Nutritional strategies to improve gastrointestinal and metabolic health

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Exploring the effects of functional foods

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Chapter 1

General introduction

General introduction

Over the last century, the world population has been growing rapidly. General health improvements such as better hygiene, living and working conditions and disease diagnosis and treatment, have contributed to a decrease in mortality and increased life expectancy. As a result, mean age and the number of elderly has increased globally.[1] In parallel, the Western diet and lifestyle is emerging and has been adopted by developed countries, thereby spreading globally. The increase in numbers of the ageing population and the adherence to this lifestyle have both been associated with an increased prevalence of chronic disorders such as obesity, metabolic syndrome, type 2 diabetes, cardiovascular disease (CVD) and liver disease.[2,3] In addition, Western diet and lifestyle related factors can also contribute to the onset and progression of diseases of the gastrointestinal (GI) tract, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).[4,5] In the Netherlands, on average more than 50% of the population suffers from one or more chronic disease(s) and this number rises to more than 95% in adults aged 75 years and older.[6] This poses a major public health concern, as costs associated with treatment of these diseases as well as with loss of *e.g*. productivity put a large burden on society.[7]

Although it is well-accepted that diet in general and the Western diet in particular, can play a role in the development of several diseases, nutrition can also be used as a strategy to improve general health and well-being and thereby decrease the risk of chronic diseases. In the following paragraphs, we will elaborate on the mechanisms by which I) nutrition can contribute to the development of chronic diseases and II) how it can be used to prevent disease and/or improve health.

Part I: Western diet as risk factor for chronic diseases

Western diet and metabolic diseases

The Western diet and lifestyle is characterized by a high intake of (high glycaemic) carbohydrates, fat and animal proteins, together with a low intake of fibres and low levels of physical activity. This lifestyle can contribute to the development of various metabolic disorders, by promoting the accumulation of adipose tissue (*i.e.* obesity) and by affecting various metabolic processes.

Metabolic diseases are generally characterized by alterations in one or more metabolic processes in the body that are needed to convert food into energy, to store and/or to utilize this energy. Although several of these diseases can also have a genetic cause,[8] diet and lifestyle are major factors implicated in the development of for example metabolic syndrome, CVD and diabetes type 2.[9]

Contributing factors

Due to the combination of a high intake of energy dense nutrition and a low energy expenditure, the Western diet and lifestyle results in a positive energy balance and storage of excess energy in the form of adipose tissue. The intake of excess energy, especially from high-glycaemic foods, will result in an increase in the post-prandial insulin response, which promotes lipid storage. Prolonged hyperinsulinemia puts a strain on the pancreatic β-cells, ultimately resulting in β-cell dysfunction and the development of insulin resistance.[10,11] Furthermore, when the mitochondrial capacity is not sufficient to process the substrate supply, nutrient excess can result in increased oxidative stress and induction of inflammatory pathways. This further contributes to the development of insulin resistance (possibly by impairing β-cell function) and endothelial dysfunction.[10,12]

An ongoing, chronically positive energy balance will not only result in new adipose cells but also in enlargement of existing adipocytes. Adipocyte hypertrophy is associated with insulin resistance and a decreased lipid buffering capacity, causing excess lipids to spill over into the blood circulation. This contributes to hyperlipidaemia and ectopic fat accumulation in other tissues such as heart, muscles and liver.[13] Increased levels of non-esterified fatty acids and ectopic fat storage in vascular and cardiac tissues may lead to vascular dysfunction and impaired cardiac function.[12,14] Increased lipid accumulation in the liver can lead to hepatic insulin resistance, resulting in an increased glucose output due to a reduction in glycogen synthesis and increased gluconeogenesis.[13,15] Furthermore, hepatic fat accumulation can impact lipid metabolism leading to dyslipidaemia, characterized by high serum levels of triglycerides and small dense low-density lipoprotein (LDL) particles combined with low levels of high-density lipoprotein (HDL) cholesterol.[15]

Adipose tissue can secrete many factors that can impact metabolic and vascular function. In obesity, the secretion of pro-inflammatory cytokines, chemokines and adipokines such as tumour necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and leptin is increased, while the secretion of adiponectin is decreased, contributing to (low-grade) systemic inflammation.[13,16,17] Pro-inflammatory cytokines can directly inhibit insulin-receptor signalling and thereby further contribute to the development of insulin resistance.[9,13] Furthermore, both cytokines and chemokines can also play a role in the initiation and progression of *e.g.* endothelial dysfunction and can thereby promote the development of atherosclerosis.[12]

Via its effects on nutrient excess and obesity, the Western diet has a deleterious impact on metabolic risk factors such as insulin resistance, (low-grade) inflammation and oxidative stress (Figure 1.1). These can in turn contribute to the development of related

co-morbid diseases, such as type 2 diabetes, CVD and non-alcoholic fatty liver disease (NAFLD).[12,13,18]

Western diet and the intestinal tract

In addition to its effects on metabolic risk factors, the Western diet also has a significant impact on different functions of the intestinal tract that are important for metabolic as well as intestinal health.

The intestinal tract, due to its many functions, plays an important role in intestinal health, but also impacts extra-intestinal organs and diseases. According to Bischoff *et al*., the hallmarks of GI health include an effective digestion and absorption, in combination with a stable microbiota, effective immune status, and state of general wellbeing in the absence of GI diseases.[19] In this context, the microbiome and the intestinal barrier are considered key factors affecting the intestine but also extra-intestinal organs.

Intestinal microbiota

The human intestinal tract harbours a vast and diverse community of microorganisms, collectively referred to as the intestinal microbiota.[20] This is a complex ecosystem, which is estimated to comprise more than 10^{14} bacteria and over 1000 species, most of which belong to only a few phyla.[21,22] In healthy adults, Bacteroidetes and Firmicutes are the two most dominant bacterial phyla. Actinobacteria, Proteobacteria and Verrucomicrobia are also present in most individuals, though generally in lower abundance.[21] There is no universal definition of a "healthy microbiota", as large variation exists between individuals.[21] However, several bacterial taxa are generally associated with beneficial effects, such as Bifidobacteria and Lactobacilli, while others are considered harmful (*e.g.* Salmonella).[22,23] Furthermore, differences in microbiota composition have been found between healthy subjects and those suffering from diseases such as (morbid) obesity, NAFLD, IBD and IBS.[21,24,25] Under physiological conditions, the relationship between the bacteria present in the intestine and the host is mutually beneficial, involving complex microbe-microbe and microbe-host interactions. The intestinal microbiota can contribute to host health *e.g*. by providing energy and essential vitamins, by protecting against pathogen colonisation and via interaction with the intestinal epithelium and immune system.[23,26] However, when alterations in the composition of the microbiota occur, these interactions may become distorted. This can result in effects that do not benefit the host, such as an increased immune stimulation and inflammation and disrupted barrier function.[27]

Intestinal bacteria rely for a large part on the fermentation of non-absorbed nutrients as an energy source, which makes the composition of the microbiota sensitive to major dietary changes. Fermentation of non-digestible carbohydrates results in the production

of short-chain fatty acids (SCFAs), primarily butyrate, acetate and propionate. These microbial metabolites exert many effects that contribute to metabolic and intestinal health, including *e.g.* beneficial effects on inflammation, on lipid and glucose metabolism and the epithelial barrier.[28] On the other hand, microbial protein fermentation results in the formation of branched-chain fatty acids and potentially toxic substances such as ammonia and hydrogen sulphide, which can damage the intestinal epithelium and contribute to mucosal inflammation.[28,29] Although results from studies that are currently available are not always consistent, evidence from multiple studies shows that major dietary changes affect the composition and especially the metabolic capacity of the microbiota within the intestine.[30-32] For example, diets high in animal protein and saturated fat, and low in fibre were associated with a reduced microbiota richness and diversity, a decrease in butyrate-producing Firmicutes and butyrate levels, and an increase in Bacteroides populations.[33]

Intestinal barrier

The intestinal barrier is the first line of defence, protecting against translocation of luminal bacteria, their products and other antigens. It is composed of a single layer of epithelial cells sealed by junctional complexes, the underlying lamina propria and a mucus layer covering the epithelial cells.[34] The junctional complex is composed of tight junctions (TJs) and adherens junctions connected to a peri-junctional ring of actin and myosin that can regulate paracellular permeability, as well as desmosomes supporting epithelial stability.[34,35] The lamina propria contains innate and adaptive immune cells, including dendritic cells, macrophages, and plasma cells. The mucus layer is composed of glycosylated mucin proteins that provide a chemical and mechanical barrier and prevent direct contact of large particles and bacteria with the epithelial cells.[34] In the colon, the mucus layer consists of an outer, loose layer containing commensal intestinal bacteria and a more dense inner layer that is largely impermeable for bacteria.[36] The intestinal barrier can be affected by the intestinal bacteria and their metabolites, pro-inflammatory mediators such as cytokines, but also by various environmental factors including for example dietary components. The intestinal microbiota plays a key role in the maintenance of intestinal barrier integrity and function by providing colonization resistance, triggering production of mucus and antimicrobial peptides, and affecting expression of junctional proteins and immune functioning.[35] Hereby, SCFAs play an important role as primary energy source for the epithelial cells and by various additional beneficial effects as will be discussed later.[35] A high intake of fat and simple sugars has been associated with an impaired barrier function in animal studies.[37] For example, a high fat diet has been shown to result in thinning of the mucus layer of the small intestine and colon and increased paracellular permeability.[38] The exact underlying

mechanisms are not clear and may in part be due to indirect effects, via alteration of *e.g.* microbiota composition and the production of cytokines[37,39] However, direct effects on TJ protein expression and intestinal permeability have been shown for several individual dietary factors (such as fructose, fat, emulsifiers and alcohol).[37,40-42] An impaired barrier function can result in an increased translocation of luminal content, eliciting an immune response locally and systemically. The Western diet can also result in inflammation by directly modulating components of the immune system.[38] For example, saturated fatty acids can promote inflammation by activation of peroxisome proliferator-activated receptor-δ (PPAR-δ) in dendritic cells and T cells.[43]

Gut microbiota perturbations, intestinal barrier dysfunction and chronic (intestinal) inflammation have been linked to both the onset and progression of GI diseases such as IBD and IBS[19,44] and various metabolic diseases, including obesity and NAFLD.[18,19] As described above, the Western diet can play an important role in the development of these diseases both by affecting metabolic processes and via its impact on different components within the intestinal tract (Figure 1.1).

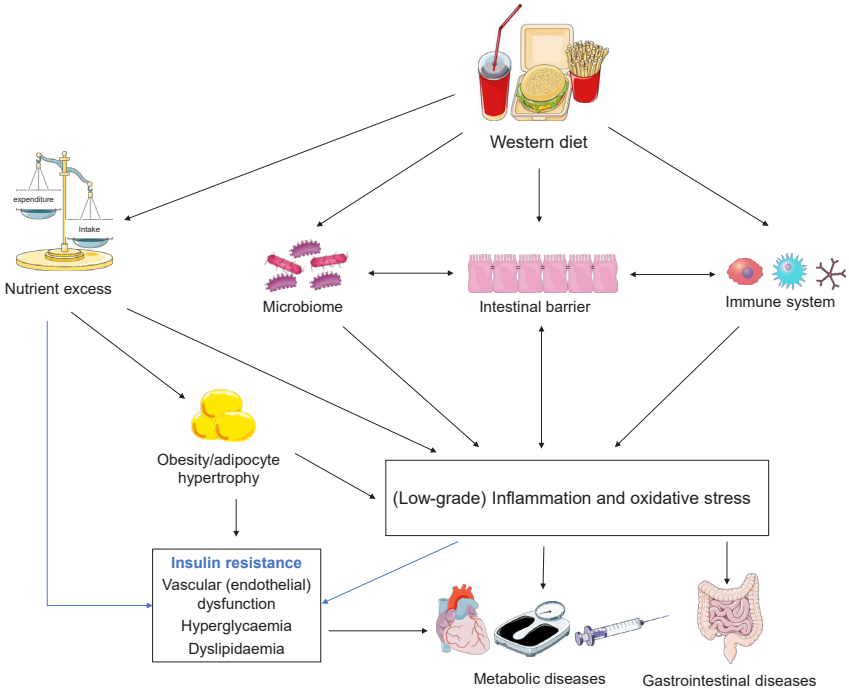

Figure 1.1 Simplified schematic overview of the relationship between the Western diet and the development of metabolic and gastrointestinal diseases. Blue arrows: pathways mainly mediated by insulin resistance. Images from Servier Medical Art (https://smart.servier.com/) were used and adapted for this figure.

Part II: The role of nutrition in improving intestinal and metabolic health

On one hand, diet can be a risk factor, but it can also positively affect intestinal and metabolic health by modulating many of the processes outlined above. Examples of beneficial nutritional components include amongst others dietary fibres, omega-3 fatty acids, vitamins and phytochemicals. These components are generally present at low levels in the Western diet. In addition to changes in habitual dietary intake, another dietary strategy to improve intestinal and metabolic health is the use of functional food products.

Beneficial nutritional components in intestinal and metabolic health

Dietary fibres

Non-digestible carbohydrates or dietary fibres are characterised by the presence of glycosidic bonds that are resistant to hydrolysis by the human digestive enzymes.[45] They are present in various plant-derived foods such as legumes, fruits, vegetables and whole grains. Dietary fibres can be classified according to their different properties, such as solubility and fermentability. Insoluble fibres cannot dissolve in water, but are able to increase bulk by absorbing water and are mostly non-fermentable. Soluble fibres on the other hand dissolve in water, may form a viscous gel and are easily fermented by the bacteria in the colon.[46] Dietary fibres that selectively stimulate the growth of beneficial bacteria and thereby provide health benefits to the host are known as prebiotics.[47] Fermentable fibres are an important energy source for the colonic microbiota. Examples are inulin, pectin, β-glucan, fructo-oligosaccharides (FOSs) and galacto-oligosaccharides (GOSs).[31] These substances can modulate the number and activity of specific bacterial groups, although effects vary between fibres (depending on their biochemical structure) and between individuals. The latter depends amongst others on the initial microbiota composition and the individual's transit and dietary pattern.[24,45] Although specific effects on the intestinal microbiota composition vary, the most consistently reported change is an increase in *Bifidobacterium* spp. abundance.[48] Furthermore, saccharolytic fermentation of dietary fibres by the microbiota results in the production of SCFAs. These metabolites, butyrate in particular, are the primary energy source for enterocytes and can increase blood flow to the colonic mucosa via vasodilatory effects. Furthermore, SCFAs can modulate the microbiota composition, improve barrier function (*e.g*. by inducing TJ assembly and increased mucus secretion), impact cell proliferation and differentiation and have anti-oxidant potential.[37,49-52] Immune-modulatory effects of SCFAs include inhibition of nuclear factor kappa B (NF-kB) activity, suppressed pro-inflammatory cytokine production and differentiation of mucosal T regulatory cells.[28,49] Furthermore, SCFAs can influence energy homeostasis and substrate metabolism via effects on *e.g.* satiety hormones and adipose tissue function, and by improving insulin sensitivity in skeletal muscle and liver.[53]

Omega-3 fatty acids

Omega-3 and omega-6 are both polyunsaturated fatty acids (PUFAs) and are classified as essential fatty acids since they cannot be synthesised by the body. Omega-3 PUFAs are mainly found in fatty fish, while vegetable oils are the major source of omega-6 PUFAs. Whereas both PUFAs have important functions within the body, a high omega-6/omega-3 PUFA ratio as is predominant in the Western diet, is associated with inflammation, alterations in microbiota composition and increased intestinal

permeability. Therefore, increasing the intake of omega-3 PUFAs is considered to be beneficial.[54] Omega-3 PUFAs exert anti-inflammatory effects by binding to G-protein-coupled receptor (GPR) 120 expressed by $CD11^+$ macrophages, which prevents the production of pro-inflammatory cytokines and inhibits Toll like receptor (TLR) expression by monocytes and macrophages.[43] Furthermore, positive effects on the microbiota include an increase in butyrate producing bacteria. Omega-3 consumption resulted in an improvement in the Firmicutes/Bacteroides ratio and an increase in *Lachnospiraceae* taxa.[31] Improvements in intestinal barrier function have also been shown, by activating PPAR-ϒ and upregulating TJ expression.[55]

Proteins

Diets high in meat protein have been linked to microbiota perturbations and a disrupted barrier function. This is mainly due to fermentation of proteins, which results in the production of metabolites with unfavourable effects.[31,37] However, the consumption of proteins from plant sources such as pea and soy have been associated with beneficial alterations in microbiota composition, such as an increase in Bifidobacteria and Lactobacilli.[31,33] This difference in effect between animal and plant proteins was also observed in a short-term dietary intervention study. This study showed that consumption of an animal-based diet affects the microbiota composition differently (*e.g.* increased abundance of bile resistant bacteria and lower levels of polysaccharide fermenting Firmicutes) when compared to an all plant-based diet.[56] Positive effects have also been shown for individual protein-derived compounds. For example, some amino acids and peptides (*e.g*. glutamine and β-casein) were able to improve intestinal barrier function *in vitro*.[57] Furthermore, protective effects on inflammation and intestinal permeability have been shown for metabolites of tryptophan.[50]

Vitamins and Phytochemicals

In addition to macronutrients, various (phyto)chemicals that constitute a much smaller part of the diet, such as vitamins, carotenoids and polyphenols, can interact with various factors involved in metabolic and intestinal health. Among these, polyphenols are the most abundant antioxidants in the diet and have gained much attention due to their beneficial health effects.[58] Polyphenols are mainly present in fruits and beverages like tea and red wine, and to a lesser extent in other food products such as vegetables, cereals and legumes.[59] They can be degraded by bacteria present in the intestinal tract, resulting in the release of smaller phenolic compounds with their own distinct properties and bioactivity. Polyphenols and their metabolites can inhibit the growth of some harmful bacteria via their antimicrobial properties, while stimulating growth of more beneficial species by serving as substrate.[50,60] In addition, polyphenols and

polyphenol metabolites can improve intestinal barrier function by inducing changes in TJ expression, can modulate the secretion of immune markers (*e.g.* inhibition of pro-inflammatory cytokines), and protect against oxidative stress by upregulating endogenous antioxidant enzyme production and scavenging of free radicals.[50,57,61] It should however be noted that the exact pathways underlying these actions are not always clear and effects differ between specific polyphenols.

Carotenoids are another class of antioxidants that are widely distributed in the diet with various positive health effects.[62-67] Reported effects of carotenoids include an increased abundance of lactic acid bacteria, increased production of SCFAs, increased expression of TJ proteins, increased mucus thickness and reduced systemic inflammation.[62-64,67] Furthermore, carotenoids such as β-carotene are important precursors for vitamin A.[65] In addition to polyphenols and carotenoids, various vitamins and other compounds naturally present in fruits and vegetables can modulate processes involved in immunity and inflammation. For example, metabolites of vitamin A and vitamin D can bind to different kinds of nuclear receptors (such as retinoic acid receptors, retinoid X receptors, liver X receptors and PPARs) expressed by immune cells present in the intestinal mucosa.[43] Phytochemicals obtained from cruciferous vegetables (*e.g.* broccoli and cabbage), such as indole-3-carbinol, can act as ligands for the aryl hydrocarbon receptor (AhR).[43,68]

Functional foods in intestinal and metabolic health

Foods designed to improve health by specifically targeting one or more of the processes outlined above can be classified as "functional foods". A widely applied approach to produce a functional food product is by adding a new ingredient with specific health benefits to a more commonly known food item.[69]

Probiotics and prebiotics

Probiotics and prebiotics are the most well-known functional foods used to promote intestinal health. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host",[70] while a prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit".[47] Products that contain a combination of both pro- and prebiotics are termed synbiotics.[71] Most probiotic formulations currently on the market contain bacteria from just a few genera, mainly *Lactobacillus* spp. and *Bifidobacterium* spp. However, other probiotics that are now available include also other genera such as *Enterococcus* and *Bacillus* or combinations of several species.[72] Prebiotics most widely studied include inulin, FOS and GOS. However, other substances such as polyphenols may also meet the criteria to be classified as prebiotics in the future.[47]

A large body of evidence shows that both pro- and prebiotics can exert various beneficial effects, of which modulation of the intestinal microbiota composition and increasing the production of beneficial metabolites are the major mechanism of action involved in intestinal and metabolic health.[70,73] Although various *in vitro* and animal studies have shown promising results, the results from human intervention studies in specific patient populations are not consistent and often even disappointing. Targeting specific underlying mechanistic effects by specific strains or products may help to improve outcomes of future studies.[74]

Polyphenols

Polyphenols are another example of dietary compounds that could potentially confer benefits on the host by targeting the intestinal microbiota, but also by their anti-inflammatory/anti-oxidant effects. A particular group of polyphenols with great potential in this regard are flavonoids from citrus fruits. Hesperidin and naringin are the two major flavonoids present in citrus fruits. Previous human, animal and *in vitro* studies have shown that these compounds can improve microbiota composition, SCFA production, intestinal barrier function and reduce intestinal inflammation.[75-85] However, evidence from well-controlled clinical studies in populations with conditions associated with low-grade inflammation and/or microbiota dysbiosis, such as IBD, IBS and overweight, is still scarce. Alterations in both microbiota composition and immune parameters have also been shown in elderly populations,[86-88] suggesting that these compounds may have beneficial effects in this population. Furthermore, the relative contribution of the phenolic compounds formed during intestinal metabolism when compared to the parent compounds (*i.e.* hesperidin and naringin) remains unclear. Finally, polyphenolic compounds with anti-inflammatory effects may also be used to exert beneficial effects beyond the intestine, for example in diseases with a metabolic component. One example of such a compound is oleuropein, a polyphenol that is mainly present in olive leaves with the potential to improve blood lipid profiles and lipid peroxidation. Although the exact underlying mechanisms are not completely clear, it has been suggested that these effects can be ascribed to anti-inflammatory effects and a modulation of bile flow.[89]

Aims and outline of the thesis

Whereas both metabolic and intestinal diseases are associated with a Western-type diet, nutritional strategies can also be used to improve many processes involved. In this thesis, the effects of functional food supplements on intestinal and metabolic health are evaluated. The first part of this thesis focuses on intestinal health domains in relation

to (intestinal) inflammation, while the second part of the thesis further addresses metabolic and intestinal health outcomes in overweight individuals.

In **Chapter 2-5,** the effects of citrus flavonoids on intestinal health are investigated in various disease models and populations associated with inflammation. **Chapter 2** provides a comprehensive overview of the intestinal fate of citrus flavonoids and their effects on intestinal health, including the intestinal microbiota, intestinal barrier function and intestinal inflammation. In order to further investigate the effect of citrus flavonoids on immune-mediated barrier disruption, a validated *in vitro* co-culture system was used (**Chapter 3**) to provide insight into the effects of the metabolites formed during intestinal metabolism, and their relative contribution compared to the parent compounds. As studies in humans are scarce, in **Chapter 4** and **Chapter 5** we studied the *in vivo* effects of citrus flavonoid supplementation in relevant target populations. First, the effects of citrus extract supplementation on intestinal inflammation, immune defence, microbiota composition and GI symptom scores was investigated in IBS patients with low-grade intestinal inflammation (**Chapter 4**). For **Chapter 5,** we chose an elderly study population, as ageing has been associated with impaired immune functioning and oxidative stress. In this study, we investigated the effects of a citrus extract on biomarkers of oxidative stress and (intestinal) immune function.

In the second part of this thesis (**Chapter 6 & 7**), the effect of functional food interventions on outcomes of intestinal and metabolic health in healthy overweight/obese subjects is explored. In **Chapter 6**, we studied the effect of a potential next generation probiotic on intestinal barrier function in a combined animal and human approach. This strain could improve intestinal barrier function both via the more traditional pathways (microbiota modulation), but also via modulation of the immune system, due to its ability to produce carotenoids. In order to explore metabolic effects of nutritional interventions beyond the intestinal tract, the effect of olive leaf extract supplementation on cardiometabolic risk factors was studied in an overweight/obese population with altered blood lipid profiles (**Chapter 7**).

Finally, in **Chapter 8**, the main findings of this thesis are summarised and new insights and implications for further research and future applications will be discussed.

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Part I

Chapter 2

The intestinal fate of citrus flavanones and their effects on gastrointestinal health

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Abstract

Citrus flavanones, with hesperidin and naringin as the most abundant representatives, have various beneficial effects, including anti-oxidative and anti-inflammatory activities. Evidence also indicates that they may impact the intestinal microbiome and are metabolised by the microbiota as well, thereby affecting their bioavailability. In this review, we provide an overview on the current evidence on the intestinal fate of hesperidin and naringin, their interaction with the gut microbiota, and their effects on intestinal barrier function and intestinal inflammation. These topics will be discussed as they may contribute to gastrointestinal health in various diseases. Evidence shows that hesperidin and naringin are metabolised by intestinal bacteria, mainly in the (proximal) colon, resulting in the formation of their aglycones hesperetin and naringenin and various smaller phenolics. Studies have also shown that citrus flavanones and their metabolites are able to influence the microbiota composition and activity and exert beneficial effects on intestinal barrier function and gastrointestinal inflammation. Although the exact underlying mechanisms of action are not completely clear and more research in human subjects is needed, evidence so far suggests that citrus flavanones as well as their metabolites have the potential to contribute to improved gastrointestinal function and health.

Introduction

Polyphenols are naturally occurring secondary metabolites found in plants, where they play an important role in the plant's defence systems by providing protection against, *e.g*., pathogens, insects, and UV radiation. Different classes of polyphenols can be identified, of which flavonoids are the largest and most studied group. Flavonoids have a widespread presence in edible plants and are a prominent part of the human diet. They can be classified based on their chemical structure into a variety of subclasses, such as flavanones, flavones, isoflavones, flavans (flavanols), anthocyanins, and flavonols. In Europe at the population level, a median daily flavonoid intake of 335.0 and 332.2 mg was found in men and women, respectively, of which about 5% could be attributed to flavanone consumption.[1] In contrast to other flavonoids that are present in a wide range of foods such as fruits, vegetables, cereals, legumes, and beverages like tea and red wine, the presence of flavanones in the human diet is mainly restricted to citrus fruits, and to a lower extent to tomatoes and aromatic herbs like mint. [2] Despite the fact that their presence is limited, flavanones contribute significantly to the dietary flavonoid intake because of the widespread consumption of citrus fruits and juices.[3,4] The main flavanone glycosides present in citrus fruits are hesperidin and naringin. Hesperidin is the principal flavanone in sweet oranges, while naringin is the most abundant flavanone in grapefruit and is primarily responsible for its distinct bitter taste.[5] Commercial sweet orange juice contains between 4.5 and 76.3 mg/100 mL hesperidin with an average of 37.5 mg/100 mL, whereas naringin concentrations in commercial grapefruit juice range between 4.8 and 119.7 mg/100 mL with an average of 43.5 mg/100 mL.[6] Overall, the average daily population intake of flavanones in Europe was found to be 25.7 ± 27.1 mg, being most often consumed as fruits (72.0%), juices (17.2%), wine (5.4%), and soft drinks (1.7%).[7]

The consumption of citrus flavanones has repeatedly been associated with a lower risk of degenerative diseases such as cardiovascular diseases and cancers.[8-13] This potentially protective effect has been related to the various properties of these compounds, which include anti-oxidative and anti-inflammatory activities.[14-16] Additionally, recent evidence has indicated that citrus flavanones could modulate the microbiota composition and activity by inhibiting pathogenic bacteria and selectively stimulating the growth of beneficial bacteria.[17,18] As inflammation, oxidative stress, and intestinal microbiota perturbations are involved in several gastrointestinal (GI) as well as metabolic diseases, consumption of citrus flavanones may contribute to the maintenance of intestinal homeostasis and may improve GI health. The intestinal microbiota has many functions that are important for host health. These include protection against pathogen colonization, maintenance of intestinal barrier function, for example, by secretion of

mucus and regulation of the junctional complex, interaction with the host immune system, and its large metabolic capacity.[19] During normal intestinal homeostasis, the balance between inhibiting invading pathogens and tolerance to commensal microbes is tightly regulated.[20,21] However, a disturbed barrier function and/or reduced tolerance, may facilitate intestinal inflammation.[22,23] As part of the inflammatory response, reactive oxygen species and reactive nitrogen species are produced, resulting in enhanced exposure to oxidative stress.[24]

Because of their putative mechanistic effects, citrus flavanones may be of interest in the prevention and/or treatment of various diseases. Most studies use extracts or purified bioactive compounds to study the effects of citrus flavanones. Their effects however, may be influenced by processes taking place in the GI tract such as bacterial metabolism and absorption. Therefore, in this review, we will provide an overview of the current evidence on the intestinal fate of citrus flavanones, including their interaction with the intestinal microbiota as well as their effects on factors contributing to GI health.

Intestinal fate and bioavailability

The basic chemical structure of flavonoids consists of 15 carbon atoms, two aromatic rings (A and B), and a pyran ring (C) (Figure 2.1). Hesperidin and naringin are both flavanone glycosides. Their aglycones hesperetin and naringenin are attached to disaccharides consisting of glucose and rhamnose at the 7-carbon position, with rhamnose as the terminal sugar (Figure 2.2A, B). The disaccharide attached to hesperitin is rutinose, *i.e.* 6-O-α-L-rhamnopyranosyl-D-glucopyranose (Figure 2.2A), while the disaccharide attached to naringenin is neohesperidose, *i.e.* 2-O-α-L-rhamnopyranosyl-D-glucopyranose (Figure 2.2B). Beneficial effects of citrus flavanones (*e.g.*, radical scavenging and anti-inflammatory activity and modulation of microbiota) are considered to be related to their biochemical structure, such as the number and specific position of hydroxyl groups on the A and B ring and the presence of the sugar moiety.[16,25-27]

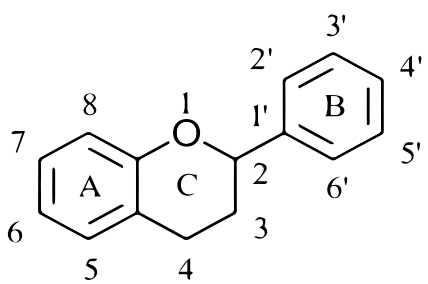

Figure 2.1 Basic chemical structure of flavonoids

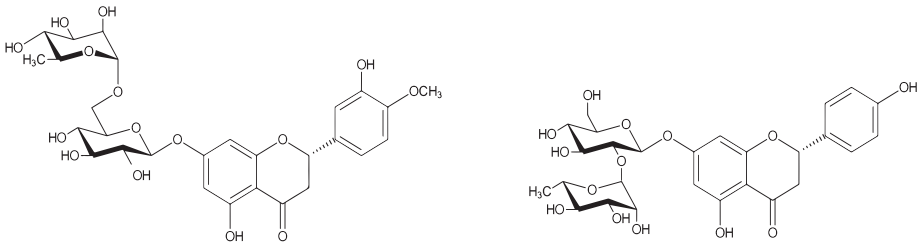

Figure 2.2 Chemical structure of hesperidin (A) and naringin (B)

The ability of citrus flavanones to exert beneficial effects strongly depends on their bioavailability, which can be affected by the structure of the compound, the food matrix, and host factors.[28] The intestinal metabolism of citrus flavanones is mainly determined by their degree of conjugation to sugar moieties[29-31] and the removal of these by intestinal bacteria.[29] Citrus flavanones such as hesperidin and naringin are considered to be largely resistant to enzymatic breakdown in the stomach and small intestine and, thereby, mainly reach the colon intact. Here, hesperidin and naringin are exposed to α-rhamnosidases secreted by the gut microbiota, which remove the rhamnose moiety followed by the removal of glucose by β-glucosidases.[31-33] Although the majority is converted in the colon, some breakdown can already take place in the distal part of the small intestine.[34] Upon release, the aglycones hesperetin and naringenin are absorbed through the intestinal epithelium by means of passive diffusion and proton-coupled active transport, or are further metabolised into phenolic acids and simple phenolics by C-ring cleavage, demethylation and dehydroxylation by bacterial enzymes.[31,35-38] Hence, the bioavailable fraction in the intestine upon citrus flavanone consumption is likely to consist of a mix of hesperetin, naringenin, and phenolic metabolites.

Intestinal metabolism: *in vitro* evidence

Because of difficulties in sampling the small intestine and proximal colon, there are only limited data available on the biotransformation of citrus flavanones in the GI tract *in vivo*. Data from studies measuring faecal excretion levels show that these compounds have been metabolized extensively within the intestine, resulting in the formation of the aglycone forms and smaller phenolics.[35,39] Data from *in vitro* studies using various GI digestion models have provided additional information. *In vitro* digestion of hesperidin, naringin, and their aglycones has been carried out either in batch-like settings using incubations with faecal slurries or isolated bacteria,[37,40,41] or in more comprehensive models, such as the TNO *in vitro* model of the colon (TIM-2) and the simulator of the

human intestinal microbial ecosystem (SHIME).[42,43] These *in vitro* studies, listed in Table 2.1, show quite consistently that during colonic microbial fermentation, hesperidin and naringin are first metabolized into their aglycones hesperetin and naringenin and subsequently into various phenolics, including dihydrocaffeic acid, isoferulic acid, 4-hydroxyphenylacetic acid, dihydroferulic acid, ferulic acid, resorcinol, phloroglucinol, 2,4-dihydroxyphenylacetic acid, 4-hydroxybenzoic acid, phloretic acid, phloroglucinic acid, hydrocinnamic acid, 3-(3′-hydroxyphenyl)propionic acid, protocatechuic acid, and hippuric acid. More information about the metabolism of citrus flavanones, including an overview of the proposed pathways, can also be found in a review by Kay *et al.*[44]

In addition to the type of metabolites formed, a recent study by Van Rymenant *et al*. provided information about the location of citrus flavanone metabolism. Using a digestion model comprising compartments representing the ascending, transverse, and descending colon (SHIME) inoculated with a faecal sample from a healthy volunteer, the metabolites formed upon hesperidin conversion were found at the highest concentrations in the compartments representing the ascending and transverse colon, while lower concentrations were measured in the descending colon.[42] These *in vitro* results suggest that microbial metabolism predominantly takes place in the more proximal parts of the colon. It should be noted that *in vivo* absorption mechanisms are lacking in these models. Furthermore, several of these studies used single or pooled samples for inoculation. Chen *et al*., investigating the *in vitro* biotransformation of naringin by intestinal bacteria from healthy human volunteers using labelled naringin, found that (intermediate) metabolites differed between subjects.[45]

Bioavailability in humans

Human studies performing analyses in plasma, urine, and/or faeces also showed the formation of their aglycone forms as well as many smaller phenolic metabolites after the consumption of citrus flavanones (see also Table 2.2).[46-50] These data indicate that citrus flavanones, like many other polyphenols, undergo extensive metabolism *in vivo* by the intestinal microbiota. Several of the metabolites identified in the *in vitro* simulations mentioned previously, were also found in these *in vivo* samples, suggesting that *in vitro* simulations have the capacity to mimic *in vivo* metabolism. It should be noted however that variations in flavanone metabolism can be found between individual subjects or donors both *in vitro* and *in vivo*, indicating that more research is needed. In addition, as most of these studies were performed in healthy volunteers or with faecal donations of healthy volunteers, it is unclear how disease states, dietary factors, and medication use may impact these findings. Although data are scarce, a few studies investigated the effects of the food matrix or medication use on the bioavailability of citrus flavanones. For example, the effects of the solubility of flavanones in orange juice and the ingestion

of yoghurt together with orange juice on excretion levels of metabolites have been reported.[46,51]

Table 2.1 Nonexclusive listing of *in vitro* studies investigating the colonic metabolism of citrus flavanones

***In Vitro* Model System**[Ref.]	**Sampling Time (Extra Sampling)**	**Treatment (Dosage)**	**Metabolites Formed**	**Comments**
Batch incubation, faecal samples from four healthy volunteers[37]	24 h (1 h, 2 h, 4 h, 6 h, 8 h, 24 h)	Hesperetin (50 µmol in 50 mL) Naringenin (50 µmol in 50 mL)	Isoferulic acid, dihydrocaffeic acid, hydrocinnamic acid, 3-(3′-hydroxyphenyl) propionic acid, phloretic acid, 4-hydroxyphenylacetic acid Phloretic acid, hydrocinnamic acid, 4-hydroxyphenylacetic acid	Metabolite concentrations vary between time points and donors
Batch incubation, bacteria isolated from fresh human faecal samples[40]	12 h	Hesperidin (5 mg in 50 mL) Naringin (5 mg in 50 mL)	Hesperetin, resorcinol, phloroglucinol, 2,4-dihydroxyphenylacetic acid Naringenin, 4-hydroxybenzoic acid, phloroglucinol, phloroglucinic acid, 4-hydroxyphenylacetic acid	
Batch incubation, probiotic bacteria (*Bifidobacterium longum* R0175 and *Lactobacillus rhamnosus* subsp. rhamnosus NCTC 10302)[41]	48 h (12 h, 24 h, 36 h, 48 h)	Hesperidin (410 nmol in 10 mL) Naringenin (430 nmol in 10 mL) Hesperetin (820 nmol in 10 mL) Naringenin (920 nmol in 10 mL)	- - Isoferulic acid, dihydrocaffeic acid, 3-(3′-hydroxyphenyl)propionic acid, hydrocinnamic acid Phloretic acid, hydrocinnamic acid	Metabolite concentrations vary between time points and bacteria
SHIME, faecal sample from one healthy volunteer[42]	3 weeks (1 wk, 2 wks, 3 wks)	Hesperidin (500 mg)	Hesperetin, dihydrocaffeic acid, isoferulic acid, 4-hydroxyphenylacetic acid, dihydroferulic acid, ferulic acid, protocatechuic acid, vanillic acid, caffeic acid	
TIM-2, faecal samples from 10 healthy volunteers (pooled)[43]	28 h (0 h, 4 h, 8 h, 12 h, 16 h, 24 h, and 28 h)	Citrus + rutin supplement (284 mg rutin, 430 mg naringin, 88 mg hesperidin, 4.4 mg eriodictyol).	Phloretic acid, isoferulic acid, dihydroferulic acid, dihydrocaffeic acid, homovanillic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 2,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, resorcinol, phloroglucinol	

In Vitro Model System[Ref.]	Sampling Time (Extra Sampling)	Treatment (Dosage)	Metabolites Formed	Comments
Batch incubation, faecal samples from 30 healthy volunteers[45]	24 h (4 h, 8 h, 12 h, 24 h)	[2′,3′,5′,6′-D4]naringin (10 µL (20 mg/mL) in 990 µL)	[2′,3′,5′,6′-D4]naringenin, 5-Oac-[2′,3′,5′,6′-D4]naringin, [2′,3′,5′,6′-D4]apiforol-7-O-rhamnoglucoside, 6/8-hydroxyl-[2′,3′,5′,6′-D4] naringin, 8/6-hydroxyl-[2′,3′,5′,6′-D4] naringin, [2′,5′,6′-D3]neoeriocitrin, [2′,3′,5′,6′-D4]apigenin, [2′,5′,6′-D3] eriodictyol, 6/8-hydroxyl-[2′,3′,5′,6′-D4] naringenin, 8/6-hydroxyl-[2′,3′,5′,6′-D4] naringenin, [2′,3′,5′,6′-D4]apiforol, 3-(4′-hydroxyphenyl)-[2′,3′,5′,6′-D4] propanoic acid, 3-phenyl-[2′,3′,5′,6′-D4] propanoic acid	Metabolism varies between donors

Various metabolites were found after *in vitro* colonic fermentation of citrus flavanones. A number of metabolites were formed in several different studies, but differences between studies have been observed, which might be due to differences in the methods and donors used. SHIIME, simulator of the human intestinal microbial ecosystem. TIM-2, TNO *in vitro* model of the colon.

Table 2.2 Nonexclusive listing of studies investigating the metabolism and bioavailability of citrus flavanones in human subjects

Population (Design)[Ref]	Treatment (Dosage/Flavanone Concentration)	Sample and Sampling Time (Extra Sampling)	Metabolites Formed	Comments
Healthy subjects, n = 7 (pre- and post-test)[46]	320 mg naringin	72 h urine (0–4 h, 4–8 h, 8–12 h, 12–24 h, 24–36 h, 36–48 h, 48–60 h, 60–72 h), 72 h faeces	Urine and faeces: 4-hydroxybenzoic acid, 4-hydroxyhippuric acid, hippuric acid, phoretic acid, phloretic acid sulphate, naringin, naringenin, naringenin diglucuronide Urine: naringenin diglucuronide, naringenin glucoside glucuronide, naringenin glucoside sulphate, naringenin glucuronide sulphate, naringin glucuronide, naringenin sulphate, hydroxylated naringenin glucuronide, naringenin glucuronide, naringenin glucuronide dimer, hydroxylated naringenin sulphate	Excretion of metabolites varied between individuals
Healthy volunteers, n = 12 (pre- and post-test)[47]	Orange juice (500 mL /398 µmol (poly) phenols, of which 246 µmol was hesperidin)	24 h plasma (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 24 h)	Plasma: naringenin-4′-O-glucuronide, naringenin-7-O-glucuronide, naringenin-4′-sulfate, hesperetin-3′,7-O-diglucuronide, hesperetin-5,7-O-diglucuronide, hesperetin-3′,5-O-diglucuronide, hesperetin-O-glucuronyl-sulphate, hesperetin-7-O-glucuronide, hesperetin-3′-O-glucuronide, hesperetin-sulhate, hesperetin-3′-sulfate, eriodictyol sulphate, eriodictyol-O-glucuronyl-sulphate, caffeic acid-3′-sulfate, caffeic acid-4′-sulfate, ferulic acid, ferulic acid-4′-O-glucuronide, ferulic acid-4′-sulfate, isoferulic acid, isoferulic acid-3′-O-glucuronide, 3-(3′-hydroxyphenyl)hydracrylic acid, 3-(3′,4′-dihydroxyphenyl) propionic acid, 3-(3′-hydroxyphenyl)propionic acid-4′-sulfate, 3-(4′-hydroxyphenyl)propionic acid-3′-sulfate, 3-(3′-methoxy-4′-hydroxyphenyl)propionic acid, 3-(3′-methoxyphenyl)propionic acid-4′-O-glucuronide, 3-(3′-hydroxy-4′-methoxyphenyl) propionic acid, 3-(4′-methoxyphenyl)propionic acid-3′-O-glucuronide, 3-(3′-methoxyphenyl)propionic acid-4′-sulfate, 3-(4′-methoxyphenyl)propionic acid-3′-sulfate, 3-(4′-hydroxyphenyl) propionic acid, 3-(phenyl)propionic acid, hydroxyphenylacetic acid-O-glucuronide, hydroxyphenyl acetic acid-3′-sulfate, methoxyphenylacetic acid-O-glucuronide, 3′-methoxyphenylacetic acid-4′-sulfate, 4′-methoxyphenylacetic acid-3′-sulfate, 3′-hydroxyphenylacetic acid, 4′-hydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, benzoic acid-4-sulfate, 3′-methoxy-4′-hydroxymandelic acid, 4′-hydroxymandelic acid, hippuric acid-O-glucuronide, 3′-hydroxyhippuric acid, 4′-hydroxyhippuric acid, hippuric acid	

Population (Design)[Ref]	Treatment (Dosage/Flavanone Concentration)	Sample and Sampling Time (Extra Sampling)	Metabolites Formed	Comments
		24 h urine (baseline, 0–5 h, 5–8 h, 8–10 h, 10–24 h)	Urine: same as plasma, but also naringenin-4′,7-O-diglucuronide, naringenin-5,7-O-diglucuronide, naringenin-4′,5-O-diglucuronide, naringenin-O-glucuronyl-sulphate, hesperetin-5-O-glucuronide, hesperetin-O-glucosyl-sulphate, 3′-hydroxycinnamic acid, coumaric acid-3′-O-glucuronide, 4′-hydroxycinnamic acid, coumaric acid-4′-O-glucuronide, coumaric acid-4′-sulfate, caffeic acid-3′-O-glucuronide, caffeic acid-4′-O-glucuronide, 3-(3′-hydroxyphenyl)propionic acid-4′-O-glucuronide, 3-(phenyl)propionic acid-4′-O-glucuronide, 3-(4′-hydroxyphenyl)propionic acid-3-O-glucuronide, 3-(3′-hydroxyphenyl)propionic acid, 3-(phenyl)propionic acid-3′-O-glucuronide, 3-(phenyl)propionic acid-4′-sulfate,3-(phenyl)propionic acid-3′-sulfate, 3′,4′-dihydroxyphenylacetic acid, hydroxyphenylacetic acid-4′-sulfate, 3′-methoxy-4′-hydroxyphenylacetic acid, 3′,4′-dimethoxyphenylacetic acid, phenylacetic acid, hydroxybenzoic acid-O-glucuronide, 3-hydroxybenzoic acid-4-sulfate, 4-hydroxybenzoic acid-3-sulfate, 3-methoxy-4-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, benzoic acid-3-sulfate, 1,3,5-trihydroxyphenol, 1,2,3-trihydroxyphenol, 1,2-dihydroxyphenol	
Healthy subjects, n = 5 (controlled cross over)[48]	Orange juice (250 mL/168 µmol hesperidin, 12 µmol narirutin)	24 h urine (0-2 h, 2-5 h, 5-10 h, 10-24 h)	3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid, 3-hydroxyhippuric acid	
Healthy volunteers, n = 12 (controlled cross over)[49]	Pulp-enriched orange juice (250 mL/537 µmol flavanones, of which 329 µmol was hesperidin)	24 h urine (0 h, 0–2 h, 2–5 h, 5–10 h, 10–24 h)	Hesperetin-O-diglucuronide, hesperetin-O-sulphate-O-glucuronide, hesperetin-O-sulphate-O-glucoside, hesperetin-7-O-glucuronide, hesperetin-3-O-glucuronide, hesperetin-3-O-sulfate, naringenin-O-diglucuronide, naringenin-4′-O-glucuronide, naringenin-7-O-glucuronide, eriodictyol-O-sulphate 3-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid, isoferulic acid, dihydroferulic acid, 3′-hydroxyhippuric acid, 4′-hydroxyhippuric acid, 3-(3′-hydroxyphenyl)hydracrylic acid, 3-methoxy-4-hydroxyphenylacetic acid, hippuric acid	Excretion of metabolites varied between individuals

Population (Design)[Ref]	Treatment (Dosage/Flavanone Concentration)	Sample and Sampling Time (Extra Sampling)	Metabolites Formed	Comments
Men at moderate CVD risk, n = 16 (controlled cross over)[50]	Orange juice, (767 mL/ 320 mg hesperidin)	5 h plasma	Hesperetin-glucuronide, naringenin-7-glucuronide, hesperetin-glucuronide, naringenin-glucuronide, hesperetin, naringenin Hippuric acid, dihydroferulic acid, dihydroferulic acid-3-glucuronide, 4-hydroxyphenylacetic acid, vanillic acid, hydroxyhippuric acid, iso/ ferulic acid-glucuronide, 3- hydroxyhippuric acid, isovanillic acid, 3-hydroxyphenylacetic acid, vanillic acid-glucuronide, isovanillic acid-glucuronide, iso/vanillic acid-glucuronide, 4-hydroxy-benzoic acid, benzoic acid-4-glucuronide	

Various metabolites were found in plasma, urine, and/or faecal samples after ingestion of citrus flavanones in humans. Although a number of metabolites were formed in several studies, differences between studies have also been observed. These differences might be due to differences in study designs and host factors, including the microbiota. CVD, cardiovascular disease.

Effects on microbiota composition

The intestinal microbiota is a complex ecosystem which varies between individuals.[52] The interaction between the gut microbiota and polyphenols is considered to be bidirectional — in addition to the ability of intestinal bacteria to metabolise polyphenols, evidence has also accumulated that polyphenols may induce changes in the microbiota towards a more favourable composition and activity, including the production of short-chain fatty acids (SCFAs) in the colon. These metabolites have many known beneficial biological effects, *e.g*., acting as fuel for enterocytes, improving barrier function, and inhibiting inflammation.[53,54] Studies investigating the effect of citrus flavanones or food products derived from citrus fruit on the intestinal or faecal microbiota have mainly focused on their ability to inhibit the growth of pathogens, to increase beneficial commensal bacteria (such as *Bifidobacterium* and *Lactobacillus* species), and to stimulate the production of SCFAs.

In vitro studies

Duda-Chodak showed *in vitro* that both citrus flavanone aglycones, hesperetin and naringenin, inhibited the growth of different bacterial species after 24 h of incubation, while the parent compounds did not have such an effect. These included effects on *Bacteroides galacturonicus*, *Enterococcus caccae*, *Bifidobacterium catenulatum*, *Ruminococcus gauvreauii*, and *Escherichia coli*. The growth of *Lactobacillus* spp. was inhibited by naringenin only. Inhibitory effects were observed at concentrations of at least 250 μg/mL.[18] The ability of naringenin to inhibit bacterial growth was also confirmed by Parkar *et al*., who tested the effects on *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium,* and *Lactobacillus rhamnosus*. In this study, even lower minimum inhibitory concentrations were reported, being 62.5 μg/mL for *S. aureus* and 125 μg/mL for the other three strains.[55] Antibacterial activities of citrus flavanones have also been shown against vancomycin-intermediate *S. aureus* (VISA) at a concentration of 400 μg/mL for naringenin and 3200 μg/mL for naringin and hesperetin,[56] and against *Aeromonas hydrophila* after 3125 μg/mL hesperidin treatment.[57] However, other studies failed to show the antibacterial activity of naringin, naringenin, or hesperidin against pathogens such as *S. aureus* and *E. coli*,[58,59] but also found that hesperidin and naringin stimulated the growth of *Bifidobacterium bifidum*.[60] In addition to citrus flavanones and citrus flavanone aglycones, phenolics that might be formed as a result of colonic microbial fermentation can also influence the intestinal microbiota.[55,60-63] For example, antimicrobial effects towards *E. coli* have been reported for ferulic acid, isoferulic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, vanillic acid, and caffeic acid, at concentrations ranging from approximately 100–1000 μg/mL depending on the specific phenolic acids and strains tested.[55,61,62] Interestingly, the previously mentioned

study by Parkar *et al*. also tested the antimicrobial effect of caffeic acid and showed that naringenin was more effective at inhibiting the growth of the four different strains than caffeic acid.[55] On the other hand, Gwiazdowska *et al*. showed that incubation with caffeic acid and vanillic acid resulted in a stronger growth stimulation of *B. bifidum* than incubation with naringin and hesperidin.[60]

The differences in the outcomes of the abovementioned studies may in part be due to differences in the experimental setup of these studies, such as the flavanone concentrations used. *In vivo*, it is possible that not the citrus flavanones themselves, but the metabolites formed as a result of microbial metabolism and cross-feeding are responsible for modulating the intestinal microbiota. Therefore, the results from studies that did not focus on single strains, but a more complete microbiota, may provide more insight into the effects that could be expected in the human GI tract.

In a study using the SHIME model, supplementation with 105 mL orange juice twice daily for 14 days resulted in a significant increase in *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., and *Clostridium* spp. populations, while a decrease in enterobacteria was found. These changes in microbiota composition were accompanied by increased levels of the SCFAs acetate, butyrate, and propionate. Unfortunately, the concentration of citrus flavanones in the orange juice was not reported.[17] Changes in SCFA levels and microbiota composition were also reported in a recent study with a similar experimental setup in the SHIME model. After a three-week treatment period with 500 mg citrus extract, containing >80% hesperidin-2S and >4% of naringin, both butyrate and total SCFA levels, and the relative abundance of the *Clostridium coccoides/ Eubacterium rectale* cluster were significantly increased.[42] This cluster, which is part of *Clostridium* cluster XIVa, includes species that are known to produce butyrate.[64] Positive effects on SCFA levels have also been shown for pure naringin at concentrations of 40 and 160 mg/L, after 24 h of incubation in a batch-culture fermentation experiment.[65]

Animal and human studies

In rats, three-week hesperetin supplementation via the diet (16.4 mmol/kg) resulted in significant increases in acetate and butyrate in the cecum. No significant effects were observed after hesperidin treatment. Similarly, hesperidin was not able to significantly affect the microbiota composition, while hesperetin administration resulted in increased proportions of *Clostridium* clusters IV and XVIII and a reduced proportion of *Clostridium* subcluster XIVa in the faeces.[66] The differential effects are remarkable as hesperidin is expected to be rapidly converted into hesperetin in the proximal colon. However, in a recent rat study, oral administration of 100 and 200 mg/kg hesperidin three times a week for a period of four weeks, did result in significant changes in microbiota

composition.[67] They found that hesperidin treatment, at the high dose, resulted in an increased *Lactobacillus* proportion, while an increased proportion of *Staphylococcus* and a decrease of *C. coccoides/E. rectale* were reported for both dosages.

The effect of hesperidin supplementation on microbiota composition and SCFAs has also been studied in humans. In a randomised, placebo-controlled trial in healthy subjects with features of metabolic syndrome, daily supplementation with 500 mg citrus extract (with >80% hesperidin-2S and >4% naringin) for 12 weeks, did result in an increase in the butyrate to total SCFA ratio but not in the absolute levels of faecal SCFAs.[68]

In healthy volunteers, consumption of a pasteurized orange juice with unknown flavanone content for two months resulted in a significant increase in *Lactobacillus* spp. and total anaerobes in faecal samples. In addition, a significant reduction in ammonium concentration and an increase in the acetate to total SCFA ratio were found compared to the baseline.[69] Daily supplementation of two orange juices with different flavanone content for seven days in healthy volunteers resulted in microbiota composition shifts, of which the most notable was an increase in the abundance of Clostridia operational taxonomic units from Mogibacteriaceae, Tissierellaceae, Veillonellaceae, Odoribacteraceae, and Ruminococcaceae families.[70]

Overall, results from available *in vitro*, animal and human studies show that citrus flavanone treatment can affect the composition of the microbiota or growth of specific taxa. Although some findings vary, growth inhibition of Enterobacteriaceae has been demonstrated repeatedly. Unfortunately, none of the human studies on the effect of flavanones on the microbiome included analyses on faecal metabolite levels.

Effects on host parameters related to gastrointestinal health

According to Bischoff *et al*., GI health comprises an effective digestion and absorption, together with a stable microbiota, effective immune status, and state of general wellbeing in the absence of GI diseases.[71] In this context, an intact intestinal barrier is considered an important factor. One of its main functions is to act as defensive barrier against intraluminal toxins, antigens, and microorganisms. Together with the intestinal microbiota and the immune system, the barrier is determined by, *e.g*., the mucus layer and an intact epithelial cell monolayer sealed by junctional complexes.[72] Two *in vitro* studies using Caco-2 cell monolayers have shown that hesperetin and naringenin were able to improve intestinal barrier function, as measured by an increase in transepithelial electrical resistance and expression levels of tight junction proteins and a decrease in fluorescein isothiocyanate (FITC)-conjugated dextran flux across the monolayer.[73,74] Emerging evidence indicates that the intestinal permeability is increased in several disorders, such as inflammatory bowel disease (IBD).[75] To study

the effects of citrus flavanones on barrier function and other features of IBD, chemically induced colitis models in rodents have been widely used, such as dextran sulphate sodium (DSS)- , trinitrobenzene sulfonic acid (TNBS)-, and dinitrobenzene sulfonic acid (DNBS)- induced colitis. The key findings of *in vitro* and *in vivo* studies investigating the effects of citrus flavanones on host parameters related to intestinal inflammation and barrier function are summarised in Table 2.3. For example, animals treated with hesperidin, hesperetin, or naringenin showed a significant improvement of chemically induced colitis symptoms and inflammatory parameters, such as pro-inflammatory cytokines and neutrophil infiltration.[76-82] Beneficial effects were also shown with regard to colonic barrier function.[76,77]

Furthermore, an improved barrier function and/or decrease in intestinal inflammation have also been reported *in vitro* for phenolics that can be formed during flavanone metabolism, such as ferulic acid, isoferulic acid, dihydroferulic acid, dihydrocaffeic acid, hydrocinnamic acid, and phloretic acid.[83-85] Studies directly comparing the effect size versus the flavanones or aglycones are not available.

At present, data from human studies regarding the effects of citrus flavanone supplementation in IBD or other GI diseases are lacking. However, in a recent human trial in healthy subjects with features of metabolic syndrome, supplementation with a citrus extract (consisting of >80% hesperidin-2S and >4% naringin) for 12 weeks showed a clear tendency to reduce levels of faecal calprotectin, a commonly used biomarker of intestinal inflammation.[68] Together with the abovementioned findings from *in vitro* and animal studies, this underlines the relevance to further investigate the effect of citrus flavanones in individuals with GI disorders characterized by intestinal inflammation, increased intestinal permeability, and/or microbial perturbations.

Table 2.3 Nonexclusive listing of studies on the effects of citrus flavanones on host parameters related to barrier function and inflammation

Model[Ref.]	Treatment	Treatment Duration	Dose and Administration	Change in Main Outcomes vs. Control (Relevant Concentrations)
Caco-2 cell monolayers[73]	Hesperetin vs. control Naringenin vs. control	24 h	100 μM	**Barrier function:** ↑ TEER, occludin expression, claudin-4 expression, cytoskeletal association of occludin and claudin-1, and -3 ↔ FITC–dextran flux **Barrier function:** ↑ TEER, occludin expression, claudin-4 expression, cytoskeletal association of ZO-2, occludin and claudin-1, -3, and -4 ↔ FITC–dextran flux
Caco-2 cell monolayers[74]	Naringenin vs. control	48 h	10, 30, 100 μM	**Barrier function:** ↑ TEER (30, 100 μM)†, claudin-4 expression (30, 100 μM)†, cytoskeletal association of occludin, claudin-1, claudin-4 and ZO-2 (100 μM) ↓ FITC–dextran flux (30, 100 μM)†
DSS-induced colitis in male BALB/c mice[76]	Naringenin vs. control diet	9 days	0.3% of the diet, oral administration	**Barrier function:** ↓ Colonic permeability, claudin-1 expression ↑ Occludin, junctional adhesion molecule-A, claudin-3 expression **Inflammation:** ↓ DAI, colonic shortening, expression of cytokines (IFN-Ɣ, IL-6, MIP-2, and IL-17A)
DSS-induced colitis in male BALB/c mice[77]	Naringenin vs. control diet Hesperetin vs. control diet	12 days	0.3% of the diet, oral administration 0.3% of the diet, oral administration	**Barrier function:** ↓ Colonic permeability ↑ Occludin expression **Inflammation:** ↓ Weight loss, colonic damage ↑ Colon length **Barrier function:** ↔ Colonic permeability, occludin expression **Inflammation:** ↓ Weight loss, colonic damage ↔ Colon length
DSS-induced colitis in male BALB/c mic[78]	Hesperidin vs. control	7 days	10, 40, 80 mg/kg, oral administration	**Inflammation:** ↓ DAI, MPO, MDA, IL-6, colonic wet weight (10, 40, 80 mg/kg)† ↓ Mucosal cell damage (80 mg/kg) ↔ IL-4 (10, 40, 80 mg/kg)

Model[Ref.]	Treatment	Treatment Duration	Dose and Administration	Change in Main Outcomes vs. Control (Relevant Concentrations)
TNBS-induced colitis in female Wistar rats[79]	Hesperidin vs. control	Twice (48 h pre- + 48 h post-colitis induction)	2.5, 5, 10, 25, 50 mg/kg, oral administration	**Inflammation:** ↓ Colonic damage, colonic weight, colonic MPO (10, 25 mg/kg) ↑ Glutathione levels (10, 25 mg/kg), colonic fluid absorption (10–50 mg/kg) ↔ MDA, LTB4 (2.5–50 mg/kg)
TNBS- induced colitis in male Wistar rats[80]	Orange juice vs. control	15 days	2 mL/kg, 5 mL/kg, 8 mL/kg, oral administration	**Inflammation:** ↓ Colonic damage (2, 5, 8 mL/kg), MPO, CRP (5, 8 mL/kg), ALP (8 mg/kg) ↑ GSH (8 mL/kg)
	Grapefruit juice vs. control		0.1 mL/kg, 0.3 mL/kg, 0.5 mL/kg, oral administration	**Inflammation:** ↓ Colonic damage (0.1, 0.3, 0.5 mL/kg) MPO, CRP (0.3, 0.5 mL/kg), ALP (0.3, 0.5 mL/kg) ↑ GSH (0.3 mL/kg)
	Combination vs. control		2 mL/kg OJ + 0.1 mL/kg GJ (low dose), 5 mL/kg OJ + 0.3 mL/kg GJ (high dose), oral administration	**Inflammation:** ↓ Colonic damage, MPO, CRP, ALP (low dose, high dose) ↑ GSH (high dose)
DNBS- induced colitis in Male CD1 mice[81]	Bergamot juice extract vs. control	4 days	5, 10, 20 mg/kg, oral administration	**Inflammation:** ↓ Colonic damage, weight loss, MPO, TNF-α, IL-1β, ICAM-1, p-selectin, nitrotyrosine, PAR, nuclear NF-kB translocation, p-JNK activation (20 mg/kg) ↑ Colon length (20 mg/kg)
LPS-challenged broiler chickens[82]	Hesperidin vs. control diet	42 days	20 mg/kg diet, oral administration	**Inflammation:** ↑ Phagocytic index, villus length, villus width, villus length/crypt depth ↓ Crypt depth ↔ Body weight gain, feed intake feed conversion ratio
Human subjects with features of metabolic syndrome[68]	Citrus extract (>80% hesperidin-2S and <4% of naringin) vs. placebo	12 weeks	500 mg, oral administration	**Inflammation:** ↔ Calprotectin

TEER, transepithelial electrical resistance. FITC, fluorescein isothiocyanate. ZO-2, zonula occludens 2. DSS, dextran sulphate sodium. DAI, disease activity index. IFN-ϒ, interferon-ϒ. IL, interleukin. MIP-2, macrophage inflammatory protein-2. MPO, myeloperoxidase. MDA, malondialdehyde. TNBS, trinitrobenzene sulfonic acid. LBT4, leukotriene B4. OJ, orange juice. GJ, grapefruit juice. CRP, C-reactive protein. ALP, alkaline phosphatase. GSH, glutathione. DNBS, dinitrobenzene sulfonic acid. TNF-α, tumour necrosis factor-α. ICAM-1, intercellular adhesion molecule-1. PAR, poly ADP-ribose. NF-kB, nuclear factor-kB. ↑, increase. ↓, decrease. ↔, no significant change. †, dose-dependent effect.

Conclusions

Following oral ingestion, citrus flavanones reach the distal part of the small intestine and the colon almost completely intact, where they interact with the microbiota. Evidence shows that citrus flavanones are extensively metabolised by intestinal bacteria resulting first in the formation of the aglycones hesperetin and naringenin and subsequently in the formation of various smaller phenolics. The microbiota composition and activity is highly variable between individuals, which can contribute to differences in metabolites formed and potential effects.[30,86,87] This can further be influenced by variations in dietary intake. Citrus flavanones and their metabolites, in turn, can also impact the microbiota composition and activity. For example, growth inhibition of Enterobacteriaceae has been reported. Beneficial effects of citrus flavanones on parameters such as GI inflammation and intestinal barrier function have repeatedly been reported, suggesting that intake of citrus flavanones can contribute to improved GI functioning and health. Based on the current evidence, this is likely a combined effect of the original compounds, their metabolites, and an interaction with the intestinal microbiome. Most of the currently available evidence is derived from *in vitro* and animal studies. Therefore, more research focusing on bioavailability and on effects in human subjects may help to improve our understanding of the effects of citrus flavanones in the human gut.

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Chapter 3

The effects of citrus flavonoids and their metabolites on immune-mediated intestinal barrier disruption using an *in vitro* co-culture model

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Abstract

Hesperidin and naringin are citrus flavonoids with known anti-oxidative and anti-inflammatory properties. Evidence from previous studies indicates that both these compounds and the metabolites that are formed during intestinal metabolism are able to exert beneficial effects on intestinal barrier function and inflammation. However, so far, studies investigating the relative contributions of the various compounds are lacking. Therefore, we assessed the effect of citrus flavonoids and their intestinal metabolites on immune-mediated barrier disruption in an *in vitro* co-culture model. Caco-2 cell monolayers were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells for 30 hours. At baseline, the citrus flavonoids and their metabolites were added to the apical compartment (50 or 100 μM per compound). After 24 hours, THP-1 cells were incubated with LPS in the basolateral compartment for 6 hours. Incubation with citrus flavonoids and their metabolites did not induce changes in transepithelial electrical resistance, FITC-D4 permeation or gene expression of barrier related genes for any of the compounds tested. After LPS stimulation, NF-κB activity was significantly inhibited by all compounds (100 μM) except for one metabolite (all $p \leq 0.03$). LPS-induced production of the cytokines IL-8, TNF-α and IL-6 was inhibited by most compounds (all $p < 0.05$). However, levels of IL-1β were increased, which may contribute to the lack of an improved barrier effect. Overall, these results suggest that citrus flavonoids may decrease intestinal inflammation via reduction of NF-κB activity and that the parent compounds and their metabolites formed during intestinal metabolism are able to exert comparable effects.

Introduction

Flavonoids are polyphenolic compounds present in vegetables, tea, red wine and fruits and are widely consumed as part of a habitual diet.[1] Flavonoids that are present in citrus fruits possess diverse beneficial effects, including anti-oxidative and anti-inflammatory properties.[2, 3] Several gastrointestinal diseases, such as inflammatory bowel disease (IBD) and celiac disease, have been associated with increased levels of oxidative stress and inflammatory mediators, contributing to intestinal barrier disruption.[4-6] Certain pro-inflammatory cytokines such as TNF-α and IFN-γ are described to increase intestinal permeability.[7, 8] An increased intestinal permeability may result in translocation of luminal content, further triggering the inflammatory response, thereby contributing to disease onset and progression. Via their anti-oxidative and anti-inflammatory properties citrus flavonoids may beneficially affect intestinal barrier function and intestinal health. Hesperidin and naringin are two important flavonoids present in citrus fruits that are largely resistant to hydrolyzation in the stomach and small intestine. Once reaching the colon, they are extensively metabolised by the gut microbiota. Here, hesperidin and naringin are first converted into their aglycones hesperetin and naringenin and subsequently into various smaller phenolics.[9-11] A recent study using an *in vitro* digestion model of the gastrointestinal tract, showed that after digestion of hesperidin, the phenolic metabolites found at the highest concentrations were dihydrocaffeic acid, isoferulic acid, 4-hydroxyphenylacetic acid and dihydroferulic acid. These metabolites were mainly present in the compartments representing the proximal parts of the colon.[12]

Beneficial effects of citrus flavonoids as well as their aglycones on intestinal health have been observed in various studies employing rodent colitis models.[13-16] Citrus flavonoids at dosages ranging from 10 mg/kg to 3 g/kg resulted in decreased colonic permeability, inflammation, oxidative stress and disease activity index scores.[13-16] Furthermore, *in vitro* studies using intestinal cell lines have shown beneficial effects of citrus flavonoid aglycones or phenolic metabolites on either intestinal barrier function[17-20] or inflammation,[21] indicating that both the parent compounds that are ingested and the (phenolic) metabolites formed are able to exert beneficial effects. However, as these *in vitro* models did not include the crosstalk between epithelial cells and immune cells, assessed only one or a few metabolites and/or did not include the parent compounds, the effects of the metabolites on immune-mediated barrier disruption and their relative contribution compared to the parent compounds remain unclear.

Therefore, the aim of this study was to assess the potential beneficial effects of hesperidin, naringin and several of the main metabolites formed by bacterial metabolism in the

intestine on immune-mediated intestinal barrier disruption, using an *in vitro* co-culture model of intestinal inflammation and barrier dysfunction.

Methods

Test compounds

The citrus flavonoid extract used in this study (MicrobiomeX®, containing >85% hesperidin and >5% naringin) and hesperidin (Cordiart®) were kindly provided by BioActor BV (Maastricht, the Netherlands). Hesperetin, naringin, naringenin, dihydrocaffeic acid, 4-hydroxyphenylacetic acid, ferulic acid, hydrocinnamic acid, phloretic acid and phloroglucinol were purchased at Sigma-Aldrich (Schnelldorf, Germany). Isoferulic acid and dihydroferulic acid were purchased at TCI Europe (Zwijndrecht, Belgium). All compounds were administered at a concentration of 100 µM based on previous *in vitro* studies[17, 18] and because it is anticipated that both the parent compounds and metabolites can reach concentrations in this order of magnitude in the intestinal lumen after oral consumption of citrus flavonoids.[12, 17, 22] Furthermore, to investigate whether similar effects can be expected at lower dosages, several experiments were also performed at a dose of 50 µM for the following compounds: hesperidin, naringin, hesperetin, naringenin, isoferulic acid and 4-hydroxyphenylacetic acid. These compounds were selected as they showed the most promising effects and represent different stages of intestinal metabolism (*i.e.* parent compounds, aglycones and phenolic metabolites). All test compounds were first dissolved in dimethylsulfoxide (DMSO; Applichem, Darmstadt, Germany) and diluted in complete Dulbecco's modified Eagle medium (DMEM; composition as described below) resulting in a final concentration of 0.1% DMSO.

Cell cultures

Caco-2 cells were derived from the American Type Culture Collection (ATCC, Rockville, USA) and were maintained in DMEM high glucose (Cat no. D5796, Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS; cat no. 26140079, Invitrogen, Breda, the Netherlands), 1% Antibiotic-Antimycotic (Anti-Anti; cat no. 15240062, Invitrogen) and 1% non-essential amino acids (NEAA; cat no. 11140035, Invitrogen) at 37°C in a 5% CO_2 atmosphere.

THP-1-Blue™ NF-κB cells (InvivoGen, Toulouse, France) were kindly provided by ProDigest BVBA (Ghent, Belgium). These cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (cat no. R8758, Sigma-Aldrich) supplemented with 10% FBS (Invitrogen), 1% Antibiotic-Antimycotic (Invitrogen) and 1% sodium pyruvate (cat no. 11360070, Invitrogen). THP-1 cells are human monocytic leukemia cells that

differentiate into macrophage-like cells after treatment with phorbol 12-myristate 13-acetate (PMA).[23] THP-1-Blue cells include an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct, enabling monitoring of NF-κB activity by use of the colorimetric QUANTI-Blue™ enzyme assay (cat code rep-qb1, InvivoGen).

Assessment of cytotoxicity

The cytotoxic effect of the test compounds was assessed by a fluorometric lactate dehydrogenase (LDH) assay (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Promega, Madison, USA) according to the manufacturer's protocol. Caco-2 cells in 96- well plates (Greiner CELLSTAR, Frickenhausen, Germany) were incubated with the test compounds for 24 hours, after which LDH release into the culture medium was determined as described previously.[7] Cytotoxicity of the test compounds was expressed as percentage of the values obtained for the 100% cell lysis control by Triton® X-100.

Caco-2/THP-1 co-culture experiments

Co-culture experiments were performed as described previously by Possemiers *et al.*[24] Briefly, Caco-2 cells (passage 30-36) were seeded into 24-well Transwell inserts (cat no. 3470, Corning, Cambridge, MA, USA) at a density of 80.000 cells per insert and cultured for 14 days until a functional monolayer with a TEER of > 330 $\Omega \bullet cm^2$ was formed,[25] before being placed in co-culture. Two days preceding the co-culture experiment, the THP-1 cells were seeded in 24-well plates at a density of 500.000 cells/well in the presence of 100 ng/mL PMA (cat no. P8139, Sigma- Aldrich) prepared in RPMI 1640 (Sigma-Aldrich). PMA-stimulated THP-1 cells produce considerable amounts of inflammatory mediators, which when placed in co-culture with Caco-2 cells, can contribute to a disruption of the cell monolayer.[25] After 2 days of PMA treatment, the THP1- cells were washed with PBS, placed in Caco-2 culture medium and the inserts containing the Caco-2 monolayer were placed on top of the THP-1 cells (Figure 3.1). Subsequently, the test products were added apically to the Transwell inserts. Cells were also treated with culture medium containing 0.1% DMSO as vehicle control, this condition will hereafter be referred to as the medium + DMSO control. After a treatment period of 24 hours, the THP-1 cells were incubated with 500 ng/mL ultrapure LPS from *E. coli* (cat code tlrl-peklps, InvivoGen) in the basolateral compartment for an additional 6 hours. Binding of LPS to toll-like receptor 4 (TLR4) results in NF-kB activation and production of cytokines.[26] After the 6-hour co-incubation period, the basolateral medium was collected and stored at -80°C.

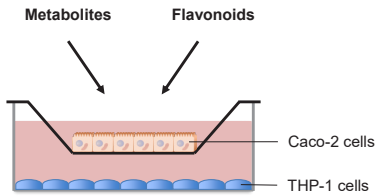

Figure 3.1 Schematic overview of the co-culture model with Caco-2 cells and THP-1- cells. Caco-2 cells cultured in transwell inserts for 14 days were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. After 24 hours of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 hours.

Measurement of barrier function

The epithelial integrity of the Caco-2 cell monolayers was assessed by measurement of transepithelial electrical resistance (TEER), fluorescein isothiocyanate–dextran 4kDa (FITC-D4) permeation and gene expression of different barrier related genes. TEER of the Caco-2 monolayers was measured at baseline, after 24 hour co-incubation with the test compounds and after another 6 hours of co-culture with the THP-1 cells (*i.e.* t=30 hours). TEER was measured using the EVOM^2 Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL, US). Monolayer resistance of treated wells was calculated by subtracting the TEER of empty inserts and multiplying these with the surface area of the filter membrane. Data are reported as TEER percentage of baseline values. FITC-D4 (1 mg/ml; Sigma-Aldrich) was added apically to the Caco-2 cells at t=30 hours and incubated for 1 hour at 37°C, after which the basolateral medium was collected and stored at -20°C for further analysis. FITC-D4 concentrations were measured by fluorimetry using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, USA) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Gene expression of barrier related genes (*i.e.* zonula occludens-1 (ZO-1), occludin, claudin-2, claudin-3, claudin-4, E-cadherin and myosin light chain kinase (MLCK)) was evaluated for the following six compounds: hesperidin, naringin, hesperetin, naringenin, isoferulic acid and 4-hydroxyphenylacetic acid at t=30 hours. RNA extraction, reverse transcription, and qPCR were performed as described previously.[7] Briefly, RNA was extracted using the Qiagen RNeasy Mini kit (QIAGEN, Germantown, USA). After cells were lysed by adding lysis buffer to the monolayers, RNA was isolated and purified with on-column DNase digestion. All qPCRs were performed with the iQ SYBR® Green Supermix (Bio-Rad Laboratories, Veenendaal, the Netherlands) on a thermal cycler (Bio-Rad Laboratories). The qPCR conditions used were 10 minutes at 95°C, followed by 40 amplification cycles of 10 seconds at 95°C and 40 seconds at 60°C. Primer sequences

used are listed in supplemental Table s3.1. Expressions of target genes were normalised to 18S ribosomal RNA (18S RNA) as reference gene as described previously.[27]

Measurement of NF-κB activity and cytokine secretion

After stimulation with LPS, NF-κB activity of THP-1 cells was measured by use of the QUANTI-Blue™ enzyme assay (InvivoGen) according to the manufacturer's instructions. In brief, 20 µl of the basolateral medium was incubated for 15 minutes with 200 µl QUANTI-Blue at 37°C. Optical density was measured at 630 nm using the Synergy HT microplate reader (BioTek instruments, Bad Friedrichshall, Germany). Secreted cytokine levels were quantified in basolateral medium using a cytometric bead array flex set assay (BD Biosciences, Franklin Lakes, USA) followed by measurement with a flow cytometer (BD FACS Canto II™), as described previously.[28] Levels of the pro-inflammatory cytokines IL-8, TNF-α, IL-6 and IL-1β were measured, with respective detection limits of 156.25 pg/ml, 39 pg/ml, 39 pg/ml and 78 pg/ml. Data are expressed as percentage of the vehicle control to correct for limited interassay variation.

Statistical analysis

Data are presented as means ± SEM of three independent experiments, with three technical replicates. In the incidental case that one of the three replicates deviated more than 30% from the median, this value was considered a technical outlier and removed from the data set. The final data was obtained from at least two independent experiments, each conducted at least in duplicate. For all cytokines, values below the detection limit were imputed with the corresponding detection limit. Statistical analyses were performed with an independent samples t-test using IBM SPSS Statistics version 25 (IBM Statistics, Chicago, IL, USA) based on the individual values of each replicate. Statistical significance was set at $p < 0.05$. For the TEER, gene expression and cytokine results P-values were corrected for multiple comparisons using the false discovery rate (FDR) of Benjamini–Hochberg.

Results

Intestinal barrier function

As citrus flavonoids have anti-inflammatory potential, a Caco-2/THP-1 co-culture model was used[24] to assess whether these compounds may be able to exert anti-inflammatory effects and thereby attenuate cytokine-induced barrier disruption. Prior to examining these effects, the cytotoxicity of all test compounds was evaluated by measurement of LDH release from cells. These results showed that there were no significant differences in percent cytotoxicity of the test compounds at the chosen working concentration of 100 µM as compared to the medium + DMSO control (all ≤ 10.7% and $p \leq 0.18$).

In this co-culture model, PMA-induced differentiation of the THP-1 cells resulted in a decrease in TEER of approximately 30% (ranging from 23.4 to 35.4%) compared to baseline (set at 100%), indicating that Caco-2 monolayer integrity was compromised after 24 hours of co-culture. Apical treatment with the citrus flavonoids, their aglycones and their metabolites at concentrations of 100 μM or 50 μM did not attenuate this decrease in TEER values (Figure 3.2A; supplemental Figure s3.1A). After incubation for an additional 6 hours in the presence of LPS, no further differences in TEER values were observed (decrease of 23.5-37.7% compared to baseline). Treatment with the citrus flavonoid test compounds did not affect barrier function after this additional incubation period at both dosages (Figure 3.2B; supplemental Figure s3.1B). Paracellular permeability of the monolayers was also evaluated by FITC-D4 permeation after incubation with citrus flavonoids at concentrations of 100 μM and 50 μM. In line with the TEER results, treatment with the test compounds did not affect FITC-D4 permeation (supplemental Figure s3.2A and s3.2B). Furthermore, no significant effects on expression levels of tight junction or adherens junction related genes were observed after treatment with citrus flavonoids and their key metabolites (Figure 3.3).

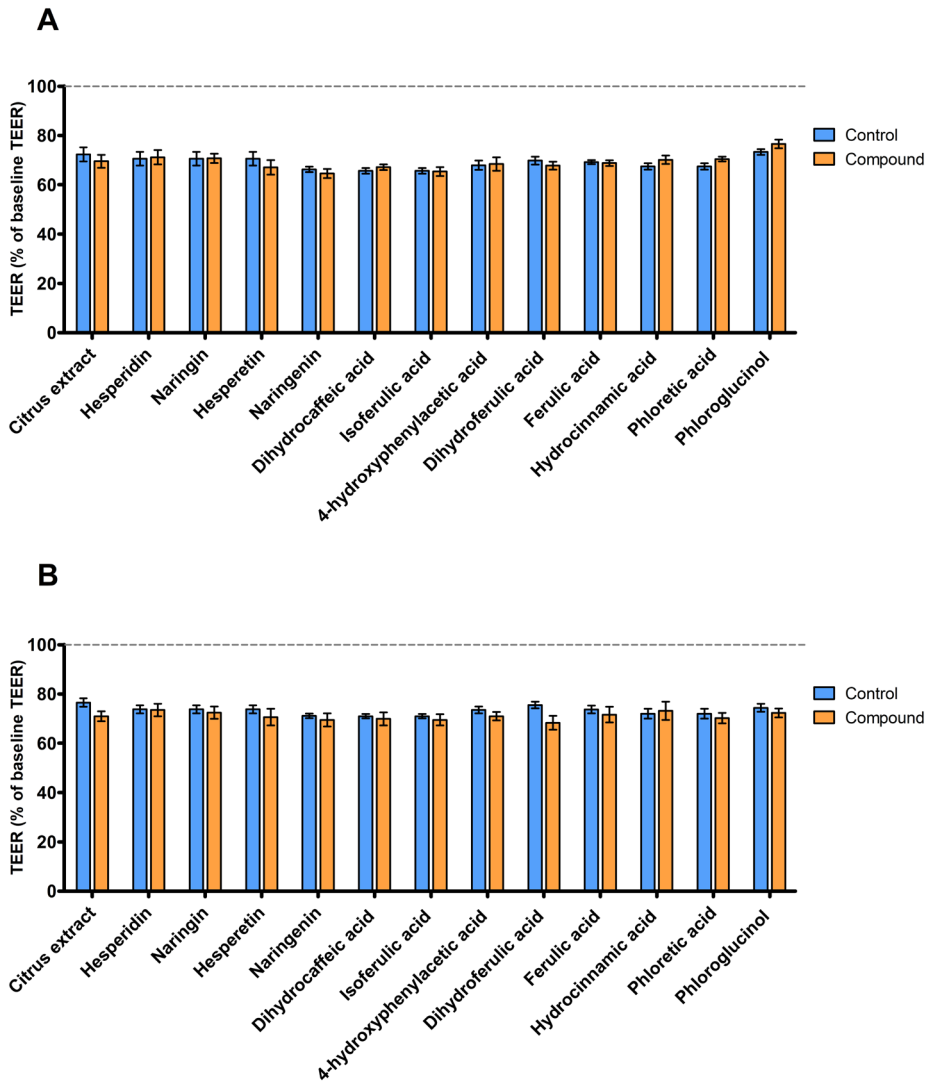

Figure 3.2 The effects of citrus flavonoids, their aglycones and their metabolites on transepithelial electrical resistance (TEER) of Caco-2 monolayers after 24 hours (A) and after 30 hours (B) of co-culture with PMA-simulated THP-1 cells. At baseline, Caco-2 cells cultured in Transwell inserts for 14 days were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either the test compounds (100 µM) or culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 hours of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 hours. TEER was measured at baseline, after 24 hours and after 30 hours of incubation. TEER values are expressed as mean percentage from the baseline value ± SEM from at least 2 independent experiments in duplicate. No significant differences were observed between the test compounds and the medium + DMSO control.

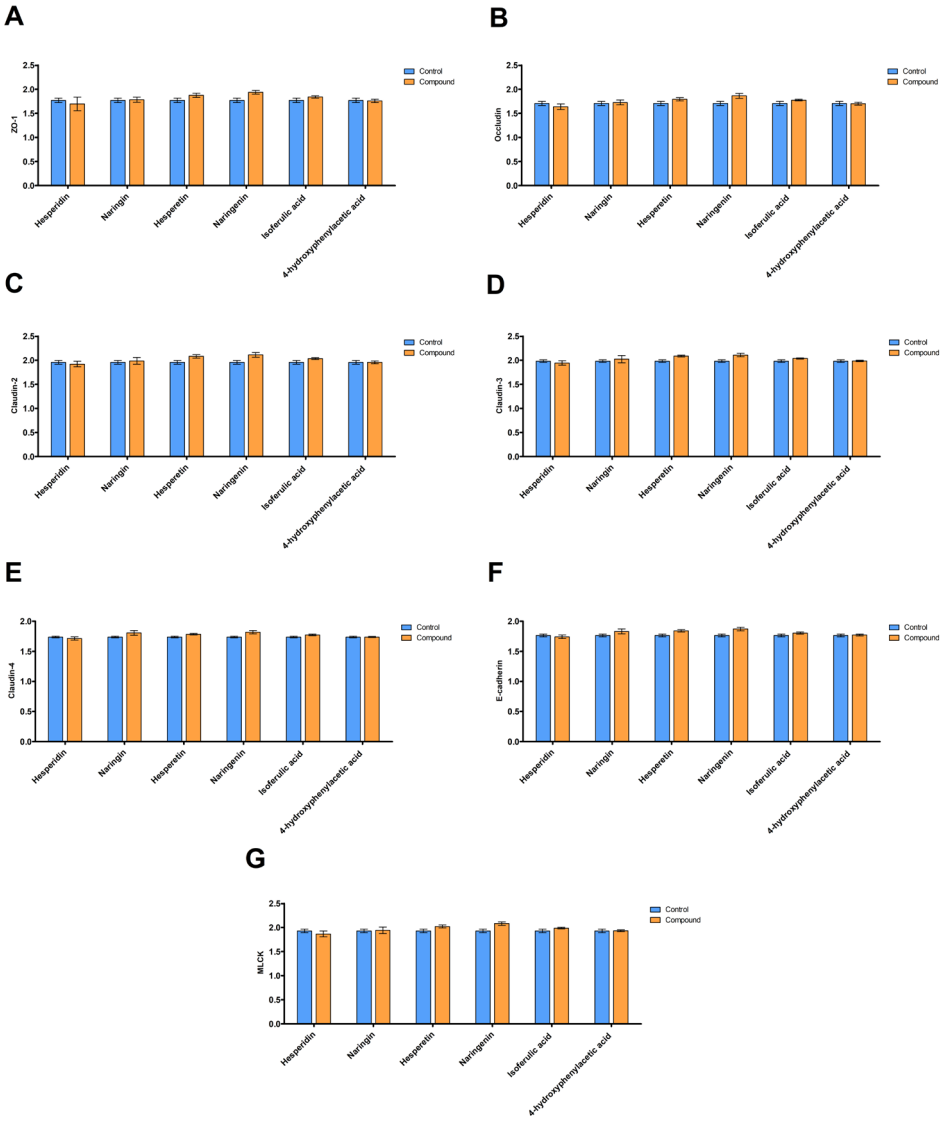

Figure 3.3 The effects of citrus flavonoids, their aglycones and their metabolites on expression levels of tight junction or adherens junction related genes of Caco-2 monolayers in co-culture with PMA-simulated THP-1 cells. Caco-2 cells cultured in Transwell inserts for 14 days were placed in co-culture with PMA-simulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either culture medium containing 0.1% DMSO (medium + DMSO control) or test compounds (100 μM) added to the apical compartment. RNA was extracted after 30 hours of incubation. Expressions of target genes were normalized to 18S ribosomal RNA (18S RNA) as reference gene. Values are represented as mean ± SEM. No significant differences were observed between the test compounds and the medium + DMSO control. A: ZO-1; B: occludin; C: claudin-2; D: claudin-3, E: claudin-4; F: E-cadherin; G: MLCK. ZO-1: zonula occludens-1; MLCK: myosin light chain kinase.

NF-κB activity and cytokine secretion

The anti-inflammatory potential of the citrus flavonoids and their metabolites was evaluated by measuring NF-κB activity and cytokine secretion in the co-culture model after the 24-hour treated THP-1 cells were incubated for 6 more hours with LPS. NF-κB activity was determined by assessment of NF-κB-inducible SEAP levels secreted into the basolateral medium. Incubation with LPS resulted in a significant increase in NF κB- inducible SEAP levels secreted by the THP-1 cells (supplemental Figure s3.3). The increase in NF-κB activity was significantly inhibited by all test compounds after treatment at a concentration of 100 μM except for phloroglucinol (Figure 3.4; all $p \leq 0.03$) and this effect seemed to be most pronounced for the phenolic metabolites isoferulic acid, 4-hydroxyphenylacetic acid, and dihydroferulic acid. Except for hesperetin ($p < 0.001$), no significant effects of the citrus flavonoid test compounds on NF-κB activity were observed at a lower concentration of 50 μM (supplemental Figure s3.4).

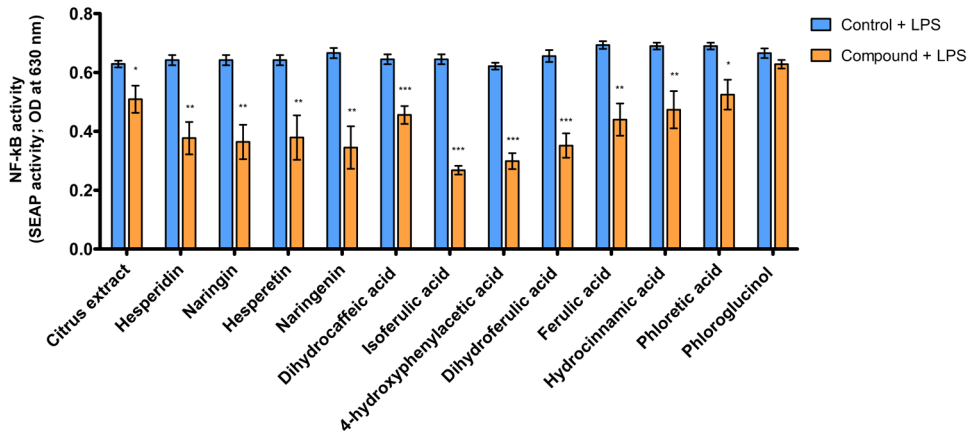

Figure 3.4 The effects of citrus flavonoids, their aglycones and their metabolites on basolateral secretion of NF-κB-inducible SEAP after LPS stimulation by PMA-simulated THP-1 cells in co-culture with Caco-2 cells. At baseline, Caco-2 cells were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either the test compounds (100 μM) or culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 hours of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 hours. Levels of NF-κB-inducible SEAP were measured in basolateral medium after 30 hours of incubation. Values are represented as mean ± SEM from at least 2 independent experiments in duplicate. * represents significant difference from medium + DMSO control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Cytokine production was determined by measuring the levels of the pro-inflammatory cytokines IL-8, TNF-α, IL-6 and IL-1β in the basolateral medium. Stimulation with LPS during the last 6 hours of incubation resulted in significantly increased levels of these cytokines as compared to the control condition (cells treated with culture medium alone) (supplemental Figure s3.5). Production of IL-8 was significantly inhibited by hesperidin,

naringin, hesperetin, naringenin, isoferulic acid, 4-hydroxyphenylacetic acid, ferulic acid and hydrocinnamic acid (Figure 3.5A; all $p \leq 0.02$). TNF-α levels were also significantly inhibited for all the aforementioned compounds and also for dihydroferulic acid (Figure 3.5B; all $p \leq 0.008$). For IL-6, a similar pattern of inhibitory activity of the different test compounds could be observed, which was significant for isoferulic acid, dihydroferulic acid, ferulic acid and hydrocinnamic acid (Figure 3.5C; all $p \leq 0.01$) and showed a trend towards significance for 4-hydroxyphenylacetic acid ($p = 0.05$). Although no IL-6 levels are depicted for hesperidin, naringin, hesperetin and naringenin, as the majority of the values were below the detection limit, the results do support the previous findings indicating that these compounds are able to inhibit the production of pro-inflammatory cytokines. In contrast, levels of IL-1β were increased when compared to the medium + DMSO control, being significant for hesperidin, naringin, hesperetin, naringenin, dihydrocaffeic acid, isoferulic acid, 4-hydroxyphenylacetic acid, dihydroferulic acid, ferulic acid and hyrocinnamic acid (Figure 3.5D; all $p \leq 0.02$).

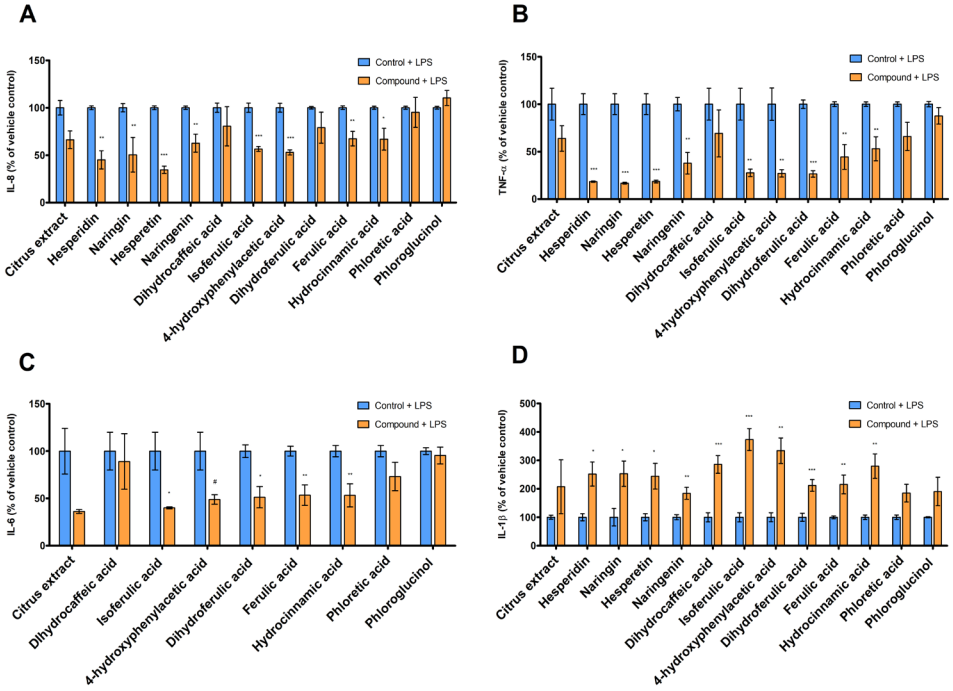

Figure 3.5 The effects of citrus flavonoids, their aglycones and their metabolites on basolateral secretion of IL-8 (A), TNF-α (B), IL-6 (C) and IL-1β (D) after LPS stimulation by PMA-simulated THP-1 cells in co-culture with Caco-2 cells. At baseline, Caco-2 cells were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either the test compounds (100 μM) or culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 hours of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 hours. Cytokine levels were measured in basolateral medium after 30 hours of incubation. Values are represented as mean ± SEM from at least 2 independent experiments in duplicate. * represents significant difference from medium + DMSO control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; #: $p = 0.05$).

Discussion

In this *in vitro* study, we assessed the potential beneficial effects of the citrus flavonoids hesperidin and naringin, their aglycone forms and several of their phenolic metabolites on immune-mediated barrier disruption. Incubation with the citrus flavonoids and their metabolites did not induce changes in TEER, FITC-D4 permeation or expression of barrier related genes for any of the compounds tested. However, LPS-induced NF-κB activity and production of the cytokines IL-8, TNF-α and IL-6 was inhibited by most compounds, whereas levels of IL-1β were found to be increased.

Chronic intestinal inflammation is characterized among others by, an increased production of pro-inflammatory cytokines, which are important mediators that can disrupt the intestinal barrier via modulation of expression and functioning of tight junction proteins.[5] Intestinal barrier disruption, associated with local or systemic inflammation has been observed in several gastrointestinal and metabolic diseases, where it can play a role in the onset and progression of disease.[29] Recently, we found plasma of Crohn's disease patients to induce epithelial barrier disruption in 3D Caco-2 cysts.[7]

In the current study, an *in vitro* co-culture system was used, mimicking immune-mediated barrier disruption by combining the epithelial Caco-2 cell line with activated macrophages.[25] Treatment with citrus flavonoids, their aglycone forms and their phenolic metabolites did not attenuate the decrease in barrier integrity that was observed after 24 hours of co-culture. In contrast to our findings, two previous studies using Caco-2 cells did show positive effects after treatment with the aglycones hesperetin and naringenin, as shown by increased expression of several tight junction proteins and a 5-15% increase in TEER.[17, 18] The aforementioned studies focused on improving barrier function in the absence of a stressor, while the current study was performed in order to assess whether citrus flavonoid treatment can attenuate an inflammation induced disruption of barrier function, as was suggested based on animal studies. Using chemically induced mice models of colitis, it was shown that oral administration with naringenin or hesperetin was able to (partly) suppress dextran sulfate sodium (DSS)-induced barrier impairment, as measured by FITC-D4 and expression of tight junction proteins.[13, 14] In these mice studies, the citrus flavonoid aglycones were given at a dose of 3 g/kg per day. This dose was estimated to correspond to a daily dose of approximately 2 g in humans,[13] while more physiological dosages of approximately 50-500 mg, that are also typically used in human intervention studies, are expected to result in luminal concentrations similar to the concentration of 100 μM that was used in the current study.[12, 17, 22]

Despite the lack of an effect on barrier disruption after treatment with the different test compounds, we wanted to evaluate whether they could have an effect on markers of immune function. All test compounds, except for phloroglucinol, were found to significantly attenuate the NF-kB activity induced by LPS after treatment at a concentration of 100 μM. The LPS-induced secretion of IL-8, TNF-α and IL-6 was also reduced by most of the tested compounds. Overall, these anti-inflammatory effects seemed most pronounced for hesperidin, naringin, hesperetin, naringenin and the phenolics isoferulic acid and 4-hydroxyphenylacetic acid. Previous *in vitro* studies with various experimental setups corroborate our findings by providing evidence that citrus flavonoids and their aglycone forms exert anti-inflammatory effects, partly by inhibiting the activation of the NF-κB signaling pathway.[30-33] Furthermore, although these effects have not been studied extensively for all phenolic metabolites that were tested here, previous studies confirm that several of these compounds possess anti-inflammatory properties.[21, 34-40]

In the present study, however, citrus flavonoid treatment resulted in increased levels of the cytokine IL-1β. These results were consistent among the various test compounds and unexpected, as activated NF-κB induces the transcription of cytokine genes, including IL-1β. Although the increased IL-1β levels in combination with an inhibition of NF-κB activity seem contradictory, a possible explanation for these findings might be an increased pro-IL-1β processing. In a study by Greten *et al*.,[34] LPS treatment of IKKβ-deficient macrophages resulted in increased IL-1β secretion, while the expression of pro-IL-1β was decreased, suggesting that the increased IL-1β levels might be due to an increase in pro-IL-1β processing that was already present in the cell. Another possible explanation may be that because the NF-κB activity was not completely inhibited by the test compounds, in contrast to the reduction in IL-8, TNF-α and IL-6 secretion, some cytokines including IL-1β may still have been upregulated. How our findings relate to the *in vivo* situation is however not clear. For example, a study using a mouse model of colonic inflammation showed reduced IL-1β levels together with a decrease in other inflammation markers such as TNF-α and nuclear NF-kB translocation after oral administration of bergamot juice, although this could also have been the effect of other compounds present in the juice.[41]

As citrus flavonoids are metabolised by the intestinal microbiota, it is also important to take the bioactivity of the large number of different metabolites that are formed and their relative contribution into account. The observed effects on NF-κB activity and cytokine production showed some variation between the different compounds that were tested. Phloroglucinol did not significantly affect NF-κB activity or cytokine production, while the citrus extract, dihydrocaffeic acid and phloretic acid significantly inhibited

NF-κB activity but not the production of all cytokines. Although it is not entirely clear why the effects on cytokine production are not in line with the NF-κB activity results for these compounds, it should be noted that a slight (non-significant) inhibition could be observed in most cases. Overall, the parent compounds, aglycone forms and the phenolic metabolites isoferulic acid and 4-hydroxyphenylacetic acid showed the most potent (anti-)inflammatory effects of which the order of magnitude was similar. This shows that the parent compounds and several of the metabolites can exert comparable effects. It should be noted however that *in vivo*, the exact concentrations in the lumen after oral ingestion of citrus flavonoids will most likely be different for each compound and it is possible that not all compounds are able to reach a luminal concentration of 100 μM. Furthermore, the anti-inflammatory effects found after treatment with a dose of 100 μM citrus flavonoids were not observed at a lower dose of 50 μM.

Although there is some variation in literature about the specific metabolites that are formed during intestinal metabolism of citrus flavonoids and their concentrations, evidence suggests that the highest concentrations of the aglycone forms and smaller metabolites (including isoferulic acid and 4-hydroxyphenylacetic acid) can be found in the more proximal parts of the colon, while the parent compounds are present at the highest concentrations in the small intestine.[42] Based on the results of the current study, it is therefore highly likely that citrus flavonoid ingestion will result in anti-inflammatory effects in the small intestine as well as the proximal colon and that especially in the colon effects *in vivo* will be more pronounced than *in vitro* as they are the result of a combination of the effects of the various compounds present in the intestinal lumen. Furthermore, similar to the *in vivo* situation, the parent compounds are very poorly transported across the epithelium while the aglycones and several phenolics can be converted into phase II metabolites by Caco-2 cells and are also transported to the basolateral compartment.[12, 43-45] As the anti-inflammatory effects of the parent compounds and the most potent metabolites are of the same order of magnitude, this is likely the result of a combination of luminal and basolateral effects. Future research assessing the absorption, metabolism, and transport of these compounds by intestinal epithelial cells may contribute to further insight in the underlying mechanisms.

In the current study, we used a validated co-culture system of Caco-2/THP-1 cells as model for intestinal inflammation and cytokine-induced barrier disruption.[24, 25] THP-1 cells in this model are treated with PMA to induce differentiation into activated macrophages, as was supported by the secretion of inflammatory markers, such as NF-κB-inducible SEAP, IL-8 and IL-1β. At the same time a significant decrease in TEER values of the Caco-2 monolayer was observed, indicating that the barrier was disrupted. In order to further induce an inflammatory environment, the activated macrophages

were stimulated with LPS after 24 hours of co-culture, resulting in an increase in NF-kB activity and (enhanced) secretion of the pro-inflammatory cytokines IL-8, IL-1β, TNF-α and IL-6. Based on data from previous studies[46, 47] it is likely that the inflammatory markers measured basolaterally before and after LPS stimulation were secreted by the activated macrophages, without additional effects of the Caco-2 cells. This increased production of pro-inflammatory mediators was not associated with further disruption of the epithelial barrier, possibly because the effect of some of the cytokines was not concentration dependent and/or the maximal decrease in TEER already occurred as a result of basal cytokine release by the activated macrophages.[48] Treatment with the citrus flavonoids and their metabolites significantly inhibited NF-kB activity and the production of IL-8, TNF-α and IL-6, but this effect did not result in an improved barrier function. For some compounds the effects on LPS-induced and basal cytokine production may not be the same[24, 40] and the citrus flavonoid compounds may therefore have only reduced the release of cytokine secretion stimulated by LPS without affecting basal levels. However, we cannot exclude that the lack of an effect on TEER, FITC-D4 permeation or the expression of barrier related genes after treatment with the test compounds may be due the increased levels of IL-1β found. IL-1β can lead to barrier disruption, as shown by multiple *in vitro* studies using Caco-2 monolayers.[48-50]

The validated co-culture model[24, 25] that was used provides a better reflection of the *in vivo* situation than a mono-culture, as it includes crosstalk between immune cells and epithelial cells. However, many components of the *in vivo* situation are not represented in this model. For instance, this model does not include other immune cells nor the intestinal microbial population, which varies between subjects and is affected by *e.g*. disease status, medication use and dietary intake.[51] The microbiota composition in particular might be important, as we know that bacteria can convert the citrus flavonoids into bioactive metabolites[9-11] and emerging evidence also shows that citrus flavonoids may induce alterations in the microbiota composition and activity.[52, 53] Therefore, the current *in vitro* findings warrant further research and validation in human subjects.

In conclusion, we have demonstrated that treatment with citrus flavonoid compounds did result in a significant inhibition of NF-κB activity and production of several inflammatory cytokines for both the citrus flavonoids and several of their metabolites, but did not protect against an immune-mediated barrier disruption. Overall, these results suggest that citrus flavonoids may decrease intestinal inflammation via reduction of NF-κB activity and that the parent compounds and their metabolites formed during intestinal metabolism are able to exert comparable effects.

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Supplemental material

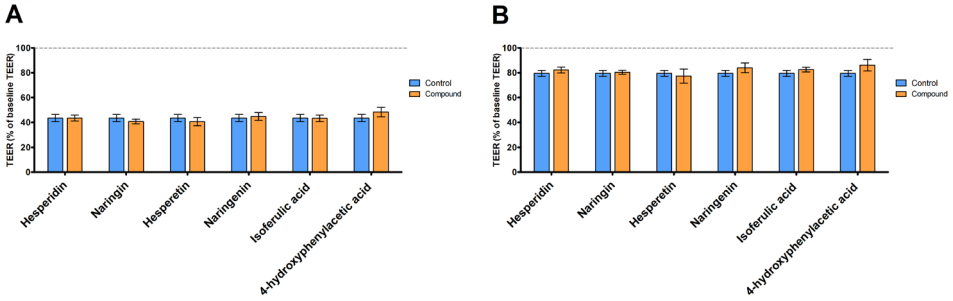

Figure s3.1 The effects of citrus flavonoids, their aglycones and their metabolites on transepithelial electrical resistance (TEER) of Caco-2 monolayers after 24 hours (A) and after 30 hours (B) of co-culture with PMA-simulated THP-1 cells. At baseline, Caco-2 cells cultured in Transwell inserts for 14 days were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either the test compounds (50 μM) or culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 hours of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 hours. TEER was measured at baseline, after 24 hours and after 30 hours of incubation. Values are represented as mean percentage from the baseline value ± SEM. No significant differences were observed between the test compounds and the medium + DMSO control.

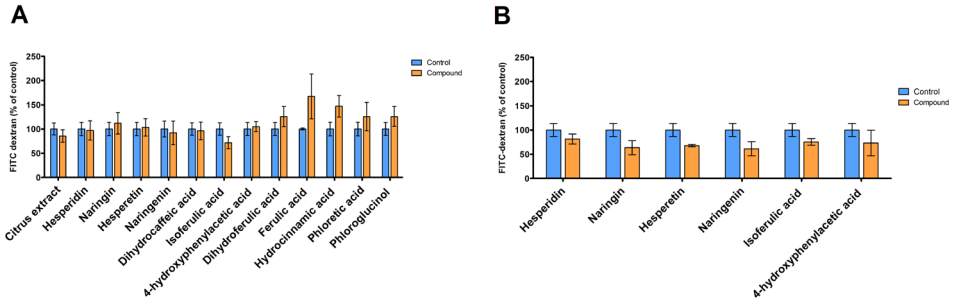

Figure s3.2 The effects of citrus flavonoids, their aglycones and their metabolites on fluorescein isothiocyanate–dextran 4kDa (FITC-D4) permeation of Caco-2 monolayers in co-culture with PMA-simulated THP-1 cells at concentrations of 100 μM (A) or 50 μM (B). Caco-2 cells cultured in Transwell inserts for 14 days were placed in co-culture with PMA-simulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either culture medium containing 0.1% DMSO (medium + DMSO control) or test compounds (100 μM or 50 μM) added to the apical compartment. FITC-D4 was added apically to the Caco-2 cells after 30 hours of incubation. FITC-D4 concentrations were measured in the basolateral compartment after 1 hour of incubation. Values are represented as mean ± SEM. No significant differences were observed between the test compounds and the medium + DMSO control.

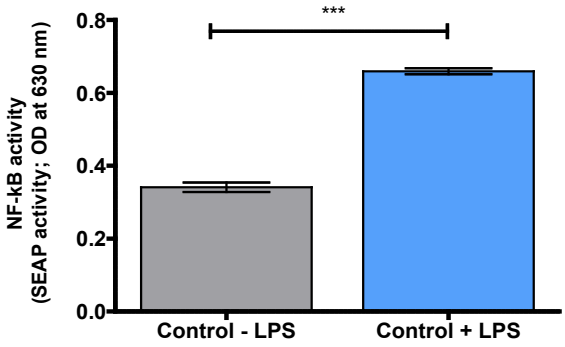

Figure s3.3 Basolateral secretion of NF-κB-inducible SEAP by PMA-simulated THP-1 cells in co-culture with Caco-2 cells alone and after stimulation with LPS. At baseline, Caco-2 cells were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 hours of incubation, THP-1 cells were incubated with culture medium containing 500 ng/ml LPS (control + LPS) or culture medium alone (control – LPS) in the basolateral compartment for an additional 6 hours. Levels of NF-κB-inducible SEAP were measured in basolateral medium after 30 hours of incubation. Values are represented as mean ± SEM. * represents significant difference (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

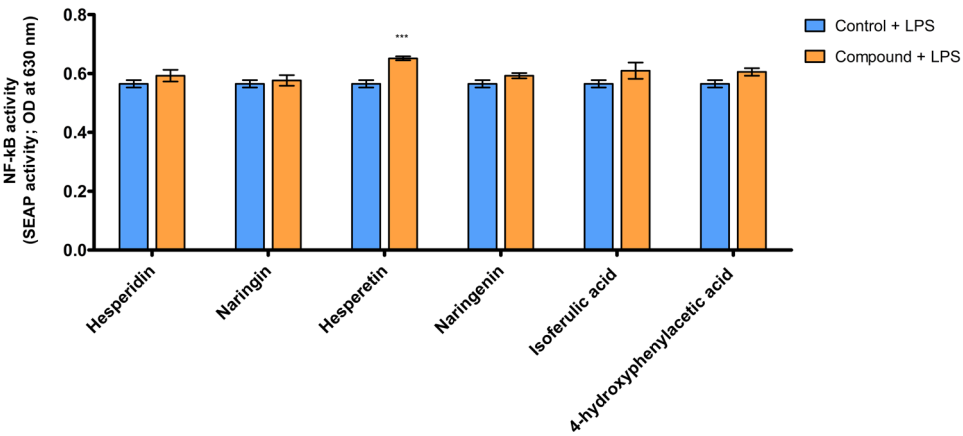

Figure s3.4 The effects of citrus flavonoids, their aglycones and their metabolites on basolateral secretion of NF-κB-inducible SEAP after LPS stimulation by PMA-simulated THP-1 cells in co-culture with Caco-2 cells. At baseline, Caco-2 cells were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either the test compounds (50 μM) or culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 hours of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 hours. Levels of NF-κB-inducible SEAP were measured in basolateral medium after 30 hours of incubation. Values are represented as mean ± SEM. * represents significant difference from medium + DMSO control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

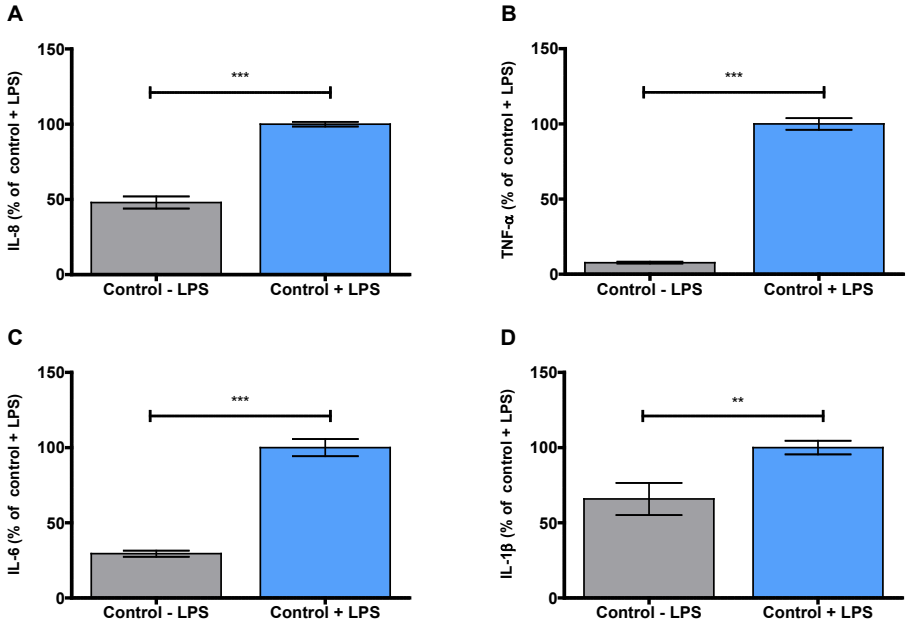

Figure s3.5 Basolateral secretion of IL-8 (A), TNF-α (B), IL-6 (C) and IL-1β (D) by PMA-simulated THP-1 cells in co-culture with Caco-2 cells alone and after stimulation with LPS. At baseline, Caco-2 cells were place in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 hours of incubation, THP-1 cells were incubated with culture medium containing 500 ng/ml LPS (control + LPS) or culture medium alone (control – LPS) in the basolateral compartment for an additional 6 hours. Cytokine levels were measured in basolateral medium after 30 hours of incubation. Values are represented as mean ± SEM. * represents significant difference (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Table s3.1 Sequences of the primers used for qPCR analysis

Gene	Forward	Reverse
18S RNA	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG
ZO-1	AGG GGC AGT GGT GGT TTT CTG TTC TTT C	GCA GAG GTC AAA GTT CAA GGC TCA AGA GG
Occludin	TCA GGG AAT ATC CAC CTA TCA CTT CAG	CAT CAG CAG CAG CCA TGT ACT CTT CAC
Claudin-2	AAC TAC TAC GAT GCC TAC C	GAA CTC ACT CTT GAC TTT GG
Claudin-3	TTC ATC GGC AGC AAC ATC ATC	CGC CTG AAG GTC CTG TGG
Claudin-4	ACA GAC AAG CCT TAC TCC	GGA AGA ACA AAG CAG AGA G
E-Cadherin	CAC CTG GAG AGA GGC CGC GT	AAC GGA GGC CTG ATG GGG CG
MLCK	GCC TGA CCA CGA ATA TAA GTT	GCT CC TTC TCA TCA TCA TCT G

18S RNA: 18S ribosomal RNA; ZO-1: Zonula Occludens-1; MLCK: myosin light chain kinase.

Chapter 4

The effect of citrus extract on intestinal inflammation: an explorative, randomised clinical trial in patients with Irritable Bowel Syndrome

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In preparation

Chapter 5

The effect of citrus extract administration on markers of oxidative stress and immune function in elderly subjects

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In preparation

Part II

Chapter 6

Effect of a carotenoid-producing *Bacillus* strain on intestinal barrier integrity and systemic delivery of carotenoids: a randomised trial in animals and humans

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Abstract

The aim of the present study was to investigate effects of the carotenoid-producing *Bacillus indicus* strain PD01 on intestinal barrier function and its ability to survive passage through the gastrointestinal tract and to assess systemic bioavailability of these carotenoids *in vivo*. As model for impaired barrier function, 16 early weaned piglets were randomly assigned to a control diet or control diet with PD01 for 23 days. In addition, 67 overweight/obese, otherwise healthy individuals were randomly assigned to groups receiving PD01 or placebo for six weeks. PD01 survived passage through the gastrointestinal tract in piglets and human subjects and resulted in significant accumulation of PD01 derived carotenoids (methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene) in human plasma after 3- and 6-weeks supplementation versus baseline (0.044 and 0.076 vs 0 μM, respectively; $p < 0.001$). PD01 supplementation resulted in higher expression levels of occludin in the distal small intestine (1.38 ± 0.31 vs 0.59 ± 0.14; $p = 0.044$) and transepithelial electrical resistance in the mid colon (34.1 ± 3.01 vs 24.3 ± 1.13 $\Omega.cm^2$; $p = 0.019$) of early weaned piglets compared to control. In overweight/obese individuals with preserved barrier integrity, PD01 did not affect sugar excretion ($p \geq 0.104$). In summary, PD01 survived transit through the gastrointestinal tract, resulted in systemic carotenoid accumulation and improved compromised barrier function outcomes.

Introduction

The intestinal epithelial barrier plays an important role in the maintenance of intestinal homeostasis and in overall human health. It acts as a selective barrier allowing the absorption of substances such as water and essential nutrients, while preventing the translocation of luminal content and pathogens. In general, the barrier consists of a mucus layer, a single layer of epithelial cells sealed by junctional complexes and underlying lamina propria. In addition to these components, other factors such as the mucosa-associated lymphoid tissue and the intestinal microbiota play a key role in maintaining the integrity and function of the intestinal barrier.[1, 2] Defects in intestinal barrier functioning have been associated with the development and progression of various gastrointestinal (GI) and metabolic diseases, including inflammatory bowel disease (IBD) and diabetes.[1, 2] Therefore, treatments targeted at reducing intestinal permeability or preventing intestinal barrier dysfunction, could contribute to improve (GI) health. In this context, nutritional interventions, for instance with probiotic bacteria, have been proposed. Probiotics may improve intestinal barrier function by stimulating the immune system, strengthening the physical barrier and by modulating the microbiota composition in favour of lactic acid bacteria.[3, 4] Globally, lactic acid bacteria and *Bifidobacterium spp.* are the most consumed bacteria included in probiotic formulations. However, species belonging to other genera such as *Enterococcus*, *Streptococcus*, *Bacillus*, along with *Saccharomyces spp.*, have also gained interest over the past years. Although not all *Bacillus* strains are considered safe for human consumption,[5] probiotic properties including the prevention of pathogen colonization and a reduction of intestinal permeability have been shown for *Bacillus spp.*.[5, 6] Within this genus, the species *Bacillus indicus* has an extra attribute which is of interest to intestinal health. It has the ability to produce carotenoids which are lipophilic isoprenoid compounds with well-known antioxidant, anti-apoptotic, and anti-inflammatory properties.[7] Furthermore, positive effects on microbiota composition have also been shown for carotenoids.[8, 9] Therefore, in addition to the strain itself, the carotenoids that are produced may also play a role in improving barrier dysfunction via their antioxidant and anti-inflammatory effects and their ability to modulate the microbiota composition. Various animal studies have indeed shown beneficial effects of carotenoids on intestinal barrier function such as an increased expression of tight junction proteins and a reduction of intestinal epithelial damage.[10-13]

The *Bacillus indicus* strain PD01 is a spore-forming bacterium originally isolated from human faeces from a healthy volunteer as HU19,[14] which fails to grow anaerobically. The bacterium naturally produces high levels of two glycosyl-apo-lycopene carotenoids (methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene) that do not degrade

during passage through the stomach and are equally or even more bioaccessible and bioavailable than dietary carotenoids currently on the market (unpublished data from ProDigest, Ghent, Belgium). In addition to the potential probiotic properties of PD01 itself, the carotenoids released in the intestine may also result in local and systemic effects. Until now, data confirming these properties *in vivo* are lacking. Therefore, the aim of the present study is to investigate the effects of PD01 on intestinal barrier function *in vivo*. We chose to study this effect in early weaned piglets and in healthy yet overweight or obese subjects, as both early weaning stress and overweight have been associated with GI alterations, including a potential reduction in intestinal barrier integrity.[15-17] Additionally, we evaluated whether PD01 is able to survive transit through the GI tract in both piglets and humans, and whether PD01 is able to release systemically absorbable carotenoids that can be detected in human plasma.

Methods

Animal study

The animal study was conducted in accordance with the ethical standards and recommendations for accommodation and care of laboratory animals covered by the European Directive 2010/63/EU on the protection of animals used for scientific purposes and the Belgian royal decree KB29.05.13 on the use of animals for experimental studies.

Animals and housing

Sixteen healthy male and female piglets (Topics hybrid x Piétrain) were weaned at the age of 19 days (approximate average weight 6.5 kg), origin Stefaan Debaerdemaeker, Evergem, Belgium. The piglets originated from four litters with four piglets/litter and were assigned to the pens according to litter of origin, sex and body weight (BW). Besides these criteria, the animals were allocated randomly to either one of the two treatments: basal control diet (CD) or basal control diet supplemented with *Bacillus indicus* strain PD01 at a concentration of $2x10^9$ spores/kg of diet (CD+PD01). The treatments lasted for a total of 23 days and were replicated in two pens of four piglets per pen (n = 8 animals per treatment). Piglets were housed at the Laboratory for Animal Nutrition and Animal Product Quality of the Faculty of Bioscience Engineering from Ghent University (Ghent, Belgium). Four piglets were housed per pen (2.10 m^2/pen) with full slatted floors, conventional ventilation scheme, starting ambient temperature at 30°C and a 24L schedule till day (d)5 post-weaning. From d6 till d23, ambient temperature was linearly adjusted to 28°C with 18L:6D light schedule. No medications in feed or water were used. Health was recorded on a daily and individual basis. No animals or data points were excluded during the study because of signs of disease or animal deaths.

Experimental diets and study product

The basal control diet was formulated to meet or exceed the piglets' requirements (adapted from Centraal Veevoeder Bureau, 1997) and was aimed to represent a human Western diet in terms of nutrients.[18] The diets were semi-synthetic, including minerals, and vitamins, but excluding supplementary organic acids, and Cu and Zn beyond animal requirements. The ingredient composition and calculated nutrient composition of the basal control diet is provided in Table s6.1. Feeds were prepared as mash. One batch was made and then split up to make the two experimental diets (CD and CD+PD01). *Bacillus indicus* strain PD01 (MRM Health, Ghent, Belgium) was grown and lyophilized on a maltodextrin (Pineflow; Matsutani Chemical Industry, Hyogo, Japan) carrier with 89% spores per vegetative cells. Strain PD01, deposited at BCCM/LMG as *Bacillus indicus* PD01, was originally isolated and characterised by Duc *et al.*[14] at Royal Holloway University of London (UK) from human faeces from a healthy volunteer (*Bacillus indicus* HU19 - NCIMB 41359). For the CD+PD01 diet, strain PD01 was added ($2x10^9$ spores/kg of diet) on top by premixing in a limited amount of basal diet, prior to complete mixing. All diets were fed *ad libitum*. No fasting period before sampling or sacrifice was included. Water was available *ad libitum* at all time.

Animal follow-up

The BW (kg) of the piglets was followed regularly: d0, d5, d14 and at the end of the trial (d23). For each period (d0-5, d5-14, d14-end and total period, d0-end) growth (g/d), feed intake (g/d) and feed to weight gain ratio (g/g) was recorded. All piglets were inspected two times a day for general health and presence of diarrhoea during the experimental period. The system used to score faecal consistency is provided in Table s6.2. To assess whether PD01 was detectable in the GI tract of the piglets, a fresh non-contaminated faecal sample was collected from the rectum of each piglet at d14 and d23. In addition, samples from the mid colon were also collected post-mortem at d23 for the same purpose .

Quantification of Bacillus indicus PD01

The samples collected at d14 and d23 from the rectum and mid colon were weighted, and 0.5 g of sample was dissolved in sterile PBS. Serial dilutions of this faecal slurry (10^{-1} to 10^{-6}) were plated in Luria Broth (LB) agar plates for colony counting (total cell count) upon incubation at 37°C for 24 h. Because the PD01 strain of *Bacillus indicus* produces carotenoids, the colonies are easily identified by their bright yellow-to-orange appearance. For spore counting, the same samples were pasteurised at 65°C for 30 minutes. Only spore-forming bacteria are able to survive this treatment. Pasteurized

samples were then plated in the same manner and colonies counted. Results are provided as colony forming units (CFU)/g of faecal sample.

Collection of samples post-mortem

All piglets were sacrificed on d23 postweaning. Just before euthanasia, the weight of the piglets was registered. Piglets were euthanized by intra-peritoneal pentobarbital (90 mg/kg BW), followed by exsanguination. After piglets were killed, the abdomen was immediately opened to collect several intestinal sections: 50% and 90% of small intestinal length and of the mid colon. Samples were either used for histomorphology, RNA extraction and quantitative real-time polymerase chain reaction (qPCR) or for Ussing chambers.

Histomorphology of intestinal segments

Five cm segments of the 50% and 90% of small intestinal length were collected for histomorphology measurements (segments were flushed with saline and immersed in formalin). In brief, after fixation in neutral-buffered formalin, intestinal tissue samples were processed under standard conditions in an automatic tissue processor, embedded in paraffin wax and subsequently 5 µm slides were stained with haematoxylin-eosin.[19] Villus length (from tip to base) and crypt depth (from base to opening) of all well-oriented villi and adjacent crypts were measured using a microscope equipped with a camera and computer with appropriate software (Olympus BX61 microscope and image analysis software, analySIS Pro, Olympus, Aartselaar, Belgium). Villus:crypt ratio was calculated as the mean value of the ratios of the obtained villus heights and adjacent crypt depths.

Intestinal permeability and ion transport analysis using Ussing chambers

Twenty cm segments of the 90% of small intestinal length and mid colon were collected for Ussing chamber measurements. Segments were flushed with saline, then cut along the mesenteric border, stripped of the muscle layers and mounted in modified Ussing Chambers (Andreas Mund Scientific Instruments, Simmerath, Germany) as flat sheets on a segment holder, with an exposed tissue area of 1.07 cm^2.[20] Immediately after mounting the tissues in the chambers, both half-chambers were filled with the Ringer's buffer solution (pH 7.4) containing (in mmol/L): 115 NaCl, 25 $NaHCO_3$, 0.4 $NaH_2PO_4.H_2O$, 2.4 $Na_2HPO_4 \cdot 2H_2O$, 5 KCl, 1.2 $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ and 12 D-glucose, however at the mucosal side of the epithelium the 12 mmol/L D-glucose in buffer was replaced by an equimolar amount of mannitol. Paracellular intestinal permeability of the tissue was assessed by the apparent permeability for FITC-dextran, 4 kDa (Sigma-Aldrich, Overijse, Belgium) in two chambers (P_{app} FD-4). Parameters of intestinal ion transport and tissue

integrity in another two chambers. Two sets of Ag/AgCl electrodes were connected to the half-chambers by 3 mmol/L KCl-agar bridges. One pair was used to record the potential differences between the half-chambers, while an external current ran through the other pair. After correction for solution resistance, the trans-epithelial potential difference was clamped to 0 mV by applying an external short-circuit current (Isc). Ion transport was evaluated by measuring the baseline Isc, and chloride- (Cl^-) stimulated secretion by the agonists carbachol (10 μM serosal-side; a Ca^{2+}-mediated secretagogue; $\Delta Isc_{carbachol}$) and theophylline (5 mM, bilateral; a cAMP/cGMP mediated secretagogue; $\Delta Isc_{theophylline}$).[20] Barrier function was determined by measuring the transepithelial electrical resistance (TEER).

RNA extraction and qRT-PCR of tight junction protein genes

Ten cm segments of the 50% and 90% of small intestinal length, and mid colon, were collected for real-time quantitative reverse-transcription (qRT)-PCR of two tight junction (TJ) protein genes: TJ protein 1 (*Tjp1*), encoding Zonula occludens 1 (ZO-1), and occludin (*Ocln*). For that, segments were flushed and mucosa was scraped, collected and stored in RNA*later®* (Sigma-Aldrich) at -80°C until further RNA isolation. Total RNA was extracted by using the RNeasy Plus Mini Kit from Qiagen (Germantown, USA) and complementary (c)DNA was synthetized by using the High-Capacity cDNA Reverse Transcription kit from Thermo Fisher Scientific (Dreieich, Germany) according to the manufacturer's instruction (0.5 μg of RNA was used for synthesis). All qPCRs were performed by using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA), the SensiMix SYBR Hi-ROX Kit from Bioline (London, UK) and by using 12.5 ng of template per reaction together with 250 nM of each primer (BioLegio, Nijmegen, the Netherlands) (all samples were tested in triplicate). Primer nucleotide sequences were as follows: *Tjp1*-Fw: 5'-ATCTCGGAAAAGTGCCAGGA-3'; *Tjp1*-Rev: 5'-CCCCTCAGAAACCCATACCA-3'; *Ocln*-Fw: 5'- CATGGCTGCCTTCTGCTTCATTGC-3'; *Ocln*-Rev: 5'-ACCATCACACCCAGGATAGCACTCA-3'. The hydroxymethylbilane synthase (*HMBS*) gene was used as internal reference for normalization (*HMBS*-Fw: 5'-AGGATGGGCAACTCTACCTG-3'; *HMBS*-Rev: 5'-GATGGTGGCCTGCATAGTCT-3').[21] Relative quantification (RQ) was performed according to the comparative $2^{-\Delta\Delta Ct}$ method[22] (for that a reference sample from the control diet group was randomly chosen for normalization) using the StepOne Software v2.3 (Applied Biosystems).

Clinical study

The clinical study was part of a larger project in which the effect of *Bacillus indicus* strain PD01 on cardiovascular health and microbial environment was investigated.[23] The study was approved by the Medical Ethics Committee of the Maastricht University

Medical Centre + (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 body mass index (BMI) between 25 and 35 kg/m^2 were recruited by advertisement. Participants were excluded from the study when meeting one or more of the following exclusion criteria: 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 the general Dutch population); abuse of alcohol (> 20 alcoholic units / week) and drugs; absence of a stable BW 3 months prior to the study (± 3 kg); plans to lose weight or to follow a hypocaloric diet during the study period; use of medication, vitamin, mineral or antioxidant supplements; consumption of pro-, pre- or synbiotics during study period and in the 30 days prior to start of the study; use of antibiotics 90 days prior to the study; pregnancy and lactation; history of any side effects towards the intake of pro-, pre-, synbiotic supplements or carotenoids. To assess the fruit and vegetable intake of the participants, a 3-day food record was completed prior to start of the study (during screening). During the study, the subjects were instructed to maintain their habitual diet. The sample size calculation was determined for the primary outcome of the original research protocol, *i.e.* lipid peroxidation, which is not included in this manuscript. Based on previous research,[24] it was calculated that a sample size of at least 60 subjects (*i.e.* 30 participants per intervention group) would be required.

Study products

Bacillus indicus strain PD01 (MRM Health, Ghent, Belgium) was grown and lyophilized on a maltodextrin (Pineflow; Matsutani Chemical Industry, Hyogo, Japan) carrier with 89% spores per vegetative cells. The study products were provided as a powder in sachets. The PD01 group received per day one sachet containing each 5x10^9 CFU PD01 with maltodextrin (3 g) as carrier material. The amount of PD01 carotenoids present inside the spores of the clinical batch was determined after lipophilic extraction by spectrophotometry at 455 nm in dichloromethane (ε = 165 000 M^{-1}.cm^{-1} and MW = 750 g.mol^{-1}). This amount was quantified as 2.81 µg per 10^9 CFU, resulting in a total of 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 a total of six weeks.

Design and intervention

This study was designed as a randomised, placebo-controlled, double-blind, parallel-group study. Each subject underwent three test days. Participants were randomly assigned to one of the two intervention arms: PD01 or placebo (maltodextrin). An independent and blinded person generated the randomization list, using a computerized procedure (http://randomizer.org). 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. After an overnight fast, subjects handed in a faecal sample on the first test day. Then, anthropometric measurements (height, BW, waist-to-hip circumference) 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 measurement. Finally, the subjects 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 six weeks. After three weeks of daily supplementation, the second test day was organised. 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 three weeks of intervention. After six weeks of daily administration of the study product, the third test day was organised, which was identical to the first test day. To assess compliance, participants were asked to collect the empty sachets and to return these at the last visit.

Quantification of Bacillus indicus PD01

Prior to analysis, faecal samples were thawed for 20 minutes and homogenized in sterile distilled water. Homogenized faecal samples were serially diluted in PBS buffer and total cell count and spore count were determined according to the methods described above.

Bioavailability of bacterial carotenoids in plasma

After 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 until further analysis. Carotenoids were extracted by the

Bligh and Dyer method.[25] Then, an enzymatic step, according to Breithaupt, was carried out to hydrolyse esterified carotenoids.[26] Carotenoids were quantified by reverse-phase HPLC as described by Gleize *et al*.[27] using an HP1100 Agilent system equipped with a 150 x 4.6 mm i.d. C30 column (YMC, Kyoto, Japan) set at 35°C. Mobile phase consisted of a gradient of methanol (A), methyl *tert*-butyl ether (B) and water (C) and the flow rate was 1 mL/min. The gradient profile of the mobile phase (A:B:C) was set at 96:2:2 and changed linearly to 18:80:2 in 27 min, and then the mobile phase was changed back to 96:2:2 from 31 to 35 min. The carotenoids were identified at 460 nm based on retention time and UV-Visible spectra (for PD01 carotenoids spectra, see Perez-Fons *et al.*[28]). The absorption spectra of each standard were measured between 300 and 550 nm in the mobile phase to cross-check the identification of sample molecules. Quantification was performed comparing peak area with standard calibration curves. Lutein, β-carotene and lycopene standards were purchased from Carotenature (Münsingen, Switzerland) and PD01 carotenoids standard was purified from PD01 spores as detailed by Sy *et al*..[29]

Gastrointestinal permeability

GI permeability was assessed by using a validated multi-sugar test,[30, 31] measuring the urinary excretion of ingested sugar probes reflecting permeability of four segments of the GI tract. The method of measurement has been described previously by Mujagic *et al*..[32] Urinary sugar probes were measured by HPLC-MS as previously described.[30, 31]

Gastrointestinal 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, diarrhoea, and constipation.[33] 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 by using the Bristol Stool Form Scale Chart.[34]

Statistical analyses

Normality of the data was evaluated by using the Shapiro-Wilk test and statistical tests were applied accordingly. For the animal study, statistically significant differences between the control and test diet were assessed by unpaired, two-tailed Student's *t*-tests for normally distributed data and Mann-Whitney U tests for data that were not normally distributed. Differences between two time points were assessed by two-tailed paired Student's *t*-tests.

For the clinical study, baseline differences between intervention groups were tested using unpaired, two-tailed Student's t-tests, Mann-Whitney U tests or Chi-square tests when appropriate. To compare the presence of PD01 in faeces and bacterial carotenoids in plasma between baseline samples and samples collected after three and six weeks of supplementation, per protocol analyses were performed based on all participants that actually consumed the study product, by using the Friedman test with post-hoc Wilcoxon Singed Rank test. For GI permeability, GI tolerance, stool consistency and stool frequency per protocol analyses were performed based on all participants that completed the study protocol. For these outcomes, differences between intervention groups (PD01 or placebo) were assessed using linear mixed models with intervention group (placebo and PD01), time (baseline, three weeks and six weeks) and intervention 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 and can be used for data showing limited skewedness providing a sufficient sample size. Final conclusions were confirmed in a sensitivity analysis using log transformed data.

All statistical analyses were performed using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY, USA). Data analysed by parametric tests are presented as mean ± SEM or medians with interquartile ranges for data analysed by non-parametric tests. Two-sided p-values ≤ 0.05 are considered significant. Correction for multiple testing was performed by Bonferroni correction based on a correction for multiple time points.

Results

Animal study

Faecal quantification of Bacillus indicus PD01

Faecal samples from the rectum and mid colon of early weaned piglets were collected at day 14 (only rectum) and day 23 (rectum and mid colon). The number of total bacterial cells and spores detected in the rectum was high and did not increase significantly from day 14 to day 23 (Table 6.1). In the faeces collected post-mortem from the mid colon, the number of total cells and spores was also high ($1.83x10^6$ and $1.38x10^6$ CFU/g faeces, respectively). This indicates that PD01 was present in a viable form in the gut of the animals that received PD01.

Table 6.1 PD01 total cells and spores (CFU/g) in the gastrointestinal tract of piglets at day 14 and at the end of the study in the CD+PD01 group

	Faeces (rectum)			**Faeces (mid-colon)**
	Day 14 (n=6)	Day 23 (n=8)	P-value	Day 23 (n=8)
Total cells	1.52E+06 ± 3.08E+05	2.62E+06 ± 6.46E+05	0.218	1.83E+06 ± 8.29E+05
Spores	1.45E+06 ± 4.24E+05	1.86E+06 ± 3.64E+05	0.541	1.38E+06 ± 2.36E+05
Percentage of total	96%	71%		75%
Vegetative cells	6.33E+04 ± 2.85E+05	7.63E+05 ± 3.90E+05	0.331	4.48E+05 ± 7.08E+05
Percentage of total	4%	29%		25%

Total cell count was performed by plating serial dilutions of collected samples in Luria Broth agar plates. Spore count was performed in the same manner and on the same samples after pasteurization at 65°C. Vegetative cells are calculated as the difference between total cell count and spore count. Average CFU/g of faecal sample are presented, and percentage from total cells were calculated for both spores and vegetative cells. All values are presented as mean ± SEM. Differences between day 14 and day 23 were tested with a paired student's t-test. CFU: colony forming unit; CD: basal control diet.

Intestinal barrier function

PD01 supplementation did improve barrier function in early weaned piglets (Table 6.2). Both in the distal small intestine and mid colon, PD01 supplementation resulted in higher TEER values ($p = 0.070$ and $p = 0.019$ at 90% of small intestinal length and mid colon, respectively), although this effect was only significant in the mid colon. To investigate whether the positive effects observed could be attributed to changes in the expression of TJ proteins, we have measured the expression of *Tjp1* and *Ocln* in the small intestine and mid colon of the piglets. A significantly higher *Ocln* gene expression in the distal small intestine was observed for the group fed PD01 when compared to the control diet (Figure 6.1).

Table 6.2 Effect of control (CD) and test diets (CD+PD01) on intestinal permeability and electrophysiological parameters as determined in the distal small intestine and mid colon sections of piglets by using Ussing chambers

		CD	CD+PD01	*P*-value
	SI length (m)	9.88 ± 0.35	10.5 ± 0.37	0.244
90% of small intestinal length	P_{app} FD-4 (10^{-7} cm/s)	13.4 ± 2.62	9.89 ± 1.91	0.303
	TEER ($\Omega.cm^2$)	36.4 ± 2.47	46.2 ± 4.30	0.070
	$\Delta Isc_{carbachol}$ ($\mu A/cm^2$)	34.8 ± 11.1	35.8 ± 10.0	0.951
	$\Delta Isc_{theophylline}$ ($\mu A/cm^2$)	30.6 ± 7.38	17.4 ± 2.28	0.100
Mid colon	P_{app} FD-4 (10^{-7} cm/s)	23.6 ± 3.58	16.9 ± 3.37	0.194
	TEER ($\Omega.cm^2$)	24.3 ± 1.13	34.1 ± 3.01	0.019
	$\Delta Isc_{carbachol}$ ($\mu A/cm^2$)	63.2 ± 9.54	71.9 ± 9.78	0.538
	$\Delta Isc_{theophylline}$ ($\mu A/cm^2$)	53.1 ± 9.18	46.1 ± 6.12	0.533

All values are presented as mean ± SEM (n=8/group/segment). Differences between control and test diet were tested with an unpaired student's *t*-test. CD: basal control diet; SI: small intestine (90% length); P_{app} FD-4: apparent permeability for FITC-Dextran 4 kDa; TEER: transepithelial electrical resistance of tissue; $\Delta Isc_{carbachol}$ and $\Delta Isc_{theophylline}$: changes in short-circuit current upon stimulation with chloride secretagogues carbachol and theophylline, respectively.

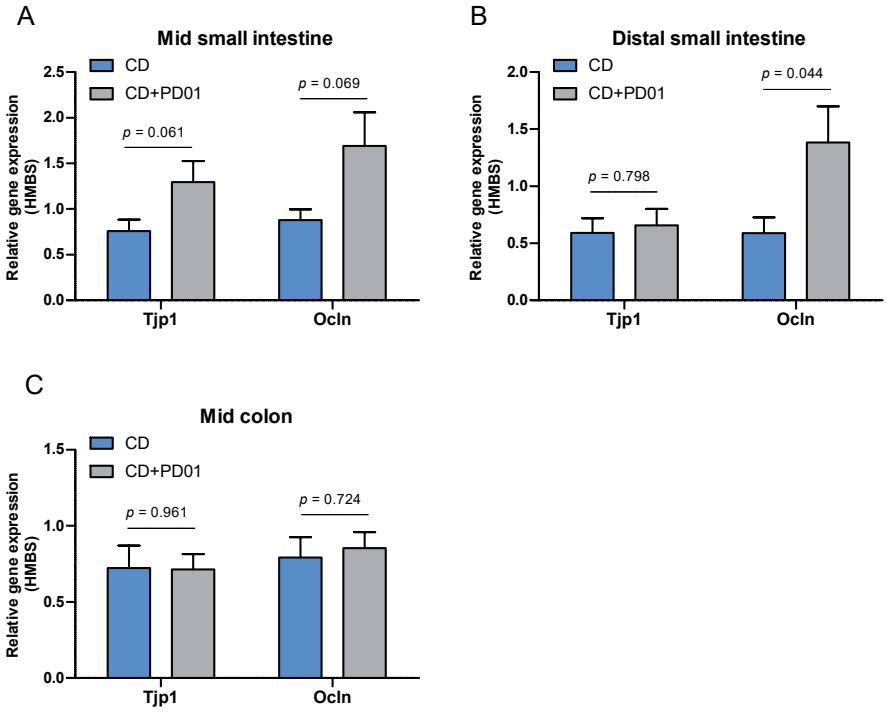

Figure 6.1 Changes in tight junction protein gene expression in the (A) mid small intestine, (B) distal small intestine, and (C) mid colon of piglets upon supplementation of diet with PD01. Means and standard errors are shown (n=8 animals/group/segment). Mid and distal small intestine correspond to the 50% and 90% small intestinal length tissues, respectively. The expression of tight junction protein 1 (Tjp1) and occludin (Ocln) were normalized to hydroxymethylbilane synthase (HMBS). Differences between control and test diet were tested with an unpaired student's *t*-test. For 90% SI length, *TJP1* expression was tested with a Mann-Whitney U test.

Gastrointestinal tolerance

Although statistical analyses could not be performed on indices per pen, the average pen data indicate that supplementation of the piglet's diet with *Bacillus indicus* PD01 did not have major impact on animal performance in terms of total BW, daily growth or feed intake (Table s6.3). The mean faecal score was also similar between the two diets. No diarrhoea was recorded for any of the groups. Also no differences were observed in histomorphology in terms of crypt depth and villus height, both at 50% and 90% small intestinal length (Table 6.3; Figure s6.1).

Table 6.3 Effect of control (CD) and test diets (CD+PD01) on histomorphology of small intestinal mucosa

		CD	CD+PD01	*P*-value
50% SI	Crypt depth (µm)	143 ± 2.51	145 ± 4.59	0.710
	Villus height (µm)	358 ± 20.1	331 ± 27.3	0.435
	Villus:crypt ratio	2.56 ± 0.13	2.31 ± 0.13	0.191
90% SI	Crypt depth (µm)	139 ± 7.02	136 ± 4.92	0.764
	Villus height (µm)	305 ± 18.1	328 ± 26.5	0.495
	Villus:crypt ratio	2.25 ± 0.12	2.44 ± 0.13	0.205

Data are presented as mean ± SEM (n=8/group/segment). Differences between control and test diet were tested with an unpaired student's *t*-test. For 90% SI length, the villus:crypt ratio was tested with a Mann-Whitney U test. CD: basal control diet; SI: small intestine (50% and 90% length).

Clinical study

Study subjects

For the clinical trial, 67 healthy overweight or obese volunteers were enrolled of which 62 completed the entire study protocol (Figure 6.2). One randomised participant did not start the study for a personal reason. Four participants dropped out during the intervention period: one due to the need for antibiotic treatment being unrelated to the current study, another for unspecified, private reasons, and the two remaining participants experienced mild GI complaints. These complaints were present already at the start of the study and were not associated to PD01 intake. From these participants only baseline characteristics were available. Baseline characteristics are presented in Table 6.4. From the total of 67 randomised subjects, 33 participants in the placebo group and 29 participants in the PD01 group completed the study protocol.

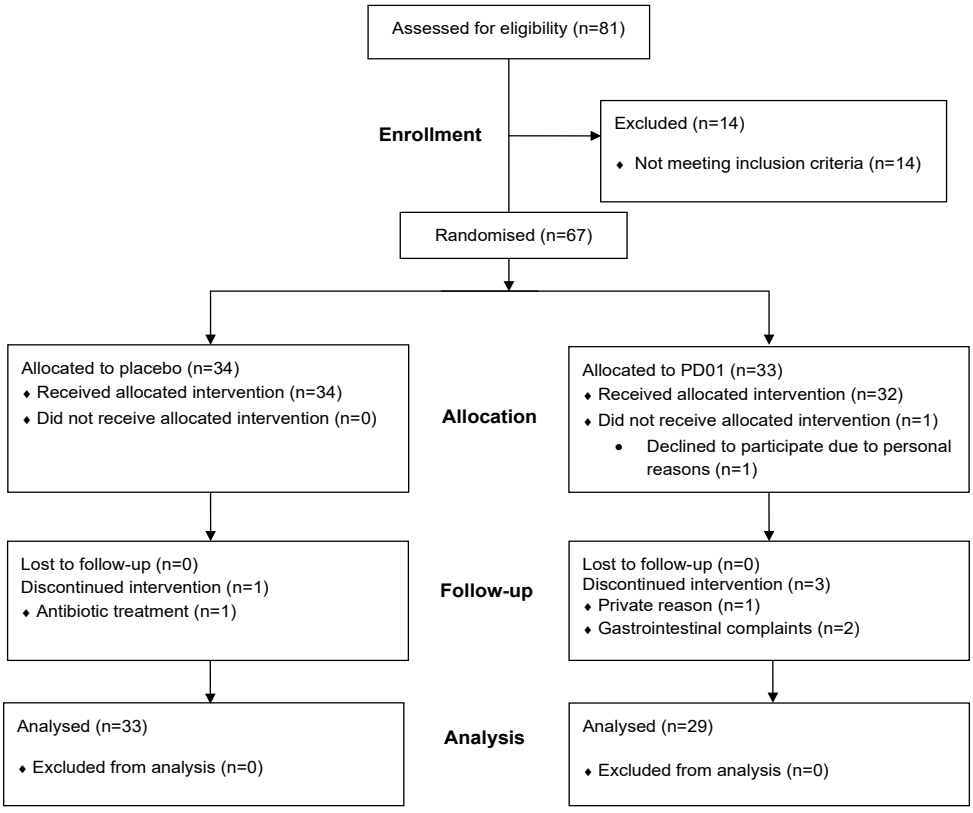

Figure 6.2 CONSORT flow diagram

Table 6.4 Baseline characteristics of the clinical study participants

	Total population (n=67)	**Placebo (n=34)**	**PD01 (n=33)**	***P*-value**
Age, years	53.0 [46.0; 64.0]	51.0 [40.5; 64.5]	58.0 [50.0; 64.0]	0.129
Sex, M/F	29/38	12/22	17/16	0.180
WHR	0.91 [0.85; 0.98]	0.90 [0.84; 0.96]	0.94 [0.86; 1.00]	0.127
BMI, kg/m^2	29.3 [26.7; 32.3]	29.4 [27.1; 34.3]	29.3 [26.3; 31.6]	0.527

Values are presented as medians [Q1; Q3] or numbers. Differences in Age and BMI between placebo and PD01 were tested with a Mann-Whitney U test. Differences in WHR between placebo and PD01 were tested with an unpaired student's *t*-test. Differences in gender between placebo and PD01 were tested with a Chi-square test. M: male; F: female; WHR: waist-to-hip-ratio; BMI: body mass index.

Quantification of Bacillus indicus PD01

In the group of subjects receiving placebo, PD01 was not detected in any of the faecal samples. In the PD01 group, PD01 was not detected in any of the faecal samples at baseline, but was found in faecal samples of all 29 subjects after three weeks of supplementation and was present in all of the subjects that consumed PD01 during the complete study period (n = 27) after six weeks. As shown in Table 6.5, PD01 total cells and spores were significantly increased both after three weeks and six weeks supplementation, compared to baseline (all $p < 0.001$). No significant differences could be observed between three and six weeks of supplementation both for total cells and spores. The total amount of viable PD01 cells in human faeces after six weeks of PD01 supplementation was quantified as $2.26x10^7$ CFU/g, of which $3.43x10^6$ CFU/g are PD01 spores. Thus, although administered as spores, PD01 was mainly present in the faeces as vegetative cells ($1.99x10^7$ CFU/g; 73% of total counts), indicating effective germination under intestinal conditions.

Bioavailability of PD01 carotenoids in plasma

In fasted plasma, PD01 carotenoids were detected in samples of all subjects after three weeks (0.044 μM) and six weeks (0.076 μM) of PD01 supplementation, but not in the baseline samples. The total plasma concentration of PD01 carotenoids were significantly increased after three weeks and continued to increase during the six weeks of daily supplementation (all $p \leq 0.027$, Table 6.6). The PD01 carotenoids detected in plasma were methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene, with methyl-glycosyl-apo-8'-lycopenoate making up 74.6% and 85.3% of the total concentration after three and six weeks, respectively. *Bacillus indicus* PD01 supplementation did not induce significant changes in the plasma content of lutein, β-carotene or lycopene during the study period (all $p \geq 0.618$, Table 6.6). The presence of (bacterial) carotenoids in plasma samples of subjects from the placebo group was not assessed, as bacterial carotenoids proved undetectable in the baseline sample of the subjects. Representative chromatograms of carotenoids found in plasma before and after supplementation are shown in Figure 6.3.

Table 6.5 PD01 total cells and spores (CFU/g) in faecal samples of the clinical study participants at baseline, after 3 weeks and after 6 weeks supplementation with PD01 (*n*=27)

	Baseline	3 weeks	6 weeks	P_1	P_2	P_3	P_4
Total cells	0 [0; 0] †	2.50E+07 [1.11E+07; 3.98E+07]	2.26E+07 [1.06E+07; 4.19E+07]	<0.001	<0.001	<0.001	0.939
Spores	0 [0; 0]	5.77E+06 [1.28E+06; 1.56E+07]	3.43E+06 [1.85E+06; 1.06E+07]	<0.001	<0.001	<0.001	0.117
Percentage of total	0	30 [11; 53]	27 [7; 39]				
Vegetative cells	0 [0; 0]	1.70E+07 [4.20E+06; 3.95E+07]	1.99E+07 [5.65E+06; 2.93E+07]	<0.001	<0.001	<0.001	>0.999
Percentage of total	0	70 [47; 89]	73 [61; 93]				

All values are presented as medians [Q1; Q3]. Differences between baseline and 3 weeks and 6 weeks supplementation were tested with Friedman test with post hoc Wilcoxon signed rank test. Correction for multiple testing was performed by Bonferroni correction. P_1 represent the p values for the overall difference between the three time points tested with the Friedman test. P_2 represent the adjusted p values for the analysis of baseline vs. 3 weeks of intervention. P_3 represent the adjusted p values for the analysis of baseline vs. 6 weeks of intervention. P_4 represent the adjusted p values for the analysis of 3 weeks vs. 6 weeks of intervention. † Values that could not be detected, depicted here as 0 (all such values). CFU: colony forming unit.

Table 6.6 Carotenoid concentrations (μM) in fasted plasma of the clinical study participants at baseline, after 3 weeks and after 6 weeks supplementation with PD01 (*n*=27)

	Baseline	3 weeks	6 weeks	P_1	P_2	P_3	P_4
PD01 carotenoids	0 [0; 0] †	0.044 [0.016; 0.073] $	0.076 [0.042; 0.14] ¥	<0.001	<0.001	<0.001	0.027
Lutein	0.13 [0.063; 0.19]	0.12 [0.084; 0.21]	0.12 [0.091; 0.22]	0.618	-	-	-
β-Carotene	0.28 [0.19; 0.54]	0.32 [0.23; 0.41]	0.32 [0.17; 0.44]	0.692	-	-	-
Lycopene	0.18 [0.091; 0.29]	0.18 [0.10; 0.26]	0.18 [0.087; 0.28]	0.936	-	-	-

All values are presented as medians [Q1; Q3]. Differences between baseline and 3 weeks and 6 weeks supplementation were tested with Friedman test with post hoc Wilcoxon signed rank test. Correction for multiple testing was performed by Bonferroni correction. P_1 represent the p values for the overall difference between the three time points tested with the Friedman test. P_2 represent the adjusted *p* values for the analysis of baseline vs. 3 weeks of intervention. P_3 represent the adjusted *p* values for the analysis of baseline vs. 6 weeks of intervention. P_4 represent the adjusted *p* values for the analysis of 3 weeks vs. 6 weeks of intervention. In case post hoc Wilcoxon signed rank test was not applicable, no p values are reported. † Values that could not be detected, depicted here as 0. $ Sum of methyl-1-glycosyl-3,4-dehydro-apo-8'-lycopenoate (74.6%) and 1-glycosyl-3,4-dehydro-apo-8'-lycopene (25.4%), non-esterified carotenoids of PD01. ¥ Sum of methyl-1-glycosyl-3,4-dehydro-apo-8'-lycopenoate (85.3%) and 1-glycosyl-3,4-dehydro-apo-8'-lycopene (14.7%), non-esterified carotenoids of PD01.

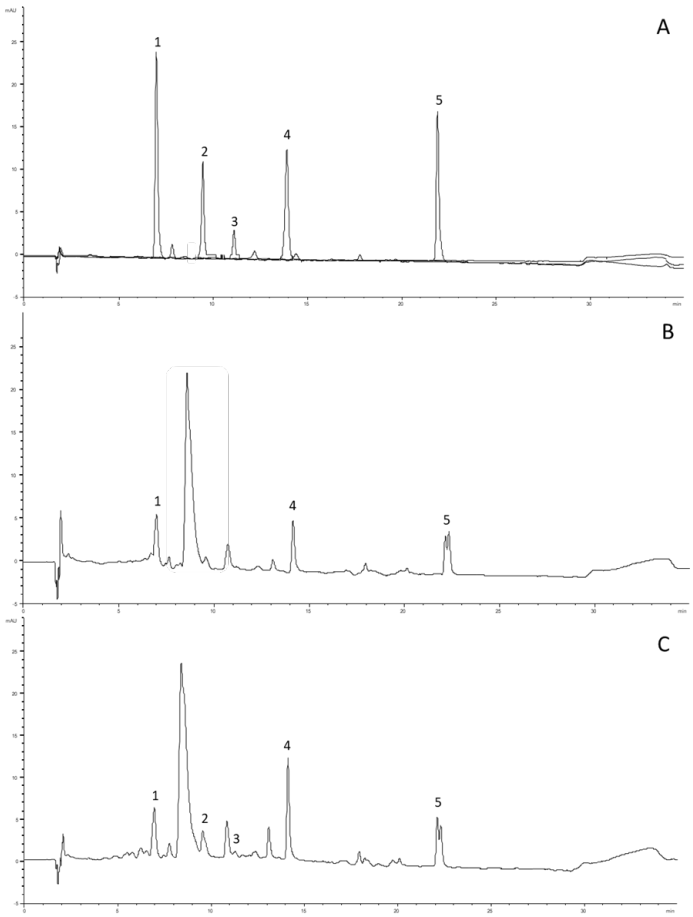

Figure 6.3 Representative chromatograms of carotenoid standards (A) and carotenoid profiles in fasted plasma at baseline (B) and after 6 weeks supplementation with PD01 (C). Carotenoids were analysed by reverse-phase HPLC on a HP1100 Agilent system using a YMC C30 column. Free forms of carotenoid were detected at 460 nm and identified by retention time compared with pure standards. The absorption spectra of each standard were measured between 300 and 550 nm in the mobile phase to cross-check the identification of sample molecules. 1: Lutein, 2: Methyl-glycosyl-apo-8'-lycopenoate (free form of orange bacterial carotenoid produced by PD01 strain), 3: Glycosyl-apo-8'-lycopene (free form of yellow bacterial carotenoid produced by PD01 strain), 4: β-Carotene, 5: Lycopene.

Gastrointestinal permeability

The GI permeability results, as determined by the multi-sugar test, are presented in Table 6.7. Neither did the excretion of sugars indicative for small intestinal ($p = 0.842$) or whole gut permeability ($p = 0.266$) differ after six weeks PD01 supplementation nor did the changes in sugar excretion reflecting gastroduodenal ($p = 0.131$) or colonic ($p = 0.104$) permeability reach statistical significance after six weeks PD01 supplementation compared to placebo.

Table 6.7 Permeability test: sugar excretion (µmol) and ratios of excreted sugars as measured in urine (0-5, 5-24 and 0-24 h fractions) of the clinical study participants at baseline and after 6 weeks supplementation (n=62)

	Placebo		PD01		*P*-value
	Baseline	6 weeks	Baseline	6 weeks	
0-5 h sucrose	8.92 ± 1.76	12.0 ± 3.22	11.4 ± 2.14	8.46 ± 2.04	0.131
0-5 h L/R ratio	0.042 ± 0.010	0.032 ± 0.003	0.049 ± 0.010	0.042 ± 0.008	0.842
5-24 h S/E ratio	0.013 ± 0.001	0.018 ± 0.004	0.051 ± 0.025	0.017 ± 0.002	0.104
0-24 h S/E ratio	0.014 ± 0.001	0.014 ± 0.002	0.031 ± 0.014	0.016 ± 0.002	0.266

All values are presented as mean ± SEM. Differences between placebo and PD01 were tested with an unstructured linear mixed model with correction for baseline values. L/R: lactulose/l-rhamnose; S/E: sucralose/erythritol.

Gastrointestinal tolerance

GI tolerance was assessed by GSRS subdimension scores at every test day. As shown in Figure s6.2, the scores for indigestion decreased in the PD01 treatment group after six weeks of supplementation (uncorrected $p = 0.045$), but this reduction did not remain statistically significant after correction for multiple testing ($p = 0.135$). For the other GI symptom scores and time points, also no significant changes were observed after PD01 supplementation compared to placebo (all $p \geq 0.183$, Figure s6.2), indicating that PD01 was well tolerated. Neither PD01 nor placebo did affect stool frequency or consistency throughout the study period (data not shown).

Discussion

This is the first *in vivo* study evaluating the effect of a carotenoid-producing *Bacillus* strain on intestinal barrier function as well as its intestinal fate and ability to release systemically absorbable carotenoids in a combined animal and human approach. We have shown that *Bacillus indicus* strain PD01 survives transit through the GI tract in both piglets and human subjects and was able to release carotenoids *in situ.* Supplementation with PD01 resulted in a significant improvement in outcomes of intestinal barrier function in early weaned piglets, but did not significantly affect intestinal barrier permeability in healthy overweight or obese human subjects. Repeated intake of PD01 did not result in any adverse effects, not in early weaned piglets nor in human subjects.

In both piglets and humans, PD01 survived passage through the GI tract and was able to germinate into vegetative cells under intestinal conditions, although germination seems

to be more efficient in humans based on the percentage of vegetative cells present in the faeces after supplementation. This difference in results between the piglets and humans may be due to interspecies differences in GI physiology, in particular gastric emptying.[35, 36] Furthermore, we have shown that in humans daily PD01 supplementation over a period of six weeks led to a significant accumulation of PD01 carotenoids in plasma. These carotenoids, methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene, are normally not present in the diet. In plasma, no changes in concentrations of the three main dietary carotenoids (*i.e.* lutein, β-carotene and lycopene) were observed, indicating that subjects did not change their dietary carotenoid intake during the study and these novel PD01 carotenoids do not interfere with the absorption and transport of other carotenoids. Plasma concentrations of the PD01 carotenoids were slightly lower in comparison with those of the dietary carotenoids. This is not surprising as in general the average intake of these carotenoids (10 mg/day) is of a much higher magnitude than the calculated daily dose of 14.05 μg PD01 carotenoids.[37] Still, the fact that bacterial carotenoid levels reached similar order of magnitude levels in plasma upon a six-week intake, despite the much lower intake levels, further suggests that PD01 is capable of local production of high amounts of bacterial carotenoids in the intestine, in combination with high bioavailability of the released carotenoids.

In early weaned piglets, we observed an improvement in intestinal barrier function in animals fed a diet supplemented with *Bacillus indicus* strain PD01. Others did previously show with this model that the probiotic strain *Lactobacillus frumenti* was able to promote intestinal barrier function, via increased expression levels of the TJ proteins ZO-1, *Ocln* and Claudin-1.[38] In addition to any potential effects of the *Bacillus indicus* strain PD01 itself, the carotenoids released in the intestine following PD01 supplementation may have been responsible or could have contributed to the observed improvement in barrier function. To date, in different mouse models associated with intestinal barrier disruption, treatment with the carotenoids β-carotene and lycopene resulted in an improvement in markers for intestinal barrier integrity and for colonic damage.[10-12] In the current study, PD01 supplementation resulted in higher expression levels of *Ocln* in the distal small intestine of the piglets. These increased expression levels were not accompanied by a significant change in TEER or transcellular permeability in the small intestine, although a slight improvement in both outcomes could be observed. In the mid colon, PD01 supplementation resulted in a significantly increased TEER, while TJ gene expression levels of *Tjp1* and *Ocln* were not affected. These findings do not exclude effects on protein levels, but can also be due to other TJ or adherens junction related factors.[39] In future studies, additional analyses on the expression of

junctional proteins and activity of signalling cascades will contribute to further insight in underlying mechanisms.

In our population of overweight or obese subjects, no significant effects on barrier function were observed as a result of PD01 intake. Intestinal barrier function was assessed by the use of a multi-sugar test measuring urinary recovery of sugars indicative of gastroduodenal, small intestinal, colonic and whole gut permeability. This method has been widely used and validated for non-invasive measurement of GI permeability in human subjects.[31] Although overweight and obesity have been associated with an impaired barrier function, a recent study in morbidly obese patients that assessed intestinal permeability using the same sugar test showed that only gastroduodenal permeability was significantly increased in obese subjects, while small intestinal and colonic permeability were not increased when compared to healthy lean subjects.[40] These recent results indicate that in overweight/obese subjects, who are otherwise healthy, intestinal permeability is not increased in contrast to previously expected. Therefore, this group may not have been the most suitable study population to study effects on this outcome. So far, data from human intervention trials investigating the effect of probiotics on intestinal barrier function in overweight or obese subjects are scarce and show varying results[41, 42] while data on carotenoids are still lacking. For example, daily supplementation with *Lactobacillus casei* Shirota for three months did not have any significant effects on intestinal permeability,[4] while the intestinal barrier was found to be significantly improved after three weeks of supplementation with *Bifidobacterium adolescentis* IVS-1 or *Bifidobacterium lactis* BB-12.[4] Altogether, the results from the piglet and human studies show that while supplementation with PD01 was not able to induce any changes in intestinal barrier permeability in a healthy overweight/obese population, it was able to do so in an animal model of impaired barrier function. Therefore, further investigations regarding the effect of PD01 supplementation in specific patient groups with a more clearly compromised barrier integrity, particularly those in which immune-mediated barrier disruption is likely, are worthwhile. Use of a stressor that is able to induce a transient disruption of intestinal barrier function could also provide additional insight.

As this was one of the first studies performed *in vivo* with this particular *Bacillus* strain (PD01), animal growth performance and GI tolerance were assessed during the intervention periods. In animals, no changes in total BW, daily growth, feed intake or faecal scores were observed as a result of PD01 feeding. In human subjects, GI symptom scores, stool frequency and stool consistency were also not significantly affected by the intake of PD01. These results show that PD01 did not result in any adverse effects and was well tolerated in early weaned piglets and in healthy overweight/obese subjects.

This is in line with data from a previous study assessing the safety profile of a similar strain (HU36) in guinea pigs and rabbits.[43] Furthermore, toxicity and safety trials in mice and healthy lean and obese human subjects with supplementation periods of up to 90 days showed that repeated intake of *Bacillus indicus* strain PD01 was not associated with any adverse effects (unpublished data from ProDigest, Ghent, Belgium).

Some potential shortcomings of our study should be mentioned. First, the diet of the participants was not strictly controlled in the clinical study. 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. Second, the study population chosen for the current study was relatively healthy, because PD01 is a new product which so far has only been tested in healthy human volunteers. Third, the required sample size of the human intervention study was not calculated based on an improvement in intestinal permeability, as this was not the primary outcome of the original study protocol. Therefore, this study might have lacked sufficient power to confirm the effects on intestinal barrier function observed in animal models. Fourth, statistical analyses could not be performed on piglet performance indices, as these were based on data per pen. Last, the barrier function results in the animal study were in some cases borderline significant or a trend toward significance, indicating that the results have to be interpreted with caution.

In conclusion, this study provides the first evidence that PD01 survives transit through the GI tract, is able to germinate and is able to release bacterial carotenoids, which are absorbed and detected in human blood plasma. Furthermore, PD01 supplementation resulted in improved barrier function outcomes in an animal model of disturbed intestinal barrier in early weaned piglets. While no significant effects on barrier function were found in an overweight/obese, but otherwise healthy population, these results warrant further research in specific target populations to investigate the specific bioactivities of PD01 in the intestine and possibly also on systemic parameters.

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Supplemental material

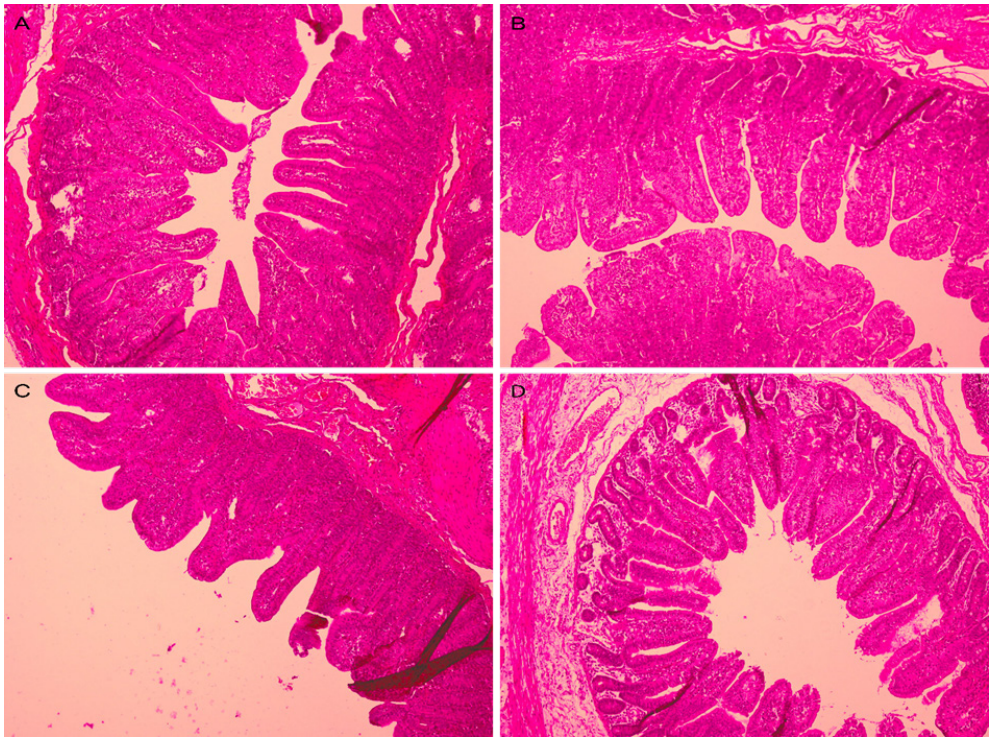

Figure **s6.1** Histomorphology of small intestinal tissue after supplementation with basal control diet and diet with PD01. Representative images of haematoxylin-eosin stained slides for 50% and 90% of small intestinal length are shown. A: 50% small intestinal length, control diet (CD). B: 50% small intestinal length, Bacillus indicus PD01 (CD+PD01). C: 90% small intestinal length, CD. D: 90% small intestinal length, CD+PD01.

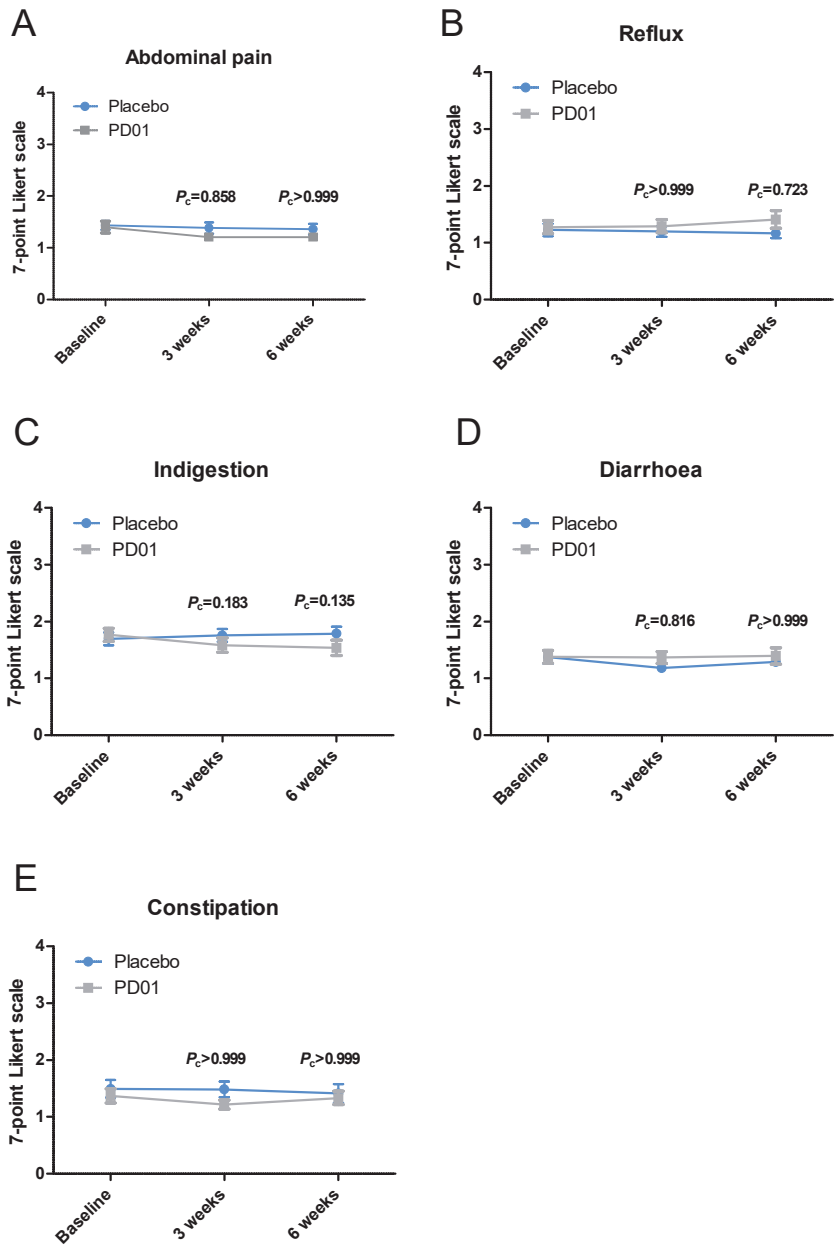

Figure s6.2 Gastrointestinal symptoms of the clinical study participants at baseline, after 3 weeks and after 6 weeks of supplementation with placebo (blue) or *Bacillus indicus* PD01 (grey). A: Abdominal pain scores. B: Reflux scores. C: Indigestion scores. D: Diarrhoea scores. E: Constipation scores. Means and standard errors are shown. Differences between placebo and *Bacillus indicus* PD01 were tested with a linear mixed model with correction for baseline values. P_c: adjusted p-values, after Bonferroni correction for multiple testing.

Table s6.1 Feed formulation of basal control diet (CD)

Ingredients	g/kg
Corn Starch	381.40
Oats Beta-Glucan	35.00
Alphacell (Cellulose)	90.00
Sucrose	50.00
Ca Caseinate	80.00
Whey Protein Concentrate 80%	140.00
Whole Egg Powder	8.00
Animal Fat	60.00
Palm Oil	60.00
Soybean Oil	50.00
Premix Trace Min &Vit (*)	10.00
Monocalphosphate	25.00
Lime	8.00
Salt	3.00
Sodium Bicarbonate	6.00
Dl-Methionine	2.30
L-Tryptophan	0.30
Calculated composition (in g/kg, unless stated otherwise)	
Dry matter	930
Crude protein	189
Ether extract	182
Sugars + starch	425
Ash	48.3
Calcium	10.5
Phosphorus	5.54
P digestible	3.97
Saturated fatty acids	54.8
Mono-unsaturated fatty acids	56.2
Poly-unsaturated fatty acids	43.6
n-6 Poly-unsaturated fatty acids	42.7
n-3 Poly-unsaturated fatty acids	0.90
Net Energy Pigs (MJ/kg)	13.36
Digestible Lysine	14.88

(*) Providing per kg of diet: vit A (retinyl acetate), 15000 IU; vit D3 (cholecalciferol), 2000 IU; vit E (all-rac-alfa-tocopherylacetate), 50.0 mg; vit K3 (menadion), 4.0 mg; vit B1 (thiamine mononitrate), 3.1 mg; vit B2 (riboflavine), 8.0 mg; vit B3 (calcium-D-pantothenate), 20 mg; vit B6 (pyridoxine hydrochloride), 6.0 mg; vit B12 (cyanocobalamine), 50.0 µg; vit PP (niacinamide), 40.0 mg; folic acid, 2.0 mg; biotin, 0.3 mg; betaine anhydrate, 285 mg; endo-1,4-beta-glucanase E3.2.1.4, 250 TGU; endo-1,4-beta-xylanase E3.2.1.8, 560 TXU; Cu (copper(II)sulphate pentahydrate), 15.0 mg; Mn (manganese(II)oxide), 48.0 mg; I (calciumjodate anhydrate), 1.9 mg; Se (sodium selenite), 200 µg; Se (selenomethionine produced by *Saccharomyces cerevisae* NCYC-R397), E306 extract of vegetable oils rich in tocopherols, tocopherols, 228 mg; 100 µg; clinoptioliet, 1.64 g, aromatic compounds, 72 mg.

Table s6.2 Piglet faecal scoring system (0-3 scale)

Score	Appearance	Description
0	No faeces	Normal
1	Normal brown soft-formed stool	Normal
2	Yellow or dark black (bloody) soft formed sticky faeces	Not unusual, check more frequently, indication of diarrhoea
3	Watery, liquid, unformed stool. Yellow or dark black (bloody) diarrhoea, wet backsides piglets	Diarrhoea. The number of diarrhoea days corresponds to the total number of piglets each day showing wet backsides

Table s6.3 Effect of control (CD) and test diets (CD+PD01) on piglet performance, faecal score and number of diarrhoea days

Period	Parameter	Control diet	CD+PD01
Day 0 to 5	Initial BW	6.48	6.56
	Final BW	6.78	6.78
	Growth	58.5	44.1
	Feed intake	196	182
	Feed to weight gain ratio	12.7	5.56
Day 5 to 15	Final BW	7.53	7.57
	Growth	83.3	87.8
	Feed intake	261	246
	Feed to weight gain ratio	3.15	2.94
Day 15 to 23	Final BW	8.38	8.21
	Growth	95.0	71.4
	Feed intake	264	280
	Feed to weight gain ratio	2.89	4.01
Total period (day 0 to 23)	Growth	82.5	71.9
	Feed intake	248	245
	Feed to weight gain ratio	3.20	3.44
Faecal score	Faecal score	1.40	1.48
Number of diarrhoea days	Number of diarrhoea days	0	0

Body weight (BW) is shown in kg. Animal daily growth and animal daily feed intake are presented in g/d. The feed to weight gain ratio is shown in g/g. All data are presented as mean values calculated for 2 pens (n=2). CD: basal control diet; BW: body weight.

Chapter 7

The effect of olive leaf extract on cardiovascular health markers: a randomised placebo-controlled clinical trial

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Abstract

Purpose: Overweight and obesity are associated with many health problems, including cardiovascular disease (CVD). Evidence from previous studies has shown that extracts from olive leaves rich in olive phenolics are able to positively affect CVD risk factors, such as high blood pressure and dyslipidaemia. The aim of this study was to investigate the effect of 8-week olive leaf extract (OLE) administration on blood lipid profiles in overweight/obese subjects with mildly elevated cholesterol levels.

Methods: In this randomised, double-blind, placebo-controlled study, 77 healthy adult overweight/obese subjects (aged 56 ± 10 years and BMI 29.0 ± 2.7 kg/m^2) with total cholesterol levels of 5.0 – 8.0 mmol/L (5.9 ± 0.7 mmol/L) were randomly assigned to receive 500 mg of OLE (n = 39) or placebo (n = 38) for eight weeks. In total, 74 subjects completed the entire study protocol. At baseline, after four weeks, and after eight weeks of supplementation, blood lipid profiles, oxidized low-density lipoprotein (oxLDL), blood pressure, glucose, and insulin levels were assessed. In addition, liver function parameters were measured at baseline and after eight weeks.

Results: OLE supplementation did not significantly affect blood lipid levels after four weeks or after eight weeks compared to placebo (all $p > 0.05$). For oxLDL, blood pressure, glucose, and insulin levels and liver function parameters, also no statistically significant differences were found between the two intervention groups (all $p > 0.05$).

Conclusions: Blood lipid profiles were not significantly affected by eight weeks OLE supplementation in overweight/obese subjects with mildly elevated cholesterol levels.

Introduction

The prevalence of overweight and obesity has increased rapidly over the last decades and poses a major public health concern, as excess body weight is associated with many health problems such as diabetes, liver disease, cardiovascular disease (CVD), and cancer.[1, 2] Atherosclerosis is an important precursor for CVD. It is considered to start with damage to the endothelial layer caused by CVD risk factors associated with obesity, such as high blood pressure and dyslipidemia.[3, 4] The latter includes increased blood cholesterol levels, which is also widely used as screening marker in clinical practice. In case of markedly elevated cholesterol levels (total cholesterol > 8.0 mmol/L), treatment with cholesterol-lowering medication is usually prescribed. For individuals with moderately or slightly elevated cholesterol levels, instead of medication, the use of lifestyle interventions such as dietary changes or dietary supplements is recommended.[5]

Evidence from epidemiological studies and randomised-controlled trials has shown that the Mediterranean diet is associated with a significantly lower incidence of CVD.[6] One of the main components of this diet, olive oil, is assumed to be, at least in part, responsible for a decreased CVD risk.[7-9] Although this effect could be due to various different constituents of olive oil, several studies have shown favourable effects on CVD risk biomarkers resulting from the intake of phenolic compounds present in olives and in olive oil.[10-13] The main phenolics present in the olive plant and olive oil are oleuropein, tyrosol, and hydroxytyrosol. Olive leaves contain the highest overall concentration of these compounds, especially oleuropein, compared to other parts of the plant.[13]

Previous studies investigating the effects of extracts from olive leaves rich in olive phenolics have shown that these extracts are able to positively affect CVD risk factors.[14-27] Although much of the evidence has been obtained from animal and *in vitro* studies,[14-22] human data are also available.[23-27] Human intervention studies in subjects with elevated blood pressure, diabetes, overweight, or osteopenia indicate that olive leaf extract (OLE) supplementation favourably affects outcomes such as blood pressure, glucose metabolism, and blood lipid profiles.[20, 23-27] Although these results are promising, the effects of OLE on blood lipid profiles were not the primary aim in any of these studies, nor were participants selected based on their cholesterol levels. Therefore, the primary objective of our study was to investigate the effect of 8-week OLE administration on blood lipid profiles in overweight/obese subjects with mildly elevated cholesterol levels. The secondary objective was to investigate the effect of OLE on lipid peroxidation and blood pressure. Additionally, effects on parameters of glucose metabolism and liver function were assessed. We hypothesize that supplementation with OLE for eight weeks will improve blood lipid profiles, lipid peroxidation, blood pressure, glucose metabolism, and liver function.

Methods

The study has been approved by the Medical Ethics Committee of Maastricht University. Medical Centre+ (MUMC+), Maastricht, The Netherlands, and was conducted in full accordance with the Declaration of Helsinki (as amended by the World Medic Association in 2013) and the Dutch Regulations on Medical Research Involving Human Subjects (WMO, 1998). All subjects gave their written informed consent before participation. This study has been registered at ClinicalTrials.gov (NCT02990637).

Subjects

Healthy subjects, aged 18 – 70 years with a body mass index (BMI) between 25 and 35 kg m^2 and elevated cholesterol levels, were recruited by means of advertisements in local newspapers and posters on notice boards at the Maastricht University buildings and from an existing cohort of eligible subjects. For this study, we sought participants with baseline total cholesterol levels in the range of 5.0–8.0 mmol/L, based on the guidelines of the Dutch heart association (https://www.hartstichting.nl/). Cholesterol values within this range are classified as elevated, while values above 8.0 mmol/L are classified as markedly elevated and generally result in the prescription of cholesterol-lowering medication. Exclusion criteria were: (history of) chronic or severe diseases that may affect study outcomes or limit participation in the study; use of medication influencing endpoints of the study; administration of investigational drugs or participation in any scientific intervention study which could interfere with the study; use of antibiotics within 30 days prior to the start of the study; use of antioxidants, minerals and vitamin supplements; pregnancy or lactation; abuse of alcohol (> 20 alcoholic units/week) or recreational drugs; smoking; recent weight gain or loss (> 3 kg in previous 3 months); high physical activity (> 4.5 hours of running/week); history of any side effects towards intake of olives. In total, 109 subjects were assessed for eligibility. Of these, 77 subjects were included in the current study (Supplemental Figure s7.1).

Study design

The study was designed as a randomized, parallel, double-blind, placebo-controlled trial, conducted at the Metabolic Research Unit of Maastricht University, Maastricht, The Netherlands. The study participants were randomly assigned to receive one of the following two interventions: placebo or OLE. The randomization list was computer-generated by an independent person, with random and concealed block sizes of two, four and eight for treatment allocation. Participants and researchers were blinded to the intervention allocations until all analyses were completed. The total treatment duration was eight weeks and measurements were performed at three different time points, *i.e.,* at baseline (test day 1), after four weeks (test day 2), and after eight weeks

(test day 3) of treatment. A total intervention period of eight weeks was chosen as this is in line with previous human intervention studies showing a significant improvement in blood lipid profiles after OLE supplementation and it has been suggested to be of sufficient duration to show a sustained effect on blood lipid levels as mentioned by the European Food Safety Authority.[24, 25, 28] The test day at four weeks was added to also assess the short-term effects of OLE supplementation on blood lipids. Before each test day, subjects were instructed to refrain from eating and drinking (except for water) after 10 pm in the evening. In addition, subjects were asked not to consume any alcohol containing beverages and abstain from vigorous physical exercise from two days prior to testing. To limit the influence of diet, participants were asked to eat the same type of meal before each test day. Furthermore, subjects were instructed not to consume any foods containing olive phenolics during the entire duration of the study and to maintain their habitual diet. All measurements were performed in the morning in a quiet, temperature controlled room (20 – 24°C). After an overnight fast, subjects arrived at the study site, where they handed in a 3-day dietary record. Next, anthropometric (height, weight, and waist and hip circumference) and blood pressure measurements were performed. Then, blood samples were collected from an antecubital vein in the forearm, and finally, a questionnaire was completed to assess gastrointestinal symptoms, stool consistency, and stool frequency. Supplementation with the study product started after completion of the baseline measurements.

The primary objective of this study was to assess the effect of daily OLE supplementation on blood lipid profile as measured by serum levels of total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, and triglycerides. The secondary objective was to assess the effect of OLE supplementation on risk markers related to the development of CVD, which were lipid peroxidation as measured by plasma oxidized LDL (oxLDL) and blood pressure. As explorative objectives, effects on glucose metabolism and liver function parameters were assessed.

Study product

The test product that was used was an extract prepared from olive leaves (*Olea europaea* L.) using a 100% water-based extraction method, standardized for its oleuropein content (> 16%), supplied by Interquim SA (Murcia, Spain). The batch used in the current study had an oleuropein content of 16.7%, providing 83.5 mg oleuropein per day. This dose was chosen based on previous studies showing a positive effect on blood lipid profiles after intake of OLEs.[24-26] As placebo, maltodextrin (Gonmisol, Barcelona, Spain) was used. The study product was provided as capsules, containing 350 mg of maltodextrin for the placebo or 250 mg of OLE in combination with 100 mg of maltodextrin. The extra 100 mg of maltodextrin was added to meet the minimum

volume requirement of the capsules. Participants were asked to ingest two capsules each morning with 200 mL water 30 minutes before breakfast. The participants were instructed to return empty packages and unused study product during the last visit for compliance assessment.

Blood lipids, glucose, insulin, and liver function parameters

Serum levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and plasma levels of glucose were measured by spectrophotometry (Cobas 8000 analyzer series, Roche Diagnostics, Mannheim, Germany). Serum levels of insulin were determined using an immunometric assay (XPi instrument, Siemens Medical Solutions Diagnostics, LA, USA). Blood lipids, glucose, and insulin levels were determined at baseline, after four weeks, and after eight weeks of supplementation. Liver function parameters were determined at baseline and after eight weeks.

For analysis of oxLDL, 8 mL blood was collected in an EDTA tube at baseline, after four weeks, and after eight weeks of supplementation. Samples were centrifuged within 30 minutes at 4°C at 1250 g for 10 minutes to obtain plasma, which was stored at -80°C until further analysis. OxLDL measurements were performed by a sandwich ELISA procedure according to the manufacturer's instructions (oxLDL, Mercodia AB, Uppsala, Sweden).

Blood pressure and heart rate

Blood pressure and heart rate were assessed using a semi continuous blood pressure monitoring device (Omron, Hoofddorp, The Netherlands) at the upper left arm after a 30-minute rest in supine position. Four measurements were conducted at 5-minute intervals. The first measurement was not used; the three other ones were averaged.

Dietary intake

To assess dietary intake, participants were asked to complete a 3-day dietary record before each test day. Volunteers were asked to record the intake of two week days and one weekend day. Before the start of the study, participants were instructed on how to record their food and beverage intake, *i.e.,* based on standard household units. During each test day, the records were checked, discussed with the participant and completed in case of missing data. Energy and nutrient intake were analyzed using the online dietary assessment tool of The Netherlands Nutrition Centre (www.voedingcentrum.nl), which is based on the Dutch Food Composition Dataset (NEVO, National Institute

for Public Health and Environment, Ministry of Health, Welfare and Sport, The Hague, The Netherlands).

Gastrointestinal tolerance, stool consistency and stool frequency

Gastrointestinal tolerance was assessed using the gastrointestinal symptom rating scale (GSRS) as described previously.[29] To assess defecation frequency and stool consistency, the Bristol Stool Form Chart was used.[30]

Statistical analysis

The sample size calculation was determined for the primary outcome of the study, which was the effect of eight weeks of OLE supplementation on blood lipid profiles. Based on a previous study in participants with normal cholesterol levels[25] and the assumption that a 5% stronger effect would be observed in the current study population, a sample size of at least 36 participants per group was required. A significance level of alpha = 0.05, a power of 80%, a difference in LDL-cholesterol of 0.4 mmol/L, and a standard deviation of 0.6 mmol/L were assumed for this calculation.

Statistical analyses were performed using IBM SPSS Statistics for Windows (version 25; IBM Corporation, Armonk, NY, USA). Baseline characteristics are presented as mean ± standard deviation (SD) for numerical variables and numbers for categorical variables. Analyses were performed on an intention to treat basis, including all subjects with baseline cholesterol levels between 5.0 and 8.0 mmol/L. Differences in blood lipid profiles, lipid peroxidation, blood pressure, heart rate, glucose metabolism, liver function parameters, anthropometrics, dietary intake, intestinal symptoms, stool consistency, and stool frequency were assessed by linear mixed model analyses (marginal model) with intervention group (OLE or placebo), time (baseline, four and eight weeks) and intervention*time as fixed factors. For this model, an unstructured covariance structure for repeated measures was used. Results obtained with this model are presented as estimated mean ± standard error of the mean (SEM), with p-values for the differences in means between groups after four and eight weeks adjusted for baseline differences, where a two-sided p-value ≤ 0.05 was considered statistically significant. For all outcomes, correction for multiple testing was performed by the false discovery rate (FDR) of Benjamini-Hochberg based on a correction for multiple time points and parameters.

Results

Subjects

In total, 77 healthy volunteers with mildly elevated cholesterol levels were included in the study, of which 74 completed the study protocol. Two participants dropped out after completing the baseline measurements, of which one due to the need to start with medication for treatment of arrhythmia and one person due to personal circumstances. One other participant dropped out after the second test day as a precaution because of a suspected olive allergy. Baseline characteristics of the study population are presented in Table 7.1. Based on capsule counts, overall compliance was 97.4%.

Table 7.1 Baseline characteristics

	Total population (n=77)	Placebo (n=38)	OLE (n=39)
Age (years)	56 ± 10	57 ± 9	56 ± 11
Male/Female, *n*	35/42	19/19	16/23
Weight (kg)	85.1 ± 11.8	86.5 ± 13.3	83.8 ± 10.3
BMI (kg/m^2)	29.0 ± 2.7	29.6 ± 2.8	28.5 ± 2.6
WHR	0.94 ± 0.08	0.95 ± 0.08	0.92 ± 0.08
Total cholesterol (mmol/L)	5.9 ± 0.7	5.7 ± 0.7	6.1 ± 0.7

All values are presented as mean ± SD or as number of participants. BMI, body mass index; WHR, waist-to-hip ratio; OLE, olive leaf extract.

Blood lipid profiles

As shown in Table 7.2, no significant differences in changes in blood lipid profiles were observed after OLE treatment for eight weeks compared to placebo. After four weeks, there were a significant decrease in triglyceride levels ($p = 0.028$) and a significant improvement in the triglyceride to HDL-cholesterol ratio in the OLE group compared to the placebo ($p = 0.018$), but these effects did not remain significant after correction for multiple testing (both $p > 0.05$). Furthermore, OLE supplementation did not significantly affect oxLDL levels compared to placebo (Table 7.2).

Table 7.2 Blood lipid and oxLDL concentrations at baseline, after 4 weeks and after 8 weeks of supplementation

	Placebo			OLE			*Uncorrected* P_1	*Uncorrected* P_2
	Baseline	**4 weeks**	**8 weeks**	**Baseline**	**4 weeks**	**8 weeks**		
Total cholesterol (mmol/L)	5.8 ± 0.1	5.7 ± 0.1	5.7 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	0.472	0.838
HDL-cholesterol (mmol/L)	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	0.575	0.490
Non-HDL-cholesterol (mmol/L)	4.1 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	4.4 ± 0.1	4.3 ± 0.1	4.3 ± 0.1	0.586	0.960
LDL-cholesterol (mmol/L)	3.6 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	0.802	0.571
Triglycerides (mmol/L)	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.028[a]	0.176
Total cholesterol/HDL-cholesterol	3.8 ± 0.2	3.8 ± 0.2	3.7 ± 0.2	3.9 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	0.501	0.852
Triglycerides/HDL-cholesterol	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.018[a]	0.155
OxLDL (U/L)	72.8 ± 2.8	73.1 ± 2.9	73.9 ± 3.1	83.6 ± 2.9	82.3 ± 2.9	83.3 ± 3.0	0.595	0.613

All values are presented as mean ± SEM. Differences between the placebo and OLE were compared with an unstructured linear mixed model with correction for baseline values. P_1 and P_2 represent the P values for the difference in estimated means after 4 and 8 weeks of intervention, respectively, between placebo and OLE, corrected for baseline differences. HDL, high-density lipoprotein; LDL, low-density lipoprotein; OxLDL, oxidized low-density lipoprotein; OLE, olive leaf extract.
[a] $p > 0.05$ after correction for multiple testing by the false-discovery-rate (FDR) of Benjamini-Hochberg (16 tests).

Table 7.3 Hemodynamic parameters at baseline, after 4 weeks and after 8 weeks of supplementation

	Placebo			OLE			*Uncorrected* P_1	*Uncorrected* P_2
	Baseline	**4 weeks**	**8 weeks**	**Baseline**	**4 weeks**	**8 weeks**		
Systolic BP (mmHg)	131 ± 2	129 ± 2	131 ± 2	128 ± 2	128 ± 2	127 ± 2	0.501	0.731
Diastolic BP (mmHg)	82 ± 2	81 ± 2	82 ± 1	80 ± 2	80 ± 2	79 ± 1	0.029[a]	0.590
MAP (mmHg)	99 ± 2	97 ± 2	98 ± 2	96 ± 2	96 ± 2	95 ± 2	0.095	0.905
Pulse pressure (mmHg)	49 ± 1	49 ± 2	49 ± 1	49 ± 1	48 ± 1	48 ± 1	0.473	0.428
HR (bpm)	66 ± 2	67 ± 2	68 ± 2	67 ± 2	67 ± 2	67 ± 2	0.803	0.532

All values are presented as mean ± SEM. Differences between the placebo and OLE were compared with an unstructured linear mixed model with correction for baseline values. P_1 and P_2 represent the *P* values for the difference in estimated means after 4 and 8 weeks of intervention, respectively, between placebo and OLE, corrected for baseline differences. BP, blood pressure; MAP, mean arterial pressure; HR, heart rate; OLE, olive leaf extract. [a] $p > 0.05$ after correction for multiple testing by the false-discovery-rate (FDR) of Benjamini-Hochberg (10 tests).

Hemodynamic parameters

Systolic blood pressure, mean arterial pressure, pulse pressure, and heart rate were not significantly altered after four weeks or eight weeks of supplementation with OLE compared to placebo (all $p \geq 0.095$; Table 7.3). Diastolic blood pressure showed a slight decrease in the placebo group after four weeks of supplementation compared to OLE ($p = 0.029$), but this effect was not significant after correction for multiple testing, nor after eight weeks of intake.

Glucose and insulin

At baseline, blood glucose and insulin levels were both within normal blood value ranges (Table 7.4). Although glucose levels showed a slight but significant increase after eight weeks of supplementation compared to placebo ($p = 0.024$), this effect did not remain significant after correction for multiple testing. No significant difference in changes were observed for glucose levels after four weeks or for insulin levels throughout the study as a result of OLE supplementation compared to placebo (all $p \geq 0.155$).

Anthropometrics and dietary intake

Anthropometrics and dietary intake were monitored during the study period to check whether participants maintained their dietary habits during the study period. No significant differences in body weight, BMI, waist-to-hip ratio (WHR) or dietary intake were observed between both interventions over time (Table 7.5).

Liver function parameters

Serum ALP, GGT, AST, ALT, and bilirubin were all within normal blood value ranges throughout the study period (Table s7.1) and were not significantly affected by OLE supplementation compared to placebo.

Gastrointestinal tolerance, stool frequency, and stool consistency

Gastrointestinal tolerance, as assessed by GSRS sub-dimension scores (*i.e.* abdominal pain, reflux, diarrhoea, indigestion and constipation), did not show a significant difference in change from baseline throughout the study period between groups (Table s7.2). Furthermore, no significant difference in changes in stool frequency or stool consistency was observed between OLE and placebo treatment (Table s2).

Table 7.4 Glucose and insulin concentrations at baseline, after 4 weeks and after 8 weeks of supplementation

	Placebo			OLE			*Uncorrected* P_1	*Uncorrected* P_2
	Baseline	**4 weeks**	**8 weeks**	**Baseline**	**4 weeks**	**8 weeks**		
Glucose (mmol/L)	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	0.229	0.024[a]
Insulin (pmol/L)	87.7 ± 21.0	97.7 ± 23.6	92.7 ± 24.8	65.1 ± 20.7	55.1 ± 23.2	62.5 ± 24.5	0.155	0.467

All values are presented as mean ± SEM. Differences between the placebo and OLE were compared with an unstructured linear mixed model with correction for baseline values. *P*1 and *P*2 represent the *P* values for the difference in estimated means after 4 and 8 weeks of intervention, respectively, between placebo and OLE, corrected for baseline differences. OLE, olive leaf extract. [a] $p > 0.05$ after correction for multiple testing by the false-discovery-rate (FDR) of Benjamini-Hochberg (4 tests).

Table 7.5 Anthropometrics and dietary intake at baseline, after 4 weeks, and after 8 weeks of supplementation

	Placebo			OLE			*Uncorrected* P_1	*Uncorrected* P_2
	Baseline	**4 weeks**	**8 weeks**	**Baseline**	**4 weeks**	**8 weeks**		
Weight (kg)	86.5 ± 1.9	86.6 ± 1.9	86.6 ± 1.9	83.8 ± 1.9	83.6 ± 1.9	83.9 ± 1.9	0.308	0.873
BMI (kg/m^2)	29.5 ± 0.4	29.5 ± 0.5	29.5 ± 0.4	28.5 ± 0.4	28.5 ± 0.4	28.6 ± 0.4	0.451	0.839
WHR	0.95 ± 0.01	0.94 ± 0.01	0.95 ± 0.01	0.92 ± 0.01	0.92 ± 0.01	0.92 ± 0.01	0.768	0.884
Energy (kCal)	2022 ± 65	1990 ± 84	1977 ± 74	1977 ± 64	2024 ± 81	1981 ± 72	0.490	0.613
Fat (g)	78.6 ± 3.6	78.3 ± 4.9	80.6 ± 4.0	79.5 ± 3.5	84.3 ± 4.7	82.3 ± 3.9	0.475	0.891
Saturated fat (g)	28.5 ± 1.6	29.2 ± 1.6	28.6 ± 1.8	28.2 ± 1.6	30.2 ± 1.6	30.4 ± 1.7	0.603	0.430
Carbohydrates (g)	219.3 ± 8.6	212.6 ± 11.1	206.0 ± 9.7	209.0 ± 8.4	215.3 ± 10.7	203.4 ± 9.5	0.425	0.525
Fibre (g)	21.3 ± 1.2	20.8 ± 1.2	20.2 ± 1.2	20.3 ± 1.2	20.7 ± 1.2	20.2 ± 1.1	0.395	0.398
Protein (g)	83.1 ± 3.5	85.1 ± 3.7	81.8 ± 3.5	80.6 ± 3.5	85.4 ± 3.6	84.9 ± 3.4	0.629	0.238
Salt (g)	6.9 ± 0.6	6.8 ± 1.2	6.6 ± 0.5	6.7 ± 0.6	8.8 ± 1.1	6.8 ± 0.4	0.231	0.580
Alcohol (g)	6.7 ± 1.7	5.2 ± 1.4	5.4 ± 1.5	9.3 ± 1.7	8.2 ± 1.4	6.7 ± 1.5	0.899	0.420

All values are presented as mean ± SEM. Differences between the placebo and OLE were compared with an unstructured linear mixed model with correction for baseline values. P_1 and P_2 represent the P values for the difference in estimated means after 4 and 8 weeks of intervention, respectively, between placebo and OLE, corrected for baseline differences. BMI, body mass index; WHR, waist-to-hip ratio; OLE, olive leaf extract.

Discussion

In this study, the effect of eight weeks of OLE supplementation on markers of CVD was investigated in overweight/obese participants with mildly elevated cholesterol levels. OLE supplementation did not significantly affect blood lipid levels after four weeks or after eight weeks compared to placebo. Furthermore, no statistically significant differences in oxLDL, blood pressure, glucose, and insulin levels or liver function parameters were found between the two intervention groups.

Increased levels of total cholesterol and LDL-cholesterol, and decreased levels of HDL-cholesterol are well known CVD risk factors.[4, 31] In addition, evidence also indicates that elevated triglyceride levels and in particular an elevated triglyceride to HDL-cholesterol ratio might be a useful marker for CVD risk, as it has been associated with plasma levels of small dense LDL particles.[32, 33] In the current study, no significant effects of OLE (containing 83.5 mg oleuropein) on lipid levels were found after eight weeks of supplementation. Our findings are in line with findings by de Bock *et al.*.[23] In their study, daily supplementation with OLE (containing 51.1 mg oleuropein and 9.7 mg hydroxytyrosol) for 12 weeks in overweight middle-aged men resulted in an improved insulin sensitivity, but had no significant effect on blood lipid profiles. Other studies, however, have found improved lipid profiles after OLE supplementation, although the outcomes and their effect sizes differ between studies. In pre-hypertensive males, a significant decrease in total cholesterol, LDL-cholesterol, and triglyceride levels was observed after OLE (containing 136 mg oleuropein and 6 mg hydroxytyrosol) intake for six weeks.[24] Supplementation with OLE containing 200 mg oleuropein for eight weeks in patients with stage-1 hypertension also resulted in reduced total cholesterol, LDL-cholesterol, and triglyceride levels.[27] A decrease in LDL-cholesterol was shown after eight weeks of supplementation with OLE at dosages containing 104 and 208 mg oleuropein in monozygotic twins with mild hypertension.[25] In all three of these OLE intervention studies, participants were selected based on their blood pressure levels, participants had slightly lower cholesterol values compared to the current study population, and different control conditions were used. These control conditions consisted of a liquid formula control designed to match the liquid OLE product in appearance, taste, texture, and aroma as closely as possible,[24] an active control (Captopril),[27] and lifestyle advice.[25] Furthermore, higher dosages of olive phenolics (104-208 mg vs 83.5 mg daily) were used, although the actual dose may have been lower than that in some cases.[24] Improvements in blood lipids have been demonstrated at much lower phenolic dosages (~12.5 and 9 mg per day) and after a much shorter interval of only three weeks of supplementation with olive oils rich in polyphenols in healthy subjects[34] and in subjects with hypercholesterolemia.[35] In both trials,[34, 35] a significant decrease in LDL-cholesterol

concentrations and various other atherogenic markers were found with olive oil rich in polyphenols compared to olive oil low in polyphenol levels but with identical fat and micronutrient composition. It is, however, remarkable that these two olive oil studies were able to show a decrease in blood lipids after supplementation at much lower phenolic dosages as compared to the current study. A clear explanation is lacking, though differences in study design should be noted. For example, olive oil and olive leaf differ in phenolic composition, with olive leaves containing significantly higher concentrations of oleuropein and glycosylated flavones, while olive oil is generally higher in flavone aglycones.[13] Furthermore, we cannot exclude that differences in factors that can influence phenolic bioavailability such as food matrix, gender, and microbiome perturbations associated with overweight and obesity have played a role.[13, 36, 37] In post-menopausal women with osteopenia, OLE supplementation containing approximately 100 mg oleuropein resulted in a significant improvement of total cholesterol, LDL-cholesterol, and triglyceride levels after 12 months.[26] In that specific trial, the primary focus was on bone health, which is why a study duration of 12 months was chosen. We have chosen a study duration of eight weeks, in line with most previous OLE trials. We also assessed the effects after four weeks, because if short-term beneficial effects can be shown, this could positively affect patient's compliance for potential future applications. Furthermore, a functional food instead of dietary intervention was chosen, as long-term adherence to dietary regimens is often poor.[38]

As previous studies have shown that certain diets or dietary components such as trans fatty acids can affect lipid levels, *e.g.,* LDL-cholesterol, HDL-cholesterol, and triglycerides,[39-41] participants were instructed to consume similar meals the night before each test day and to maintain their dietary habits throughout the entire study period. It is therefore unlikely that the results of the current study were influenced by the diet of the participants. Data from the anthropometric measurements and the food records do corroborate this, as there were no significant changes over time between the intervention groups with regard to body weight, WHR, and dietary intake.

In close relation to dyslipidaemia, hypertension and oxLDL are important risk factors for the development of atherosclerosis and CVD.[3, 42] Previous studies have shown improvements in systolic and diastolic blood pressure after intake of OLEs with a phenolic content of ~100-200 mg per day for six or eight weeks, using both 24 h ambulatory blood pressure and blood pressure measurements at a single time point.[24 ,25, 27] These studies all have in common that the participants had elevated blood pressure levels. One study that did not include participants based on blood pressure levels failed to show significant effects on systolic or diastolic blood pressure after 12 weeks of intake.[23] In the current study, we also did not observe a significant effect of OLE

supplementation on systolic or diastolic blood pressure compared to the placebo, nor on oxLDL levels.

Elevated glucose and/or insulin levels and altered liver function parameters are associated with overweight and obesity.[2, 43] As mentioned previously, an improvement in insulin sensitivity as a result of 12 weeks of OLE supplementation has been shown in middle-aged overweight men.[23] Using an oral glucose tolerance test, a reduction in the area under the curve for both glucose and insulin was observed. Furthermore, supplementation with 500 mg OLE for 14 weeks in patients with type 2 diabetes resulted in significantly lower HbA1c levels.[20] We did not find any significant improvements in either glucose or insulin levels after intake of OLE. When taking into account the baseline values within our population, these findings were to be expected, as glucose and insulin levels were all within normal ranges and our study design did not include a glucose challenge. Similarly, no significant effects on liver function parameters were observed.

Gastrointestinal symptom scores were assessed to monitor gastrointestinal tolerance during the supplementation period. OLE had no significant impact on gastrointestinal symptom scores, indicating that the study product was well-tolerated. Furthermore, with the exception of one participant (allergic symptoms) no adverse events were reported as a result of the intake of the study product.

Some limitations of the current study have to be addressed. First, for several outcome measures, there were slight differences in baseline values between the two intervention groups. However, these differences did not have an impact on our conclusions as statistical analyses were performed with correction for baseline values. Second, the changes in blood lipids, blood pressure, and glucose levels were very small, and do not appear to be clinically relevant. Third, based on the dietary food records, it was not possible to calculate the intake of trans fatty acids within our study population. However, as there were no significant changes in the intake of the major food components over time, we do not expect that the intake of trans fatty acids could have affected our outcomes. Fourth, the dose that was used in the current study was chosen based on a previous study using an OLE at a dose of approximately 100 mg oleuropein, which showed an effect after 12 months of supplementation.[26] We aimed to confirm these longer term effects on blood lipid profiles at this dose of oleuropein in a relevant target population during a much shorter lasting supplementation. It should be taken into account that due to differences in the extraction method applied (water vs ethanol/water) and the resulting oleuropein content between extracts, the final dose of the current study product was 83.5 mg per day instead of 100 mg per day in the long-term supplementation study.[26] In previous studies of similar or shorter duration, positive effects on blood lipids have been shown

for OLE at daily dosages of approximately 100 mg of oleuropein or even lower dosages for olive oil phenolics.[25, 34, 35] In our study, all subjects were selected based on *mildly elevated* baseline total cholesterol levels. In patients with higher total cholesterol levels, outcomes might have been different, but in these patients, cholesterol-lowering medication is indicated.

In conclusion, we have shown that OLE supplementation at a dose of 83.5 mg OLE phenolics per day for eight weeks in overweight/obese participants with mildly elevated cholesterol had no significant effect on blood lipid profiles as primary parameter or any of the other markers related to CVD as secondary parameters.

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Supplemental material

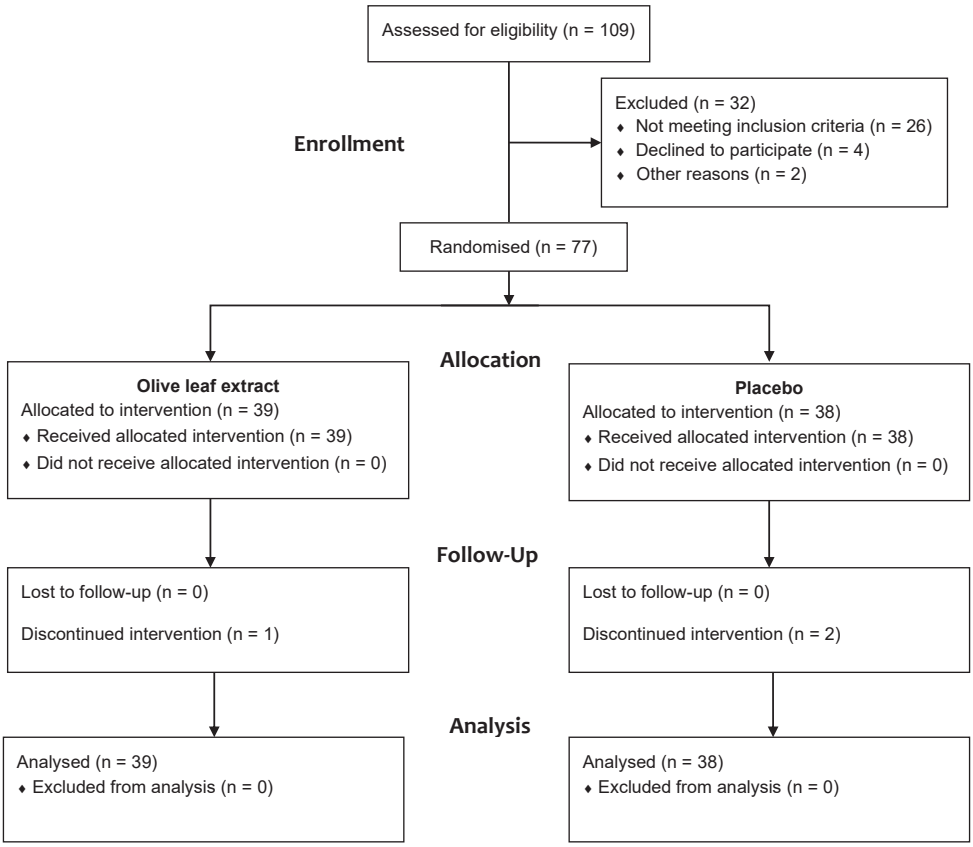

Figure s7.1 CONSORT flow diagram

Table s7.1 Liver function parameters at baseline and after 8 weeks of supplementation

	Placebo		OLE		*Uncorrected P* value
	Baseline	**8 weeks**	**Baseline**	**8 weeks**	
ALP (U/l)	72.9 ± 3.0	72.5 ± 3.3	77.0 ± 2.9	78.1 ± 3.2	0.425
GGT (U/l)	32.6 ± 3.8	32.0 ± 3.8	25.1 ± 3.7	25.1 ± 3.8	0.792
AST (U/l)	23.8 ± 1.1	22.4 ± 1.4	23.7 ± 1.1	23.7 ± 1.4	0.320
ALT (U/l)	27.3 ± 2.1	26.7 ± 2.9	27.1 ± 2.1	29.3 ± 2.9	0.246
Bilirubin (µmol/l)	10.3 ± 1.0	11.6 ± 1.1	9.0 ± 1.0	9.6 ± 1.1	0.600

All values are presented as mean ± SEM. Differences between the placebo and OLE after 8 weeks of supplementation were compared with an unstructured linear mixed model with correction for baseline values. ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table s7.2 GSRS sub scores, stool frequency and stool consistency at baseline, after 4 weeks and after 8 weeks of supplementation

	Placebo			OLE			*Uncorrected* P_1	*Uncorrected* P_2
	Baseline	**4 weeks**	**8 weeks**	**Baseline**	**4 weeks**	**8 weeks**		
Abdominal pain	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	0.941	0.768
Reflux	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	0.750	0.215
Diarrhoea	1.4 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	0.589	0.379
Indigestion	1.9 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	0.137	0.202
Constipation	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	0.798	0.652
Stool frequency	1.6 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.2	1.4 ± 0.1	1.3 ± 0.1	0.195	0.160
Stool type[a]	3.7 ± 0.2	3.8 ± 0.2	3.8 ± 0.2	3.5 ± 0.2	3.7 ± 0.2	3.6 ± 0.2	0.557	0.879

All values are presented as mean ± SEM. Differences between the placebo and OLE were compared with an unstructured linear mixed model with correction for baseline values. P_1 and P_2 represent the P values for the difference in estimated means after 4 and 8 weeks of intervention, respectively, between placebo and OLE, corrected for baseline differences. GSRS, gastrointestinal symptom rating scale. [a] Values represent average self-reported scores over a period of 1 week.

Chapter 8

General discussion

General discussion

Nutrition plays an important role in the development of chronic diseases, such as cardiovascular disease, non-alcoholic fatty liver disease and inflammatory bowel disease (IBD), by influencing metabolic processes and various functions of the gastrointestinal (GI) tract. On the other hand, nutrition is also capable of improving the course of chronic diseases and of general health and well-being via the same mechanisms. In this thesis, we aimed to assess the effect of various functional food supplements on intestinal and metabolic health.

Citrus flavonoids and their metabolites may contribute to improved GI functioning and health via their effects on the microbiota composition and activity, intestinal inflammation and intestinal barrier function. Further research, especially in human subjects, is needed to improve our understanding on the effects of citrus flavonoids in the human gut. Based on the currently available evidence, this is composed of a combined effect of the original compounds, their metabolites, and an interaction with the intestinal microbiome (**Chapter 2**). In **Chapter 3**, we showed that the parent compounds as well as their metabolites formed during intestinal metabolism were indeed able to exert comparable anti-inflammatory effects. However, no significant effects were found on immune-mediated intestinal barrier disruption. In human subjects, supplementation with citrus extract rich in flavonoids did not significantly affect markers of intestinal inflammation and microbiota composition in irritable bowel syndrome (IBS) patients with low-grade intestinal inflammation (**Chapter 4**). Citrus extract did also not improve biomarkers of oxidative stress and (intestinal) immune function in healthy elderly (**Chapter 5**). In addition to citrus flavonoids, many other functional food interventions may have the potential to beneficially affect GI and metabolic health outcomes. The effects of a carotenoid-producing *Bacillus* strain on intestinal barrier function in a combined human and animal approach was studied in **Chapter 6**. Supplementation with this potential next generation probiotic resulted in improved outcomes of barrier function in animals with impaired intestinal barrier function, while no such effect was seen in overweight/obese, but otherwise healthy human subjects. In **Chapter 7**, the metabolic effects of a functional food with olive polyphenols were investigated. Olive leaf extract supplementation did not significantly improve blood lipid profiles in overweight/obese subjects with mildly elevated cholesterol levels.

In general, it is challenging to demonstrate beneficial health effects of food in general and of single food items in particular. This is especially difficult in individuals with only mild disturbances and in conditions associated with a multifactorial aetiology. In the following sections, the main findings of the previous chapters will be put into perspective and implications for further research and future applications will be discussed.

Assessing intestinal function and health

In addition to its role in diseases of the GI tract, such as IBD and IBS, the intestine and its microbiome can also affect various extra-intestinal organs and metabolic processes and can contribute to the development and progression of conditions such as obesity and metabolic syndrome. In **Chapters 3, 4, 5, 6** and **7,** the effect of different food interventions on GI and/or metabolic health were studied using non-invasive outcome measures. Overall, these studies showed limited effects on main outcome parameters. This may be related to limitations associated with the use of these (non-invasive) parameters and how they represent GI processes and/or pathways.

Barrier function

The intestinal epithelial barrier plays a key role in the maintenance of intestinal homeostasis and of overall health and disease. It is a complex structure that is composed of different components that contribute to its function as a selective physical, chemical and immunological barrier. In **Chapters 3** and **6**, we have mainly focused on measuring functional markers of intestinal barrier function reflecting paracellular and transcellular permeability. Analysis of intestinal barrier function is important, given the highly dynamic character of the junctional complex. *In vitro* and *ex vivo*, barrier function was assessed by measuring transepithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC)–dextran permeation, while a multi-sugar absorption test was used in human subjects. In **Chapter 6**, recovery ratios between sugar probes of different sizes were used. In our opinion this combined parameter gives a better indication of GI permeability then a single marker as it corrects for factors such as gastric emptying, dilution by intestinal secretions, intestinal transit time and renal clearance.[1] Furthermore, in contrast to the classical dual sugar test to determine small intestinal permeability, the method we applied also included probes that allow us to assess gastroduodenal, colonic and whole gut permeability.[2] Although it remains challenging to determine the exact sensitivity and specificity of this test, previous studies have shown that it is able to pick up an effect of *e.g*. 1-2 glasses of alcohol or an acute bout of exercise on intestinal permeability.[3, 4]

The methods applied in the current thesis are widely used and have previously been validated. However, measurement of functional effects in human subjects remains challenging due to the dynamic nature, and thereby adequate timing of sample collection, and resilience of the intestinal barrier.[5] Additional markers that may be considered for future human studies include blood biomarkers that measure the consequences of barrier dysfunction such as bacterial products measured in plasma indicative of endotoxemia.[6, 7] Increased levels of lipopolysaccharides (LPS), indicating bacterial translocation, have been associated with increased intestinal permeability

and several diseases. However, due to the risk of contamination by LPS, measurement of LPS binding protein, being relatively stable, can be considered.[7] Measurement of plasma zonulin, a protein that has been proposed to modulate tight junction (TJ) proteins in the intestinal epithelium, could also provide valuable information.[7] Plasma D-lactate may also be a useful marker. In healthy individuals, this bacterial fermentation product is prevented to enter the circulation, but in case of intestinal barrier function loss the levels of plasma D-lactate will increase.[6, 8]

Although the aforementioned parameters provide information on the function of the intestinal barrier, they do not provide insight into the underlying mechanisms of action. In the current thesis, gene expression levels of different TJ proteins were analysed (**Chapters 3** and **6**). In case of observed barrier disruption, this could be followed by analyses at the protein level as well as of the signalling pathways involved, such as the myosi light chain kinase (MLCK) or mitogen-activated protein kinase (MAPK) pathways.

Microbiota

The microbiota confers many functions, being highly relevant for maintaining intestinal homeostasis. The microbiota also has a large metabolic capacity resulting in the production of important metabolites such as short-chain fatty acids (SCFAs) and specific vitamins. Although many studies to date apply modern sequencing techniques to assess the composition of the intestinal microbiota, most studies have focused primarily on variations in taxonomic composition. Characterisation of the metagenome does provide additional information on the functional potential of the microbiota.[9] Differences in the microbial metabolic potential were found to be associated with disease states such as IBD and insulin resistance.[10] A major class of bacterial metabolites that likely play an important role in this context are SCFAs. However, interpretating changes in faecal SCFA levels remains challenging, as the majority is produced in the proximal colon, due to the preference of the microbiota for carbohydrate fermentation and ready absorption by transporters (*e.g*. proton-coupled monocarboxylate-transporter SLC16A1 and sodium-coupled monocarboxylate-transporter SLC5A8).[11, 12] The lack of an effect on SCFA concentrations measured in faecal samples in **Chapter 5,** does therefore not necessarily exclude effects in the proximal colon. In a recent study in pre-frail elderly subjects, no changes in faecal SCFA production were shown after oral galacto-oligosaccharide (GOS) supplementation and SCFA production was not different between pre-frail elderly and healthy adults.[13] On the other hand, an *in vitro* batch fermentation (inoculated with a subset of faecal samples from the same study population) did reveal significant differences in metabolite production between elderly and adults after GOS treatment.[14]

In light of these shortcomings and based on the crucial metabolic function of the microbiota, it is important to consider inclusion of state-of-the-art microbiota metabolomic and metagenomic analyses in future studies.

Inflammation and immune function

The immune system of the GI tract, together with the intestinal barrier and microbiota are key components of whole-body immune defence, providing protection against the continuous threat of different chemical substances and microorganisms from the luminal environment. However, constant or repetitive activation of the immune system can lead to a chronic inflammatory state characterized by amongst others increased levels of oxidative stress and inflammatory mediators such as pro-inflammatory cytokines.

In human (explorative) studies with repeated measurements, the use of non-invasive markers is important to limit the burden for participants. Non-invasive markers that are commonly measured in biomaterials such as faeces, urine and plasma include calprotectin (in faeces), inflammatory mediators such as cytokines and chemokines, markers of lipid peroxidation such as malondialdehyde (MDA) and markers of antioxidant potential such as trolox equivalent antioxidant capacity (TEAC). In **Chapter 5**, oxidative stress biomarkers and cytokine levels were determined in blood. In addition to measurement of the widely used plasma markers MDA and TEAC, glutathione and glutathione disulphide (GSH/GSSG) concentrations in erythrocytes were also assessed to provide information on both extra- and intracellular redox status in the circulation.[15] Cytokines are generally active at very low pharmacological doses and circulating concentrations in healthy volunteers are often below the detection limit. In **Chapter 5**, we measured cytokine concentrations after stimulating whole blood samples with LPS using a commercially available electrochemiluminescence immunoassay, which resulted in increased cytokine levels. The resulting pattern of cytokine production may provide some insight into the inflammatory response, but the absolute levels are still difficult to interpret. Furthermore, although levels of non-invasive blood markers can reliably be measured in biomaterials, the general position of the European Food Safety Authority (EFSA[1]) on this matter is that health claims on the functions of the immune system cannot be based solely on the measurement of outcome variables (such as cytokines) that do not refer to a benefit on specific functions in the body.[16] The EFSA recommends that these outcomes should be accompanied by evidence of a physiological effect or clinical outcome (*e.g*. incidence, severity and/or duration of symptoms). An example of a study design that enables this is a study in which all subjects receive a vaccine. The effect on immune defence can then be determined by comparing the number of

[1] An agency funded by the European Union that provides scientific advice.

responders to the vaccination (*i.e*. number of subjects attaining antibody titres above a previously determined cut-off) between intervention groups. Changes in non-invasive biomarkers may still be useful and relevant, but are at best supportive evidence with respect to the mechanisms of action.

Model systems and populations

Choosing the most suitable outcome parameters to establish an effect on the various components of intestinal health is crucial and may explain some of the limited effects found in the studies reported in this thesis. In addition, it is important to focus on the different model systems and patient populations. Effects of treatments in human subjects do provide the most compelling evidence, but this is not always feasible for ethical or technical reasons. Therefore, drug discovery and proof of concept on efficacy generally starts with relatively simple *in vitro* assays and continues with increasingly complex models (*i.e. ex vivo* models, *in vivo* animal studies and finally human studies).

One of the advantages of using an *in vitro* cell culture model is that well characterised single cells are used. This enables high-throughput experiments under well-controlled conditions. Furthermore, they can provide mechanistic insight with limited ethical concerns about animal and human welfare. In **Chapter 3**, we made use of a validated *in vitro* co-culture system combining intestinal epithelial cells (Caco-2) with activated macrophages (THP-1) in Transwell plates. This model provided us the possibility to mimic local effects of citrus flavonoids within the intestine and to compare the relative contribution of various compounds under identical conditions. Although no significant effects were found on immune-mediated barrier disruption, citrus flavonoid compounds exerted significant anti-inflammatory effects and we were able to show that the parent compounds and several of the metabolites did have comparable effects. A major drawback of this model however is that both the epithelium and the immune compartment comprise single cell types that are grown in a 2D structure and are often of cancerous origin. Furthermore, these models are not able to accurately capture the complexity of a living organism, which involves interactions between different organs, all of the different cell types making up the intestinal barrier and the microbiota present in the intestine.[17] This can in part be overcome by using organoid cultures or organ-on-a-chip models. Intestinal epithelial organoids can be grown from stem cells isolated from patient materials into an epithelial monolayer surrounding a functional lumen. They are able to mimic the physiology of the barrier more closely and may even include features specific to the host, making it a valuable model to study the pathophysiological mechanisms underlying (GI) diseases like IBD.[18, 19] The recently developed intestine-on-a-chip models combine biopsy-derived organoids and (duodenal or colonic) endothelial cells with mechanical forces, resulting in an intestinal epithelium that resembles human

colon or small intestinal tissue more closely. Because of the accessibility of both the lumen and basolateral compartments, this system might be especially relevant for medium-throughput screening of responses to nutritional compounds.

In **Chapter 6**, we used early weaned piglets as model of intestinal barrier disruption, as early weaning is known to result in alterations in morphology and function of the intestine, such as villus atrophy and crypt deepening, a reduction in absorptive capacity and decreased barrier integrity.[20] Other examples of widely used animal disease models of intestinal barrier function and/or inflammation include chemically induced rodent models of colitis, such as dextran sulphate sodium (DSS)-, trinitrobenzene sulfonic acid (TNBS)-, and dinitrobenzene sulfonic acid (DNBS)-induced colitis. In addition to species-specific differences, it remains difficult to capture all disease aspects and these models often lack interindividual variation. In **Chapter 6**, supplementation with a potential next generation probiotic resulted in improved barrier outcomes in early weaned piglets, but not in healthy overweight/obese subjects. This may in part be due to interspecies differences in microbiome, GI anatomy (*e.g.* of the stomach, small intestine and cecum) and differences in gastrointestinal physiology (*e.g.* gastric emptying and immune responses),[21] but could also be because the barrier function in the animal model was more disrupted than in the human subjects.

Because extrapolation of results from *in vitro* and animal studies to humans remains challenging, human studies are essential to translate the knowledge obtained from such models into clinical practice. In this thesis (**Chapters 5, 6** and **7**), we chose to include healthy elderly individuals and healthy overweight/obese subjects, as they were (relatively) healthy, but at risk for mild disturbances in metabolic, immunological or GI conditions that could be prevented by nutritional interventions. In all three studies, however, no significant effect of the intervention on the main outcomes (*i.e*. oxidative stress, intestinal barrier function and blood lipid profiles) was observed. Retrospectively, the effect sizes that could be reached may have been too small to be detected by the non-invasive outcome markers used in these relatively healthy study populations. It is therefore important for these types of studies to consider whether the internal validity (the extent to which the trial supports a cause and effect relationship) can be improved, while also keeping the applicability of the study findings to the "real world" situation and final target population in mind. Examples of ways to increase the internal validity are selection of specific patient populations (with altered levels of specific biomarkers) or using a stressor. In **Chapter 4**, IBS patients with a mild intestinal inflammatory disturbance (based on baseline calprotectin levels 15 - 150 μg/g) were included. In these patients, citrus extract supplementation did not result in a significant decrease in faecal calprotectin levels, indicating that patient populations with a more pronounced

disturbance may be needed to detect a significant effect. An example of a study population with severe inflammatory disturbances are patients with IBD. In a previous study it was shown that a nutritional intervention of oligofructose-enriched inulin in addition to maintenance therapy with mesalazine, resulted in a significantly more pronounced reduction in faecal calprotectin levels after seven days of supplementation in patients with active ulcerative colitis (average baseline calprotectin levels of 4377 ± 659 μg/g) when compared to mesalazine alone.[22] When patient recruitment is a limiting factor, use of a stressor can be considered. Examples of stressors that have been used successfully by our group and by others to induce intestinal barrier dysfunction in healthy subjects include ethanol and indomethacin. Administration of these compounds has been shown to result in increased small intestinal and colonic permeability measured by multi-sugar tests.[3, 23]

Human intervention studies with functional foods that can improve intestinal and metabolic health

In this thesis, the effects of two classes of widely applied functional food interventions have been studied: probiotics and polyphenols. They were chosen because of their potential to improve GI and metabolic health via various mechanisms of action. For example, the citrus extract (**Chapters 3, 4** and **5**) contains flavonoids that have direct anti-inflammatory and anti-oxidative effects, but may also act as a prebiotic. PD01 (**Chapter 6**) is a next-generation probiotic that, in addition to its probiotic properties, produces carotenoids in the intestine. The results of the human intervention studies described in this thesis (**Chapters 4, 5, 6** and **7**), did not show major differences between intervention and control. Unfortunately, this is true for many studies with functional foods reported in literature.

Especially in the context of prevention and early treatment, the use of functional foods is often preferred over pharmaceutical drugs, in particular by consumers, because of their natural origin and the relatively low risk of side effects.[24] In general, the beneficial effects that can be obtained with such interventions are however modest. This may in part be due to the origin of the intervention, which often includes one active ingredient at a physiological but relatively low dose. In addition, several other factors can have an impact on the effects observed in humans, which need to be taken into account when designing a study with a functional food intervention (see also Table 8.1). To start, the use of a placebo is important to ensure adequate blinding of both investigators and participants. This is particularly important for studies using populations which generally have high placebo response rates such as IBS patients. Furthermore, the complete diet and lifestyle should be taken into consideration. As lifestyle strategies such as dietary and/or exercise interventions may be equally or even more effective than functional

food supplements,[25-27] potentially small effects may be masked by overall dietary intake and lifestyle factors. One way to take diet into consideration is to standardize the diet of the participants as part of the trial. The human studies described in this thesis (**Chapters 4, 5, 6** and **7**) were all double blind, randomised, placebo-controlled trials. Furthermore, participants were instructed to avoid foods with properties similar to the intervention (*i.e.* probiotics, prebiotics and foods high in polyphenols) and participants were instructed to maintain their habitual diet and lifestyle throughout the study period (being checked by food diaries or questionnaires). Although effective, long-term compliance with dietary and exercise regimens is usually poor.[28] Therefore, the use of functional food interventions remains an attractive approach to explore further. One of the most important aspects for studies with a functional food is to know what specific health outcome is to be targeted and to choose a food that acts via the expected mechanisms of action. Based on this, outcome parameters and appropriate study populations or models have to be selected that will enable measurement of these outcomes. Ideally, a combination of multiple clinical and mechanistic outcomes should be used to determine the expected health effect. Due to the many challenges associated with studying the effects of functional food supplements, it may be more relevant to make use of supplements that combine multiple active ingredients, if possible at a relatively high dose, thereby increasing the potential beneficial impact of these interventions.

Table 8.1 Summary of recommendations for future human intervention studies with functional food interventions

	Recommendation
Study design and intervention	• Adequate blinding by use of placebo • Take total diet and lifestyle of participants into consideration • Choose health outcome and intervention based on expected mechanism of action of the food • Combination of multiple relevant ingredients • Use relatively high, but safe, dose of active ingredient
Outcome parameters	• Focus on underlying mechanism of action in addition to functional markers • Use of multiple state-of-the-art and robust biomarkers • Use of clinical outcomes in combination with non-invasive biomarkers
Study populations and models	• Use of patient populations with pronounced disturbances • Use of a stressor when studying participants that are healthy or with a mild disturbance

Concluding remarks and future perspectives

Functional foods are a widely studied type of nutritional intervention that have the potential to promote short and long-term health. Although various *in vitro* and animal studies have shown promising results, the results from human intervention studies are often inconsistent or negative. To improve the results of these studies, human intervention trials that combine clinical outcome parameters with (state-of-the-art) biomarkers providing information about the underlying mechanism(s) of action are needed. Furthermore, it is important to study effects in a population with more pronounced pathophysiological disturbances or use relevant disease specific model systems (*e.g. in vivo* stressors or *ex vivo/in vitro* organoid cultures). More focus on functional food interventions that combine multiple active ingredients may improve their effectiveness and could thereby contribute to our understanding of the role of these interventions in human health and disease.

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Addendum

Summary

Nutrition plays an important role in the development of chronic diseases, such as cardiovascular disease (CVD), non-alcoholic fatty liver disease and inflammatory bowel disease (IBD), by influencing metabolic processes and various functions of the gastrointestinal (GI) tract. On the other hand, nutrition can also be used as a strategy to improve general health and well-being and thereby decrease the risk of chronic diseases via the same mechanisms. The studies described in this thesis investigated the effect of various functional food supplements on intestinal and metabolic health.

In **Chapter 2**, we provided a comprehensive overview of the intestinal fate of citrus flavonoids and their effects on intestinal health. Evidence shows that the citrus flavonoids hesperidin and naringin are metabolised by intestinal bacteria, mainly in the (proximal) colon, resulting in the formation of their aglycones hesperetin and naringenin and various smaller phenolics. Studies have also shown that citrus flavonoids and their metabolites are able to influence the microbiota composition and activity and can exert beneficial effects on intestinal barrier function and GI inflammation. Based on the currently available evidence, this is composed of a combined effect of the original compounds, their metabolites, and an interaction with the intestinal microbiome.

In order to further investigate the effect of citrus flavonoids and their intestinal metabolites on immune-mediated barrier disruption, we used a validated *in vitro* co-culture system in **Chapter 3**. Caco-2 cell monolayers were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells for 30 hours. At baseline, the citrus flavonoids or their metabolites were added to the apical compartment (50 or 100 μM). After 24 hours, THP-1 cells were incubated with LPS in the basolateral compartment for 6 hours. We found that both incubation with the citrus flavonoids and their metabolites resulted in a significant inhibition of NF-κB activity and production of several inflammatory cytokines, but did not protect against an immune-mediated barrier disruption.

In **Chapters 4** and **5**, the *in vivo* effects of citrus extract (CE) supplementation were studied in relevant target populations. In **Chapter 4**, the effect of CE was investigated in irritable bowel syndrome (IBS) patients with (low-grade) intestinal inflammation. In this explorative, randomised, double-blind, placebo-controlled, parallel study, 29 IBS patients with faecal calprotectin levels between 15 and 150 μg/g faeces received 500 mg CE/day or placebo for a duration of eight weeks. Faecal calprotectin, faecal secretory immunoglobulin A (sIgA), faecal microbiota composition, gastrointestinal symptoms, stool consistency and stool frequency were assessed at baseline, after four weeks and after eight weeks of administration. Supplementation with CE for eight weeks did not affect faecal biomarkers of inflammation and immune defence, overall microbiota

composition or disease symptoms in this study population. Subsequently, the effect of daily supplementation with CE in elderly subjects was investigated in **Chapter 5**, as ageing has been associated with impaired immune functioning and oxidative stress. In this randomised, double-blind, placebo-controlled, cross-over study, 37 healthy elderly (aged 70-85 years) received 500 mg CE/day or placebo. Blood biomarkers of oxidative stress, stimulated cytokine production, faecal markers of immune defence and faecal microbial metabolites were assessed at baseline and after four weeks of supplementation. Four weeks of CE administration did not significantly affect biomarkers of oxidative stress and immune function, nor faecal metabolite production. This may, at least in part, be due to the fact that the elderly participating in this study appeared to be relatively healthy and did not have baseline levels outside the normal range.

In addition to citrus flavonoids, many other functional foods may have the potential to beneficially affect GI and metabolic health outcomes. The effect of the carotenoid-producing *Bacillus indicus* strain PD01 on intestinal barrier function in a combined human and animal approach was studied in **Chapter 6**. As a model for impaired barrier function, 16 early weaned piglets were randomly assigned to a control diet or control diet with PD01 for 23 days. In addition, 67 overweight/obese, otherwise healthy individuals, were randomly assigned to groups receiving PD01 or placebo for six weeks. PD01 survived transit through the GI tract and resulted in systemic carotenoid accumulation. Furthermore, PD01 supplementation resulted in improved barrier function outcomes in an animal model of disturbed intestinal barrier in early weaned piglets, while no significant effects on barrier function were found in an overweight/obese, but otherwise healthy population.

In **Chapter 7**, the effect of olive leaf extract (OLE) supplementation on cardiometabolic risk factors was studied in an overweight/obese population with altered blood lipid profiles. In this randomised, double-blind, placebo-controlled study, 77 healthy overweight/obese adults with total cholesterol levels of 5.0-8.0 mmol/L were randomly assigned to receive 500 mg OLE or placebo for eight weeks. At baseline, after four weeks, and after eight weeks of supplementation, blood lipid profiles, oxidised low-density lipoprotein (oxLDL), blood pressure, glucose, and insulin levels were assessed. In addition, liver function parameters were measured at baseline and after eight weeks. We showed that OLE supplementation for eight weeks in overweight/obese participants with mildly elevated cholesterol levels had no significant effect on blood lipid profiles (as primary parameter) or any of the other markers related to CVD (as secondary parameters).

In **Chapter 8**, we summarised the main findings of this thesis. Furthermore, we discussed new insights and implications for further research and future applications of functional food interventions to improve metabolic and GI health.

Samenvatting

Voeding speelt een belangrijke rol bij het ontstaan van chronische ziekten zoals hart- en vaatziekten, niet-alcoholische leververvetting en inflammatoire darmziekten, door het beïnvloeden van metabole processen en verschillende functies van het maagdarmstelsel. Aan de andere kant kan voeding ook gebruikt worden als strategie voor het verbeteren van algehele gezondheid en welbevinden en kan het daardoor het risico op chronische ziekten verminderen, deels via dezelfde mechanismen. In de studies die in dit proefschrift beschreven worden, werd gekeken naar het effect van verschillende functionele voedingssupplementen op darm- en metabole gezondheid.

In **Hoofdstuk 2** geven we een uitgebreid overzicht van wat er bekend is over de afbraak en lokale beschikbaarheid in de darm en de effecten op darmgezondheid van citrus flavonoïden. Onderzoek laat zien dat de citrus flavonoïden hesperidine en naringine gemetaboliseerd worden in het (proximale) colon, wat resulteert in de vorming van de aglyconen hesperetine en naringinine en verschillende kleinere fenolen. Studies hebben ook laten zien dat citrus flavonoïden en hun metabolieten de compositie en activiteit van de microbiota kunnen beïnvloeden en mogelijk gunstige effecten hebben op darmbarrière functie en darmontsteking. Op basis van het bewijs dat op dit moment beschikbaar is lijkt dit een combinatie te zijn van effecten van de originele stoffen, hun metabolieten en een interactie met het darmmicrobioom.

Om de effecten van citrus flavonoïden en hun metabolieten op immuun-gemedieerde barrière verstoring verder te onderzoeken, hebben we een *in vitro* co-culture model gebruikt in **Hoofdstuk 3**. Caco-2 cellen werden uitgegroeid tot een enkele cellaag en in cultuur geplaatst met PMA-gestimuleerde THP-1-Blue™ NF-κB cellen gedurende 30 uur. Op baseline werden de citrus flavonoïden of hun metabolieten toegevoegd aan het apicale compartiment (50 of 100 μM). Na 24 uur werd in het basolaterale compartiment LPS toegevoegd aan de THP-1 cellen gedurende zes uur. We vonden dat incubatie met zowel de citrus flavonoïden als hun metabolieten resulteerde in een significante inhibitie van NF-κB activiteit en de productie van verschillende inflammatoire cytokinen, maar niet beschermde tegen immuun-gemedieerde barrière verstoring.

In **Hoofdstukken 4** en **5** zijn de *in vivo* effecten van citrus extract (CE) inname onderzocht in relevante doelgroepen. In **Hoofdstuk 4** werd het effect van CE onderzocht in patiënten met het prikkelbare darm syndroom (PDS), waarbij aanwijzingen waren voor aanwezigheid van (laaggradige) ontsteking in de darm. In deze exploratieve, gerandomiseerde, dubbelblinde, placebo-gecontroleerde, parallelle studie kregen 29 PDS patiënten met een fecale calprotectine waarde tussen de 15 en 150 μg/g feces gedurende acht weken 500 mg CE/dag of placebo. Fecaal calprotectine, fecaal secretoir

immunoglobuline A (sIgA), de fecale microbiota compositie, maagdarmklachten, ontlastingsconsistentie en -frequentie werden onderzocht bij start en na vier en acht weken inname. Toediening van het citrus extract gedurende acht weken bleek geen effect te hebben op de fecale biomarkers voor ontsteking en afweer, de algemene microbiota compositie of klachten in deze studiepopulatie. Vervolgens werd in **Hoofdstuk 5** het effect van dagelijkse CE-toediening onderzocht in ouderen, omdat er aanwijzingen zijn dat het verouderingsproces geassocieerd is met een verminderde afweerfunctie en toename van oxidatieve stress. In dit gerandomiseerde, dubbelblinde, placebo-gecontroleerde, cross-over onderzoek kregen 37 gezonde ouderen (met een leeftijd van 70-85 jaar) 500 mg CE/dag of placebo. Bij start en na vier weken toediening werden markers voor oxidatieve stress en gestimuleerde cytokineproductie gemeten in bloed en markers voor afweer en microbiële metabolieten in ontlasting. De inname van CE gedurende vier weken bleek echter geen significante invloed te hebben op de markers voor oxidatieve stress en afweer of op metaboliet productie. Mogelijk kwam dat deels door het feit dat de ouderen die deelnamen aan de studie relatief gezond bleken en bij start geen duidelijk afwijkende waardes hadden.

Naast citrus flavonoïden zijn er ook verschillende andere functionele voedingsmiddelen die de potentie hebben om maagdarmgezondheid en metabole gezondheid gunstig te beïnvloeden. Het effect van de carotenoïde-producerende *Bacillus indicus* stam PD01 op darmbarrièrefunctie werd onderzocht in **Hoofdstuk 6**, waarbij gebruik werd gemaakt van zowel humane als diermodellen. Als model voor verstoorde barrièrefunctie werden 16 vroeg gespeende biggetjes op willekeurige volgorde toegewezen aan een groep die een controle dieet of een controle dieet met PD01 toegediend kregen gedurende 23 dagen. Daarnaast werden 67 mensen met overgewicht/obesitas, die verder gezond waren, in willekeurige volgorde toegewezen aan groepen die PD01 of placebo kregen gedurende zes weken. PD01 overleefde passage door het maagdarmstelsel en resulteerde in systemische opname van carotenoïden. Verder zorgde PD01 inname voor een verbetering van barrièrefunctie uitkomsten in de vroeg gespeende biggetjes als model voor verstoorde darmbarrièrefunctie, maar werden er geen significante effecten gevonden in mensen met overgewicht/obesitas die verder gezond waren.

In **Hoofdstuk 7** werd het effect van toediening van olijfblad extract (OBE) op cardiometabole risicofactoren onderzocht in mensen met overgewicht/obesitas met een verstoord bloedlipidenprofiel. In deze gerandomiseerde, dubbelblinde, placebo-gecontroleerde, parallelle studie werden 77 mensen met overgewicht/obesitas en totaalcholesterol waarden van 5.0-8.0 mmol/L willekeurig aan groepen toegewezen die 500 mg OBE of placebo kregen gedurende acht weken. Bij start, na vier en na acht weken toediening werd het bloedlipidenprofiel, geoxideerd LDL (oxLDL), de bloeddruk,

glucose en insuline waarden gemeten. We hebben laten zien dat OBE-toediening gedurende acht weken geen significant effect had op het bloedlipidenprofiel (als primaire uitkomstmaat) of op de andere markers gerelateerd aan hart- en vaatziekten (als secundaire uitkomstparameters), in mensen met overgewicht/obesitas en mild verhoogde cholesterol waarden.

In **Hoofdstuk 8** hebben we de hoofdbevindingen van dit proefschrift samengevat. Verder hebben we nieuwe inzichten en implicaties besproken voor verder onderzoek en toekomstige toepassingen voor functionele voedingsinterventies voor het verbeteren van darm- en metabole gezondheid.

Impact paragraph

Over the last century, the mean age and the number of elderly individuals in the general population has increased substantially, in parallel with a global increase in adherence to the Western diet and lifestyle.[1] Both factors have been associated with an increased prevalence of chronic conditions and disorders such as obesity, metabolic syndrome, type 2 diabetes, cardiovascular disease and liver disease.[2, 3] In addition, nutrition and lifestyle related factors can also contribute to the onset and progression of diseases of the gastrointestinal (GI) tract, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).[4, 5] These chronic metabolic and/or inflammatory diseases are associated with a lower quality of life and an increased risk of disease-related comorbidity.[6, 7]

On the one hand, diet can be a risk factor, while on the other hand, nutrition can also be used as a strategy to improve general health, well-being and the course of chronic diseases. A class of nutrition specifically designed to improve health by targeting one or more of the processes involved in disease development and progression are the functional foods. These are foods that offer health benefits beyond their nutritional value. In this thesis, we assessed the effects of two different classes of functional food supplements on intestinal and metabolic health, *i.e*. probiotics and polyphenol-rich extracts. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".[8] Polyphenols are naturally occurring metabolites found in plants with known anti-inflammatory and anti-oxidant effects, of which flavonoids are the largest and most studied group. These interventions were chosen as they have the potential to improve GI and metabolic health via various mechanisms of action. The main mechanistic targets we focused on were intestinal barrier function, microbiota composition, inflammation and blood lipid profiles. Functional foods are merely used as strategy to improve health and well-being or to reduce disease risk. To study the effects in humans, we chose to include elderly individuals and overweight/obese subjects, as they are (relatively) healthy, but at risk for mild disturbances in these outcomes. Furthermore, we included IBS patients with low-grade intestinal inflammation and performed *in vitro* experiments using a model that mimics disruption of intestinal barrier function, which is induced by inflammation.

The studies we performed can contribute to further insight into the health effects of the products we focused on and can provide leads for future studies that are relevant to scientists. In addition, by studying various mechanism related to chronic metabolic and inflammatory diseases, these types of studies can also provide more general insight into the factors that can contribute to the development of certain (pre-) disease conditions,

which is valuable information for researchers but also for healthcare workers involved in direct patient care.

Scientific impact

In the current thesis, we provided an extensive overview of the currently available evidence on citrus flavonoids and their effects on intestinal health (**Chapter 2**). We concluded that citrus flavonoids and their metabolites may contribute to improved GI functioning and health, likely due to a combined effect of the original compounds, of their metabolites, and an interaction with the intestinal microbiome. So far, most of the currently available evidence comes from animal and *in vitro* studies. To improve our understanding of these effects in the human gut, more research, especially in human subjects, is needed. Furthermore, studies are needed that directly compare the effect size of the flavonoids with the metabolites formed during intestinal breakdown, to provide more insight into their relative contribution to the beneficial effects.

In this thesis, we performed four randomised, placebo-controlled clinical trials with functional food supplements in subjects with a mild metabolic or inflammatory disturbance (**Chapters 4-7**). These well-controlled trials are the first step in translating the beneficial effects found in *in vitro* and animal studies into clinical practice. So far, many human studies have selected study populations that are convenient, but not necessarily the target population for the final application, while we wanted to study the effects of food interventions in populations that could ultimately benefit from such products. We performed these studies in subjects that were mainly at risk for metabolic or GI diseases and asked them to maintain their dietary and lifestyle habits. With these studies, we aimed to mimic the real-world settings as closely as possible, providing evidence of effects that can realistically be expected. Because we wanted to gain more insight into several different processes that may be involved, we made use of a combination of various non-invasive measurements in these studies. Overall, the observed effects were modest, but still do provide valuable information. In **Chapter 6**, we found that the carotenoid-producing *Bacillus* strain PD01 did not significantly affect GI permeability in overweight/obese subjects. However, we were able to show that PD01 survived transit trough the GI tract and resulted in systemic carotenoid accumulation, without causing adverse events. In **Chapters 4, 5** and **7,** we did not find significant effects of the interventions (Citrus extract and olive leaf extract) on the main outcome parameters studied. All these studies had in common that the subjects included were still relatively healthy or had only mild disturbances. To determine whether these interventions, but also other functional foods, can contribute to improved metabolic or GI health, we advise future studies to test the application of these interventions in populations with

more pronounced disturbances and to consider using more mechanistic and clinical outcomes.

In addition to performing human studies, we wanted to focus in more detail on the relative contribution of citrus flavonoids and their metabolites to the observed effects. Therefore, we studied the effects of the two main citrus flavonoids hesperidin and naringin and their main metabolites formed during intestinal metabolism on immune-mediated barrier disruption in a well-controlled environment (**Chapter 3**). In this study, we showed that *in vitro*, the flavonoids as well as their metabolites were indeed able to exert comparable anti-inflammatory effects. These findings combined with what we already know about intestinal citrus flavonoid metabolism, provide additional information about where effects can be expected in the intestine after oral intake. Citrus flavonoids are rapidly metabolised by the intestinal microbiota once they reach the colon, with the highest levels being produced in the proximal colon. Therefore, anti-inflammatory effects, due a combination of direct effects of the (metabolised) compounds themselves and those mediated by the microbiota, will likely be most pronounced in the proximal colon. Human intervention studies applying methods to confirm these local effects, *e.g*., by sampling biopsies or faecal material in the different intestinal compartments, are needed. In addition, further studies should also focus on how these effects can be influenced by the composition of the microbiota and potential other (host related) factors (*e.g.,* disease status, intestinal transit time and age).

Societal impact

In the Netherlands, on average more than 50% of the population suffers from one or more chronic disease(s) and this number rises to more than 95% in adults aged 75 years and older.[9] This poses a major public health concern, as the costs associated with treatment of these diseases and societal costs, such as loss of productivity and increased disability, place a large burden on society.[6] The use of functional foods is often preferred as treatment option over pharmaceutical drugs, in part because of their natural origin and the lower risk for side effects. In addition, consumers have become increasingly interested in how food and lifestyle can contribute to their health and well-being. Especially in those cases where it proves difficult to maintain lifestyle changes, such as strict dietary regimen long-term, intake of functional foods may be a more feasible option for consumers. Thereby, effective functional food products can have a significant impact on individual consumer well-being, but also on society in general, by contributing to a healthy lifestyle and the prevention of chronic diseases.

An important aspect contributing to the societal impact of research is effective communication and dissemination of study findings. The findings of studies in this

thesis have been presented during both national and international meetings and congresses. Furthermore, the articles presented in this thesis were all published in peer-reviewed open access journals, ensuring that the study findings are accessible not only to scientists within the field, but also to the general public. This was also the case for the clinical trials with final results that did not confirm or were not in line with our conceived hypothesis. However, we do feel that it is also very important to learn from negative findings. Findings described in this thesis have also been covered by an online news source for the nutrition industry (NutraIngredients) and in press releases of BioActor (the ingredient supplier and funder of the research), which both summarize newly published research in layman's terms. Communication about the effects of functional foods may even improve the awareness of consumers about the importance of nutrition and the role certain foods can play in health and disease in general.

Performing studies with functional foods and publishing the results of these studies is also important for companies focusing on the development of these products and the food industry as a whole. On one hand, scientific evidence, for example from well-controlled intervention studies, supporting the safety and clinical efficacy of products is important for companies to be able to comply with regulations (*e.g.,* to be allowed to substantiate and claim a certain health effect), but also for marketing and product development. On the other hand, collaboration of science and industry will contribute to the translation of scientific findings to successful products by taking factors into account that will impact the applicability of the intervention. This thesis is a typical example of a long-term collaboration between industry and academic partners, providing us with the opportunity to perform several well-designed clinical trials with relevant products. A crucial factor that determines how successful functional foods can become at preventing or improving a disease course, is consumer acceptance. This factor was taken into consideration by us already in the conceptualisation phases of the research performed within this thesis. Not only by performing consumer research in parallel to the clinical trial, but also by choosing a study product that can realistically be ingested on a daily basis (both in terms of convenience and price). The polyphenol interventions in particular are suitable for the development of products with high compliance and consumer acceptance rates. By using extracts with a standardized high content of the bioactive compounds, the total volume can be kept low. Furthermore, the extracts we used can be stored at room temperature for extended periods of time. Together, this will increase the potential product applications as the extract can be easily offered as capsules, effervescent tablets or added to products such as drinks with limited impact on palatability.

Conclusion

In this thesis steps have been taken in the search for effective nutritional strategies to improve GI and metabolic health, by performing several trials with different functional food supplements in relevant populations and models and by communicating these findings via various channels accessible to both scientists and the general public. This thesis has shown that more human studies with food interventions are needed, not only in populations with mild but also with more pronounced health disturbances, and should include more mechanistic and clinical outcomes.

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Stevens Y, de Bie T, Pinheiro I, Elizalde M, Masclee A, Jonkers D. The effects of citrus flavonoids and their metabolites on immune-mediated intestinal barrier disruption using an *in vitro* co-culture model. Br J Nutr. 2022:1-24.

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Curriculum Vitae

Yala Ria Alexandra Jan Stevens was born on August 2nd 1989 in Maastricht, the Netherlands. After graduating from the Bernard Lievegoed College in Maastricht in 2009, she studied Health Sciences at Maastricht University. During her bachelor she was awarded the Top 3% award 2010/2011 and participated in the Honours programme "International Health" including the facultative course "orientation to the health system in India", which took place in Bangalore, India. In 2012, she obtained her BSc degree and started the two-year MSc programme Health Food Innovation Management at Maastricht University, Campus Venlo. For this programme, she was selected as a UM scholarship student. After receiving her MSc degree in 2014, she started working as a clinical research scientist at BioActor BV. In 2016, she started as PhD candidate at the department of Internal Medicine, division Gastroenterology and Hepatology, within NUTRIM, School of Nutrition and Translational Research in Metabolism, in addition to her position at BioActor. This PhD track was part of a collaboration with BioActor BV and was supervised by Prof. Dr. D.M.A.E. Jonkers and Prof. Dr. A.A.M. Masclee. Currently, she is working as Chief Scientific Officer at BioActor BV.