aged 20 years and older were overweight and 13.7% were obese.[6] Overweight and obesity are associated with a broad spectrum of gastrointestinal and systemic metabolic alterations, serving as major risk factors for the development of chronic metabolic diseases such as diabetes mellitus type 2 and cardiovascular disease.[7,8] As a consequence, obesity imposes a tremendous economic burden on society through increased total direct (treatment obesity-associated diseases) and indirect (productivity losses) medical costs. In the Netherlands, it has been calculated that in general the direct health care costs of obesity account for up to total 2.2% of national health expenditure.[9] The indirect health care costs could amount to twice the direct health care costs.[10] Significant reductions in these costs can be accomplished by prevention of overweight and obesity or by a significant delay in the onset of overweight, obesity and associated diseases.

Besides diseases linked to undernourishment and overnutrition, there exists another class of diseases related to food: food allergies and food intolerances. Important and epidemiologically relevant phenomenon are the gluten related disorders, such as wheat allergy, celiac disease and gluten sensitivity. The number of individuals embracing a gluten-

free diet is increasing. However, a gluten-free diet is hard to comply with as gluten-free products may not always be correctly labelled, are expensive, and may not always be at hand during social events or travelling. Accordingly, the market for gluten-free products is constantly growing and research identifying alternative therapies is ongoing.

The aim of this thesis was to investigate the effects of several nutritional interventions on gastrointestinal and metabolic health in healthy, overweight and obese individuals. The nutritional interventions we studied included a prolyl endoprotease, a putative prebiotic supplement, a putative probiotic species, and two different phytonutrients.

Gluten is the main structural protein complex of wheat with equivalent toxic proteins present in barley and rye. These gluten-containing cereals have always been a main component of the Western diet, but their consumption is increasing in Eastern countries as well. Ingestion of gluten can induce a wide range of gluten related disorders with different pathogenic pathways. For all conditions, strict adherence to a gluten-free diet is still the only effective treatment. As such, the number of individuals on a gluten-free diet is increasing worldwide. However, the high cost and often poor availability and palatability of gluten-free products makes it difficult to maintain such diet on the longer term. Even in case of strict adherence, a subgroup of individuals may remain symptomatic due to inadvertent gluten indigestion. In addition, a gluten-free diet is nutritionally suboptimal, it may predispose to an undesirable high intake of fat and carbohydrates and lead to poor vitamin status and detrimental weight gain or weight loss. Due to these problems with gluten-free dieting a strong interest in the development of alternative therapies exists. A possible alternative treatment is enzyme supplementation therapy targeted at degradation of immunogenic gluten peptides before entering the intestinal epithelium. In this thesis, we evaluated the *in vivo* gluten-degrading potential of *Aspergilles Niger*-derived prolyl endoprotease (AN-PEP). To standardize each meal intake, we intragastrically administered the test meal via a triple lumen nasogastroduodenal tube. AN-PEP showed to be able to efficiently degrade gluten from a meal in the stomach of healthy volunteers within the time period a meal normally resides in the stomach. The gluten-degrading efficacy of AN-PEP was independent of meal caloric density and gastric emptying rate. These *in vivo* results are promising but to optimise AN-PEP for clinical application it is important that future studies investigate the efficacy of AN-PEP in an actual meal setting, focusing on subjects intolerant to gluten. Furthermore, it is anticipated that these supplements will not fully replace the gluten-free diet, but will be marketed as a supplement that in conjunction with a gluten-free diet may help digest unintentional ingested gluten for subjects intolerant to gluten.
To measure gastric emptying rate in the above-mentioned study, we used paracetamol as post-prandial marker molecule. This method is considered a well-established method and is extensively applied in research. It is assumed that paracetamol, mixed trough a meal and

ingested, will be absorbed immediately in the duodenum after leaving the stomach and that its passage time is identical to that of the meal. The gastric emptying rate can be derived from the time course of the plasma paracetamol concentration. However, in our study we obtained data which were in conflict with this widely accepted model. We observed that paracetamol was divided in two fractions; one fraction dissolved in the liquid content of the meal and another fraction encapsulated by meal contents that precipitate in the acid stomach environment. The aqueous phase is expected to leave the stomach quickly and the paracetamol is readily absorbed in the duodenum, the encapsulated paracetamol is slowly released from the bolus and not instantaneously taken up once it reaches the duodenum. Both fractions did not accurately reflect gastric emptying rate, instead these data show the effect of food on the rate of absorption of paracetamol. The absorption of a drug in the gastrointestinal tract bears major clinical relevance. Food-drug interactions are thus not limited to an effect of food on gastric emptying, but it also includes bioaccessibility. Bioaccessibility, *i.e.* the release of a compound from the matrix in the intestine, has an impact on both the onset and duration of the pharmacotherapeutic effect of a drug.

The human gut is host to a diverse and dynamic community of several trillion microbes that play an important role in intestinal physiology, nutrient digestion, luminal metabolism and immune function.[11] Colonization of the gastrointestinal tract is influenced by genetic background, gestational age, mode of delivery, type of feeding, exposure to antimicrobials and, very important, diet throughout life.[12] Long-term perturbations to the intestinal environment can induce changes in the gut microbiome, contributing to the development of a variety of gastrointestinal diseases[13] as well as systemic metabolic diseases including diabetes mellitus type 2 and cardiovascular disease.[14] Thus, targeting the gut microbiome seems to offer a promising strategy to ameliorate or even prevent the development of above-mentioned diseases. A number of dietary strategies potentially modulating the gut microbiome are available; pre- and probiotics are among the most well established, while recent data suggest that also different types of polyphenolic compounds may influence the gut microbiome.

In this thesis we evaluated the effect of a high-molecular weight arabinoxylan (AX) concentrate, a potential prebiotic product, on intestinal microbiota, gut barrier and immune function in overweight and obese subjects. AX form an interesting novel class of potential prebiotics as, due to their structure, they are more gradually fermented along the colon resulting in a more distal fermentation and activity. A limitation of most other prebiotics is their rapid fermentation (and thus activity only) in the proximal colon. Previous *in vitro* and animal studies have shown promising effects of this product on the gut barrier, gut microbiota, immune system and metabolic markers. In humans, AX administration did not result in a change in the overall microbiota composition, but AX resulted in significantly increased fecal concentrations of short-chain fatty acids (SCFA) and lower fecal pH. The

effects of AX on the gut barrier were explored at various levels. At gene transcription level, we found a significant upregulation of the tight junction (TJ) proteins occludin, claudin-3 and claudin-4, important transmembrane components regulating paracellular permeability. However, these changes in TJ gene expression were nog accompagnied with changes in TJ protein expression or in gastrointestinal permeability. Furthermore, results of this study suggest a capacity of AX to influence the immune function. At last, daily intake of AX for several weeks proved to be well-tolerated by the participants. Altogether, these data suggest that also in humans high-molecular weight AX modulates the gut microbiome and thereby may positively affect gut barrier and immune function. While more research in large, homogeneous study populations is required to confirm its *in vivo* microbiota modulating - and potentially gut barrier and immune system modulating- properties, AX is considered a promising prebiotic compound conferring health effects along the entire length of the colon.

In this thesis, we also studied the effects of a putative probiotic product, a *Bacillus indicus* strain called PD01, on the intestinal microbiota and gut barrier in healthy, yet overweight and obese individuals. Several studies have already shown potential health benefits of bacillus strains. In addition, bacillus species as probiotics are particularly attractive for functional reasons as they can form robust endospores that are able to survive transit through the stomach, are stable to many food processing steps and can be long-term stored at room temperature. Previous *in vitro* data showed that this strain was able to survive and germinate in the gastrointestinal tract and to modulate the intestinal environment. *In vivo*, PD01 also survived transit throughout the digestive tract, but did not significantly influence the gut microbiome or gut barrier when compared to placebo. Repeated intake of PD01 was well-tolerated by the participants, and was even accompanied with a decrease in reported gastrointestinal symptoms such as borborygmi, abdominal distension, eructation and increased flatulance. Previous studies in IBS patients showed that changing the gut microbiome by probiotics may induce quantitative and qualitative decreases in bacterial groups with gas-producing abilities.[15,16] Possibly, a similar mechanism played a role in our study results. Although no significant effects of PD01 on gut microbial environment or gut barrier function could be established in this study, it does provide the first evidence in humans that PD01 survives transit through the gastrointestinal tract and is well-tolerated. To determine whether PD01 possesses additional probiotic properties, it is important that more *in vivo* studies will be conducted in future, including a homogeneous, more diseased study population or that a stressor is applied during the test period inducing an imbalance in homeostasis.

As said before, also polyphenols may have the potential to affect the intestinal environment. We evaluated the relationship between the polyphenol hesperidin and SCFA production. Although we did not observe an effect on the quantity of SCFA production, twelve weeks hesperidin intake modulated the intestinal environment as reflected by a shift from the SCFA acetate to the health-promoting SCFA butyrate. These results strengthen the

hypothesis that also polyphenols are able to influence the gut microbiome. Research investigating the interactions between the gut microbiome and phenolic compounds is still in its early days. More human intervention studies are required to understand the underlying mechanisms and to establish the potential prebiotic-like effects of hesperidin and other polyphenols.

It has been demonstrated that fruits, vegetables and grains exert protective effects against the development of chronic metabolic diseases such as diabetes mellitus type 2 and cardiovascular disease. This protective role can be mainly attributed to the antioxidant phytonutrients in them. Today, about 10,000 phytonutrients have been identified. In this thesis we studied the bioavailability and systemic effects of two main kinds of phytonutrients: polyphenols and carotenoids.

Hesperidin, a polyphenol abundantly present in the peels of citrus fruits, has drawn attention due to its positive effects on metabolic and cardiovascular health. In order to exert systemic health effects, it is essential that a compound is bioavailable and absorbed from the gastrointestinal tract into the systemic circulation. However, the bioavailability of hesperidin in humans is low. We demonstrated that the bioavailability of a micronized hesperidin 2S formulation, having a similar enantiomer configuration as natural hesperidin, is higher than that of a non-micronized standard hesperidin product. The enhanced bioavailability may positively affect its biological activity. Furthermore, this study provided evidence that there exists a high inter-individual variation in polyphenol bioavailability, most likely the result of a great inter-individual diversity in the gut microbiome.

Based on above results, we investigated the effect of micronized hesperidin 2S supplementation on cardiovascular and metabolic parameters in healthy, yet overweight and obese individuals. Results indicate a promising preventive role for hesperidin in cardiovascular disease. Given the high inter-individual variability of polyphenol bioavailability in humans, it would be particularly important and interesting to correlate the magnitude of observed changes with plasma polyphenol concentrations in future studies. Regarding glucose and lipid metabolism we did not observe hesperidin-induced beneficial effects in our generally healthy study population. Consequently, long-term intervention studies in subjects with(pre)diabetes, metabolic syndrome or hypercholesterolemia are warranted to establish and confirm beneficial hesperidin-induced effects on systemic metabolic health markers.

Carotenoids are lipophilic antioxidants, synthesized mainly in plants, but also by a number of bacterial species. In contrast to plant-derived carotenoids, bacterial carotenoids are gastric-stable, are equally or even more bioaccessible and bioavailable, and have a higher antioxidant capacity. In this thesis we evaluated the bioavailability of carotenoids, produced by a spore-forming *Bacillus* strain, in overweight and obese individuals. The bacterial carotenoids were present in the plasma and levels increased throughout the study period.

These results indicate that the bacillus strain was able to produce systemically absorbable bacterial carotenoids *in vivo*. However, no beneficial effects of the bacterial carotenoids regarding cardiovascular and metabolic parameters were observed, probably due to the fact that participants had normal baseline values for most parameters. It is clear that more and larger human intervention studies are needed to investigate and confirm an improved bioavailability, antioxidant capacity and systemic health effects of bacterial carotenoids over plant-derived carotenoids.

In conclusion, beyond its nutritional value food can also play an important role in disease prevention and health promotion. All of the here studied nutritional interventions may affect one or more functions in the body in a positive way and therefore can be considered a functional food. Health-conscious consumers are increasingly seeking functional foods in an effort to control their own health and well-being. However, the field of functional foods is still in its infancy. Many functional foods showed to have beneficial effects in *in vitro* and in animal studies, but their efficacy in humans remains equivocal. A number of factors complicate the establishment of a strong scientific foundation for a functional food. Factors include complexity of the food substance, compensatory metabolic changes that may occur with dietary changes, lack of surrogate markers of disease development and, very important, the existing large variation between individuals regarding general health, genetic make-up and expression, gut microbiome and environmental factors. The development of nutritional therapies that target on prevention or on improvement of chronic gastrointestinal, cardiovascular and metabolic diseases is important and is considered an attractive alternative for medication use. Therefore, it is essential that in future more large, well-designed, placebo-controlled randomized trials exploring the effects of nutritional interventions in well-defined study populations will be performed. Besides scientifically proven health effects, also consumer acceptance is key success factor for use of functional foods in the future. As a result, within the food industry, consumer behavior is currently an important research topic.

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Curriculum vitae

Bouke Nicolette Hubertus Salden was born on June 17th 1987 in Nieuwstadt, The Netherlands. After graduation from the Trevianum Gymnasium in Sittard in 2005, she started medical school at the Maastricht University, Faculty of Health, Medicine and Life Sciences. During medical school she finished clinical and scientific traineeships at the Division of Gastroenterology and Hepatology, Maastricht University Medical Center. After graduating in 2011, Bouke started as PhD-fellow affiliated to the division of Gastroenterology and Hepatology of Maastricht University Medical Center, and to NUTRIM, the School of Nutrition and Translational Research in Metabolism, Maastricht University, under the supervision of prof. dr. A.A.M. Masclee, Dr. F.J. Troost and Dr. S. Possemiers. Part of her PhD was conducted in collaboration with BioActor BV, where she acted as Medical Affairs Manager. In September 2016 she started her residency Gastroenterology-Hepatology under supervision of Prof. dr. A.A.M. Masclee. She is currently working at the Internal Medicine department at the Catharina Hospital Eindhoven under supervision of Dr. C.J.A.M. Konings.

Dankwoord

Eindelijk krijg ik dan de kans om de mensen te bedanken die – direct of indirect – hebben bijgedragen aan dit proefschrift. Het is een uitdaging om de waarde van jullie bijdrage en mijn dank hiervoor in een paar regels uit te drukken, maar ik ga een poging wagen!

Prof. Masclee, beste *Ad*, ik ben ontzettend blij dat u mij de mogelijkheid heeft gegeven te promoveren. Ik kan me herinneren dat wij beiden in het begin twijfelden of een promotietraject voor mij wel de juiste keuze was op dat moment. Met het AN-PEP project gaf u mij de kans om een tijdje van het leven als onderzoeker te proeven. Daarna was het duidelijk voor mij: ik wilde dit uitbouwen tot een promotietraject. Ik wil u bedanken voor alle hulp, de goede begeleiding en steun, en voor het snelle nakijken van manuscripten, zeker in de laatste fase van mijn promotie.

Dr. Troost, beste *Freddy*, bedankt voor al je hulp en gezelligheid in de afgelopen jaren. Met name in de opstartperiode heb ik veel van je geleerd, daarna gaf je me de vrijheid mijn eigen pad te volgen. Onze vaste dinsdagmiddagbespreking werd steeds minder noodzaak, maar bleef altijd een goed excuus om gezellig samen een kop koffie te drinken.

Dr. Possemiers, beste *Sam*, naast co-promotor was je ook mijn wetenschappelijk supervisor binnen BioActor. Ik heb in de afgelopen jaren zowel op wetenschappelijk als op commercieel vlak veel van je geleerd. Bedankt dat ik altijd voor alles bij je terecht kon, en je meestal snel en constructief commentaar gaf op mijn vragen, METC-protocollen en manuscripten. Daarnaast was je altijd fijn gezelschap op de vele BioActor-tripjes die we samen gemaakt hebben.

Graag wil ik de leden van de beoordelingscommissie, *prof. dr. K. Venema*, *dr. D. Jonkers*, *prof. dr. C.J.J. Mulder*, *prof. dr. J. Plat* en *prof. dr. R.F. Witkamp* bedanken voor het kritisch beoordelen van mijn proefschrift, het zitting nemen in de promotiecommissie en voor hun bijdrage aan deze bijzondere dag.

BioActor BV wil ik bedanken voor de mogelijkheid om als promovendus tegelijkertijd de functie van clinical research manager binnen dit bedrijf te bekleden.
Hans, als managing director van BioActor heb ik veel met jou samengewerkt. Niet altijd waren wij het met elkaar eens, maar we kwamen er uiteindelijk toch altijd uit. Ik bewonder je om je tomeloze energie, dat zich onder andere uit in altijd nieuwe onderzoeksideeën. Nooit zit je lang bij de pakken neer als de data niet zijn zoals gehoopt. Met mijn functie als clinical research manager maakte jij me verantwoordelijk voor alle onderzoeksprojecten binnen het bedrijf. Ik leerde zelfstandig werken, delegeren, grenzen stellen en bewaken, ik

mocht naar meetings van Europese projecten en moest vele presentaties geven. Ik leerde wat het inhoudt om voor een bedrijf te werken en het heeft ervoor gezorgd dat ik de afgelopen jaren zelfverzekerder, zelfstandiger en assertiever ben geworden. Bedankt daarvoor!

Uiteraard ben ik ook dank verschuldigd aan de onderzoeksschool *NUTRIM*, voor het raamwerk waarin ik mijn promotieonderzoek heb mogen uitvoeren.

DSM, in het bijzonder *Maaike* en *Luppo*, bedankt dat ik degene was die het AN-PEP project mocht uitvoeren. Het was een fijne samenwerking met mooie resultaten, we hebben zelfs de nationale en internationale pers gehaald!

Ik had mijn onderzoeksprojecten niet kunnen uitvoeren zonder hulp van alle *MDL-artsen*, *MDL-assistenten*, *Ton Mestrom* en het *endoscopieteam*. Hartelijk dank voor jullie hulp en geduld bij het afnemen van vele biopten in onvoorbereide darmen.
Dan niet te vergeten, *Mietsie* en *Elly*. Altijd gezellig om even bij jullie langs te lopen en daarbij direct een antwoord te krijgen op al mijn vragen. *Tiny*, dank voor al je hulp bij de lay-out van mijn proefschrift. Ik ben trots op ons eindresultaat.

Kira: bedankt voor het ontzettend gave design van dit boekje en de presentatie.
Ik geloof in je!

Mijn promotietijd was niet half zo leuk geweest zonder een fantastische groep onderzoekscollega's. *Mariëlle, Eveline, Kirsten P., Kirsten v/d B., Elhaseen, Tim, Bas, Zsa Zsa, Lisa, Pauline, Marin, Martine, Montserrat, Daisy, Annemiek, Annick, Selwyn, Dirk* en *Bram*, bedankt voor de gezellige lunches, borrels, congressen en de interesse in de voortgang van mijn projecten. *Daniel, Samefko* en *Mark:* bedankt voor het onvergetelijk maken van mijn eerste DDW in San Diego. *Mark*, ik wil jou in het bijzonder bedanken voor alle hulp tijdens de afgelopen jaren. Je leerde me duodenumsondes plaatsen, galblaasecho's maken en kwam menig keer opdraven als er weer een infuus geprikt moest worden. *Zlatan, Givan, Marlou, Fedde, Maarten, Steven, Fabienne, Sander* en *Paul*, met een grote grijns denk ik terug aan Orlando 2013. De avondjes in Senõr Frog's en de snake op onze hotelgang zijn mijn favorieten. Wanneer is de volgende reünie? *Roel*, roomie, zoals je weet waren Chantal en ik niet heel blij toen we hoorden dat jij op onze kamer erbij kwam. Maar gelukkig pakte het bijzonder goed uit. Met jou erbij waren we altijd gegarandeerd van een luisterend (rood) oor en kritisch, doortastend mannencommentaar. *Hao Ran*, Harry, het laatste half jaar van mijn promotie vrolijkte jij onze werkkamer nog even op. Bedankt voor je grappige verhalen, de moestuintjes en de verkeerd geadresseerde WhatsApp berichten.

Ook wil ik mijn collega's bij BioActor bedanken voor alle hulp bij de verschillende onderzoeksprojecten. In het bijzonder *Yala*, bij bijna ieder project in dit boekje ben je wel betrokken geweest. Heel veel succes met jouw eigen promotietraject.

Mestreechter maedjes: *Cor*, *Ief*, *Jans*, *Maart* en *Ray*. Een mengeling van meisjes, onafscheidelijk sinds jaar 1 van onze studie. Maandagavond, 'Greys Anatomy'-avond! Niks kwam hiertussen, zelfs geen man. Woensdagavond borrelen en op stap. Vanaf de co-schappen werd deze doordeweekse stapavond gewoon verplaatst naar het weekend. We hebben samengewoond en zijn naar de meest prachtige oorden afgereisd. Bedankt voor deze vriendschap, voor het feit dat ik altijd alles met jullie kan delen, voor de goede etentjes en gezellige borrels, de mooie feestjes en fantastische vakanties. Ik mis onze maandagavonden nu al een tijdje, maar wees gerust, ik kom gewoon terug.
Lieve *An*, ook jou hebben we sinds kort weer terug in het Maastrichtse. Fijn dat je er weer bent en dat we elkaar weer wat vaker zien.

En dan niet te vergeten de oude garde van O.D.D. Missdaad: *Cerès*, *Dree*, *Inge*, *Kim*, *Kira* en *Luus*. Samen a-tijd gelopen en actief lid van O.D.D. Missdaad geweest. Zonder jullie had ik nooit zo'n fantastische studententijd gehad. Inmiddels een stuk rustiger, maar niet minder gezellig. Fijn dat we na al die jaren nog steeds zo close zijn. Ook al is het tegenwoordig niet meer zo vaak, ik geniet iedere keer weer van onze koffiedates, etentjes en borrels.
Lieve *Bel*, ook jou wil ik bedanken voor de fijne vriendschap. Ondanks dat we elkaar niet zo vaak zien weten we altijd wel van elkaar wat ons op dat moment bezighoudt. Laten we vooral niet ophouden met het plannen van leuke festivals.

Chantal en *Ellen*, allerliefste paranimfen, ik ben heel blij dat jullie naast mij staan deze belangrijke dag, maar uiteraard hebben jullie ook veel voor mij betekend in de afgelopen jaren.
Chantal, onze eerste avonturen samen beleefden we in Nepal. De vierdaagse trekking, de jungletocht en de achtervolging door een neushoorn. Erna volgden er gelukkig nog vele andere. We deden gezamenlijk onze WESP/GEZP bij de MDL, en begonnen tegelijk aan onze promotie. We werden naast kamergenoten en collega's ook goede vriendinnen, reisbuddy's, feestmaatjes en heuse wijnkenners. Ik benijd je om jouw doorzettingsvermogen, gedrevenheid en doelgericht werken. Maar lieve Chantal, wees alsjeblieft niet altijd zo streng voor jezelf. Wat was ik trots dat ik zo'n 1,5 jaar geleden naast jou als paranimf mocht staan! Ik kijk ernaar uit dat we binnenkort weer collega's zijn.
Ellen, ik leerde je kennen als een enthousiaste, leergierige, kritische en assertieve control freak. Heerlijk dat dat mijn eerste stagiaire was. Ik was destijds nog niet zo goed in delegeren, maar al snel durfde ik veel onderzoekstaken aan jou over te laten. Sorry, ze waren niet altijd even leuk of dankbaar (denk aan de keren dat jij weer met een portie

urine of ontlasting in je rugzak op pad ging), maar nooit hoorde ik je klagen en altijd deed je je taken met evenveel toewijding. Ik herinner me vooral dat we, ondanks het meestal vroege tijdstip, altijd veel lol hadden. Heel blij was ik dus ook dat je niet veel later m'n directe collega werd bij de MDL. We hebben elkaar nog veel geholpen in de afgelopen jaren met onze afzonderlijke studies. En naast collega werd je ook een goede vriendin, mijn motivatie om te gaan spinnen en bestudeerden we samen de kunde van de wijn.

Lieve *Jorrit*, mijn grote kleine broer. Vroeger waren we onafscheidelijk; speelde ik met de poppen, wilde jij ook; droeg ik nagellak, moest jij ook; lustte ik ineens paprika, vond jij het ook lekker. Toen we ouder werden, gingen we ieder onze eigen weg. Zie hier, ik ben (bijna) gepromoveerd en werk in het ziekenhuis; jij woont in Azië en runt je eigen internetbedrijf. Twee totaal verschillende werelden, maar toch voor altijd verbonden door onze lieve ouders en mooie jeugdherinneringen. Ik mis je soms wel, zou willen dat we weer meer herinneringen samen zouden maken. Maar het is goed zo. Ik ben trots op jou en op alles dat je hebt bereikt.

Papa en *mama*, lieve ouders, jullie zijn de oorsprong van dit alles. Ik zou natuurlijk nooit gepromoveerd zijn als jullie me niet op deze wereld gezet hadden, en het zou me ook nooit gelukt zijn zonder jullie onvoorwaardelijke liefde, opvoeding, steun, vertrouwen en interesse. Ook al is het soms lastig uitleggen wat promoveren nu exact inhoudt, altijd zijn jullie even geïnteresseerd, trots en enthousiast. Sorry mam, ik had je eigenlijk al eerder een samenvatting van m'n promotie beloofd, maar bij deze verwijs ik je graag naar het vorige hoofdstuk. Pap en mam, bedankt voor alles, ik ben er trots op dat jullie mijn ouders zijn en hoop nog lang van jullie te genieten.

Lieve *Bas*, lief vriendje.
Bedankt dat jij in mijn leven bent. Ik vind je prachtig. Ik bewonder je doorzettingsvermogen, daadkracht en eerlijkheid, je vermogen tot relativeren, het feit dat je niet bang bent om fouten te maken en niet doet aan twijfelen. Je geeft me de ruimte en het vertrouwen mezelf te ontwikkelen. En allerbelangrijkst, je bent er voor me, iedere dag opnieuw. Ik hou van je.