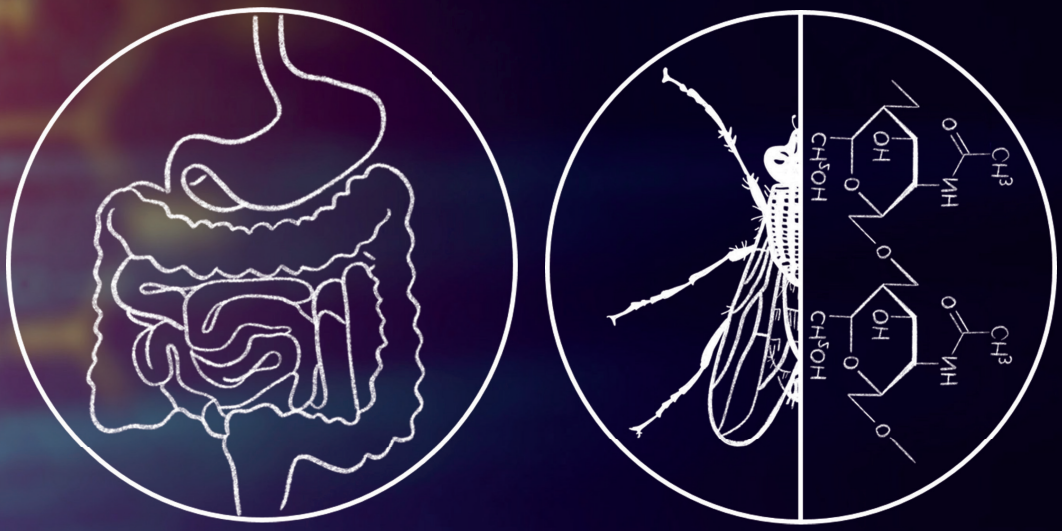
Intestinal health supportive effects of insect derivatives

In vitro studies towards immune activity, epithelial protection and microbiota modulation



Liyou Dong

Propositions

1. Insects are preferred alternatives to antibiotics to treat foodborne diseases.
   (this thesis)
2. The size of chitin particles is its most defining characteristic regarding immunomodulatory activities.
   (this thesis)
3. Not recommending vitamin D supplementation by the Dutch Health Council to decrease the chance of contracting COVID-19 (Dutch Health Council, Report number 2021/09) is a missed opportunity to lower the reproduction number by 1/3 (Ma H, American Journal of Clinical Nutrition, 2021).
4. To prove that faecal microbiota transplantation is a potential treatment to resolve multiple diseases in one shot, this should be tested on an appropriate patient group (Smits, Gastroenterology, 2013).
5. Food science requires a better communication because additives still cause consumer's panic.
6. COVID-19 is more efficient than science to change people's behaviour.

Propositions belonging to this thesis, entitled

Intestinal health supportive effects of insect derivatives: In vitro studies towards immune activity, epithelial protection and microbiota modulation.

Liyou Dong

Wageningen, 21 May 2021

Intestinal health supportive effects of insect derivatives: *In vitro* studies towards immune activity, epithelial protection and microbiota modulation

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Intestinal health supportive effects of insect derivatives: *in vitro* studies towards immune activity, epithelial protection and microbiota modulation

Liyou Dong

Thesis

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Chapter 1

General Introduction

1. Insects as sustainable and health food

According to the Union Nations Department of Economic and Social Affairs (UN-DESA) report, the world population is expected to reach 9.7 billion in 2050 [1]. To feed this huge population, the Food and Agricultural Organization of the United Nations (FAO) claimed that the world food production needs to rise with 70% [2]. The increased requirement for food production is not only a challenge to the current food production system, but also for our environment. With this increased requirement for food production, an intensification of agriculture is needed, which will lead to even increased environmental burdens such as land degradation, water usage, greenhouse gas emission and global warming [3]. All these problems put constraints on food production and threaten the future generations.

To resolve these problems, food industries, government regulators and academic researchers focus on low environment-impact, or sustainable, food production systems. A sustainable food system is defined as the delivery of foods that have a sustainable effect on economic, social and environmental systems and do not compromise available resources for future generations [4]. The use of insects as a source of food or food ingredient is expected to contribute to sustainable food supplies. The production of insects has a relatively low environmental impact and is more sustainable than the conventional food production system. Insects are cold blooded and do not require much energy to maintain their body temperature, so the food conversion ratio is more efficient than for warm-blooded animals [5]. Moreover, rearing insects leads to fewer greenhouse emission when compared to the production of milk, pork, chicken and beef production [6, 7].

In addition to the sustainability of food production, also the health impact of food on human is an important parameter to take into consideration. With the increase of the quality of our life, the requirements of food are already shifting from the old concept of avoiding nutrient deficiency to the concept of optimal and beneficial nutrition. In particular driven by the growing awareness that a large number of diseases, such as obesity, diabetes and cardiovascular diseases are linked to western-type diets and can be prevented or treated by consuming healthier and more balanced foods [8]. Hence, we need food that can meet our nutritional requirements but also support health or decrease the risk of diseases. Insects might be a relevant novel food source from this perspective as they contain many bioactive compounds that are beneficial for human health.

2. Nutritional content of insects

Human insect consumption is common in most parts of the world, and this habit is termed entomophagy [9]. Since prehistoric times, humans have consumed the eggs, larvae, pupae and adults of insects as food and this trend has continued in modern time [10]. One-third of the world population, located mainly in Central and South America, Africa, and Asia consumes nearly 2000 different species of insects [11]. Insects have high market values because their high nutritional value [10]. Insects are rich in protein, fats and fibre which are indispensable nutrients for humans. Even though the nutrient content varies per species, researchers have concluded that insects in general can provide enough energy, protein (*i.e.* meeting the requirements of essential amino acid), fats, fibres and other micronutrients for humans [10].

2.1 Insect proteins

The crude protein content of insects varies from 20-76% of dry mass depending on the species and developmental stage [10]. Insect protein has been extensively investigated as an alternative protein source to conventional animal-based proteins. These protein sources, such as eggs and milk, are considered to be high quality protein sources because of their attractive amino acid composition and high digestibility [12]. Protein from insects match this high quality as they are also efficiently digested and contain high concentrations of essential amino acids [13]. According to the overall nutritional value, some insect protein isolates are comparable or superior to soy protein, which is a plant protein commonly consumed by humans [14]. Of note, insect protein has already been used as a protein source for different livestock species [15, 16], so is indirectly already contributing to our nutrition. In addition to the nutritional value, proteins from insects were reported to have many beneficial health effects [17]. For example, protein hydrolysates or peptides from *B. mori*, *B. terrestris*, *S gregaria* and *S. littoralis* were demonstrated to have an antihypertensive effect by inhibiting angiotensin converting enzyme binding [18] and male silkmoth protein hydrolysates contain antioxidant activity due to strong radical scavenging ability [19]. In addition, insect peptides were also characterized to exert antimicrobial and immunomodulatory properties [17], of which a more detailed description is outlined in section 4. Thus, insect-derived protein does not only provide high quality proteins, but also supports the health of the consumer by serving as a source of bioactive peptides.

2.2 Insect fats

Fat is another main component of insects and the fat content varies from 10-60% of dry matter, depending on the species and developmental stage of insects [10, 20]. Insect-derived fats contribute to human health by supplying energy and essential fatty acids such as omega-3 and omega-6 fatty acids, which cannot be synthesized in human but needed for biological process [21]. The energy content of insects is on average comparable to that of meat, except for pork because of its particularly high fat content [21]. According to the fatty acid profile of insects, they are a good source of unsaturated fatty acids which are able to influence activity of cell membrane proteins such as receptors and ion transporters and modify the activity of transcription factors include NF-κB [22]. The ratio of unsaturated fatty acids to total fatty acids in insects is similar to that in poultry and fish, but insects contain more polyunsaturated fatty acids [23].

In general, insect fatty acids are associated with multiple health effects and have been reported to contain anti-microbial and anti-inflammatory effects. Regarding to the antimicrobial effects, a recent *in vitro* study showed that black soldier fly fat from prepupae suppressed the growth of D-Streptococci [24]. Also, lauric acid that can be found from black soldier fly has been shown to possess anti-inflammatory effects against *Propionibacterium acnes* infection [25]. More detailed information on beneficial effects of insect fatty acids will be described in the section 4.

2.3 Insect fibre

Insects contain significant amounts of fibre of which chitin is dominant in insects [10]. Chitin is a polymer of N-acetyl-glucosamine and constitutes the main component of the exoskeleton of insects [13]. Solid nuclear magnetic resonance (NMR) and gravimetric analysis showed that chitin content in insects can reach up to 40% of the dry mass depending on the species of insects [26]. In addition to insects, chitin can also be found in the shell of crustaceans as well as the cell wall of bacteria and fungi [27].

Chitin has been extensively studied for its beneficial impacts for humans and animals. Clinical studies have demonstrated that chitin can reduce the risk of cardiovascular diseases by lowering the serum cholesterol and support wound healing by enhancing the formation of fibroblasts [28]. For animals, many *in vivo* studies have reported that chitin can activate the immune system

through different mechanisms and exert anti-inflammatory effects through reducing the secretion of pro-inflammatory cytokines such as IL-1β and IL-6 [29]. Moreover, an *in vitro* study showed that chitin promotes the proliferation of beneficial bacteria *Lactobacillus rhamnosus* GG and inhibited the growth of pathogenic bacteria *Escherichia coli* TG [30, 31]. More detailed beneficial effects of insect chitin will be described in the section 4.

2.4. Insect species available as feed and food

Despite that insects have been highlighted as a food source to address environmental, economic and health concerns regarding nutrition, and nearly 2000 insect species are consumed, very few insects are currently reared on an industrial scale. The ones that are bred on a large scale include mealworms (*Tenebrio molitor*), crickets (*Grylloidea*) and black soldier fly (*Hermetia illucens*) larvae [32]. In particularly for rearing of mealworm it is known that this requires less water, produces less greenhouse gas emission, and results in a more efficient feed conversion ratio when compared with conventional meat production [6, 7]. Similarly, black soldier fly is also characterized with an efficient feed conversion rate and it can be reared on low-value organic by-products [33]. The latter is also a benefit considering that the global annual production of organic by-products was estimated to be 1.3 billion tons [33]. So far, *H. illucens* larvae have been extensively used for feeding animals [6, 34]. As a result, these two species were studied in this thesis and the nutritional composition of these two insects is listed in Figure 1.

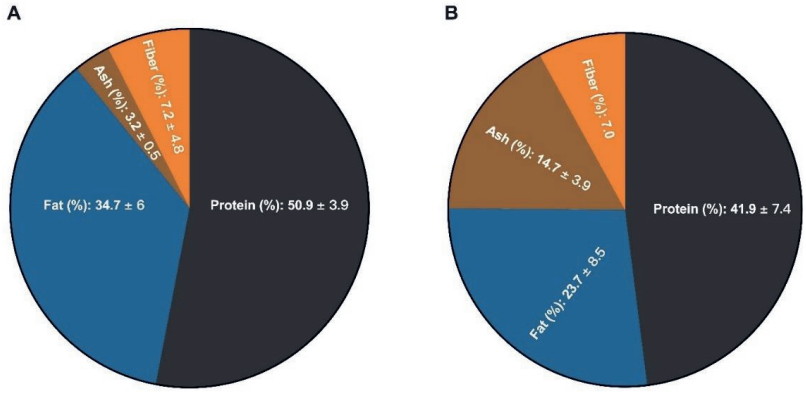

Figure 1: Nutritional composition of mealworm larvae (n=7) (A) [13, 21, 35, 36] and black soldier fly larvae (n=6) (B).

3. Maintaining immune homeostasis in the gastrointestinal tract

The human gastrointestinal (GI) tract is composed of hollow organs, jointed in a long twisted tube spanning from mouth to anus [37]. The major function of the GI tract is to transport, and at the same time digest and absorb food components. Digestion through enzymes occurs in the mouth, stomach and intestine and nutrients are absorbed in the intestine by intestinal epithelium and transported to different parts of the body via the bloodstream. Food components that are not directly absorbed reach the colon and they can be fermented by microbiota presented in the lumen of the gut. The GI tract is efficient in extracting nutrients from food and excreting non-nutritional components and uses a large surface area to do so [37]. In addition to nutrient uptake in which the epithelial cells play a crucial role, this large surface area also needs to be protected from pathogenic entry. Again, epithelial cells are crucial by providing a physical (physicochemical) barrier by forming a mucus layer which covers the intestinal epithelial cells and a cellular layer composed of intestinal epithelial cells including enterocytes, M cells, Paneth cells, goblet, enteroendocrine cells and microfold (M) cells. Below this first line of defence, there is an immune barrier that contains immune cells lining every part of the gut. Moreover, host and microbiota cooperate to maintain an ecosystem that can prevent outgrowth of pathogenic or invasive microbiota [38].

3.1 Intestinal mucus barrier

The mucus layer is the first layer of the intestinal barrier, covering the intestinal epithelial cells and protecting the intestine from pathogens. It is a hydrated gel, formed by mucins that are secreted by goblet cells and enterocytes located in the epithelium [39]. Mucins are highly viscous proteins that can trap luminal content [40]. The morphology of the mucus layer, in particularly the thickness, is different in small and large intestine. The mucus layer is the thinnest in the small intestine and the thickest in the colon (Figure 2) [41]. The mucus contains two layers being the outer layer and inner layer. The outer layer is loose and penetrable which provides a habitat for gut microbiota, such as *Akkermansia* [42]. The inner layer is more dense and impenetrable and it is rich in antimicrobial peptides, such as human α-defensins, and immunoglobulins such as IgA, that provides protection of the epithelium against luminal bacteria [39, 43]. The importance of the mucus layer has been shown in a murine study. In these mice, a main constituent of the mucus layer, the Muc2 protein, was knocked-out which resulted in the spontaneous development of colitis [44].

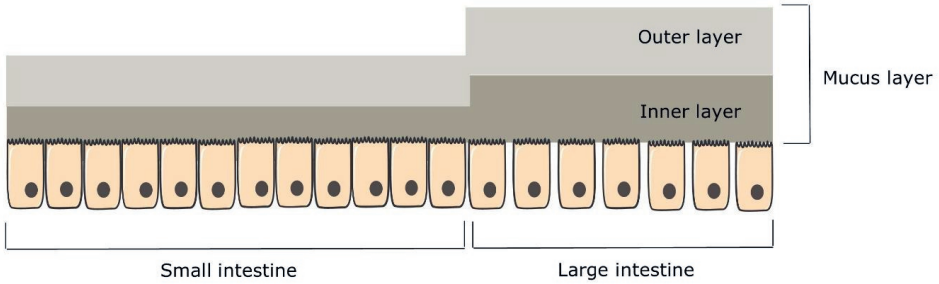

Figure 2: Structure of the mucus layer in small and large intestine. The intestinal mucus layer is composed of an inner and an outer mucus layer. The thickness of mucus layer varies with regions in intestine of which small intestine contains a thinner mucus layer when compared with the large intestine. Figure adapted from [41].

3.2 Intestinal cellular barrier

The intestinal epithelium is the largest surface area of the body that is exposed to the external environment [45]. It is a single cell layer that provides a physical and biochemical barrier to separate the lumen content from the underlaying lamina propria [46]. The intestinal epithelial cells are composed of absorptive enterocytes, secretory Paneth-, goblet- and enteroendocrine cells and antigen sampling microfold cells (M cells) (Figure 3). Each of these cells has a specific function in the maintenance of intestinal health and homeostasis. Specifically, intestinal enterocytes, the dominant population of intestinal epithelial cells (>80%), are essential for maintaining the cell polarity which forms the basis of integrity of the epithelial barrier [47]. Moreover, intestinal enterocytes also participate in digestion, transport of macromolecules, endocytosis of nutrients and regulation of mucosal immune response [47]. Intestinal enterocytes express pattern recognition receptors (PRRs) allowing these cells to interact with pathogen or microbe-associated molecule patterns (PAMPs or MAMPs respectively) which are the conserved structures on pathogens or commensal microbes [48]. Binding of luminal molecules to PRRs on epithelial cells could also induce the production of cytokines and chemokines including TSLP, IL-8, CXCL10, IL-18, IL-33, TGF-β, which are critical for maintaining the intestinal immune homeostasis [49, 50]. Through these molecules, intestinal enterocytes possess the potential to directly signal to the immune cells residing in the lamina propria.

Enteroendocrine cells, a type of secretory cells making up 1% of the intestinal epithelial cell population, support the physical and homeostatic function of the intestinal epithelium by secreting multiple hormones and peptides such as glucagon-like peptide 2 (GLP-2) [51]. GLP-2 supports the intestinal barrier function through different mechanisms, including enhanced proliferation of intestinal epithelial cells or the expression of tight junction proteins that linked epithelial cells together [52]. Paneth cells, another type of secretory cells, secretes antimicrobial peptides (AMPs), such as defensin, cathelicidins and lysosome, that prevents bacterial overgrowth or translocation [53, 54]. The third type of secretory cells are goblet cells which mainly contribute to the formation of gel-like mucus by synthesizing and secreting mucins.

Finally, microfold (M) cells are non-specific cells playing a key role in the intestinal immune response. They are located in follicle associated epithelium (FAE) and cover mucosa associated lymphoid tissue such as Peyer's patches [55, 56]. Even though it is uncertain whether M cells sampling or transcytosis of luminal microorganisms predominantly occurs in a non-specific manner, in a specifically receptor mediated endocytosis or both, many studies showed that M cells express a variety of receptors on their apical site [57]. For example, M cells recognize luminal pathogenic microorganisms such as *E. coli* by expressing glycoprotein 2 as a surface receptor and actively engulf pathogens via the apical surface and exocytose them through the basolateral membrane. This process facilitates the delivery of luminal pathogens to mononuclear phagocytes such as dendritic cells, macrophages, and B cells that are located in the Peyer's patches to initiate immune responses when necessary [55].

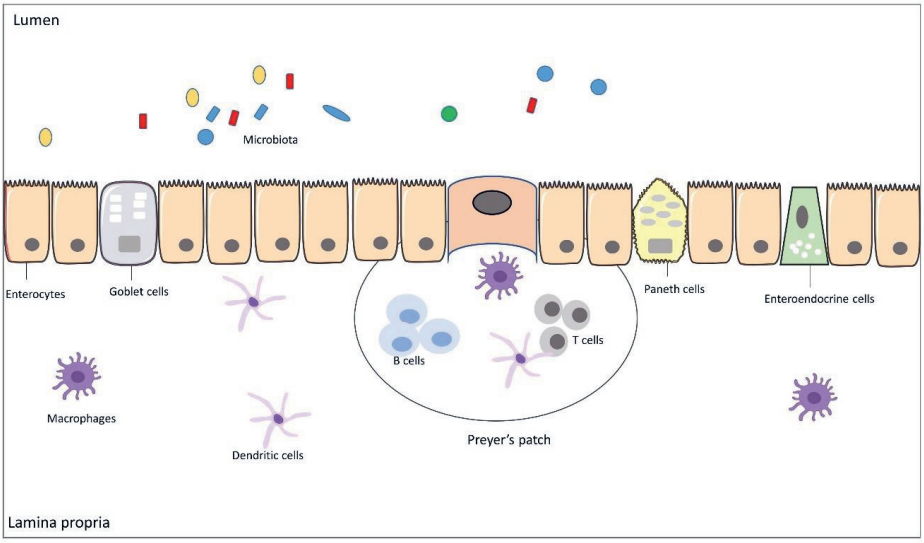

Figure 3: Composition of intestinal epithelial cell lining in the gut. Picture adapted from [50].

To prevent leakage of luminal content towards the lamina propria, the intestinal epithelial cells are tightly connected and sealed by a complex of proteins and junctional systems such as tight junction proteins, adhesion proteins and desmosomes [58] (Figure 4). Tight junction proteins are composed of transmembrane proteins such as claudins, occludins and zona occludens [59]. Tight junction proteins are situated at the luminal part of the junction between cells and act as a fence to seal the paracellular space. Through the tight junction proteins, cells can block the free diffusion of proteins and lipids and regulate the entry of nutrients, ions, water and intestinal microbes [60]. Compared to tight junction proteins, adherens junction proteins and desmosomal proteins are thought to be more important in the mechanical connection of adjacent cells. The structure of these junctional complexes are constantly shaped by the external stimuli and associated with the health status of the host [61, 62]. Disruption of tight junctions results in an increased permeability of the intestinal barrier which consequently elevates the translocation of luminal contents to the lamina propria, which could contribute to intestinal-related diseases such as inflammatory bowel disease and Crohn's disease [63].

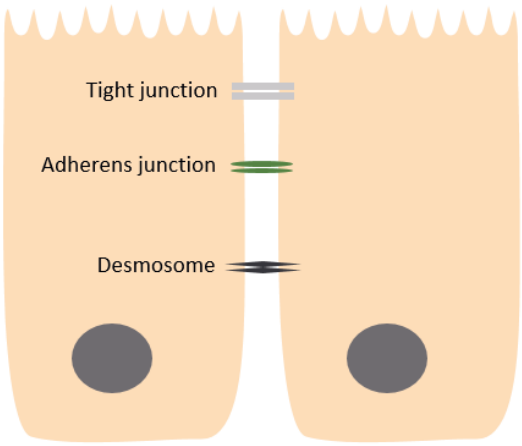

Figure 4: Intestinal epithelial cells junctional complexes. Intestinal epithelial cells are connected by junctional complexes, including tight junction proteins, adherens junction proteins and desmosomes. Tight junctions and adherens junctions are located near the luminal side, and desmosomes are located close to the lamina propria side. Figure adjusted from [64].

3.3 Intestinal immune barrier

Our intestine contains the largest number of immune cells of any tissue in the body [56]. These immune cells are located in several different structures in and around the gut, such as Peyers' patches, isolated lymphoid follicles and mesenteric lymph nodes, but are also present within the local environment in the lamina propria, and intraepithelial lymphocyte compartment [65]. As indicated above, M cells are linked to Peyers' patches to sample the luminal content. However, exposure to immune cells also occurs in the luminal space as CX3CR1+ dendritic cells or macrophages can directly sample luminal pathogens or microbes [66]. Macrophages, in fact, represent the largest population of mononuclear phagocytes in the lamina propria [67]. Like other tissue macrophages, intestinal macrophages are essential for housekeeping functions such as clearance of apoptotic cells and debris [68]. In addition, intestinal macrophages are also important for regulating intestinal homeostasis by secreting various soluble factors, such as PGE2, which can promote the proliferation of intestinal epithelial cells [69]. Furthermore, the protective function of intestinal macrophages is also important. Resident intestinal macrophages are highly phagocytic which allows them to take up pathogens or microbes that

breach the intestinal epithelial monolayer. Macrophages express a multitude of receptors that can rapidly recognize conserved structures of invading pathogens with their wide array of toll-like receptors (TLRs), such as lipopolysaccharide (LPS) on the surface of Gram-negative bacteria, RNA/DNA fragments, and bacterial flagellins [70]. In addition, due to the high surface expression of the MHC-II molecule and endocytic capacity, macrophages are also one of the local antigen-presenting cells and can impact differentiation of CD4+ T cells [71]. Furthermore, it has been suggested that intestinal macrophages may contribute to the maintenance of other T cells in the mucosa. For example, intestinal macrophages can produce IL-1β or IL-10 upon TLR stimulation, and these cytokines can support the development of Th17 and Treg cells, respectively, in the steady state intestine [72].

Taken together, the mucus, cellular and immune barrier of the intestine serves as a gatekeeper for harmful substances and pathogens from the external environment and control host defences to maintain intestinal homeostasis.

3.4 Gut microbiota

The human body coexists with a vast number of microbes, including bacteria that colonize the skin and gut, archaea, viruses and unicellular eukaryotes, commonly referred to as the microbiota [73]. Given all the microbiota in the human body, more than 70% of these inhabit our gut [74]. Gut microbiota can be divided into at least 7 phyla, of which *Bacteroidetes* and *Firmicutes* are the majority of the population (60% - 90%), and *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria* and *Verrucomicrobia* represent a minor proportion of the total microbial load [75, 76]. The number of gut microbiota varies in different regions of the GI tract and ranges from 10^1 to 10^3 bacteria per gram in the stomach, increasing to 10^4 to 10^7 per gram in the small intestine and reaching 10^{11} to 10^{12} per gram in the large intestine [76].

Gut microbiota live in a mutualistic relationship with the host. The hosts provide a habitat and energy for the normal growth and development of intestinal microbiota. Some bacterial species can use mucins in the mucus layer as the energy sources and some species can obtain energy from the fermentation of indigestible carbohydrates [77]. Fermentation of these carbohydrates results in production and secretion of short chain fatty acids (SCFAs) such as acetate, butyrate and propionate, which in turn are absorbed by the host or utilized by other bacterial species [78]. SCFAs are associated with many beneficial health effects for the host. For instance, butyrate is

used as energy source by the intestinal epithelial cells to support proliferation. Furthermore, acetate can be absorbed and enter the peripheral circulation to be metabolized by muscles or liver and improve the insulin sensitivity and oxidative capacity. Finally, propionate can be absorbed by enterocytes, passing to the portal vein circulation and being metabolized in the liver for glycogenesis [79, 80]. The importance of microbiota for hosts has been confirmed by studies carried out with germ-free mice. These mice suffer from vitamin (B, K) deficiencies, decreased total mass of intestine, decreased activity of intestinal macrophages, decreased IgA secreting plasma cells, lower immunoglobulin level, and increased susceptibility to pathogenic bacteria such as *Shigella flexneri*, and *Bacillus anthracis*-infection when compared with non-germ-free mice [81, 82].

Even though gut microbiota are associated with many beneficial health effects, they, however, can also be a potential driver for pathogenesis [83]. Gut microbiota are consistently shaped by both internal factors such as gene expression and age and external factors such as diets and drugs [84]. In particularly, external factors such as drugs and antibiotics can disrupt the ecological balance of gut microbiota, resulting in the overgrowth of some pathogenic or opportunistic bacteria and consequently cause intestinal diseases. A disruption of balance in gut microbiota has been identified in many inflammatory and metabolic disorders including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), obesity, diabetes, and colon cancer [85].

3.4.1 Intestinal pathogens

Intestinal diseases are normally a result of overgrowth by pathogenic or opportunistic bacteria that are a member of a normal microbe community, such as *E. coli*, *Salmonella* and *Clostridium*. Intestinal pathogenesis often is accompanied with symptoms of diarrhoea, fever and acute abdominal pain [86]. In particular the *Clostridium* genus contains many opportunistic and pathogenic species with significant societal impact through a burden on healthcare through diarrhoea and life-threatening colonic inflammation in humans [87]. *C. difficile* is such a species with the ability to survive the harsh environment (*e.g.* low pH) in the upper digestive tract. Once the bacteria reach the gut, the pathogenesis is linked to its germination and toxin secretion. *C. difficile* produces two enterotoxins, called toxin-A and toxin-B, which target epithelial cells. Following the endocytosis of toxins, epithelial cells undergo necrosis, resulting in loss of barrier integrity [88]. With the increase of intestinal barrier permeability, the luminal microbes can invade the lamina propria where immune cells are patrolling and incite inflammation [88].

C. perfringens is another pathogenic species from the *Clostridium* genus that can induce both systematic and intestinal diseases. It is one of the most common pathogenic bacteria that causes foodborne diseases including food poisoning and necrotic enteritis due to the production of toxins [89]. All types of *C. perfringens* combined can produce more than 20 toxins (Table 1) including enterotoxins, alpha, beta, epsilon, iota, enterotoxin and necrotic enteritis B-like (Net-B) toxin [90]. The variability of the production of these toxins provides the basis for the classification of these bacteria. *C. perfringens* can be classified into seven types [89, 91]. Both A type and C type *C. perfringens* can induce food poisoning, and A type *C. perfringens* induces intestinal diseases that rank as the second most common foodborne disease in most developed countries [92]. Similar as to *C. difficile*, A type *C. perfringens* produces enterotoxins that breaches the intestinal epithelial cells by damaging tight junction proteins and therefore cause intestinal pathogenesis [91]. In addition, A type *C. perfringens* also produces Net-B toxin which is associated with the pathogenesis of necrotic enteritis in broilers and pigs. Necrotic enteritis is a devastating disease with a high mortality rate for animals and consequently leads to a significant economic loss in farm industries [93]. When compared to A type *C. perfringens* infections, C type *C. perfringens* food poisoning is relative rare but a more severe illness with a mortality rate up to 25%, even when treated. Over the years, antibiotics have been used to control *C. perfringens* infections, but along with the ban of antibiotic usage in livestock industry, searching for effective alternatives to antibiotics target *C. perfringens* is needed [94].

Table 1: Classification of *C. perfringens* based on toxins production

C. perfringens type	α-toxin	B-toxin	E-toxin	I-toxin	CPE	Net-B
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	±	-
D	+	-	+	-	±	-
E	+	-	-	+	±	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

4. Beneficial effects of insect-derived fractions on intestinal health

Both clinical, animal and *in vitro* studies have demonstrated that inclusion of insects in diets supports intestinal health [95-97]. The supportive impact is mainly due to the improvement of intestinal barrier function, regulation of mucosal immune response, reduction of pathogenic bacteria infection and enhancement of gut microbiota fermentation. Meanwhile, crosstalk between these elements further prevents pathogen infections. The impact of insects or insect-derived bioactive compounds on each element is discussed below.

4.1 Impact of insect-derived bioactive compounds on intestinal barrier function

Insect-derived peptides have been shown to improve intestinal barrier integrity via various mechanisms. First, the CopA3 peptide, derived from the coprisin peptide from the Korean dung beetle, was shown to increase the proliferation of colonocytes in both *in vitro* and *in vivo* settings [98]. After 48 h exposure of human colonocytes (HT29 cells) to CopA3 peptides, the proliferation of the cells increased by 20% when compared with control. In line with that, oral administration of CopA3 to mice has significantly increased the proliferation of colonic epithelial cells in the crypt region. Additionally, the authors found that the mucosal barrier function was significantly increased in CopA3 treated mice when compared with control mice, which was indicated by a reduced translocation of fluorescein-labelled dextran from colonic loop to blood. The underlying mechanism was due to the ability of CopA3 to reduce the expression of $p21^{Cip1/Waf1}$, a cyclin-dependent kinase inhibitor in colonic epithelial cells to trigger cell cycle arrest and apoptosis [98, 99]. Second, this group identified another peptide coprisin in Korean dung beetle and found a coprisin analogue that can prevent *C. difficile*-mediated mucosal damage due to its anti-*C. difficile* property [100]. Third, the cecropin A peptide from *Hyalophora cerropia,* was also found to increase the integrity of intestinal epithelial cells [101]. In this *in vitro* study, cecropin A increased the transepithelial electrical resistance (TEER) and decreased paracellular diffusion of fluorescein-labelled dextran. The significantly increased expression of tight junction proteins (*i.e.* ZO-1, claudin-1 and occludin) were attributed to cecropin A activity. The underlying mechanisms result from the inhibition of mitogen activated protein kinase (MEK)/extracellular signal regulated kinase (ERK) signalling pathway induced by cecropin A. The MEK/ERK signalling pathway is important to regulate cell proliferation, differentiation and apoptosis [102]. It has been shown that inhibition of MEK/ERK pathway increased expression tight junction proteins in Caco-2 cells [101]. So far,

limited studies have been performed on insect-derived chitin and fatty acids. **In chapter 4,** we have shown that chitin from shrimp did not affect the integrity of colonic and small intestinal epithelial cells by using a Caco-2 cell line.

Taken together, insect-derived peptides were the main compounds that support the barrier function of intestinal epithelial cells. However, the intestinal barrier function also can be affected by the intestinal immune response [103] and gut microbiota metabolism [104].

4.2 Modulation of intestinal immune response by insect-derived bioactive compounds

An increased permeability of the intestinal barrier is commonly observed for intestinal inflammatory diseases such as IBD [103]. Many insect proteins have been studied for their modulatory effect of the intestinal immune response [98, 101]. A murine study claimed that the CopA3 peptide can ameliorate the symptoms of dextran sodium sulphate (DSS) induced colitis [98]. The authors showed that CopA3 reduced the production of proinflammatory cytokines TNF-α and IL-6 in the colon when compared with control group. The anti-inflammatory effect has also been found in another insect-derived peptide cecropin, a peptide from *Hyalophora cerropia.* The peptide mitigated *E. coli*-mediated inflammatory responses by reducing the expression of proinflammatory cytokine genes such as TNF-α, IL-6 and IL-8. The authors attribute the anti-inflammatory effects to the ability of cecropin to bind *E. coli*, resulting in a reduced *E. coli* adherence [101].

In addition to insect protein, chitin has been extensively researched for its ability to beneficially impact intestinal health by modulating immune responses [105]. Many murine studies have demonstrated that chitin, depending on its physicochemical properties and source, can support intestinal health [105-107]. Kazuo and colleagues have found that chitin nano-fibrils have an anti-inflammatory impact on mice that were treated with dextran sodium sulphate (DSS) to induce acute ulcerative colitis (UC). The chitin acts by decreasing serum monocyte chemotactic protein-1 (MCP-1) secretion via suppressing NF-κB activity, indicated by an immunohistochemical detection. Moreover, the authors also showed that pre-treatment with chitin nano-fibrils, before DSS-mediated UC, can improve clinical symptoms, prevent tissue injury and inhibit inflammation. This was shown to be mediated by a suppression of myeloperoxidase activation in the colon and decreasing serum IL-6 concentrations [106, 108]. Taken together, the anti-inflammatory effects demonstrated for chitin make this compound, and insects in general, promising agents as feed and food.

Finally, insect fatty acid has been shown to support intestinal immune barrier function as well. Different insect species were found to contain different types of fatty acids. For instance, the black soldier fly was found to be particularly rich in medium-chain lauric acid and mealworm was found to be rich in long-chain linoleic acid [109]. Lauric acid has been demonstrated to possess anti-inflammatory activity [25]. It significantly suppressed *Propionibacterium acnes*-mediated inflammation through reducing mitogen-activated protein kinase (MAPK) phosphorylation and NF-κB expression, and thereby regulated proinflammatory responses [110].

In summary, insects contain bioactive components that can regulate the intestinal immune response. Together with the supportive effect on intestinal barrier, insect diet was shown to have the potential to support the intestinal health.

4.3 Antimicrobial effects of insect-derived bioactive compounds

The antimicrobial effects of insect-derived protein, fatty acids and chitin has been extensively studied and these bioactive components have been reported to contain antimicrobial properties. For insect proteins, around 300 antimicrobial peptides have been identified. These antimicrobial peptides (AMPs) have been classified into 4 subtypes based on their structure: alpha-helical peptides (*e.g.* cecropin and coprisin), cysteine-rich peptides (*e.g.* insect defensin), proline-rich peptides (*e.g.* apidaecin and drosocin) and glycine-rich peptides (*e.g.* gloverin) [111]. The mechanism by which AMPs exert their antimicrobial activity involves a variety of models including membrane disruption, interference with bacterial metabolism, and depletion of cytoplasmic components [112]. The primary antimicrobial action of AMPs is to target the bacterial membrane lipids. As most AMPs are cationic, they can bind to anionic phospholipids and phosphate groups of LPS of Gram-negative bacteria, as well as to the peptidoglycan layer of Gram-positive bacteria [112]. The efficiency of their antimicrobial activity depends on differences in lipid composition and negative electrical potential of the target membrane.

In addition, insect fatty acids have been demonstrated to contain antimicrobial properties. For instance, lauric acid can supress the growth of Gram-positive bacteria such as *Streptococcus mitis* and Gram-negative bacteria like *Fusobacterium nucleatum* [113]. Even though the underlying mechanism is not fully understood, it is known that the antimicrobial activity of fatty acids is linked to the reduction of cellular pH, and the ability to disassociate. The undissociated forms of medium-chain fatty acid can penetrate the lipid membrane of bacterial

cells. Once internalized, the neutral pH of the cell cytoplasm induces the disassociation of the medium-chain fatty acids into anions and protons. To maintain a neutral pH, bacterial cells will export excess protons, which drains their energy resources [114].

Different from insect AMPs and fatty acids, chitin, as the main compound of insect exoskeleton, exert its antimicrobial effect though indirect modes. An *in vivo* study showed that daily administration of chitin to Atlantic salmon (*Salmo salar*) could increase the abundance of *Lactobacillus* spp., a major part of lactic acid producing bacteria. As a result, an increase in lactic acid producing bacteria suppressed the growth of four fish pathogens [115]. Moreover, the immunomodulatory ability of chitin can enhance the anti-pathogenic capacity of innate effector cells such as macrophages. In example, it was shown that chitin can improve the phagocytic and lysozyme activity of immune cells to prevent *Aphanomyces invadans* infection in fish [116].

4.4 Effect of insect-derived bioactive compounds on gut microbiota metabolism

Gastrointestinal microbiota also play a key role in regulating immune responses and intestinal barrier integrity to prevent infection of pathogenic microorganism [117]. Many animal studies showed that adding insects to diets can positively regulate the metabolism of gut microbiota and stimulate the colonization of probiotics and commensal bacteria such as *Bifidobacterium* and secretion of SCFAs [96, 117, 118]. For example, a broiler study demonstrated that inclusion of *H. illucens* in diets increased the diversity of gut microbiota, relative abundance of SCFAs producing bacteria such as *Bacteroides* and *Firmicutes*, and SCFAs secretion [96]. SCFAs secretion, in particularly butyrate, supports intestinal health and reinforces the intestinal barrier function through two different mechanisms. Firstly, as described in 3.4, butyrate serves as energy substrate for colonocytes, which promotes their proliferation and differentiation [119]. Secondly, butyrate has an anti-inflammatory effect and it mitigates mucosal inflammation by reducing the expression of NF-κB, a transcription factor regulating many cellular genes involved in inflammatory response, such as IL-1β, IL-6, IL-8, IL-12 and TNF-α [120]. Even though the underlying mechanisms for the beneficial effects associated with insect diet were not clear, it was proposed that insect-derived chitin plays an important role. Insect chitin might serve as the substrate for gut microbiota as demonstrated in a broiler study [96]. The authors found that the expression of enzymes that degrade and deacetylate chitin were significantly increased when compared with the control group. Moreover, the bacterial species (*i.e.*

F. plautii, *C. minuta* and *A. transvaalensis*) that contribute to the increased enzyme levels, were also associated with increased secretion of SCFAs, indicating chitin might be fermented by certain species of microbiota and induce the secretion of SCFAs.

5. Outline of the thesis

Insects or insect-derived products have been used as feed for livestock industries for a long time because their highly nutritional value. With the rapid increase of the human population on this planet, an increasing amount of insects or insect-derived products has been applied as food. However, there are limited studies reporting the effect of insects and their components on human health, particularly on intestinal health. In this thesis, we investigated the interaction of insect-derived preparations, and chitin in particular, with intestinal *in vitro* models to identify potential beneficial health effects. Through this thesis, we aim to clarify the effect of insect-derived products and chitin on human intestinal health, so that we can provide knowledge for food or pharmaceutical industries to develop novel foods or therapeutic agents.

First, **in chapter 2**, we *in vitro* digested insect-derived fractions to mimic the digestion after oral consumption. The impact of the digests on the viability of *C. perfringens* and growth and fermentation of faecal-derived microbiota was measured. It was found that digests of protein and protein/chitin enriched black soldier fly larvae reduced the viability of *C. perfringens*, but also limitedly, but significantly, the diversity of microbiota. Next, **in chapter 3**, we extend our research to investigate the effect of the digests on the intestinal immune barrier function using *in vitro* models to mimic small intestinal-like epithelial cells and residing macrophages. We observed that digests of insect-derived fractions can reduce the barrier damage of bacterial toxins and modify immune responses of small intestinal-like Caco-2 cells. To extend on the immunomodulatory findings, **in chapter 4**, we exposed a set of *in vitro* models representing the epithelium, immune cells and microbiota to pure chitin with different physicochemical properties. We found that small chitin fractions led to production of a set of cytokines by macrophages via a multi-receptor and clathrin-mediated endocytosis. This immunomodulatory activity of chitin makes it a promising agent for subjects in need of immune support. Additionally, chitin has also been described to be beneficial for our health in a broader perspective. In **Chapter 5**, we reviewed literature regarding the reported beneficial effects of chitin, and its derivative chitosan, in clinical studies, animal studies and *in vitro* studies. This demonstrated that the immunomodulatory, anti-pathogen, and anti-tumour activities of chitin

and chitosan are closely linked to their physicochemical properties. Furthermore, the *in vivo* results reveal that dedicated follow-up studies towards beneficial health effects are needed to validate the many putative effects that have been described. Finally, i**n Chapter 6**, all results obtained from previous studies were integrated and discussed in an overall perspective.

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Chapter 2

Clostridium perfringens suppressing activity in black soldier fly protein preparations

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Abstract:

Clostridium perfringens is an opportunistic pathogen, present in the intestine of humans and animals in homeostasis, that can lead to lethal foodborne diseases as a result of overgrowth when homeostasis is disrupted. The current course of treatment is the application of antibiotics. However, with increasing antibiotic resistance and limitations in antibiotic applications, alternatives are required. We investigated the antimicrobial capacity of digest from different black soldier fly- and mealworm-derived fractions towards *C. perfringens* by using *in vitro* models. Culturing *C. perfringens* with digest of insect-derived fractions showed that in particular fractions containing black soldier fly larvae protein significantly ($p< 0.05$) inhibited the growth of *C. perfringens*. In relation to this effect, many small (<5 amino acids) anti-microbial peptides were identified. The impact on healthy microbiota was also investigated through 16S rRNA sequencing and SCFA secretion following exposure of digests to healthy faecal-derived microbiota. This revealed a small but significant reduction in abundance and diversity of microbiota, that was reflected by a strong reduction in *Firmicutes* and increased abundance of *Proteobacteria* and *Klebsiella*. These changes coincided with increased levels of acetic, propionic and butyric acid secretion. The combined impact of black soldier fly larvae protein on these *in vitro* assays suggest it can be a promising antibiotic alternative to combat *C. perfringens* infection.

Key words: black soldier fly; antimicrobial; *Clostridium perfringens*; microbiota; SCFAs

1. Introduction

The human intestinal microbiome is a complex microbial ecosystem [1]. This ecosystem, when in homeostasis, acts in a symbiotic relationship with their host in healthy humans and supports intestinal health via supporting food digestion and fermentation, providing key nutrients, and preventing pathogenic infection [2]. However, a disbalance in the intestinal microbiota can lead to disease and illness as a result of overgrowth of pathogenic or opportunistic bacteria [3]. *Clostridium* is such a genus containing many opportunistic and pathogenic species with significant societal impact through a burden on healthcare and economy [4]. In homeostasis, *Clostridium* species naturally reside at low frequency in the human intestinal tract. *C. difficile* and *C. perfringens* can, however, demonstrate dramatic opportunistic growth following the use of antibiotics and induce diarrhoea and intestinal necrotic enteritis [4].

C. perfringens is a Gram-positive, rod shaped, spore forming, anaerobic pathogenic bacteria, present in the intestine of humans and animals [5]. This bacterium can be responsible for lethal foodborne diseases such as food poisoning and necrotic enteritis [6] through the production of a number of toxins (*i.e.* alpha-, beta-, epsilon-, iota-toxin, enterotoxin and necrotic enteritis B-like toxin) with membrane disruptive, actin-depolymerization, and neurotoxic capabilities [7]. The activity of *C. perfringens* can also lead to microbiota dysbiosis. In a clinical study including elderly Irish subjects, it was shown that the presence of *C. perfringens* in faeces correlated with a reduced presence of commensals *Bifidobacterium* and *Lactobacillus* [8]. Lowered levels of these commensals are also observed in intestinal bowel disease (IBD) and irritable bowel syndrome (IBS) patients [9, 10], indicating the potential impact of such a dysbiosis. Pets, such as dogs and cats, are also susceptible to *C. perfringens* infections. A study indicated that up to 28 % cases of diarrhea in dogs may occur due to *C. perfringens* poisoning [11]. In livestock, such as chicken and pigs, *C. perfringens* infection is associated with significant economic losses in livestock industries [12]. Over the years, antibiotics have been used to control *C. perfringens* infection. The ban of antibiotic usage in livestock industry, however, spurred a search for effective alternatives to target *C. perfringens* [12]. Many alternatives to antibiotics are being developed through novel biochemical and genetic technologies, including bacteriophages, antibodies, and probiotics. However, several alternatives that can be directly incorporated into feed already exist in nature, such as insects [13].

Insects are not only being considered as an alternative and sustainable protein source [14] but have also been characterized for their antimicrobial properties as they contain antimicrobial

peptides (AMPs), fatty acids and chitin [15, 16]. Currently, around 300 AMPs have been identified in insects, which act through disruption of the bacterial membrane, interference with metabolism and targeting of cytoplasmic components [17, 18]. Insect fatty acids are also known for their antimicrobial properties. Medium chain fatty acid derivatives, that are found in high concentration in larvae of the black soldier fly (*Hermetia illucens*), were reported to show activity against Gram-positive bacteria such as *Staphylococcus aureus* [19, 20]. The antimicrobial activity of fatty acids is linked to the reduction of cellular pH, and the ability to dissociate [21]. Furthermore, different isolates from insects, such as AMPs, fatty acids, and chitin (the main component of insect's skeleton) also exert their bactericidal effects via modulating the host immune response. For instance, chitin was shown to prevent *Leishmania major* infection in mice and the mechanism was putatively associated with the activation of macrophages [16, 22]. Because insects provide multiple antimicrobial compounds and can easily be implemented in feed and food, they constitute an interesting alternative to current antibiotics.

Even though insects contain these three major antimicrobial components, the antimicrobial properties of insects vary between species and life stage. In this study, we explored the bactericidal effects on *C. perfringens* of five different insect-derived products, being black soldier fly larvae protein meal, mealworm larvae powder, chitin rich black soldier fly larvae protein meal, black soldier fly larvae trilaurin fraction, and black soldier fly cocoon meal using *in vitro* models.

2. Materials and Methods

2.1 Nutritional composition of insect derived products

Black soldier fly larvae protein meal (BP), mealworm larvae powder (MP), black soldier fly cocoon meal (BC), black soldier fly larvae trilaurin fraction (BT) and chitin rich black soldier fly larvae protein meal (BchP) were provided by Protix (Dongen, The Netherlands). The details of the nutritional composition, production process and commercial availability are indicated by the supplier (Protix) and mentioned in Table 1.

Table 1. Details of five insect-derived products used in this study (as indicated by supplier). BC: black soldier fly cocoon meal, BchP: chitin rich black soldier fly larvae protein meal, BP: black soldier fly larvae protein meal, BT: black soldier fly larvae trilaurin fraction, MP: mealworm larvae powder.

Product	Nutritional composition			Production process	Commercial availability
	Protein (%)	Chitin (%)	Fat (%)		
BP	56	10	15	Produced by mincing, pasteurization, partial defatting and drying of black soldier fly larvae	Yes
BC	63	20	7	Produced by drying and grinding of black soldier fly pupal exoskeleton	No
BchP	56	15	18	Produced by mincing, pasteurization, partial defatting, standardization and drying of black soldier fly larvae	No
BT	0	0	100	Fat fraction obtained as co-product of BP during processing	Yes
MP	56	8	29	Produced by pasteurization, drying and grinding of mealworm larvae	Yes

2.2 *In vitro* digestion

In vitro digestion was performed based on the consensus INFOGEST standardized protocol [23], with the adaptation of reducing enzyme concentrations 10-fold to support cell viability upon exposure to digests. In brief, this *in vitro* digestion protocol is a three-phase process to simulate the oral-, gastric- and small intestinal digestion. The required simulated salivary fluid (SSF) was prepared by mixing 15.1 mM KCl (Merck, Darmstadt, Germany), 3.7 mM KH_2PO_4 (Merck), 13.6 mM $NaHCO_3$ (Merck), 0.15 mM $MgCl_2(H_2O)_6$ (BOOM, Meppel, The Netherlands) and 0.06 mM $(NH_4)_2CO_3$ (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 1 L MilliQ. The simulated gastric fluid (SGF) was prepared by dissolving 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$ and 0.5 mM $(NH_4)_2CO_3$, and

the simulated intestinal fluid (SIF) was prepared by dissolving 6.8 mM KCl, 0.8 mM KH_2PO_4, 123.4 mM NaCl (Sigma-Aldrich) and 0.33 mM $MgCl_2(H_2O)_6$ in 1 L MilliQ (Biopak® polisher; Merck). All insect-derived products except BT were fractioned with an IKA mill (model A11 B, IKA-Werke GmbH and Co. KG, Staufen, Germany), mimicking the chewing process and 1 gram of fractioned insects, 1 gram of MilliQ (blank) or 0.1 gram of BT and 9 or 9.9 grams of MilliQ water were mixed to reach a final mass of 10 gram. Subsequently, the mixtures were transferred to a thermal water jacket vessel to maintain a temperature of 37 °C through circulating water. The vessel is mounted on a magnetic stirring apparatus (model 888 Tritrando, Metrohm, The Netherlands) allowing a constant mixing. Samples were continuously stirred at level 3 (on a scale from 1 to 8) during all steps of digestion. Next, 8 ml of SSF, 50 µl of 0.3 M $CaCl_2$ (Sigma-Aldrich) and 1.95 ml MilliQ were added to the bottle reaching a total volume of 20 ml thereby completing the oral phase. Gastric digestion was initiated by adding 16 ml of gastric fluid (SGF), 0.6 ml pepsin (Sigma-Aldrich) solution (200 units per ml) and 10 µl of 0.3 M $CaCl_2$. The pH of the total fluid was adjusted to 3 using 1 M HCl (Sigma-Aldrich) after which the total volume of the mixture was adjusted to 40 ml and incubated for 2 h at 37 °C. Immediately after gastric digestion, the small intestinal digestion phase was started by adding 27 ml of SIF, 4.02 ml of bile extracts (1 mM) (Sigma-Aldrich), 80 µl of 0.3 M $CaCl_2$ and 5 ml of pancreatin solution (10 units/ml) (Sigma-Aldrich). The pH of the mixture was adjusted to 7 using 1 M NaOH (Sigma-Aldrich). The total volume of the mixture was adjusted to 80 ml followed by 2 h of incubation. During incubation, the pH of the total fluid was monitored and kept at 3 for the gastric digestion phase or 7 for the small intestinal digestion phase by using 1 M HCl or 1 M NaOH when necessary. Digest of empty control (MilliQ control) (ED), BT, BP, BC, BchP and MP were aliquot and stored in -80 °C immediately after *in vitro* digestion until further analysis.

2.3 Faecal sample preparation

Faecal samples were donated by a healthy adult male donor. Fresh faecal samples were collected in a box containing an anaerobic sponge (AnaeroGenTM 2.5L, Thermo Scientific, Ochten, Netherlands) to keep an anaerobic atmosphere. Immediately after collection, the faecal sample was transferred to a stomacher bag (Voor't Labo, The Netherlands) and 20% (w/v) autoclaved phosphate buffer was added containing per litre: 8.8 g K_2HPO_4 (Merck), 6.8 g KH_2PO_4 (Merck), 0.1 g sodium thioglycolate (Sigma-Aldrich) and 15 mg sodium thionate

(Merck). The sample was homogenized for 5 min at a speed of 230 rpm in a Stomacher® 400 Circulator (Seward, Hampshire, UK) after which it was poured into a 50 ml falcon tube. The tube was centrifuged at 500 g for 5 min and the supernatant was collected. Part of the supernatant was aliquoted to prepare a glycerol stock and stored at -80 °C and some was used for *in vitro* fermentation immediately.

2.4 Proliferation of *C. perfringens* and faecal derived microbiota

A glycerol stock of *C. perfringens* NCTC8238 was kindly provided by Prof.dr. T. Abee (Food Microbiology department, Wageningen University and Research) and a glycerol stock of faecal derived microbiota was prepared as described above. All manipulations were performed under anaerobic conditions. *C. perfringens* was recovered from the glycerol stock by incubating on a tryptose sulphite cycloserine (TSC) (Merck) agar plate at 37 °C for 24 h. One *C. perfringens* colony or 0.2 ml microbiota glycerol stock solution were emulsified with 10 ml fluid thioglycolate (FTG) broth (Oxioid, Wesel, Germany) or brain heart infusion (BHI) broth (BD Bioscience, Vianen, The Netherlands), respectively, at 37 °C O/N. Next, 0.2 ml of each inocula was incubated with 10 ml broth at 37 °C for 2 h after which 0.2 ml was transferred to tubes containing 10 ml FTG (*C. perfringens*) or BHI (healthy microbiota) broth and 1 ml of digest (*i.e.* empty digest as control and digest of BT, BP, MP, BC or BchP). Immediately after mixing (t=0) and after 3, 6, and 24 h, 1 ml of the solution was diluted with peptone physical salt (pfz) solution (Tritium Microbiologie, Eindhoven, The Netherlands) to a 10, 1, 0.1, 0.01 and 0.001% mixture of which 100 µl from each dilution was plated on a TSC (*C. perfringens*) or BHI (healthy microbiota) agar plate for O/N incubation. Colonies were counted after 24 h and calculated as a log CFU/ml value. The remaining solution of microbiota that were exposed to digests for 24 h was centrifuged at 10000 rpm for 3 min at 4 °C. The pellet was mixed with 1 ml DNA/RNA shield (Zymo Research, Freiburg im Breisgau, Germany) and sent to Baseclear B.V. (Leiden, The Netherlands) for microbiota composition analysis.

2.5 Microbiota composition analysis

The bacterial RNA was extracted for each pellet and the 16S rRNA gene sequencing was performed by Baseclear B.V. (L457; NEN-EN-ISO/IEC 17025). The genomic RNA was

extracted and a PCR amplification of the V3- V4 region was conducted on an Illumona MiSeq System (Illumina, San Diego, CA, USA). After amplification, the raw paired ends FASTQ files were trimmed and converted by bcl2fastq2 Conversion Software (version 2.18, Illumina). The resulting data was analysed by using CLC Genomics Workbench (Microbial Genomics toolbox version 20.0, Qiagen). The operational taxonomic units (OTUs) table was prepared based on a 99% sequencing similarity and the taxonomy was assigned using Silva database (version 132). A phylogenetic tree was created by using MUSCLE (version 3.8.425) to evaluate the alpha and beta diversity of each microbiota community. To exclude the influence of dead cells on the microbiota composition analysis, the analysis was based on RNA level.

2.6 *In vitro* fermentation

The in *vitro* fermentation was performed in a biological quadruplicate using microbiota from a healthy adult donor. Each fermentation vessel contained 21.5 ml basal medium, 10 ml PBS (control) or 10 ml of empty digest or BP, MP, BC, BCP or BchP digests. The basal medium was prepared and contained per liter: 2 g peptone (Duchefa Biochemie, Haarlem, The Netherlands), 2 g yeast (Sigma-Aldrich), 0.5 g L-cysteine (Sigma-Aldrich), 5.22 g K_2HPO_4 (Merck), 16.32 g KH_2PO_4 (Merck), 2 g $NaHCO_3$ (Merck), 1 g mucin (Sigma-Aldrich) and 2 ml Tween 80 (Sigma-Aldrich). Anaerobic processing of the fermentation vessels was achieved by consistently sparging with oxygen free nitrogen followed by transferring 3.5 ml of freshly prepared faecal-derived microbiota. Subsequently, the fermentation vessels were incubated at 37 °C whilst shaking (200 rpm) and samples (2 ml) were taken after 0, 3, 6, and 24 h for analysis. The samples were centrifuged at 20800 g at 4 °C and the supernatant was collected and stored at -80 °C.

2.7 SCFA analysis

The collected supernatants from fermentation were diluted with 16.6 mM sulphuric acid at a ratio of 1:1 (v/v) and subjected to a SCFA analysis by using high performance liquid chromatography (HPLC) (model Acquity Arc^{TM}, Waters, Eschborn, Germany) equipped with a refractive index detector (model R2414, Waters) and an AMINEX HPX-87H column (Aminex HPX-87H, 300x7.8 mm, Bio-Rad Laboratories, Richmond, VA, USA). The column

was maintained at 35 °C using an integral column heater (Waters) and sulphuric acid (8.3 mM) (Sigma-Aldrich) was used as the eluent for analysis. The standard samples were prepared at a concentration of 13.83 mM for lactic acid (Sigma-Aldrich), 18.15 mM for acetic acid (Merck), 13.82 mM for propionic acid (Sigma-Aldrich), 4.14 mM for isobutyric acid (Fluka, Cheniou, Gmblt, Germany), 12.12 mM for butyrate acid (Sigma-Aldrich), 9.75 mM for isovaleric acid (Fluka), 9.73 mM for valeric acid (Acros Organic^{TM}, Geel, Belgium) and 27.07 mM for formic acid (Merck) in 1 L of 8.3 mM sulphuric acid (Sigma-Aldrich). The standard was injected after every 5 samples, repeatedly, and the calibration curve was constructed by plotting the peak area against the molarity of standard solutions.

2.8 LC-MS/MS analysis

In vitro protein digests ED, MPD, BPD and BchPD were analyzed in duplicate on a UPLC-MS system (Dionex Ultimate 3000 online connected to QexactivePLUS (Thermofisher, Waltham, U.S.A)). Samples were injected (10 ml) both on a pentafluorophenyl F5 core-shell column (Kinetex F5, 15 cm × 2.1 mm, 2.6 mm particles, Phenomenex, Torrance, USA) and separately also on a HSS T3 column (15 cm × 2.1 mm, 1.8 mm particles, Waters, Millford, USA), operated at 40 °C, or respectively 60 °C, and flow rate of 0.2 ml (0.15 ml for T3) per minute. Total run time was developed over a 40 min time window: Starting with buffer A (0.1% formic acid in water) for 5 min and separated with a gradient of 0 to 30% buffer B (0.1% formic acid in 100% Acetonitrile) during 20 min, increasing to 80% B in 5 min, stable at 80% B for 3 min and back to 0% B during 2 min, and stable at 0% B for 5 min. Separated peptides were on-line injected into the Q ExactivePLUS using the standard ESI source in positive mode, with 3.5 kV spray voltage, 290 °C capillary temperature, nitrogen sheath gas flow 40 and auxiliary gas flow 10 heated at 60 °C. MS spectra were collected with two alternative methods; first within a m/z range of 220–1400, alternatively with a m/z range of 380-1200. MS scans were at 70000 resolution (profile) and AGC target of $3x10^6$ ions maxIT for 100 ms; followed with data-dependent switch to MS/MS mode at 17500 resolution (centroid) at AGC target 10^5 ions, NCE 27, minimum AGC 10^4, maxIT 110 millliseconds and 4 m/z isolation window, loop count 5, a dynamic exclusion of 10 s without further charge exclusion.

2.9 Sequence data analysis

Several approaches were followed to match sequence information to the acquired LC-MS/MS data. Data were searched with MaxQuant software (V1.6.5) versus a protein sequence database of Hermetia (UniProt-tax_343581), Tenebrio (UniProt-tax_7066), without or complemented with Musca_domestica Uniprot proteome_UP000095301 and Drosophila UNiprot proteome_UP000000803. Peptide tables from both searches were filtered to remove contaminants (*e.g.* collagen, trypsin), low scoring matches (Score< 20, PEPscore> 1%), and filtered peptide tables were merged together.

Alternatively, LC-MS/MS data were converted to.mgf format using MSconvert (ProteoWizard V3.0.20178). Mgf converted data were interpreted by pNOVO3 *de-novo* search algorithm separately per sample group (ED, MPD, BPD and BchPD). The resulting peptide sequence tables per sample were matched to the sequence database of LAMP2 containing 22534 peptide sequences of antimicrobial peptides (per July 3rd 2020). Only matches with complete and identical sequence fit between pNOVO output and LAMP2 db were retained. Subsequently, only peptides with a maximum of peptide spectrum matching (PSM) score > 50 were considered.

2.10 Statistic analysis

Data is presented as mean or mean + SD and statistically significant differences between parameters were analysed by one-way ANOVA (Graphpad Prism 8, La Jolla, CA, USA) or Kruskal-Wallist test (Figure 5).

3. Results

3.1 *C. perfringens* growth inhibition activity of digested insect-derived fractions

To investigate whether digest of insect-derived fractions exhibit anti-*C. perfringens* activity, *C. perfringens* growth and viability was studied upon exposure to these digests. Exponentially growing *C. perfringens* were exposed to medium control (PBS) or digest of BP, MP, BT, BchP, BC or empty digest (ED). The CFU of *C. perfringens* were measured after 0, 3, 6 and 24 h of

exposure (Figure 1A). Growth of *C. perfringens* was significantly inhibited after 3 h incubation with a digest of MP (MPD) and after 6 h incubation with a digest of BP (BPD), digest of BchP (BchPD) and MPD. After 24 h, BPD and BchPD significantly inhibited viability of *C. perfringens* by 68.5% and 84.6%, respectively (Figure 1B). Moreover, a digest of BC (BCD) and BT (BTD), although not significantly ($p > 0.05$), was demonstrated to reduce growth of *C. perfringens* over time as well.

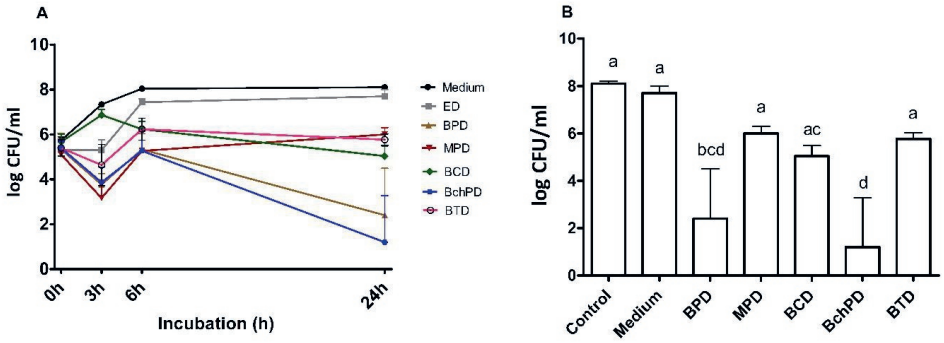

Figure 1: Impact of digest of insect-derived fractions on *C. perfringens* growth and viability. Insect-derived fractions BP, MP, BC, BchP and MilliQ (empty control) were digested and added separately, as well as medium as control, to *C. perfringens* during its exponential growth phase. The CFU of *C. perfringens* were measured at t=0, 3, 6, and 24 h of incubation by counting colonies and reported here as log CFU/ml (A). The log CFU/ml values after 24 h were shown as bar charts (B). Both line and bar charts represent the mean of 3 independent experiments + SD. Statistical analysis was performed by one-way ANOVA and different letters indicate significant ($p < 0.05$) differences; BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; BTD: black soldier fly larvae trilaurin fraction; CFU: colony-forming units; ED: empty digest; MPD: digest of mealworm larvae powder.

3.2 Overview of peptides in the digest of protein-containing insect-derived fractions

As the protein enriched insect-derived fractions BPD and BchPD demonstrated to significantly reduce the viability of *C. perfringens*, we performed a proteomics analysis to identify the presence of antimicrobial peptides. First, to identify peptide sequences matching an insect

protein sequence database, a standard MS/MS search was performed using MaxQuant software with trypsin as the digestion enzyme [24, 25]. Using the available proteins from the Uniprot database from genus *Hermetia* (Black Soldier fly, 92 sequences), *Tenebrio* (mealworm, 620 sequences), *Musca domestica* (housefly, 16984 sequences) and *Drosophila* (fruit-fly, 4974 sequences), only 54 peptide sequences were identified with a good score (Supplementary Table 1). The peptide length distribution was between 6 and 20 amino acids and none of these peptides had a sequence identical to sequences in the LAMP2 database (data not shown).

As the MaxQuant search algorithm is designed to match longer peptide sequences, whereas in our sample the majority of peptides had very short sequences, we applied a separate approach for identifying short peptides. *De novo* interpretation of MS/MS spectra was performed using the pNOVO3 algorithm applied to the multiple LC-MS/MS data per sample group [26]. From pNOVO3 a large number of peptide sequences were derived, not all of them having a perfect score. In total, 1639, 43368, 51198 and 57426 peptides were identified in the ED, MPD, BPD and BchPD, respectively. Among these peptides, 23.9%, 19%, 19.4%, 23.1% of peptides in ED, MPD, BPD and BchPD, respectively, had a sequence smaller than 5 amino acids (Figure 2A). After obtaining all the peptides in insect digests, we searched for identical matches with peptide sequence in the LAMP2 antimicrobial database. A total of 23 peptides were identified in BPD, 24 peptides in MPD and 24 peptides were found in BchPD (Figure 2B). Moreover, 14 identical peptides were found both in the MPD, BPD and BchPD, 6, 2 and 3 unique antimicrobial petides were found in MPD, BPD and BchPD respectively. After quality control of antimicrobial peptides, using a cut-off value of a max peptide spectrum matching (PSM) score > 50 or max average amino acid (Avg AA) score > 50, we found 17, 19 and 16 peptides in MPD, BPD and BchPD respectively and 10 peptides were shared among these three digests (Supplementary Table 2).

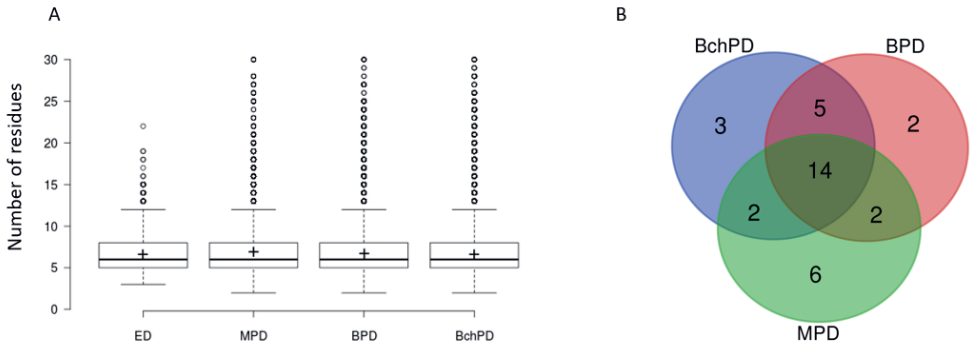

Figure 2: Peptide distribution in digest of protein-containing insect-derived fractions. Insect-derived fractions BP, MP BchP and Milli-Q (empty control; ED) were digested and the supernatant was used to analyse the presence of antimicrobial peptides. LC-MS/MS was performed. All peptide sequences were *de-novo* derived (using pNOVO3) and the distribution of all peptides based on their length. In total, 1639, 43368, 51198 and 57426 peptides were identified in the ED, MPD, BPD and BchPD, respectively (A). *De-novo* derived peptides were matched on identity with sequences in LAMP2 database and a venn diagram indicates the overlap and differences of the antimicrobial peptides in MPD, BPD or BchPD (B). Box plots show the number of residues. Boxes represents the second and third quartiles, and whiskers represent 1.58x interquartile range (IQR). BchPD: digest of chitin rich black soldier fly larvae protein meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder.

3.3 Impact of digest of insect-derived fractions on healthy microbiota viability

As the digest of insect-derived fractions decreased the viability of *C. perfringens* over time to a different extent, we examined whether this effect also applied to gut microbiota in general. To this end, we harvested microbiota from a faecal sample from a healthy donor and exposed these during their exponential growth phase to the digest of insect-derived fractions, empty digest and medium control at the same concentration as for the *C. perfringens* assay. Microbiota CFU/ml was measured after 0, 3, 6, and 24 h of exposure (Figure 3A). This revealed that the digests had minimal effect on the growth and viability of the microbiota. However, upon 24 h

incubation, the viability of microbiota was slightly, but significantly ($p < 0.05$), reduced by BPD, BCD and BchPD when compared with both medium and empty digest control (Figure 3B).

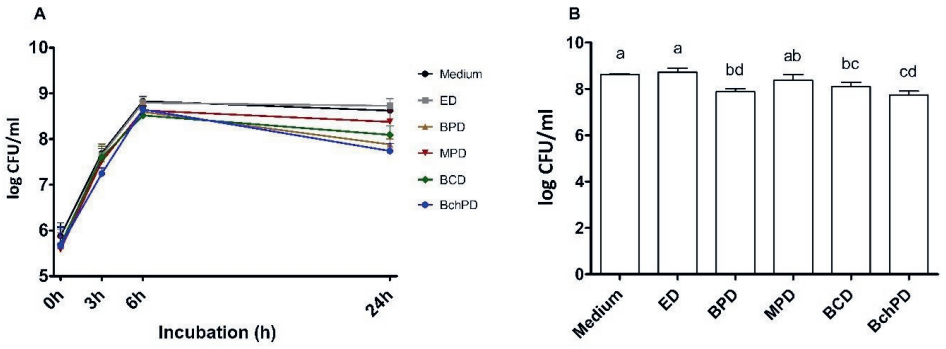

Figure 3: Impact of digest of insect-derived fractions on growth of faecal-derived microbiota. Insect-derived fractions BP, MP, BC, BchP and MilliQ (empty digest) were digested and added, as well as medium control, to faecal-derived microbiota during its exponential growth- and plateau phase. The growth of microbiota was measured at t=0, 3, 6, and 24 h by counting colonies and reported here as log CFU/ml after overnight incubation (A). The log CFU/ml values after 24 h are shown as a bar chart (B). Both line and bar charts represented the mean of 3 independent experiments + SD. Statistical analysis was performed by one-way ANOVA and different letters indicate significant ($p < 0.05$) differences; BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; CFU: colony-forming units; ED: empty digest; MPD: digest of mealworm larvae powder.

3.4 Microbiota compositional changes after exposure to digest of insect-derived fractions

To gain insight on the specific impact of BPD and BchPD on the healthy microbiota, we performed 16S rRNA sequencing to analyse the composition of microbiota after 24 h exposure to medium, empty digest, or the digested insect-derived fractions. This revealed that ED neither significantly changed the total operational taxonomic units (OTUs) (Figure 4A) nor the diversity indicated by Shannon index (Figure 4B) when compared to medium control. In contrast, BPD and BchPD induced a significantly decrease of 16.7% and 20.3%, respectively, in the total OTUs when compared to medium (Figure 4A). In addition to OTUs, BPD and

BchPD led to a significant reduction in the Shannon index when compared with medium control (Figure 4B).

To explain the decreased diversity induced by BPD and BchPD, we evaluate the changes in the relative abundance at phylum (Figure 4C) and genus (Figure 4D) level after exposure to ED, BPD and BchPD. Again, no significant changes were detected in bacteria phyla and genera after exposure to ED. At phylum level, *Proteobacteria* and *Firmicutes* were dominant in microbiota after exposure to control and ED. However, the relative abundance of *Proteobacteria* increased from 51% to 95% and *Firmicutes* decreased from 45% to 5% after exposure to BPD or BchPD when compared with ED. At genus level, *Escherichia-Shigella*, *Enterococcus* and *Lactobacillus* were the dominant genus after incubation with medium or ED. When comparing BPD and BchPD to ED, the genus of *Escherichia-Shigella* increased from 54.5% to 84.5% and 83.9%, respectively, *Megamonas* increased from 0.5% to 9.7% and 11.3%, respectively, *Enterococcus* decreased from 25.6% to 9.7% and 11.3%, respectively, and *Lactobacillus* decreased from 10% to 1% and 0.9%, respectively (Figure 4D). Statistical analysis revealed that among the 26 genera detected in all microbiota samples, 12 genera were significantly changed after incubation with BPD or BchPD when compared with ED (Figure 5). Moreover, it showed that BPD and BchPD induced a 2704- and 2760- fold increase of *Klebsiella*, respectively, although only reaching a 2% relative abundance, and 123- and 2678-fold decrease of *Enterobacter*, respectively, when compared with ED. Of note, we did not observe any changes in the abundance of the *Clostridium* genus upon incubation with BPD or BchPD, which might be due to the absence of *Clostridium* in the medium control (data not shown).

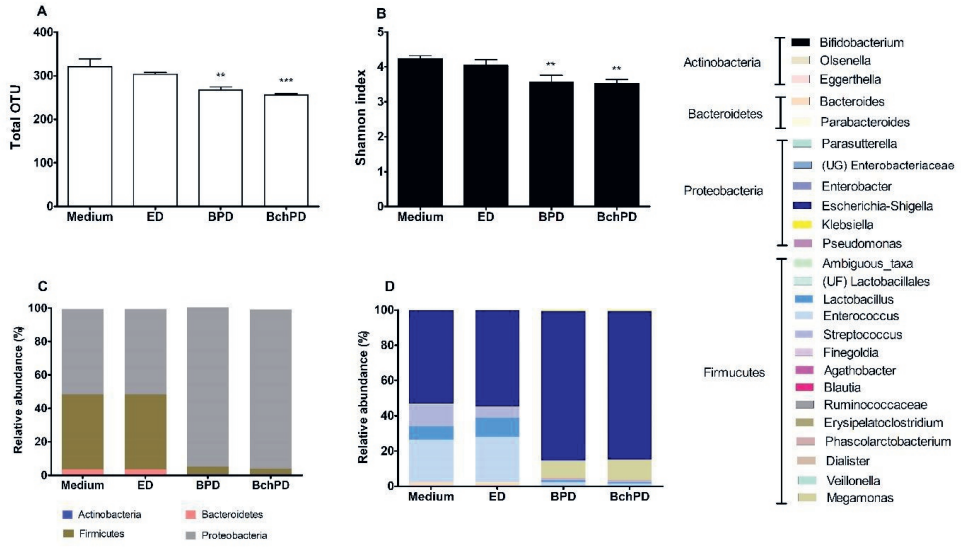

Figure 4: Microbiota compositional changes after 24 h exposure to BPD or BchPD. Insect-derived fractions BP, BChP and MilliQ (empty digest) were digested and added, as well as medium control (BHI broth), to faecal-derived microbiota during its exponential growth phrase. After 24 h exposure, the microbiota was collected and subjected to 16S rRNA sequencing to analyse the composition. The observed species were indicated as total OTUs (A) and the evenness of microbiota distribution was indicated as a Shannon index (B). The relative abundance of microbiota was showed at phylum (C) and genus (D) level. Statistical analysis was performed by one-way ANOVA with **: $p< 0.01$; ***: $p< 0.001$. BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; OTU: operational taxonomic unit; UF: unknown family; UG: unknown genus.

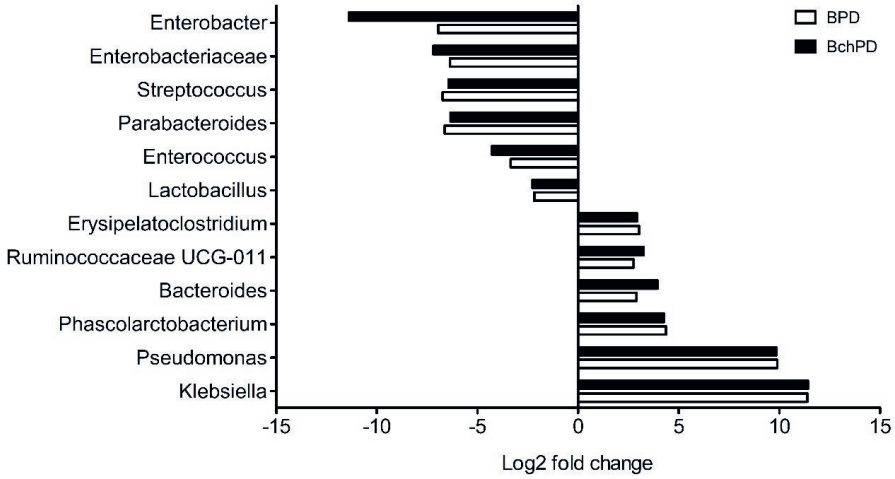

Figure 5: Changes in the microbiota at a genus level after 24 h incubation with BPD or BchPD. Only the bacteria significantly (FDR $p < 0.05$) changed after incubation with BPD and BchPD when compared with ED were showed. Statistical analysis was performed using Kruskal-Wallis test. Bar charts show the mean of 3 independent experiments. BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest.

3.5 Fermentation of digest of insect-derived fractions increased SCFA release by faecal-derived microbiota

Short chain fatty acids (SCFAs) are a group of metabolites secreted by the microbiota that support a healthy gut [27]. A change in SCFA levels, regardless of a change in microbiota composition, can therefore also impact health. To this end, the levels of acetate, butyrate, propionate, valerate, formic acid, iso-butyrate and iso-valerate were measured after 24 h incubation of the microbiota with BPD, MPD, BCD, BchPD and ED (Figure 6). Analysis revealed that MPD, BPD and BchPD significantly increased the total SCFA secretion by microbiota when compared with ED. In particularly, incubation with MPD induced a significant increase in acetate, propionate, iso-butyrate and iso-valerate release by microbiota when compared to ED. Incubation of microbiota with BPD or BchPD also led to a significantly

increased acetate, propionate and butyrate secretion and BCD induced a significantly elevated acetate and iso-butyrate secretion.

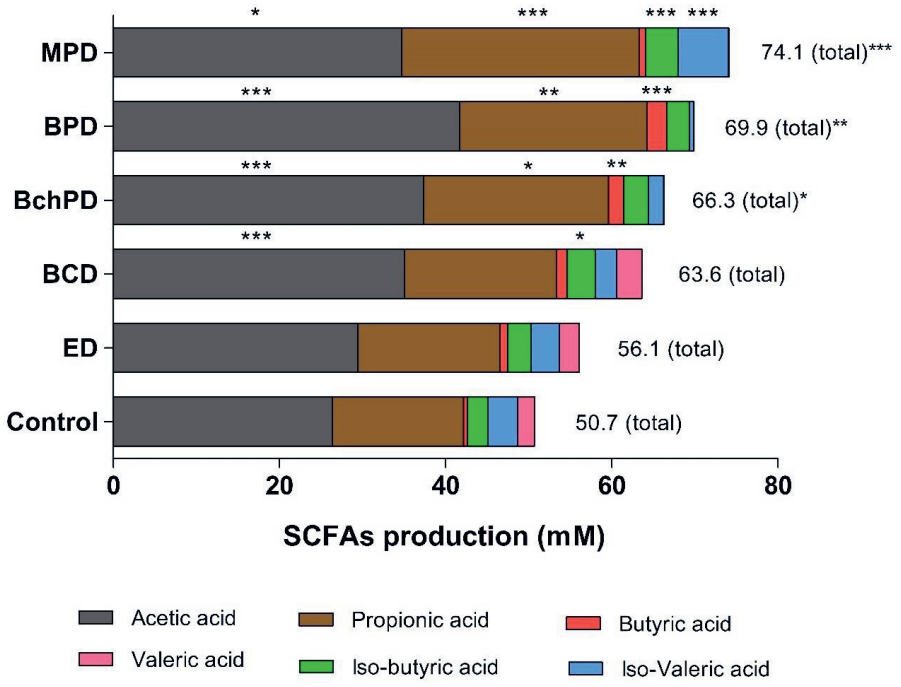

Figure 6: SCFAs secretion by faecal-derived microbiota following exposure to digest of insect-derived fractions. Insect-derived fractions BP, MP, BC, BchP and MilliQ (empty digest) were digested and added, as well as medium control (PBS), to faecal-derived microbiota. The secretion of acetate, propionate, butyrate, iso-butyrate, iso-valerate and valerate was measured after 24 h of incubation. Stacked bar shows the mean of 4 independent experiments. Statistical analysis was performed by one-way ANOVA with *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder.

4. Discussion

C. perfringens is a commensal bacterium, but also an opportunistic pathogen that can cause severe health issues in humans, pets (*e.g.* dogs and cats), and livestock (*e.g.* chickens) [4, 28]. Here, we set out to identify whether specific insect-isolates could act as alternatives to antibiotics that target *C. perfringens*. Insects are already applied as human food, pet food and livestock feed due to their high nutritional value [29]. After the ban of using antibiotic growth promoter in livestock industries, the antimicrobial property of insects such as mealworm and black soldier fly acquired increasing attention [12]. Insects have also gained popularity as sustainable and health promoting pet food ingredients [30, 31] and the pet food industry is currently the biggest outlet for insect proteins [32]. Even though the bactericidal effects of insect as a whole have been extensively researched, studies on the antimicrobial property of insect-derived fractions are still scarce. We tested five insect-derived fractions, being black soldier fly larvae protein meal (BP), mealworm larvae powder (MP), chitin rich black soldier fly larvae protein meal (BchP), black soldier fly trilaurin fraction (BT) and black soldier fly cocoon meal (BC) and digested them according to the adapted INFOGEST consensus protocol [23].

The *in vitro* digestion method used in this study aims to mimic the oral, gastric, and intestinal digestion in human by simulating parameters such as electrolytes, enzymes, bile, pH and digestion time based on available physiological data. The digestive system in humans contains stomach, small intestine and large intestine and some physiological similarities were found between human and animals [33]. For instance, the colon morphology appears similar in humans and pigs, the stomach morphology and empty characteristics are similar in humans and dogs and the pH of gastric fluid is similar in humans, dogs and pigs [33]. Hence, we need to be aware of that, in addition to human, our *in vitro* data might also indicate the effect of insect-derived fractions in certain species of pets or livestocks.

When incubating *C. perfringens* with digest of the insect-isolates, we observed that growth was strongly inhibited by BPD and BchPD with 69% and 85%, respectively. The antimicrobial effects of insects as a whole result from different bioactive components, being antimicrobial peptides (AMPs), chitin and fats [34, 35]. For the latter, *in vitro* studies have demonstrated that both black soldier fly- and mealworm-derived fats have an antimicrobial effect on *Pasteurella multocida*, *Yersinia enterocolitica*, *Listeria monocytogenes* [36]. Black soldier flies are naturally rich in lauric acid [37], which it is known to exhibit activity against both Gram-

positive bacteria such as *Staphylococcus aureus* and Gram-negative bacteria *Fusobacterium nucleatum* [38]. Lauric acid can be released after lipase digestion of trilaurin, a triglyceride that contains a significant amount of lauric acid [39]. However, the results showed that BTD did not significantly ($p < 0.05$) inhibit the growth of *C. perfringens* (Figure 1). This could result from insufficient or ineffective hydrolysation during *in vitro* digestion as the INFOGEST digestion model has yet to be optimized for digestion of fat. Next, the antimicrobial activity of chitin depends on its physiochemical property. The deacetylated form of chitin, chitosan, was demonstrated to be effective against a broad range of microbes [16]. It permeates the membrane by binding to negatively charged components on the bacterial surface with its positive charge [40]. Unlike chitosan, the antimicrobial effects of chitin with a low deacetylation level (<50%), the form found in black soldier fly [41], was limited and increasingly associated with immunomodulatory activity [16]. The results revealed that digest of BC, which has a relative higher amount of chitin (Table 1), did not significantly decrease the viability of *C. perfringens*. Taken together, the bactericidal effect on *C. perfringens* of BPD and BchPD were most likely due to the presence of AMPs, rather than lauric acid or chitin.

As our samples were derived by *in vitro* digestion simulating the gastric and ileum conditions, the majority of proteins were digested into very small peptides and single amino acids. The identification of natively digested peptides, using proteomics, is not a trivial task. Most proteomics database search algorithms are designed to identify peptides with a length of minimally 6 amino acids. Using a *de-novo* approach, a large number of potential sequence matches, with very short sequences, were generated. Following the application of a stringent filter (PSM score>50) sequences were matched on identical sequences with the LAMP2 database. We identified 23 and 24 AMPs in BPD and BchPD, respectively, and 19 AMPs that were common in both BPD and BchPD (Figure 2B). So far, studies to identify AMPs in black soldier fly larvae were performed through bioinformatic analysis based on gene transcription or chromatography analysis of insect extracts [42-44]. Up until recently, more than 3000 proteins with antimicrobial property have been found in different organisms and around 300 AMPs have been identified in insects [17]. Most active AMPs are small peptides of 20-50 residues [15]. However, AMPs found in our study are smaller than 5 amino acids as a result from the *in vitro* digestion procedure. To interpretate these small peptides in the digest of insect-derived fractions and control, they are annotated based on the sequence with an identical match with database of antimicrobial activity and structure of peptides (DBAASP) (General discussion: Table 6.1). Even though the AMPs in the digests were determined by its identical

sequence, we need to note that the antimicrobial properties of AMPs are also dependent on the structure [45, 46]. For instance, a peptide (LPLP) was identified in both BPD and BchPD and it possess the same sequence of a peptide cyclo-[LPLP] extracted from Thai sponge *Halisarca ectofibrosa* but their structure is different [47], which might lead to different functionality. Therefore, further studies are needed to verify the antimicrobial activity of individual peptides or peptides present in major concentrations found in BPD and BchPD.

Next to *C. perfringens*, the microbiota community consists of healthy microbiota which we investigated using microbiota derived from a faecal sample from a healthy donor. Generally, human microbiota composition shares some similarity with that of some animals (*i.e* dog, pigs, etc.) and differences mainly relate to variation in the relative abundance of microbiota [48-50]. Hence, results obtained from this *in vitro* study with human microbiota could also be predicable for monogastric animals. Our results revealed that, in addition to inhibiting growth and viability of *C. perfringens*, we also found that BPD and BchPD significantly decreased diversity, richness and viability of faecal-derived microbiota (Figure 3 and 4), albeit the latter only with 10.2% and 8.6% respectively. These effects were not a result of a decrease in *Clostridium* species abundance or presence, due to the absence of *Clostridium* species in both control and insect digests treated groups. Our results showed that the incubation of faecal-derived microbiota with BPD and BchPD decreased *Firmicutes* abundance from 45% to 5%, respectively, and increased *Proteobacteria* abundance from 51% to 95% (Figure 4C). A bloom of *Proteobacteria* in the gut microbiota reflects an unstable microbiota community as can be observed in the neonatal period, in patient after gastric bypass surgery and in patients with intestinal diseases [51]. At genus level, the relative abundance of dominant genus *Escherichia-Shigella* was increased 30% and *Enterococcus* decreased around 10% after exposure to BPD or BchPD when compared with ED (Figure 4D). Increased opportunistic bacteria such as *Escherichia-Shigella* has been found in patients with inflammatory bowel disease and higher abundance of this bacteria genera is recognized as the signature of the intestinal microbiota dysbiosis of patient with Crohns diseases [52, 53]. In contrast to these findings, an animal study performed on rainbow trout reported that oral administration of black soldier fly larvae increased the diversity of gut microbiota as reported an increased total OTU, richness and Shannon diversity [54]. Furthermore, a broiler chicken study also showed that replacement of soybean diet by black soldier fly diet increased the total OTUs as well as the Shannon diversity [55]. The difference between our study and these animal experiments might be explained by the presence of AMPs in the digest of insect-derived fractions. Generally, most proteins will be

digested and absorbed in the small intestine and only a fraction may reach the proximal colon in an *in vivo* setting [56]. Therefore, the observed impact on OTU and diversity by BPD and BchPD might be less pronounced *in vivo*. Furthermore, the observed reduction in OTU and diversity when incubating faecal-derived microbiota with BPD or BchPD is limited when compared to a clinical study that demonstrated that commonly used antibiotics fluoroquinolones and β-lactams reduced the diversity of faecal-derived microbiota with 25% and the core phylogenix taxa by more than 50% [57]. So, despite the fact that a strong decrease in *Firmicutes* in general and increase of *Proteobacteria* is undesirable in healthy microbiota, the total impact is less invasive than currently applied antibiotics in humans.

Next to the changes in microbiota composition, we also observed an increase of SCFAs secretion after faecal-derived microbiota exposure to the digest of insect-derived fractions. SCFAs are associated with many beneficial effects and our findings were consistant with animal studies [55, 58]. Most notably, we found an increase in butyrate secretion by microbiota after BPD or BchPD exposure (Figure 6). Butyrate is the major energy source for colonocytes and it can promote the differentiation and proliferation of intestinal epithelial cells. Hence, butyrate has been demonstrated to be beneficial for intestinal health, ameliorate mucosal inflammation, reinforce intestinal barrier function and modulate intestinal motility [59]. Even though peptides are not the major substrate for gut microbiota, they can be fermented by a range of microbiota such as *Bacteroides* and *Fusobacterium* [56]. Therefore, animal studies are required to further characterize the impact of insect-derived fractions on SCFAs production and gut microbiota metabolism.

Taken together, *C. perfringens* infections significantly impact health of pets, livestock and humans. Furthermore, increasing antibiotic resistance in animal husbandry is being observed [60], and there are well established negative implications of the use of antibiotics [57, 61, 62]. The results of these *in vitro* studies on *C. perfringens* and healthy microbiota viability and metabolism indicated that black soldier fly-derived protein might be an effective anti-*C. perfringens* product and alternative to antibiotics. Further *in vivo* studies are required to investigate the optimal dosage, and/or the combination with probiotics, to ensure reduction of the viability of *C. perfringens* without affecting the composition of the commensals.

Data availability

The authors declare that all data supporting the findings are available upon reasonable requests.

Competing interests

A.P. is employed by Protix B.V.

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Author contributions

L.D, C.G, and J.J.M conceived and designed the experiments. L.D, R.A and T.A performed the experiments. L.D, R.A and T.A performed data analysis and interpretation. L.D, A.P, C.G, T.A wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary information

Table S1: Peptides (good score) identified in digest of insect-derived fractions

Sequence	Length	Max of MS/MS Count	Min of PEP	Max of Score
TTEENPK	7	34	0.0E+00	138.85
NREEVDDPK	9	3	0.0E+00	107.69
EEVEAEA	7	1	7.7E-04	104.25
EQLEEEK	7	1	1.1E-03	102.71
AGFAGDDAPR	10	10	0.0E+00	101.25
SISGEEK	7	1	2.2E-05	100.74
VVEIAPAPR	9	8	7.9E-08	100.45
SLEVSEEK	8	1	0.0E+00	98.46
DDSLYPK	7	2	0.0E+00	97.07
GMDFQPR	7	4	0.0E+00	95.96
APIEIPK	7	2	1.0E-212	94.41
TLPDISGAKCFSR	13	9	1.4E-11	88.95
LELNGCTIK	9	5	2.7E-03	84.57
NGEEVDDPK	9	1	0.0E+00	84.21
IDSDLEGHPTPR	12	1	6.8E-08	79.47
SDDILPK	7	0	7.5E-03	79.31
LITSFTKTGK	10	22	2.3E-247	75.11
NWDDMEK	7	10	3.7E-100	74.42
ALGLPIERPK	10	5	1.2E-04	71.35
SVSLVCPGPK	10	13	1.9E-03	68.92
HLEACINSLK	10	3	8.3E-03	67.98
QWDIPK	6	1	4.3E-06	67.04
KMLENLPPK	9	1	7.0E-03	64.71
KDQHTMAMR	9	3	3.7E-03	63.57
TAEDAEECFR	10	5	4.6E-03	63.57
QAVEDAGDK	9	2	2.6E-41	62.10
LSEEFIPHQR	10	1	4.5E-06	61.96
HVIEGYKLR	9	2	9.6E-03	60.79
IDLPVVPR	8	9	7.0E-75	58.92
IDIGPVMHSKPK	12	5	1.2E-03	57.96
IEDLELKLSLYK	12	0	2.2E-08	57.96
YTNWCMLCDK	10	1	9.7E-03	57.28
FSEENTK	7	5	3.7E-09	54.53
EQATVVPEGSRPPIEIPK	18	4	9.3E-69	52.73
KIDLPVVPR	9	4	2.0E-06	52.49
EIGATSVASQR	11	0	1.6E-06	49.42
VGAPNMAQMPGGK	13	2	1.6E-03	47.82

EEVDDPK	7	1	4.1E-03	46.81
QLQRSQQELLEIR	13	3	6.2E-03	45.37
LNAERGGQK	9	2	6.2E-22	43.25
LSGAEPK	7	1	9.4E-06	43.12
LLKSWR	6	2	4.6E-03	42.77
SDKHPPK	7	2	1.8E-14	41.65
LTYGDQVK	8	2	2.4E-10	40.10
LLKDNPK	7	2	2.8E-03	38.79
TIIAEHEK	8	1	8.5E-03	38.79
TLGVNFYR	8	1	4.0E-04	38.24
NEFYMMGK	8	1	1.9E-04	37.02
NSALITTHSWQKGKR	15	0	1.8E-06	36.59
HVIAPIK	7	3	2.9E-03	35.11
VAPQTRD	7	2	6.9E-03	33.69
VFIDNPRA	8	1	9.9E-03	32.47
TILAADNHFRIRGYNFENPR	20	0	3.7E-03	24.89
LEDASLHFNFPEHASEK	17	0	5.8E-03	24.85

Table S2: Antimicrobial peptides in digest of insect-derived fractions.

Insects	**No. of peptides**	**Peptide sequences**
BPD BchPD MPD	10	WE DDL EW YR DDF LLE FF LLK EEL EDL
BPD BchPD	4	LPR YDL LPLP WR
BPD MPD	3	WW WP LWE
BPD	2	GFPY FW
BchPD	2	KR EDF
MPD	4	FPLP DEL KLL WF

Chapter 3

Clostridium difficile toxin A-mediated Caco-2 barrier damage was attenuated by insect-derived fractions and corresponded to increased gene transcription of cell junctional and proliferation proteins

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Abstract:

Pathogenesis of *C. difficile* in the intestine is associated with the secretion of toxins which can damage the intestinal epithelial layer and result in disease, such as diarrhoea. Treatment for *C. difficile* infection consists of antibiotics which, however, have non-specific microbiocidal effects and may cause intestinal dysbiosis which results in subsequent health issues. Therefore, antibiotic alternatives to treat *C. difficile* are required. We investigated whether different black soldier fly- and mealworm-derived fractions, after applying the INFOGEST digestion protocol, could counteract *C. difficile* toxin A-mediated barrier damage of small intestinal Caco-2 cells. Treatment and pre-treatment with insect-derived fractions significantly ($p< 0.05$) mitigated the decrease of the transepithelial electrical resistance (TEER) of Caco-2 cells induced by *C. difficile* toxin A. In relation to these effects, RNA sequencing data showed an increased transcription of cell junctional and proliferation protein genes in Caco-2 cells. Furthermore, transcription of genes regulating immune signalling were also increased. To identify whether this resulted in immune activation we applied a Caco-2/THP-1 co-culture model where the cells were only separated by a permeable membrane. However, the insect-derived fractions did not change basolaterally secreted IL-8 levels in this model. To conclude, our findings suggest that black soldier fly- and mealworm-derived fractions can attenuate *C. difficile* induced intestinal barrier disruption and they might be promising treatment options to counteract the pathogenesis of *C. difficile* infections.

Key words: black soldier fly; barrier function; *Clostridium difficile*; gene transcription; tight junction; immune signalling.

1. Introduction

Our intestinal homeostasis is dependent on interactions between host defensive mechanisms and commensals [1]. This hosts defence is composed of various layers and include the mucus, epithelium and local immune system [2]. The epithelium layer is composed of secretory Paneth-, goblet- and enteroendocrine cells, antigen sampling microfold cells (M cells) and mainly absorptive enterocytes. Intestinal epithelial cells provide a physical and biochemical barrier to separate luminal microbiota from immune cells in lamina propria. Whilst doing so, the luminal content is screened by enterocytes through receptor recognition [3] and M cells that are specialised in transporting luminal content for immune monitoring. This is necessary as the intestinal lumen is prone for enteral pathogenic entry.

Some commensals contain pathogenic features, especially when overgrown. *Clostridium* is such a genus that contains many opportunistic species including *C. difficile* [4, 5]. *C. difficile* is a Gram-positive, obligate anaerobic and spore forming bacterium [6, 7]. In homeostasis, *C. difficile* naturally resides at a low frequency in the human intestinal tract. Upon dysbiosis of the microbiome, *C. difficile* frequencies can increase dramatically and induce intestinal diseases such as diarrhoea and intestinal necrotic enteritis [8]. These pathologies are a direct result of the secretion of toxins during sporulation and facilitate dissemination of the spores.

C. difficile produces two main virulence toxins called toxin A and toxin B and these two toxins exhibit enterotoxicity and cytotoxicity, respectively [9]. Toxin A and toxin B contain RHO and RAC glucosyl transferase domains at the amino terminus allowing them to inactivate host RHO and RAC GTPases in the cytosol through glycosylation [6]. This disrupts the continuous (re)formation of the cytoskeleton and leads to the disassociation of tight junctions between epithelial cells, resulting in an increased permeability of the intestinal epithelial barrier. The increased intestinal epithelial permeability results in exposure of intestinal microbiota and microbiota-associated molecular patterns to intestinal immune cells, which exacerbates intestinal symptoms with inflammation and contributes to many intestinal diseases such as inflammatory bowel diseases [10]. Even though some antibiotics such as cephamycin were demonstrated to be effective to treat recurrent *C. difficile* infections, the strong microbial inhibitive effect consequently induces a dysbiosis of gut microbiota, resulting in adverse health effects [11]. Therefore, alternatives for antibiotics to treat *C. difficile* infections are needed.

In animal studies, insects have been demonstrated to promote the proliferation of intestinal epithelial cells and prevent *C. difficile*-mediated mucosal damage [12]. The underlying

mechanism is attributed to the presence of anti-microbial peptides (AMPs) (*i.e.* defensins and cecropins) and fatty acids (*i.e.* lauric acid) that can aid in controlling pathogenic bacteria growth, including *C. difficile* [12] and *Streptococcus mitis* [13]. Moreover, insects contain fibres such as chitin that could be fermented by intestinal microbiota and produce SCFAs such as butyrate which supports proliferation of intestinal epithelial cells [14].

In this study, we explored *C. difficile* toxin A counteractive potency of four different insect-derived factions with varying content of AMPs, fatty acids and polysaccharides that were *in vitro* digested to mimic intestinal bioactivity. Support of the barrier integrity of small intestinal human epithelial cells and the potential mechanism was investigated using black soldier fly larvae protein meal, mealworm larvae powder, chitin rich black soldier fly larvae protein meal, and black soldier fly cocoon meal.

2. Materials & Methods

2.1 Nutritional composition of insect-derived fractions

Black soldier fly larvae protein meal (BP), mealworm larvae powder (MP), black soldier fly cocoon meal (BC) and chitin rich black soldier fly larvae protein meal (BchP) were provided by Protix (Dongen, The Netherlands). A detailed producing process and nutritional composition of these insect-derived products was described in a previous study (**Chapter 2**). Briefly, BP contains 56% of protein, 10% of chitin and 15% of fat; BC contains 63% of protein, 20% of chitin and 7% of fat; BchP contains 50% of protein, 15% of chitin and 18% of fat; and MP contains 56% of protein, 8% of chitin and 29 % of fat.

2.2 *In vitro* digestion

In vitro digestion was performed according to the consensus INFOGEST standardized protocol with a minor modification [15]. The enzyme concentration in the consensus INFOGEST protocol was decreased 10-fold to support cell viability upon exposure to digests. The *in vitro* digestion involved the exposure of insect-derived fractions to three successive digestive processes: oral, gastric and intestinal, each containing a dilution component with specific fluids. Simulated salivary fluid (SSF) was prepared by mixing 3.7 mM KH_2PO_4 (Merck, Darmstadt,

Germany), 15.1 mM KCl (Merck), 13.6 mM $NaHCO_3$ (Merck), 0.15 mM $MgCl_2(H_2O)_6$ (BOOM, Meppel, The Netherlands) and 0.06 mM $(NH_4)_2CO_3$ (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 1 L MilliQ (Biopak® polisher; Merck). Simulated gastric fluid (SGF) was prepared by dissolving 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl (Sigma-Aldrich), 0.1 mM $MgCl_2(H_2O)_6$ and 0.5 mM $(NH_4)_2CO_3$ in 1L MilliQ. Finally, simulated intestinal fluid (SIF) was prepared by dissolving 6.8 mM KCl, 0.8 mM KH_2PO_4, 123.4 mM NaCl and 0.33 mM $MgCl_2(H_2O)_6$ in 1 L MilliQ. The oral digestive phase involved the fractionation of insects by IKA mill (model A11 B, IKA-Werke GmbH and Co. KG, Staufen, Germany) to mimic the chewing process, dilution of insects samples by mixing 1 gram of fractioned insects or MilliQ (empty digest control) with 9 grams of MilliQ water. Subsequently, 10 grams of insect suspension was mixed with 8 ml of SSF, 50 µl of 0.3 M $CaCl_2$ (Sigma-Aldrich) and 1.95 ml MilliQ reaching a total volume of 20 ml and the mixture was transferred to a thermal water jacket vessel to maintain a temperature of 37°C through circulating water to complete the oral digestive phase. For the gastric digestive phase, 16 ml of SGF, 0.6 ml pepsin (Sigma-Aldrich) solution (200 units per ml) and 10 µl of 0.3 M $CaCl_2$ were added to the bottle. The pH of the total fluid was adjusted to 3 using 1 M HCl (Sigma-Aldrich) after which the total volume of the mixture was adjusted to 40 ml and incubated for 2h at 37°C. Next the small intestinal digestive phase was performed by adding 27 ml of SIF, 4.02 ml of 199 mM bile extracts (Sigma-Aldrich), 80 µl of 0.3 M $CaCl_2$ and 5 ml of pancreatin solution (10 units/ml) (Sigma-Aldrich). The pH was adjusted to 7 using 1 M NaOH (Sigma-Aldrich) after which the total volume was adjusted to 80 ml, reaching a final insect fraction concentration of 12.5 mg/ml, and incubated for 2h at 37°C. Samples were continuously stirred at level 3 (on a scale from 1 to 8) during all steps of digestion and the pH of the total fluid was monitored and kept at 3 for the gastric digestion phase or 7 for the small intestinal digestion phase by means of the pH-stat titration apparatus (model 888 Titrando, Metrohm AG, Switzerland). Immediately after *in vitro* digestion, digests of the control (empty digest (ED)) BP, BC, BchP and MP were aliquoted and stored at -80°C.

2.3 Caco-2 cells culture and treatment

Caco-2 cells (American Type Culture Collection, Rockville, USA) were seeded on the apical side of transwell inserts of 33.6 mm^2, 0.4 µm pore size and $1x10^8$ pores/cm^2 (Greiner Bio-one, Frickenhausen, Germany). The Caco-2 were added at a concentration of $3.375x10^4$ cells in 150

µl DMEM (Gibco, Bleiswijk, The Netherlands), and 700 µl basolateral medium, containing 4.5 g/l D-glucose, L-glutamine, 25 mM HEPES and supplemented with 10% of heat inactivated fetal bovine serum (FBS; Hyclone PerBio, Etten-Leur, The Netherlands) and cultured at 37˚C with 5% CO_2. The cells were incubated for 21 days to allow differentiation into small intestinal-like epithelial cells. Both the apical and basolateral medium was replaced three times per week and one day before exposure to the samples. Before addition to differentiated Caco-2 cells, the digests of all samples were diluted 4 times with medium. This constitutes the minimal required dilution to prevent digestive enzymes to affect Caco-2 viability (data not shown). FITC-dextran 4 kDa (FD4, Sigma-Aldrich) was added to the apical compartment of differentiated Caco-2 at a concentration of 250 µg/ml and translocation to the basolateral compartment was measured by a spectrophotometer at 490 nm excitation and 525 nm emission wavelength (model Infinite 200 PRO, TECAN, Giessen, The Netherlands). Differentiated Caco-2 cells were exposed to 0.25 µg/ml *Clostridium difficile* toxin A (List Lab, Campbell, CA, USA) and/or digests of insect-derived fractions and the integrity of the Caco-2 monolayer was measured by transepithelial electrical resistance (TEER) by using a MilliCell-ERS (Millipore, Amsterdam-Zuidoost, The Netherlands) apparatus before and after addition of toxin A or any of the stimuli. TEER value was normalised to medium control and starting time point of individual transwells.

2.3 RNA extraction and sequencing

RNA isolation was performed as described previously [16]. Briefly, Caco-2 cells were lysed with 0.2 ml TRIzol® (Invitrogen, Bleiswijk, The Netherlands) after 3h exposure to medium, ED, BPD, MPD, BCD or BchPD and RNA was isolated using the RNeasy mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. The isolated RNA was sent to Baseclear B.V. (Leiden, The Netherlands) for RNA sequencing. The cDNA library was sequenced on the the NovaSeq 6000 system (Illumina, San Diego, CA, USA) to produce 150-bp paired-end reads. The raw paired-ends FASTQ files were trimmed and converted by bcl2fastq2 Conversion Software (version 2.18, Illumina). In this step, the raw data was filtered to remove adapter and low-quality sequences and only reads with average Q-score >30 were imported to CLC Genomics Workbench (Microbial Genomics toolbox version 20.0, Qiagen) for further analysis. The paired-end reads were mapped to Homo sapiens HG38 reference genome (v99) to generate counts of genes and transcript hits as RPKM (reads per kilobase of exon model per million mapped reads) and TPM (transcripts per million). The counts were

normalized by using the TMM (Trimmed Mean of M values) normalization according to the method of Robinson [17]. Normalized counts in treated and control sample were subjected to differential expression analysis which was determined by using a negative binomial Generalized Linear Model [18]. Differentially expressed genes with absolute p value < 0.05, an expression value >10.0 and a fold change ≥ 1 were uploaded into Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) to perform the gene and pathway analysis.

2.4 Gene transcription and pathway analysis

Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) was used to perform a core analysis to analyse significantly activated ($-\log_{10}$(p-value)> 1.3) canonical pathway in Caco-2 cells after exposure to insect-derived fractions when compared to that of empty digest. For analysis, limitations were set to: "Human" in the "Species" category, and "Caco-2 cells" in the category of "Tissue & Cell lines".

2.5 THP-1 cell culture

The human monocytic leukaemia cell line (THP-1; American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) containing 10% of FBS (Hyclone PerBio) and 1% of penicillin/streptomycin (Invitrogen) at 37˚C under 5% of CO_2. THP-1 cells were passaged every 3 days at a concentration of $0.25x10^6$/ml in 20 ml medium in T75 culture flask (Corning®, Amsterdam, The Netherlands). To differentiate THP-1 cells into macrophages, THP-1 cells were seeded onto a 24-well plate (Greiner Bio-one) at a concentration of $1x10^6$ cells/ml in 0.5 ml RPMI medium and exposed to 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 3 days. Following extensive washing to remove residual PMA, the cells were rested for 72h before subsequent stimulations.

2.6 Caco-2 and THP-1 co-cultured model

In the co-culture model, Caco-2 cells were cultured on the apical side of the membrane in transwell inserts and THP-1 macrophages were cultured on the basolateral side of the membrane. Transwells containing Caco-2 that were differentiated for 20 days were placed up-

side-down in a container with sufficient surface area to contain up to 12 transwell insert and sufficient volume to ensure the Caco-2, now inverted, to remain in continuous contact with DMEM containing additional 1% of penicillin/streptomycin as described before as part of generating M-cells [19]. Of note, inverting the transwells is done in such a way that no air pocket is trapped with the Caco-2. A silicon ring was attached to the transwells to create a compartment on the basolateral side of the membrane. In this compartment, 0.5 ml of THP-1 macrophages, harvested after 72h of rest, at a concentration of $0.4x10^6$ cells/ml were seeded onto the transwell membrane and cultured O/N at 37˚C under 5% of CO_2 to support cell adhesion. The next day, the silicon ring was removed and the co-culture system was put into a new 24-well plate with 150 µl and 700 µl fresh DMEM medium in the apical and basolateral compartment, respectively, followed by a 2h recovery period at 37˚C under 5% of CO_2. Typically, 80% of the macrophages remain attached to the basolateral side of the membrane (data not shown). After recovery, medium in the apical compartment was removed and replaced by 4x diluted digests of insect-derived fractions or fresh medium (control) and incubated at 37˚C under 5% of CO_2 for 24h. The integrity of the monolayer was measured by TEER before and 24h after addition of stimuli. After 24h exposure, the basolateral medium was collected and stored at -80 ˚C.

2.7 Measurements of IL-8 secretion

In collected supernatants the secretion of IL-8 was measured by using the IL-8 ELISA kit (BioLegend, Koblenz, Germany) according to the manufacturer's instruction.

2.8 Statistical analysis

Data is presented as mean or mean + SD and statistically significant differences between parameters were analysed by one-way ANOVA (Graphpad Prism 8, La Jolla, CA, USA).

3. Results

3.1 Digested insect-derived fractions did not affect the barrier integrity of small intestinal-like Caco-2 cells

Caco-2 cells, mimicking small intestinal epithelial cells, were exposed to digested insect-derived fractions, being black soldier fly larvae protein meal digest (BPD), mealworm larvae powder digest (MPD), black soldier fly cocoon meal digest (BCD) and chitin rich black soldier fly larvae protein meal digest (BchPD). The insect-derived fractions were digested according to the consensus INFOGEST protocol, with the adaptation that enzyme levels were reduced by a factor 10. This did not result in a reduced protein hydrolysis following a full digest (*i.e.* 120 minutes. of intestinal digestion) and represented an enzyme concentration at which small intestinal-like Caco-2 viability was not impeded (data not shown). All applications of insect-derived fractions were following this digestion protocol.

Changes in barrier integrity of small intestinal-like Caco-2 cells were monitored using TEER analysis during a period of 24h (Figure 1A) and by determining basolateral FD4 translocation over a period of 24h (Figure 1B). Not empty digest (ED), containing solely the enzymes added during digestion, nor any of the insect-derived fractions induced significant changes in TEER or FD4 translocation levels when compared to medium control.

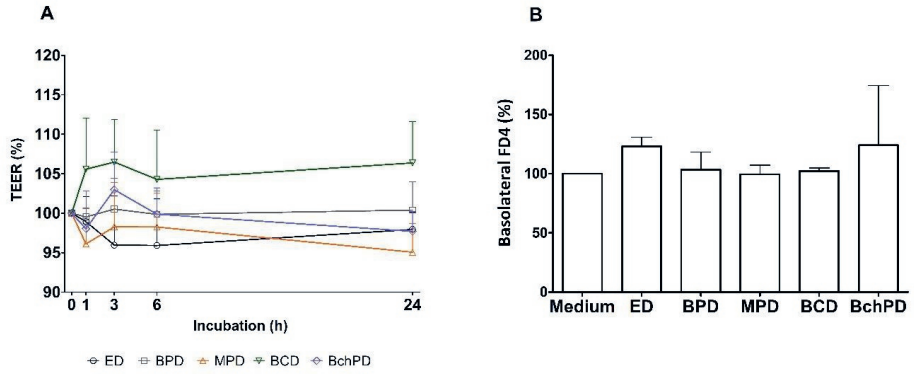

Figure 1: Effect of insect-derived fractions on barrier integrity of small intestinal-like Caco-2 cells. Caco-2 cells were differentiated into small intestinal-like cells and exposed to ED, BPD, MPD, BCD and BchPD at a concentration of 3.125 mg/ml (4x times dilution of digests) for a period of 24h. The barrier integrity was evaluated by measuring TEER directly before and 1, 3, 6 and 24h after addition of the digests (A). FD4 translocation was measured by determining the

accumulated fluorescent signal in the basolateral compartment by spectroscopy (B). Both analyses were represented in percentages relative to medium (set as 100%). Line charts and bar charts show the mean of 3 independent experiments + SD. Statistical analysis was performed by one-way ANOVA and no significant differences were observed. BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; FD4: FITC-Dextran 4 kDa; MPD: digest of mealworm larvae powder.

3.2 Insect-derived fractions mitigated toxin A-induced Caco-2 barrier disruption

Having established that the insect-derived fractions did not reduce the barrier integrity, we investigated whether they can counteract harmful effects of *C. difficile*-derived toxins on the intestinal epithelium. To this end, we applied toxin A to the small intestinal-like Caco-2, which is known to disrupt the barrier integrity [20] and indeed significantly reduced TEER levels with approximately 50% after 3 hours and reaching approximately 75% after 24 hours of exposure (Figure 2).

Next, differentiated Caco-2 cells were simultaneously exposed to toxin A and medium (no treatment) or insects-derived fractions or empty digest control (Figure 2). The addition of ED or BCD did not alter the TEER of Caco-2 cells when compared to exposure to toxin A alone. In contrast, the addition of BPD, MPD or BchPD to the toxin A challenged Caco-2 led to a significant ($p < 0.05$) increase in TEER values. After 3 hours of incubation the impact of toxin A on TEER was reduced by 27.1%, 18.2% or 21.5% through the addition of BPD, MPD or BchPD, respectively. After 6 hours of incubation BPC, MPD or BchPD continued to reduce the impact of toxin A by 9.2%, 7.1% or 8.1%, respectively.

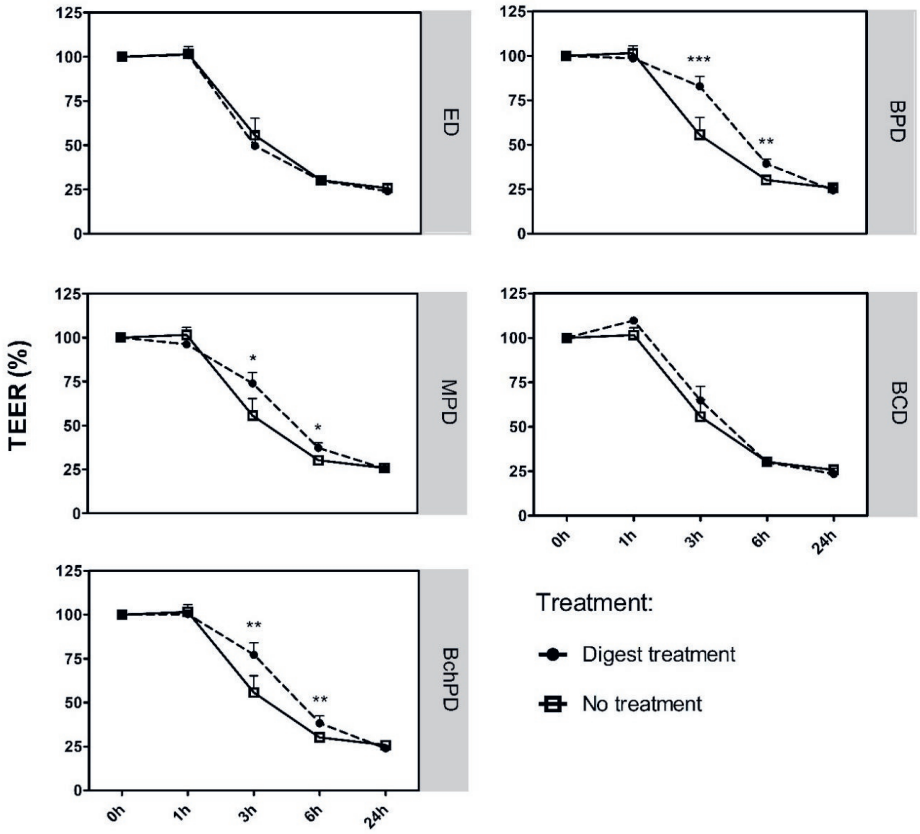

Figure 2: Insect-derived fractions reduced the Caco-2 barrier damaging effect of *C. difficile*-derived toxin A. Caco-2 cells were differentiated into small intestinal-like cells and exposed to toxin A in the presence of medium (no treatment), ED, BPD, MPD, BCD or BchPD. TEER was measured over time and the medium control before addition of digests or toxin (t=0) was set as 100%. Line charts show the mean of 3 independent experiments + SD; Statistical analysis was performed by one-way ANOVA with *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$; BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder.

3.3 Insect-derived fractions demonstrated a preventive effect towards toxin A-mediated Caco-2 barrier disruption

To study whether pre-treatment of Caco-2 cells with insect-derived fractions could also limit the impact of toxin A on the Caco-2 monolayer, we exposed Caco-2 cells to ED, BPD, MPD, BCD and BchPD for a period of 3h prior to addition of toxin A (Figure 3). Before the addition of toxin A, the digests were removed through aspiration. Similar as shown in Figure 1, none of the pre-treatments altered the TEER values during the 3 hours before addition of toxin A. In this preventive setting, again BPD, MPD or BchPD demonstrated significantly ($p < 0.05$) increased TEER levels when compared to medium treatment of toxin A. ED and BCD again did not counteract the impact of toxin A. In addition to pre-treatment, the effects of the insect-derived fractions were also tested in a curative setting. When added 2h post the addition of the toxin A, medium nor any of the insect-derived fractions significantly mitigated the toxin A mediated Caco-2 barrier disruption (Supplementary Figure 1).

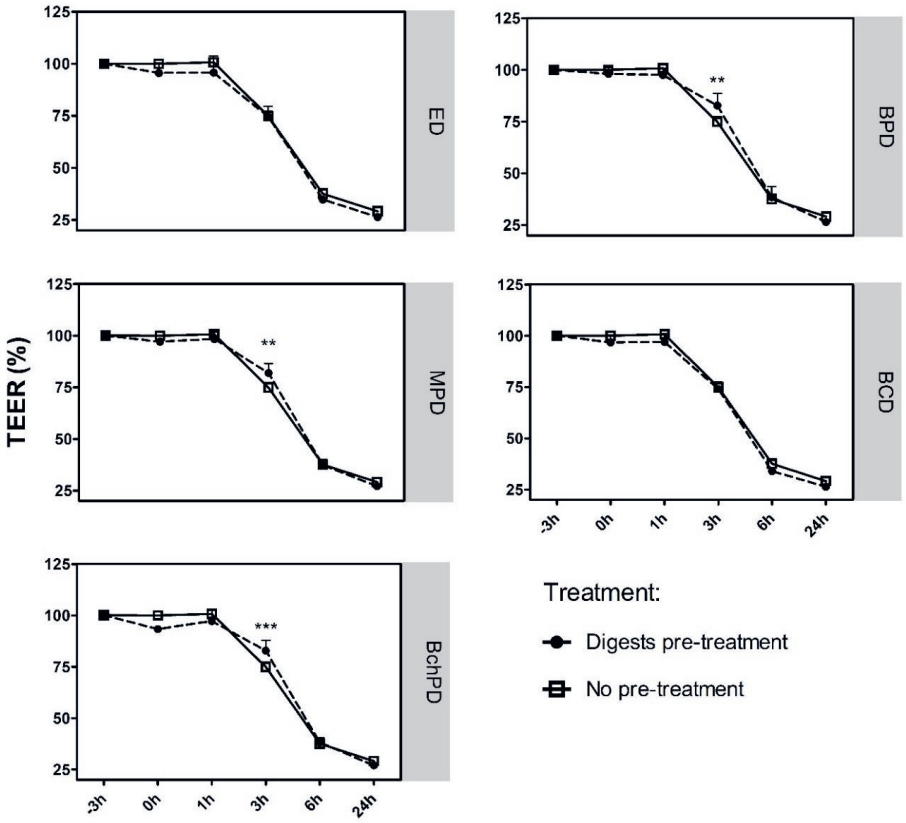

Figure 3: Pre-treatment with insect-derived fractions reduced Caco-2 barrier damaging effect of *C. difficile*-derived toxin A. Caco-2 cells were differentiated into small intestinal-like cells and exposed to medium (no treatment), ED, BPD, MPD, BCD or BchPD for 3h prior to the toxin A challenge. Before the addition of toxin A, the digests were removed. TEER was measured at the beginning (-3h), immediately after toxin A exposure and at 1, 3, 6, and 24h. Analysis was represented in percentages relative to medium at t=-3h (set as 100%). Line charts showed the mean of 3 independent experiments + SD; Statistical analysis was performed by one-way ANOVA with **: $p < 0.01$, ***: $p < 0.001$; BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder.

3.4 Insect-derived fractions induced significant changes in gene transcription of Caco-2

To investigate the mechanism by which the insect-derived fractions counteract the toxin A-induced Caco-2 barrier damage, the transcriptional changes of Caco-2 following stimulation with the fractions was investigated. The RNA was extracted from cells treated for 3h with ED, BPD, MPD, BCD or BchPD and analysed via RNA sequencing to provide a transcriptional overview. Gene expression was investigated as a fold change compared to ED treated Caco-2. Most significant ($p< 0.001$) changes in gene expression when compared to ED were observed following BchPD stimulation (*i.e.* 229) and for the least amount of genes following BCD stimulation (*i.e.* 78) (Figure 4A). Among all the genes that were significantly increased or decreased in transcription by the individual stimuli, expression of one gene was downregulated and expression of 34 genes was upregulated by BPD, MPD, BCD as well as BchPD (data not shown). Of note, transcription of 138 genes was altered by both BPD and BchPD, reflecting the homogenicity between these two samples (Figure 4B). Genes of which transcription was commonly or stimulation-specifically activated were listed in the Supplementary Table 1.

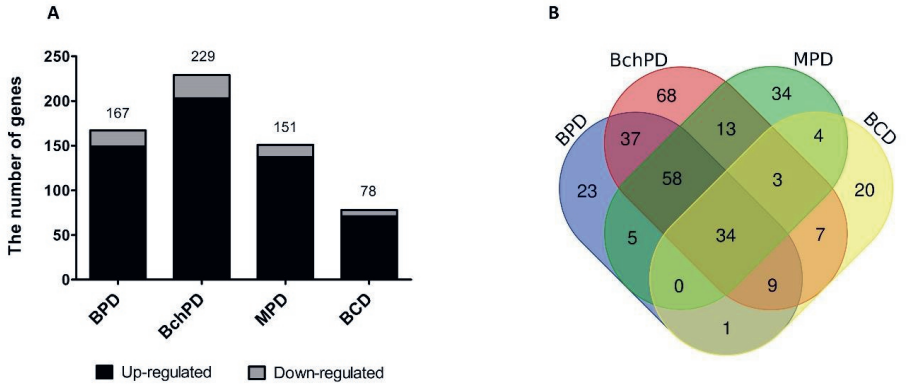

Figure 4: Transcriptional changes in small intestinal-like Caco-2 cells after exposure to BPD, BchPD, MPD or BCD. Caco-2 cells were differentiated into small intestinal-like cells and exposed for 3h to ED, BPD, MPD, BCD or BchPD. Gene transcriptional changes of Caco-2 cells were analysed by RNA sequencing. The number of significantly ($p < 0.001$) up- and down-regulated genes when compared with ED were indicated with a bar chart (A) and the number of genes of which transcription was significantly altered that were shared between BPD, BchPD, MPD and/or BCD were indicated with a Venn diagram (B). Stacked bars and Venn diagram show the mean of 3 independent experiments. BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder.

3.5 Processes to support barrier integrity appeared to be stimulated by treatment of Caco-2 with insect-derived fractions

Genes coding for proteins involved in forming and maintaining the Caco-2 monolayer barrier integrity were investigated for changes in transcription following stimulation with the insect-derived fractions. Genes related to tight junctions, non-classical junctions (*i.e.* immunoglobulin superfamily cell adhesion molecules and tetraspanin-enriched microdomains), desmosomes and focal adhesions [21] with significant ($p< 0.05$) changes in expression when compared to ED were listed in Table 1. In general, all insect-derived fractions demonstrated increased gene transcription of junction-related genes when compared to ED. Of note, only claudin-1 was found to be significantly ($p< 0.05$) increased in ED when compared with medium among all the epithelial cell junction-related genes (data not shown). Next, all genes that were significantly

($p < 0.05$) changed in transcription following Caco-2 treatment with ED, BPD, MPD, BCD or BchPD were integrated and analysed using ingenuity pathway analysis (IPA). Analysis of activated or inhibited canonical pathways (Figure 5A) showed that only BPD and BchPD were predicted with high significance ($(-\log_{10}(\text{p-value}) > 1.3$) to activated pathways related to barrier function. Both BPD and BchPD were predicted to activate "Signalling pathways of Sertoli cell-sertoli cell junction" and "Regulation of epithelial mesenchymal transition (EMT)" in Caco-2, with BPD shown to be more active on regulation of these pathways when compared to BchPD. Neither MPD or BCD activated pathways related to barrier function, indicating that genes with a significantly altered transcription level insufficiency overlapped with genes involved in barrier function-related pathways.

In a similar fashion, ingenuity pathway analysis was applied to identify the activation of pathways related to cell proliferation, as this also represents a manner through which barrier integrity can be supported. The significantly ($-\log_{10}(\text{p-value}) > 1.3$) activated canonical pathways and genes in Caco-2 cells that are related to cell proliferation following the exposure to ED, BPD, MPD, BCD or BchPD were analysed (Figure 5B and supplementary table 2). This revealed that BPD was predicted to activate the "integrin-linked kinase (ILK) signalling" pathway and BchPD was predicted to activate the "p21-activated kinase (PAK) signalling", "Cell cycle control of chromosomal replication", "Aldosterone signalling in epithelial cells", "ErbB2-ErbB3 signalling" and "Extracellular signal-regulated kinases/ Mitogen-activated protein kinases (ERK/MAPK) signalling" pathways in Caco-2 cells. Neither MPD or BCD activated pathways related to cell proliferation.

Table 1: Transcriptional changes in barrier function-related genes following Caco-2 exposure to insect-derived fractions.

Gene acronym	Gene name	BPD	MPD	BCD	BchPD
CEACAM1	CEA cell adhesion molecule 1	1.20	1.25	N. D	N. D
CLDN1	claudin 1	1.56	1.25	N. D	1.36
CLDN2	claudin 2	N. D	1.38	N. D	N. D
CLDN4	claudin 4	1.55	1.29	N. D	1.48

CLDN7	claudin 7	N. D	N. D	-1.28	-1.30
CLDND1	claudin domain containing 1	1.56	1.31	1.38	1.69
CXADR	CXADR Ig-like cell adhesion molecule	1.37	N. D	1.27	1.57
DSG2	desmoglein 2	1.21	N. D	N. D	N. D
EPCAM	epithelial cell adhesion molecule	N. D	1.30	N. D	1.35
ITGA6	integrin subunit alpha 6	1.30	1.29	N. D	1.40
ITGAV	integrin subunit alpha V	N. D	N. D	N. D	1.30
OCLN	occludin	1.28	1.34	1.27	1.31
PVR	PVR cell adhesion molecule	N. D	-1.40	-1.39	-1.32
TJP3	tight junction protein 3	N. D	N. D	-1.34	N. D
TSPAN12	tetraspanin 12	1.33	1.47	1.28	1.46
TSPAN13	tetraspanin 13	1.28	1.31	N. D	1.38

Caco-2 cells were differentiated into small intestinal-like cells and exposed for 3h to ED, BPD, MPD, BCD or BchPD after which gene transcription was analysed using RNA sequencing. Genes related to epithelial cell junctions were listed with fold changes following exposure to BPD, MPD, BCD or BchPD when compared to ED. N. D: not detected.

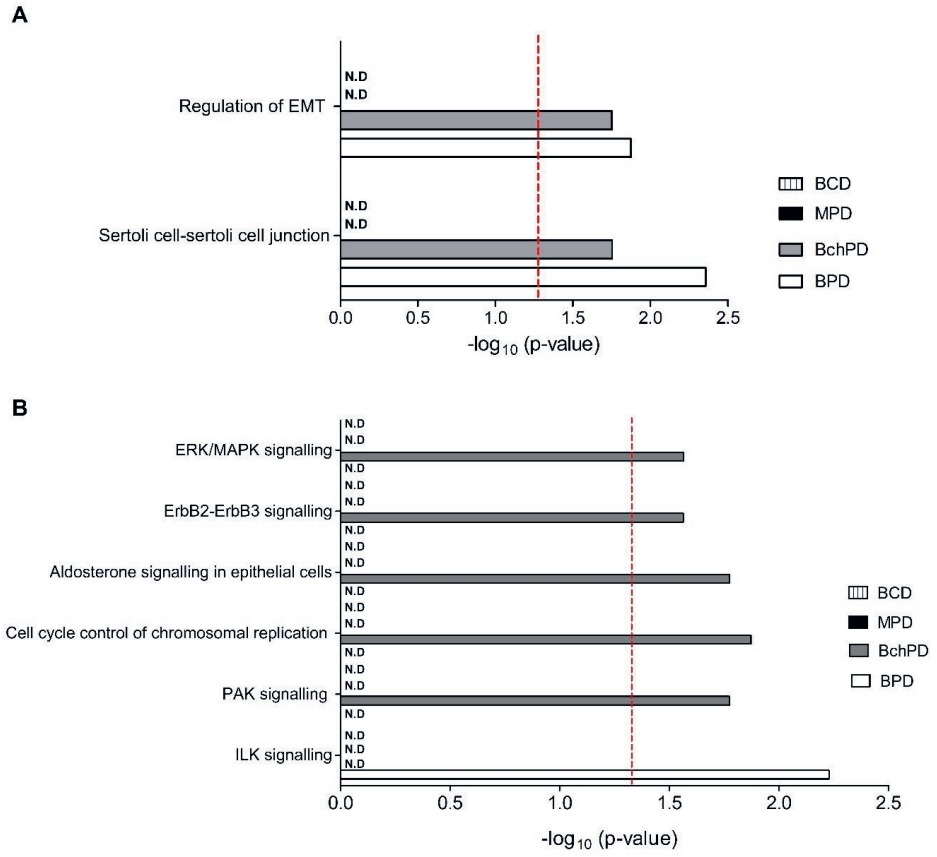

Figure 5: Caco-2 pathways related to epithelial junctions and cell proliferation were predicted to be activated by insect-derived fractions. Caco-2 cells were differentiated into small intestinal-like cells and exposed for 3h to ED, BPD, MPD, BCD or BchPD after which gene transcription was analysed using RNA sequencing. Genes that were significantly ($p < 0.05$) changed in transcription profiles were imported into IPA for canonical pathway analysis. Significantly ($-\log_{10}$(p-value)> 1.3; red dashed line) altered barrier function-related pathways (A) or cell proliferation-related pathways (B) when compared with ED were listed. BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder; N.D: not detected.

3.6 Effect of insect-derived fractions on immune function-related genes expression and pathway analysis

Genes coding for proteins that are related to the immune response of Caco-2 cells were studied for transcriptional changes following exposure to the insect-derived fractions. Genes that were significantly ($p< 0.05$) changed in expression when compared with ED and involved in immune signalling pathways, including chemokines and cytokines, cytokine receptors, and transcription factor that regulate inflammatory or anti-inflammatory responses, were listed in Table 2. In general, the expression of most of these genes was increased after exposure to BPD, MPD or BchPD when compared with ED. Of note, expression of none of the listed genes was significantly ($p< 0.05$) changed following ED exposure when compared with medium (data not shown).

Analysis of significantly ($-\log_{10}$(p-value)> 1.3) activated or inhibited canonical pathways related to immune responses revealed that BPD was predicted to activate cytokine signalling pathways "IL-2-, IL-6- and IL-17 signalling" and chemokine signalling pathway "Chemokine signalling" and BchPD was predicted to activate chemokine signalling pathway "C-X-C chemokine receptor type 4 (CXCR4) signalling" and transcription factor signalling pathway "Nuclear factor activated T-cells (NFAT) signalling" (Figure 6). Similar as for barrier function- and cell proliferation-related pathways, neither MPD or BCD activated pathways related to immune function.

Table 2: Transcriptional changes in immune function-related genes following Caco-2 exposure to insect-derived fractions

Gene acronym	Gene name	BPD	MPD	BCD	BchPD
CCL20	C-C motif chemokine ligand 20	3.11	2.04	2.96	5.62
IL17RB	interleukin 17 receptor B	-1.36	-1.46	N. D	N. D
IL22RA1	interleukin 22 receptor subunit alpha 1	N. D	-1.44	-1.35	-1.44
IL6ST	interleukin 6 signal transducer	1.28	N. D	1.42	1.35

MYD88	MYD88 innate immune signal transduction adaptor	1.26	1.26	N. D	N. D
NFKBIA	NFKB inhibitor alpha	1.62	N. D	1.40	2.02
TAB2	TGF-beta activated kinase 1 (MAP3K7) binding protein 2	1.31	1.28	1.29	1.29
TNFRSF1A	TNF receptor superfamily member 1A	1.75	1.78	N. D	1.68
TNFRSF21	TNF receptor superfamily member 21	1.61	1.70	N. D	1.73
TRAF7	TNF receptor associated factor 7	N. D	-1.36	N. D	N. D

Caco-2 cells were differentiated into small intestinal-like cells and exposed for 3h to ED, BPD, MPD, BCD or BchPD after which gene transcription was analysed using RNA sequencing. Genes related to immunity were listed with fold changes following exposure to BPD, MPD, BCD or BchPD when compared to ED. N. D: not detected.

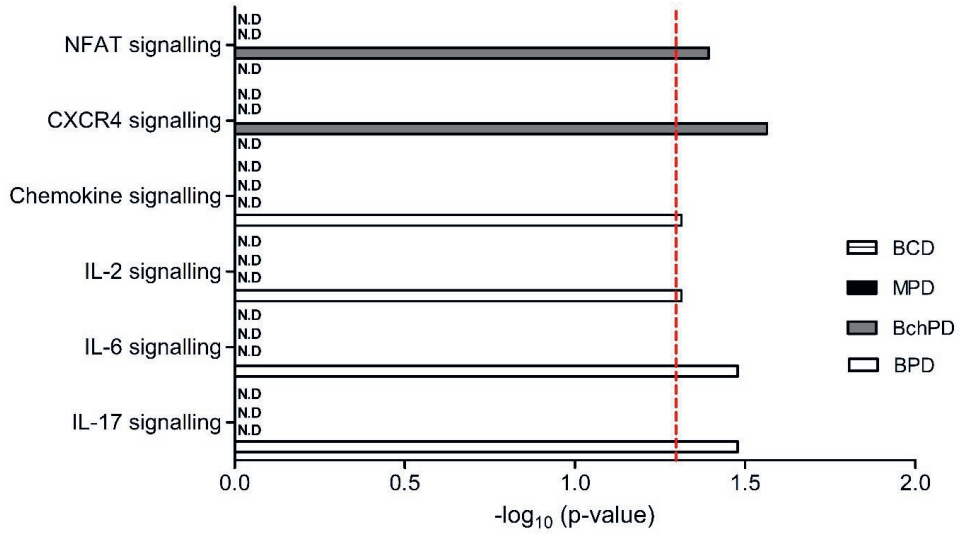

Figure 6: Caco-2 pathways related to immune response that were predicted to be activated by insect-derived fractions. Caco-2 cells were differentiated into small intestinal-like cells and exposed for 3h to ED, BPD, MPD, BCD or BchPD after which gene transcription was analysed

using RNA sequencing. Gene that were significantly ($p< 0.05$) changed in transcription profiles were imported into IPA for canonical pathway analysis. Significantly ($-\log_{10}$(p-value)> 1.3; red dashed line) altered immune function-related pathways when compared with ED were listed. BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder; N.D: not detected.

3.7 Testing immunomodulatory effects of insect-derived fractions on a co-culture of Caco-2 and THP-1 macrophages

To further characterize Caco-2 immune signalling, a transwell model was employed in which Caco-2 were co-cultured with THP-1 macrophages. THP-1 macrophages were adhered to the basolateral side of the membrane of small intestinal-like Caco-2 cells. As a result, the Caco-2 and THP-1 macrophages were physically separated only by a porous membrane, potentially allowing direct cell-cell contact. The model was stimulated with apical addition of medium, ED, BPD, MPD, BCD or BchPD and analysed for basolateral presence of IL-8 (Figure 7). The results revealed that not ED exposure, nor exposure to any of the insect-derived fractions, induced significant changes in the basolateral IL-8 concentration of the co-cultured Caco-2 and THP-1 macrophages when compared with medium control.

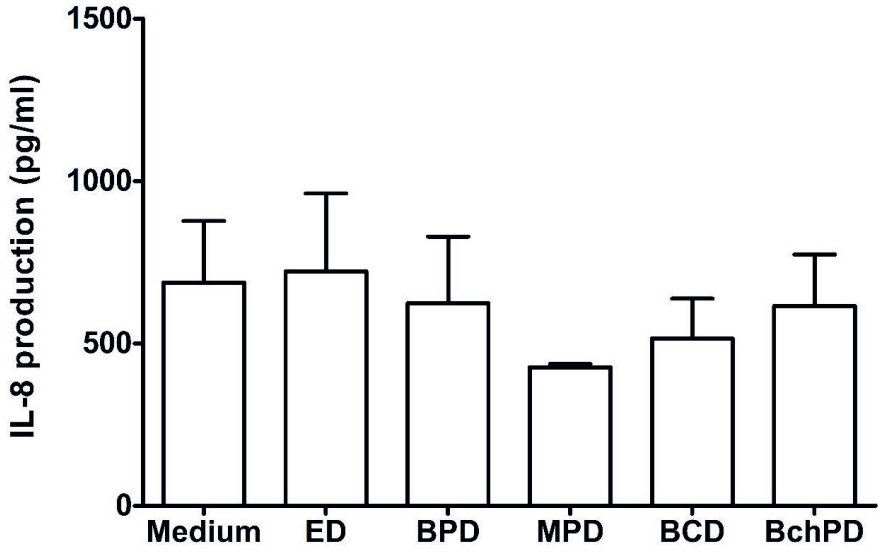

Figure 7: Exposure of the Caco-2/THP-1 macrophage co-culture model to insect-derived fractions did not alter basolateral IL-8 levels. Caco-2 cells were differentiated to small intestinal-like cells in transwells to which THP-1 macrophages were adhered on the other side of the membrane. The co-culture model was exposed to medium, ED, BPD, MPD, BCD and BchPD for 24h after which the basolateral IL-8 levels (pg/ml) were measured. The bar charts show the mean of 3 independent experiments + SD. Statistical analysis was performed by one-way ANOVA and no significant differences were found. BCD: digest of black soldier fly cocoon meal; BchPD: digest of chitin rich black soldier fly larvae protein meal; BPD: digest of black soldier fly larvae protein meal; ED: empty digest; MPD: digest of mealworm larvae powder.

4. Discussion

Pathogenesis of *C. difficile* is associated with enterotoxin secretion [6, 7] and for many years, studies towards mitigating these effects have mainly focused on the large intestine as *C. difficile* colonization was initially thought to occur only in the colon [22]. However, *C. difficile* infection was also demonstrated in the small intestine [23]. Here, *in vitro* models mimicking the small intestinal enterocytes were applied to investigate whether insect-derived fractions can mitigate the barrier integrity reducing effect of *C. difficile* toxin A and explore the underlying mechanisms. We tested four different insect-derived fractions, being black soldier fly larvae protein meal (BP), mealworm larvae powder (MP), chitin rich black soldier fly larvae protein meal (BchP) and black soldier fly cocoon meal (BC), after digestion according to the INFOGEST consensus protocol with lowered enzyme concentrations [15].

We have found that our insect-derived fractions did not impede the integrity of small intestinal-like Caco-2 cells (Figure 1). Moreover, BPD, BchPD and MPD significantly attenuated the Caco-2 barrier integrity damage induced by *C. difficile* toxin A (Figure 2). In a preventive model, we also found BPD, BchPD and MPD to reduce the detrimental effects of toxin A on the Caco-2 barrier integrity (Figure 3). The insect fractions contain a matrix of protein hydrolysates, fibres and fatty acids all with varying bioactivity, complicating the identification of active components and mechanism(s). The intestinal epithelial barrier integrity is critically controlled by tightly connected junctional complexes including tight junctions, adhesion

junctions, gap junctions, desmosomes and tetraspanin-enriched microdomains proteins [21]. Maintenance of the junctions is associated with various signalling molecules such as protein kinase C, mitogen-activated protein kinase and Rho GTPases, which are targeted and rendered ineffective by post-translational modification by *C. difficile* toxins [6, 24]. Junctions are highly dynamic structures that are consistently shaped by both external (*i.e.* food compounds) and internal (*i.e.* secreted hormones and peptides by enteroendocrine cells) factors [24, 25]. To investigate the mechanisms by which the insect fractions support the Caco-2 barrier, RNA sequencing was applied to map the transcriptome. This revealed that on single gene level and on canonical pathway level, BPD, BchPD and MPD supported junctional complexes. In particular, CEACAM1, CLDN1, DSG2, OCLN, TJP3 and TSPAN12, involved in various junctional complexes, and the Sertoli cell-Sertoli cell junction canonical pathway were significantly upregulated and predicted to be activated, respectively, after Caco-2 exposure to BPD, BchPD or MPD (Table 1 and Figure 5A). Lower levels of the tight junction protein 3 (TJP3) and claudin 1 and 2 (CLDN1, 2) were actually found in *C. difficile* toxin A-challenged Caco-2 cells using proteomics [26]. This indicates that BPD, BchPD and MPD might directly counteract the toxin activity. Furthermore, a murine study reported a significantly decreased transcription of SLC20A1 in the cecum after exposure to *C. difficile* toxin A [27]. As SLC20A1 (solute carrier family 20 member 1) encodes a transmembrane protein that can regulate cell proliferation [28], its reduced detection might not be surprising given the apoptosis-inducing activity of *C. difficile* toxin A [29]. Another study demonstrated that an anti-microbial peptide, CopA3 isolated from the Korean dung beetle, prevented *C. difficile* mediated intestinal inflammation by enhancing the proliferation of colonic epithelial cells through reducing the expression of $p21^{Cip1/Waf1}$, a cyclin-dependent kinase inhibitor that triggers cell cycle arrest and apoptosis, in colonic epithelial cells [30, 31]. Transcriptional analysis of Caco-2 demonstrated that BPD, BchPD, MPD and BCD significantly enhanced the transcriptional expression of SLC20A1 (Supplementary Table 2) and were predicted to activate various proliferation pathways after exposure to BPD or BchPD (Figure 5B). Taken together, our data reveal that Caco-2 treatment with BPD, BchPD or MPD increases transcription of genes that support junctional complexes and cell proliferation, which might directly counteract the mechanisms through which *C. difficile* toxin A exerts its intestinal barrier integrity reducing actions.

The barrier function of intestinal epithelial cells is closely linked with the intestinal immune homeostasis [2]. A breakdown of intestinal immune homeostasis can lead to increased invasion of luminal microbial pathogens and microbiota-associated molecular patterns, resulting in

inflammation and tissue damage. Intestinal epithelial cells developed defensive strategies, including immunomodulatory capabilities, to limit damage and promote effective barrier function [3]. Their immunomodulatory capabilities are closely linked to the receptors expressed on both apical and basolateral side. In particular, they can express microbial pattern-recognition receptors including toll-like receptors (TLRs) (*i.e.* TLR2) and C-type lectin receptor (CLRs) (*i.e.* Dectin-1) on their membrane which allow intestinal epithelial cells to respond to their luminal environment [32, 33] and produce signalling molecules such as IL-1β or TGF-β to instruct lamina propria residing immune cells [2, 34]. To investigate the immunomodulatory effects of insect fractions on Caco-2 cells, we studied the transcriptional changes of immune signalling genes of Caco-2 cells following exposure. Transcription of immune signalling genes, including CCL20, MYD88 and NFKBIA, was significantly increased upon exposure of Caco-2 to either BPD, BchPD, MPD or BCD (Table 2). Moreover, cytokines pathways of IL-2-, IL-6- and IL-12 signalling and the chemokines pathways were activated after exposure of Caco-2 to BPD and CXCR4 signalling and NFAT signalling were activated after exposure to BchPD (Figure 6). In general, these transcriptional changes are related to proinflammatory responses [35, 36].

In contrast, insect-derived components have demonstrated anti-inflammatory intestinal activities. CopA3, in addition to supporting proliferation, also significantly reduced LPS induced IL-6 and TNF-α production by murine macrophages through inactivating transcription factor STAT1 and STAT5 [37]. Another anti-microbial peptide, apidaecin isolated from honeybee, inhibited LPS induced IL-6 and TNF-α production by human primary macrophages [38]. Chitin, however, has also been extensively reported for its immune supportive effects [39]. These conflicting findings in pro- and anti-inflammatory effects might be related to obvious differences, as we tested a homeostatic system and applied a matrix of bioactive compounds, where these studies focussed on individual components in an inflammatory system. The tested insect-fractions also demonstrated a strong anti-microbial, and specifically an anti-*C. perfringens*, activity (**Chapter 2**), which is in agreement with immune activating properties as observed here. To determine the cumulative activity of the insect fractions, *in vivo* testing is required.

To limit animal trials, *in vitro* complex cell systems development has gained a lot of attention [40]. In line with that we applied a Caco-2 and THP-1 macrophages co-culture model in which intracellular signalling is not limited by spatial separation or dilution. None of the insect-derived

fractions significantly changed basolateral IL-8 levels in the co-culture model when compared to ED (Figure 7). This suggests that the observed immunomodulatory signalling in Caco-2 did not result in THP-1 macrophage inflammation. However, analysing the impact of the insect-fractions in a physiological inflammatory setting in this model, similar as to the CopA3 and apidaecin studies, would provide additional insights.

Taken together, the results of the *in vitro* studies on Caco-2 cells indicated that black soldier fly protein and mealworm powder effectively mitigate a part of the *C. difficile* toxin A-mediated damage in small intestinal-like Caco-2 cells. The underlying mechanism appears to be associated with the ability to enhance the transcription of cell junctional and proliferation genes in Caco-2 cells. The observed increases in immune signalling might provide an additional mechanism by which the insect fractions, in a more complex system, could support the control and eradication of *C. difficile* infections. In conclusion, these findings support the notion that insects contain many bioactive molecules and could support intestinal health *in vivo*.

Data availability

The authors declare that all data supporting the findings are available upon reasonable requests.

Competing interests

The authors report no conflict of interest.

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Author contributions

L.D, C.G, J.J.M, T.V and H.J.W conceived and designed the experiments. L.D, E.O and R.A performed the experiments and data analysis. L.D, R.A, C.G, J.J.M wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary information

Figure S1: Post-treatment with insect-derived fractions did not significantly ($p < 0.05$) reduce Caco-2 barrier damaging effect of *C. difficile*-derived toxin A.

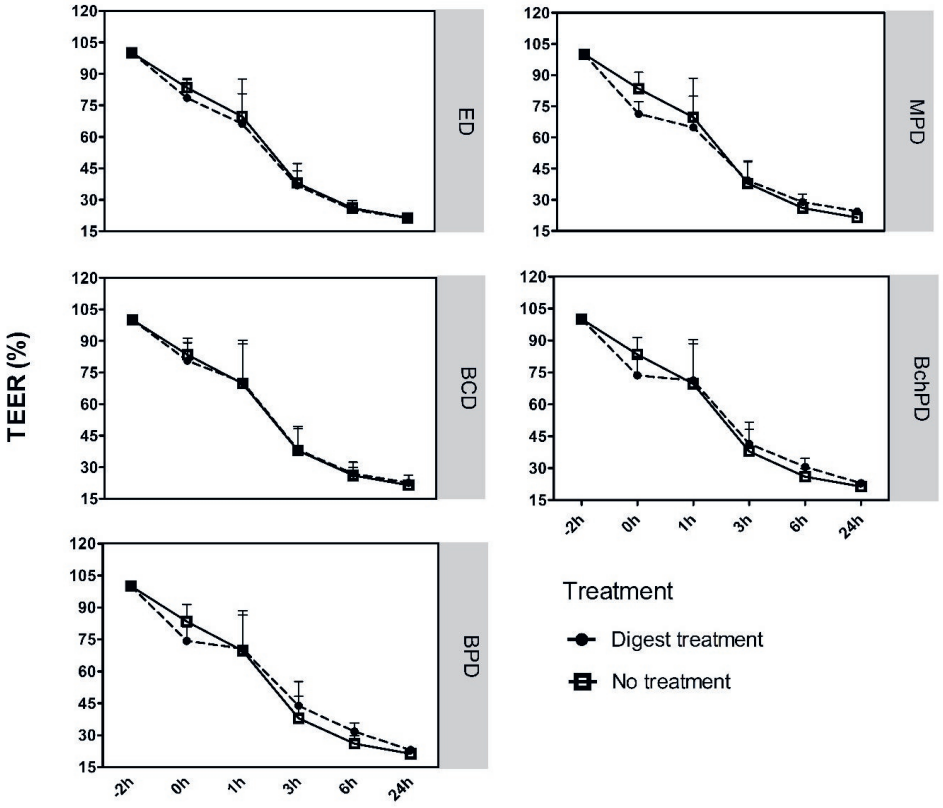

Table S1: Transcription of genes that are commonly or stimulation-specifically activated in Caco-2 cells after exposure to insect-fractions.

Insects	No. of genes	Genes
BCD BPD BchPD MPD	34	HMGCR UGCG LMO7 TM4SF1 JUND ARHGAP18 HSD3B1 DUSP6 PRELID3B ACSL3 CCL20 RSRC2 SQLE SPRED1 PCK1 NUDT4 ENC1 ZFP36L1 SC5D ARL5B BMP2 HMGCS1 EREG FAM107B TIPARP ACSL5 IDI1 AREG SLC20A1 CYP1A1 SLC30A1 INSIG1 BET1 MSMO1
BPD	23	KRCC1 MT1F ENPP4 CDKN1A DENND4C TCIM STAM CLDN4 ADM CD2AP PPP1R15B CHTF8 CD55 MUC13 CCND1 BPNT2 MAPK1IP1L TXNIP ERG28 MT-CYB PPTC7 JMJD1C ZFP36L2
BchPD	68	SMIM24 PPP1CC YIPF6 MCM3 AZIN1 PTPRR VMP1 FAM3C IER3 MTA2 CYCS INSIG2 HSPA13 MAP3K7 DDTL GGPS1 ARF6 ANP32E TBCC CXADR TUBAL3 PEX13 RPL21 LYPLA1 SCOC PTBP3 RSL24D1 PPIL4 TMEM135 FOXN2 PRR15L PFDN4 LRRC19 PM20D2 TAF1D CETN3 TMED5 GCH1 CHMP1B XPO1 BLCAP ETNK1 NCOA7 ARFIP1 G0S2 CREBZF NOP2 EIF2A UBE2B GLYCTK RNF128 TMF1 KCTD9 MCL1 PI4K2B PPIG SELENOI TMEM106B GMFB BTG3 TST RPF2 PERP RYBP GPBP1 CHMP2B NEK7 CLK1
MPD	34	EMP1 RRAS2 CDC42SE1 EPB41L4B TSPAN12 AK3 HIGD1A DNAJA2 FAT1 CSTF2 FAM102A SMIM3 MAF TSKU ATF3 GPRC5A AKIRIN2 SNTB1 FGA CPT1A EPAS1 KLF10 RHEB GNG10 AMACR WDFY1 RESF1 TOB1 GOLIM4 OBI1 NCR3LG1 SLC46A1 NADK2 KLF4
BCD	20	BLOC1S5-TXNDC5 SOS1 GTF2A1 BMF MAN1A1 PAXBP1 SLC35A3 SVIP RAB9A ATP8B2 TRIM2 EDC3 C2orf72 ESF1 SLC5A3 DCDC2 MOB4 LYSMD3 GLRX SLC25A24

Table S2: Transcriptional changes (Fold changes compared to ED >1.6) in cell proliferation function-related genes following Caco-2 exposure to insect-derived fractions.

Gene acronym	Gene name	BPD	BchPD	MPD	BCD
EREG	epiregulin	3.13	3.84	2.74	1.97
AREG	amphiregulin	2.76	3.28	2.31	1.77
GDF15	growth differentiation factor 15	2.17	2.70	N.D	N.D
SLC20A1	solute carrier family 20 member 1	2.40	2.56	2.60	1.48
JUND	JunD proto-oncogene, AP-1 transcription factor subunit	2.24	2.49	1.79	1.87
BMP2	bone morphogenetic protein 2	2.16	2.44	2.32	1.45
KLF6	Kruppel like factor 6	2.10	2.36	1.43	1.37
RND3	Rho family GTPase 3	1.55	1.92	1.40	N.D
HMGB2	high mobility group box 2	1.42	1.83	1.41	N.D
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	2.24	1.81	N.D	N.D
TCIM	transcriptional and immune response regulator	2.35	N.D	N.D	N.D
KLF4	Kruppel like factor 4	N.D	N.D	2.04	N.D
FZD5	frizzled class receptor 5	1.54	1.76	1.69	N.D
GMFB	glia maturation factor beta	1.34	1.65	1.31	1.43
ARHGAP18	Rho GTPase activating protein 18	1.57	1.63	1.53	1.59
HBP1	HMG-box transcription factor 1	1.43	1.62	1.33	N.D
MAPK6	mitogen-activated protein kinase 6	1.42	1.60	N.D	1.44
KLF11	Kruppel like factor 11	1.66	2.01	1.71	N.D
CCNL1	cyclin L1	1.60	1.58	1.47	1.40
RHOB	ras homolog family member B	1.89	1.37	1.98	N.D
RHPN2	rhophilin Rho GTPase binding protein 2	1.80	1.63	1.89	N.D

Chapter 4

In vitro studies towards the use of chitin as nutraceutical: impact on the intestinal epithelium, macrophages and microbiota

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Abstract

Scope: Chitin, the most abundant polysaccharide found in nature after cellulose, is known for its ability to support wound healing and to lower plasma oxidized LDL levels. Studies have also revealed immunomodulatory potential but contradicting results were often impossible to coalesce through usage of chitin of different or unknown physicochemical consistency. In addition, only a limited set of cellular models have been used to test the bioactivity of chitin.

Methods and Results: We investigated chitin with well-defined physicochemical consistency for its immunomodulatory potency using THP-1 macrophages, impact on intestinal epithelial barrier using Caco-2 cells, and fermentation by faecal-derived microbiota. Results showed that chitin with a degree of acetylation of ~83%, regardless of size, does not affect the intestinal epithelial barrier integrity. Large-sized chitin significantly increased acetic acid production by gut microbiota without altering the composition. Exposure of small-sized chitin to THP-1 macrophages led to significantly increased secretion of IL-1β, IL-8, IL-10 and CXCL10 in a multi-receptor and clathrin-mediated endocytosis dependent manner.

Conclusions: These findings indicate that small-sized chitin does not harm the intestinal barrier nor affects SCFA secretion and microbiota composition, but does impact immune activity which could be beneficial to subjects in need of immune support or activation.

Key words: chitin; intestinal epithelium; immunomodulation; microbiota; SCFAs

1. Introduction

Diseases that are associated with a disbalance in immune homeostasis are increasing in frequency over the past decades [1]. In cases of autoimmune disorders and allergy, the immune system is overreacting to harmless antigens [2]. Whereas in case of inflammatory bowel disease, the inflammation is of chronic nature with a failed feedback loop to resolve it [3]. In contrast, the immune system is not sufficiently reactive in case of cancer [4] or secondary opportunistic disease [5].

The immune balance is a result of genetic activity, stress, nutrition and other environmental factors. Many studies focus on nutrition to skew immune responses into a supposedly beneficial direction [6]. An example of immunomodulatory food components or nutraceuticals are dietary fibres. In particular non-digestible dietary fibres were shown to support health by strengthening the intestinal barrier, modifying microbiota composition and short-chain fatty acid (SCFA) production and supporting immune function. Research towards these effects mainly include *in vitro* and animal studies, but also clinical trials. For example, a clinical study reported that a four week intervention with arabinoxylan or resistant starch type 2 in adults with metabolic syndrome supported a healthy microbial diversity. They observed significantly increased acetate and butyrate concentrations and levels of *Bifidobacterium*, while simultaneously reducing the proportion of species that are associated with a dysbiosis [7]. Similarly, a 16-week intervention with inulin in obese children resulted in reduced IL-6 levels in serum and increased *Bifidobacterium spp.* and decreased *Bacteroides vulgatus* levels [8]. The observed effects on microbiota are possibly directly related to the type of dietary fibre, which can vary greatly based on source and extraction method [9]. In general, however, dietary fibres increase the abundance of bifidobacteria and lactobacilli, which appear to be beneficial for health and reduce infections with pathogenic bacteria, such as *Escherichia coli* [10, 11]. Next to increasing beneficial and reducing harmful bacteria concentrations, changes in intestinal metabolite concentrations also appear to support beneficial health effects [12]. The observed increases in SCFA, and butyrate in particular, have been related to improved health as they beneficially impact host metabolism and intestinal immunity [13]. Dietary fibres have also been shown to impact peripheral immunity by increasing frequency and activity of circulating B cells, NK cells and/or monocytes [14]. Furthermore, there might be a direct interaction between dietary fibres or SCFAs and the gut-associated-lymphoid tissue as there are many receptors described to bind both [15].

A compound similar to commonly consumed or tested non-digestible dietary fibres, with potency to improve intestinal health, is chitin. Chitin is a linear polymer of β-(1-4)-linked N-acetyl-glucosamine and is the most abundant polysaccharide found in nature after cellulose. So far, clinical trials have reported that orally consumed chitin lowers plasma oxidized LDL levels and therefore reduces the risk of cardiovascular diseases [16]. Furthermore, many studies described the potency of chitin to impact the immune system [16]. Results have been contrasting with studies on the one hand demonstrating inflammatory effects, such as chitin-mediated release of IL-12, TNFα, IFNγ by spleen cells and the induction of an oxidative burst in macrophages [17-19]. On the other hand, studies report on anti-inflammatory properties, such as reduced secretion of IL-1β, IL-6, TNFα, nitric oxide and prostaglandin E2 by microglia cells, but also increased IL-10 secretion by macrophages [20, 21]. The conflicting results may stem from the differences in physicochemical properties of chitin fractions used in these studies. Despite this, these studies do demonstrate the potency for chitin to support intestinal health. Furthermore, chitin is readily available from large side-streams of shrimp and insect production securing practical availability. To consider chitin as nutraceutical to support intestinal health similar to dietary fibres, we need to improve the understanding of its functional impact. This requires analysis of parameters that are relevant to intestinal health and immune balance (*i.e.* the intestinal epithelial barrier, immune system and microbiota), but also the need for detailed description of chitin characteristics, which is lacking in many studies.

In this study, we used chitin particles with a known degree of acetylation and size fragmentation and evaluated the interaction with several models of the intestinal immune barrier and with microbiota.

2. Experimental section

2.1 Chitin preparation and characterization

Fine chitin powders were prepared from a commercial chitin from shrimp shells in coarse flakes form (Sigma-Aldrich, Zwijndrecht, The Netherlands) by two continuous milling processes. Firstly, chitin coarse flakes were cracked with the IKA mill (model A11 B, IKA-Werke GmbH and Co. KG, Staufen, Germany) while frozen by liquid nitrogen to obtain chitin particles followed by another milling process with a PM100 planetary ball mill (Retsch, Haan, Germany)

in a 50 ml zirconium dioxide jar containing 17 φ10 mm zirconium dioxide balls at a frequency of 500 rpm for 1.5 h. A 10 min interval was set among every 15 min ball milling to prevent overheating to obtain a fine chitin powder. Next, the chitin powder was separated based on particle size using the Airjet Sieve (model e200LS, Hosokawa-Alpin, Augsburg, Germany) with 100 µm, 50 µm, and 20 µm sieves. Finally, the sample size was validated by the Mastersizer 3000 equipped with a laser diffraction practice analyser (Malvern Panalytical, Worcestershire, UK). In addition to size, the degree of acetylation (DA) of chitin fractions was determined by using the first derivative UV method and the formula: DA (%) = ((m1/203.21)x100) / ((m1/203.21)+((M-m1)/161.17)) [22]. In summary, glucosamine hydrochloride (Glc) (Sigma-Aldrich) and N-acetyl-D-glucosamine (GlcNAc) (Sigma-Aldrich) were dissolved in 0.85% phosphoric acid (Sigma-Aldrich) at a concentration of 0, 10, 20, 30, 40, 50 µg/ml, and the first derivative of UV value at 203 nm (H203) was measured by a UV-vis spectrophotometer (model Shimadzu 1800, Shimadzu Corporation, Kyoto, Japan) using a far UV cuvette with a 10 mm pathway. A calibration curve was constructed by plotting the H203 as a function of different concentrations of GlcNAc and Glc. To evaluate the DA of chitin, the amount of GlcNAc in chitin samples was identified from the calibration curve. Specifically, 100 mg (M) chitin samples were suspended in 20 ml of 85% phosphoric acid and incubated at 60˚C for 40 min to fully dissolve the chitin. Next, 1 ml of this solution was diluted with 99 ml of MilliQ water and heated for another 2 h at 60˚C. After this, the H203 of the solution was measured immediately in technical triplicates and the mass of GlcNAc in 1 ml solution (m1) was calculated according to the calibration curve described above.

2.2 Endotoxin measurements

HEK-Blue^{TM} human TLR4 (Invitrogen, Bleiswijk, The Netherlands) cells were used for testing the endotoxin content in chitin. HEK-Blue hTLR4 cells were seeded on a poly-D-lysine-coated 96-well flat bottom plate (Greiner Bio-one, Frickenhausen, Germany) at a concentration of $0.5x10^6$ cells/ml (200 µl) and incubated at 37˚C for 24 h. The cells were next exposed to medium, lipopolysaccharide (LPS) (titration of 0.0001 ng/ml – 100 ng/ml (Figure S1) dissolved in medium and a homogeneous chitin suspension (0.1 mg/ml) in medium, achieved by thorough mixing through vortexing and resuspending, and incubated for 24 h at 37˚C. After incubation, 20 µl of the supernatant was mixed with 180 µl of Quanti-Blue ^{TM} (InvivoGen, Toulouse, France) in a 96-well flat bottom plate and incubated at 37˚C for 2 h after which the absorbance was

measured at 655 nm using a spectrophotometer (model Infinite 200 PRO, TECAN, Giessen, The Netherlands).

2.3 THP-1 cell culture and treatment with chitin fractions

The human monocytic leukaemia cell line (THP-1; American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) containing 10% of foetal bovine serum (FBS; Hyclone PerBio, Etten-Leur, The Netherlands) and 1% of penicillin/streptomycin (Invitrogen) at 37˚C under 5% of CO_2. Cells were sub-cultured twice per week and set at $0.25x10^6$/ml in 20 ml medium in a T75 culture flask (Corning®, Amsterdam, The Netherlands). To differentiate the cells into macrophages, $0.5x10^6$ cells were exposed to 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) in 0.5 ml RPMI medium for 72 h in 24-well cell culture plate (Greiner Bio-one). Following extensive washing to remove residual PMA, the cells were rested for 72 h. Rested THP-1 macrophages were exposed to medium (supplemented RPMI) as control or a homogeneous chitin suspension in medium, achieved by thorough mixing through vortexing and resuspending, at a concentration of 0.1 mg/ml and incubate at 37˚C for 24 h.

2.4 Caco-2 cell culture and treatment with chitin fractions

Caco-2 cells (American Type Culture Collection) from passage 30 to 40 were cultured in DMEM (Gibco, Bleiswijk, The Netherlands) containing 4.5 g/L D-glucose, L-glutamine, 25 mM HEPES, and supplemented with 10% of heat-inactivated FBS at 37˚C with 5% CO_2. Transwell inserts of 33.6 mm^2, 0.4 μm pore size and $1x10^8$ pores/cm^2 (Greiner Bio-one) were seeded on the apical side with $3.375x10^4$ cells in 150 μl and suspended in a 24-well flat bottom plate (Greiner Bio-one) with 700 μl basolateral medium and cultured for 7 or 21 days at 37˚C with 5% of CO_2 to differentiate in colon-like or small-intestinal-like epithelial cells, respectively [23]. Apical and basolateral medium was replaced three times a week and one day prior to the addition of samples. Differentiated Caco-2 were exposed to medium or a homogeneous chitin suspension in medium, achieved by thorough mixing through vortexing and resuspending, of 0.1 mg/ml and incubated at 37˚C for 24 h. The transepithelial electrical Resistance (TEER) was measured by using a MilliCell-ERS (Millipore, Amsterdam, The Netherlands) apparatus directly before treatment of the Caco-2 to verify viability of the cells

and directly after treatment of the Caco-2 and at 1, 3, 6 and 24 h, and normalised to the starting time point (set at 100%).

2.5 RNA isolation, cDNA synthesis and Real-time Quantitative PCR

RNA isolation, cDNA synthesis and RT-qPCR were performed as described previously [24]. Briefly, both THP-1 and Caco-2 cells were lysed with 0.2 ml TRIzol® (Invitrogen) after 24 h and RNA was isolated using the RNase mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Subsequently, cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, CA, Hercules, USA) and 5 µl of cDNA was mixed with 10 µl of IQ™ SYBR Green Supermix (Bio-Rad) and 5 µl of primer pairs. The RT-qPCR program consisted of 90 s preheating at 95˚C, 10 s denaturing at 95˚C, 10 s annealing at 58˚C and 15 s elongation at 72˚C for 40 cycles and was run on a CFX96 touch Real-Time PCR detection system (Bio-Rad). Reference genes beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalisation.

2.6 Measurements of cytokine secretion

In the collected supernatants from THP-1 macrophages the secretion of Arginase, CXCL10, IL-1β, IL-1RA, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-23, IFNγ, TARC and TNFα was measured using the LEGENDplex kit (BioLegend, Koblenz, Germany). This is a fluorescent bead-based immunoassay that shares the same principle with sandwich ELISA and allows measuring multiple analytes simultaneously using flow cytometry for detection. The data was analysed using the CytoFlex flow cytometer (Beckman Coulter, Woerden, The Netherlands) with CytoExpert software. IL-8 and IL-10 ELISA kits (BioLegend) were used to measure the individual cytokine levels according to the manufacturer's instruction.

2.7 Endocytosis assay

The endocytosis assay was based on a method described elsewhere [25] with a slight modification. Briefly, chitin fractions at a concentration of 10 mg/ml were incubated with 1 mg/ml fluorescein isothiocyanate (FITC; Sigma-Aldrich) in 0.1 M sodium bicarbonate (Sigma-

Aldrich) for 1.5 h at RT in the dark. Next, the chitin was washed five times with MilliQ water through centrifugation at 20800 g for 5 min and dialyzed using a membrane with a pore size of 12 to 14 kDa (Medicell Membranes Ltd, London, UK) and 5 L MilliQ for 24 h, with the dialysate solution being refreshed after 8 h. The FITC-labelled chitin was lyophilized and stored at RT in the dark. FITC-labelled chitin fractions were suspended in medium and a homogeneous mixture was ensured through vortexing and resuspending and incubated with THP-1 macrophages at a concentration of 0.1 mg/ml for 1 h at 37˚C after which the cells were washed with PBS (Gibco). Subsequently, THP-1 macrophages were incubated with 0.2 ml of trypsin (Gibco) at 37˚C for 10 min and upon collection 0.8 ml of FBS was added to the cells and they were centrifuged at 450 g for 5 min. Cells were re-suspended and stored in 0.2 ml paraformaldehyde (PFA; Sigma-Aldrich) at 4˚C. Before measurement the fluorescence from particles attached to the outer cell membrane was quenched by adding 40 µl of 0.25% trypan-blue (Gibco). To block specific endocytic routes of chitin uptake inhibitors were added for 2 h at 37˚C prior to chitin addition: Cytochalasin B (10 µg/ml; Sigma-Aldrich) to block phagocytosis, nystatin (25 µg/ml; Sigma-Aldrich) to block caveolin-mediated endocytosis, and chlorpromazine (10 µg/ml; Sigma-Aldrich) to block clathrin-mediated endocytosis. Similarly, to determine involvement of receptors in uptake THP-1 macrophages were exposed to 15 µg/ml of anti-TLR2 antibody (Clone: TL2.1, BioLegend), 20 µg/ml of anti-human CD206 antibody (Clone: 19.2, BD Bioscience, Vianen, The Netherlands), 1 mg/ml of laminarin (Sigma-Aldrich) or 10 mM lactose (Sigma-Aldrich) for 30 min at 37˚C prior to chitin incubation, to block TLR2, mannose receptor, dectin-1 or galectin-3 respectively. Cells were washed with medium after blocking routes of endocytosis or receptors before addition of FITC-labelled chitin as described above. Intracellular FITC-signal was measured using the Accuri flow cytometer (BD Biosciences) and Accuri C6 software. The relative uptake of FITC-labelled chitin was determined by subtracting the mean fluorescence intensity (MFI) levels of the background fluorescence of cells treated with RPMI medium from the MFI of cells exposed to FITC-labelled chitin.

2.8 *In vitro* fermentation

The batch *in vitro* fermentation was conducted in a biological duplicate using two healthy adult microbiome donors. Each fermentation vessel contained 43 ml of autoclaved medium (containing per litre: 2 g peptone (Duchefa Biochemie, Haarlem, The Netherlands), 2 g yeast

(Sigma-Aldrich), 0.5 g L-cysteine (Sigma-Aldrich), 5.22 g K_2HPO_4 (Merck, Darmstadt, Germany), 16.32 g KH_2PO_4 (Merck), 2 g $NaHCO_3$ (Merck), 1 g mucin (Sigma-Aldrich) and 2 ml Tween 80 (Sigma-Aldrich) to which 20 ml PBS (control) or PBS containing 350 mg chitin was added. Continuous sparging with O_2-free N_2 resulted in anaerobic fermentation vessels to which 7 ml of microbiota was added. The microbiota was sampled from the distal colon vessel reactor of the Stimulator of Human Intestinal Microbial Ecosystem (SHIME) reactor (ProDigest, Gent, Belgium) after 2 weeks of stabilization [26]. After adding microbiota, the vessels were incubated at 37˚C at constant shaking (200 rpm) and samples (3 ml) were taken after 0 and 24 h for analysis. The samples were centrifuged at 10000 rpm for 3 min at 4˚C and both the supernatant and the pellet were collected and stored at -80˚C. Pellets obtained after 0 h (control) or 24 h (control and chitin fractions) incubation were mixed with 1 ml DNA/RNA shield (Zymo Research, Freiburg im Breisgau, Germany) and sent to Baseclear B.V. (Leiden, The Netherlands) for microbiota composition analysis.

2.9 SCFA analysis

The production of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and lactic acid was determined by high performance liquid chromatography (HPLC) (model Acquity Arc™, Waters, Eschborn, Germany) equipped with an AMINEX HPX-87H column (Aminex HPX-87H, 300x7.8 mm, Bio-Rad Laboratories, Richmond, VA, USA) and the column was maintained at 35°C using an integral column heater (Waters). The target compounds were detected by using a refractive index detector (model R2414, Waters) and the eluent for analysis was 8.3 mM sulphuric acid (Sigma-Aldrich) at a flow rate of 0.5 ml/min. Standards were prepared at a concentration of 11.47 mM for lactic acid (Sigma-Aldrich), 16.55 mM for acetic acid (Merck), 13.96 mM for propionic acid (Sigma-Aldrich), 4.24 mM for isobutyric acid (Fluka, Cheniou, Gmblt, Germany), 11.53 mM for butyrate acid (Sigma-Aldrich), 10.51 mM for isovaleric acid (Fluka), and 10.44 mM for valeric acid (Acros Organic™, Geel, Belgium) in 1 L of 8.3 mM sulphuric acid (Sigma-Aldrich). The standard samples were injected repeatedly every 5 measurements. The calibration curve was constructed by plotting the peak area against the molarity of standard solutions. Tested samples for HPLC were prepared by diluting the collected supernatant with 16.6 mM sulphuric acid at a ratio of 1:1 (v/v).

2.10 Microbiota composition analysis

Bacterial genomic DNA was extracted from each pellet and the 16S rRNA gene sequencing was performed by Baseclear B.V. (L457; NEN-EN-ISO/IEC 17025). Briefly, the microbial DNA was extracted and the V3-V4 region of the 16S rRNA gene was amplified and sequenced by using MiSeq System (Illumina, San Diego, CA, USA). Subsequently, the raw paired-ends FASTQ files were trimmed and converted by bcl2fastq2 Conversion Software (version 2.18, Illumina). The analysis on the resulting data was conducted using CLC Genomics Workbench (Microbial Genomics toolbox version 20.0, Qiagen). 16S rRNA gene paired read sequences were used to prepare an operational taxonomic units (OTUs) table at 99% reference of sequence similarity from Silva 16S/18S gene database (version 132). To evaluate the alpha diversity or beta diversity of each microbiota community, a phylogenetic tree was created by using MUSCLE (version 3.8.425). The phylogenetic tree together with the OTU abundance table were used for alpha and beta diversity clustering.

2.11 Statistics

Microbiota data is presented as the mean of 2 independent experiments with 2 different donors and statistical analysis between parameters was performed using the Kruskal-Wallis test (Figure 2B and C) or the Wald test (Figure 2D). Other data is presented as mean + SD and statistically significant differences between parameters were analysed by one way ANOVA (Graphpad Prism 8, La Jolla, CA, USA).

3. Results

3.1 Physicochemical characterization of size-separated chitin fractions

Fine chitin powder was fractionated by size, resulting in fractions with 80% of the size distributed between 4.8 to 25.5 μm termed small chitin (SC), 19.8 to 71.6 μm termed intermediate chitin (IC) and 50.4 to 140.2 μm termed large chitin (LC) (Table 1). Next to size, the DA was determined and was found to be 80±0.7%, 81.3±2.7%, and 87.5±3.9% for SC, IC and LC, respectively (Table 1). In addition, chitin fractions were analysed for contamination

with the strongly immunomodulatory lipopolysaccharide (LPS), which was not detected in any of the samples (<1pg/0.1mg; Table 1).

Table 2: Physicochemical and biochemical properties of chitin.

	SC	IC	LC
Size distribution (80%)[a]	4.8-25.5 µm	19.8-71.6 µm	50.4-140.2 µm
Degree of acetylation	80±0.7%	81.3±2.7%	87.5±3.9%
LPS contamination[b]	n.d	n.d	n.d

The degree of acetylation of chitin fractions was described as the mean (n=2) ± SD. [a]10% of the sample was smaller and 10% larger than the provided size range; [b]detection limit of the assay was 1 pg/ml as determined with a titration experiment (Figure S1) and the applied chitin concentration 0.1 mg/ml. Statistical analysis on the DA among chitin fractions was performed by one way ANOVA but no significant differences were observed. Abbreviations: IC: intermediated chitin; LC: large chitin; LPS: lipopolysaccharide; n.d: not detected; SC: small chitin.

3.2 Chitin fractions did not affect the barrier integrity of Caco-2 in a transwell system

Caco-2 cells, mimicking small- or large-intestinal epithelial cells, were exposed to the different chitin fractions and the barrier integrity and transcriptional changes of both tight junction and adherence junction proteins were measured using TEER and qPCR. SC, IC and LC fractions increased the TEER levels of colonic epithelial-like Caco-2 cells, albeit non-significantly, following 24 h of exposure (Figure 1A). Using qPCR, the transcription levels of tight junction proteins zona occludens 2 (ZO2) and intercellular adhesion molecule 1 (ICAM-1) were measured. None of the chitin fractions induced significant changes in the transcriptional profile of these genes (Figure 1B).

Similar as for colonic epithelial-like Caco-2 cells, the barrier integrity and transcription of tight junction and adherence junction proteins were investigated of small intestinal epithelial-like

Caco-2 cells following exposure to the chitin fractions. Again, no significant effects were observed on TEER (Figure 1C) nor on transcription levels of ZO2 and ICAM-1 (Figure 1D).

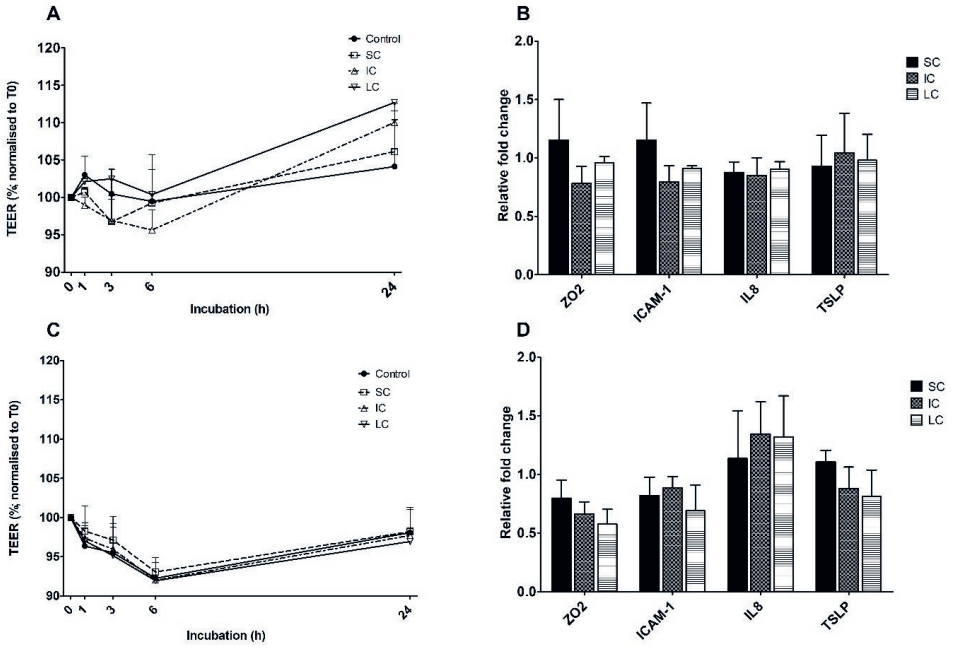

Figure 1: Effect of chitin fractions on the barrier integrity of small- and large-intestinal epithelial-like Caco-2 cells models. Caco-2 cells differentiated into colonic (7 day culture on transwell inserts; A, B) and small-intestinal-like (21 day culture on transwell inserts; C, D) epithelial cells were exposed to 0.1 mg/ml SC, IC and LC chitin for 24 h. Changes in barrier integrity were monitored using TEER analysis (A, C) in percentages relative to the starting values (set at 100%) during a period of 24 h and gene expression analysis with qPCR (B, D) after 24 h in fold change to medium (set at 1). Line charts and bar charts show the mean of 3 independent experiments + SD. Statistical analysis was performed by one way ANOVA but no significant differences were observed. IC: intermediate chitin; LC: large chitin; SC: small chitin.

3.3 Incubation of microbiota with large chitin resulted in increased levels of acetate but no change in composition

To investigate whether chitin interacts with gut microbiota an *in vitro* fermentation with the different chitin fractions was conducted. Following 24 h of anaerobic incubation of microbiota

with control medium containing only PBS or PBS with SC, IC or LC chitin fractions, the production of lactic acid and SCFAs acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid were assessed (Figure 2A). There was a significant increase in the production of acetic acid following microbiota incubation with LC when compared to the control. To follow up on this finding, we investigated the impact of chitin on gut microbiota composition after 24 h incubation. This revealed that chitin fractions did not significantly change the total number of species (Figure 2B) or the abundance of gut microbiota (Figure 2C) when compared to control. Of note, 24 h incubation of microbiota with the control medium also did not significantly change the microbiota composition (Figure S2). In addition, we examined the relative abundance of different bacterial groups of total microbiota at the class (Figure 2D) and genus level (data not shown) following 24 h of incubation which again revealed no significant differences.

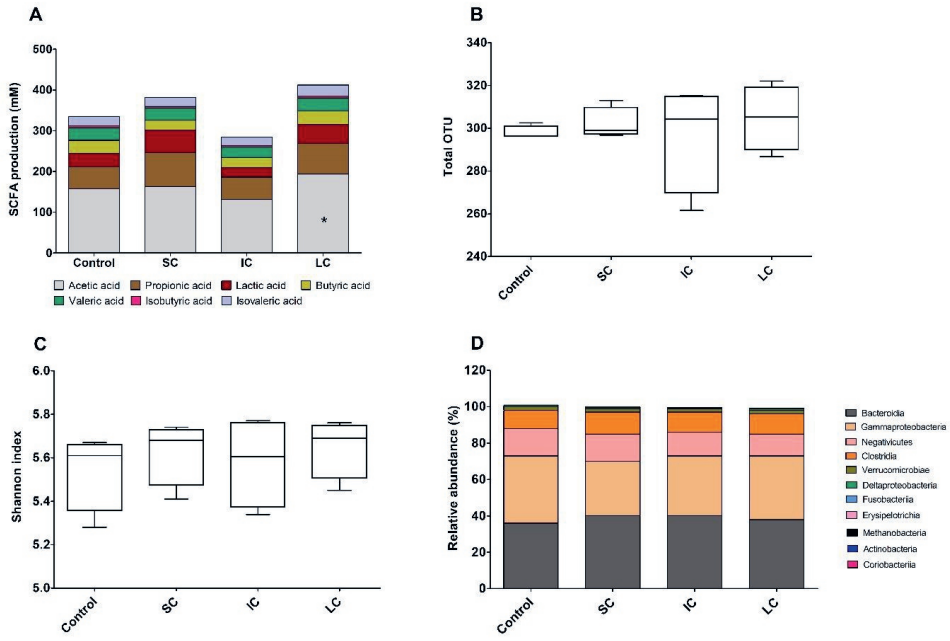

Figure 2: SCFAs, lactic acid and gut microbiota composition changes following exposure to chitin fractions. Gut microbiota cultures were incubated for 24 h with PBS (control), SC, IC or LC after which lactic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid production were measured (A). The alpha diversity of the gut microbiota was determined and indicated as total OTU count (B) and Shannon index (C). The

relative abundance of different bacterial groups of the total microbiota were tested and shown at the class level (D). Stacked bar and box plots show the mean of 2 independent experiments using 2 different donors. Statistical analysis was performed by Kruskal-Wallis test (B, C) and the Wald test (D) but no significant differences were found, and one way ANOVA (A) with *: $p < 0.05$; IC: intermediate chitin; LC: large chitin; SC: small chitin; OTU: operational taxonomic unit.

3.4 Immunomodulatory effects of chitin fractions on Caco-2 and THP-1 macrophages

The intestinal epithelium represents an important mediator for immune function [27]. To investigate the effects of chitin fractions on the intestinal immune system, we analysed the transcription levels of IL-8 and TSLP in Caco-2 cells. Neither colonic nor small-intestinal-like Caco-2 cells demonstrated altered transcription levels of IL-8 or TSLP following exposure to any of the chitin fractions (Figure 1B and D).

Next, THP-1 macrophages were tested for their response to chitin by analysing transcriptional changes and protein secretion of various signalling molecules. Using qPCR, the differential transcription of the cytokines IL-1β, IL-8, TNFα, IL-10 and TGFβ and chemokines CCL1, CCL5, CCL15, CCL18, CCL22, CCL24 were determined (Figure 3A, B). This revealed that SC significantly increased the transcription of IL-1β, IL-8, TNFα, IL-10, CCL1, CCL15, CCL18 and CCL24 compared to the medium control. Furthermore, IC significantly increased transcription of IL-1β and CCL1 whereas exposure of LC only resulted in a significantly increased transcription of CCL1. The THP-1 macrophages were also investigated for their secretion of IL-8 and IL-10 following exposure to the chitin fractions. In line with the transcription data, IL-8 and IL-10 secretion was chitin-size dependent and only SC significantly stimulated the production of both IL-8 and IL-10 (Figure 3C). To further explore the impact of SC on THP-1 macrophage activity, the secretion of IL-1β, CXCL10, IL-1RA, IL-4, IL-6, IL-12p40, IL-12p70, IL-23, TNFα, TARC and arginase were examined using a multiplex assay. This revealed that SC significantly increased IL-1β and CXCL10 secretion by THP-1 macrophages in addition to IL-8 and IL-10 (Figure 3D).

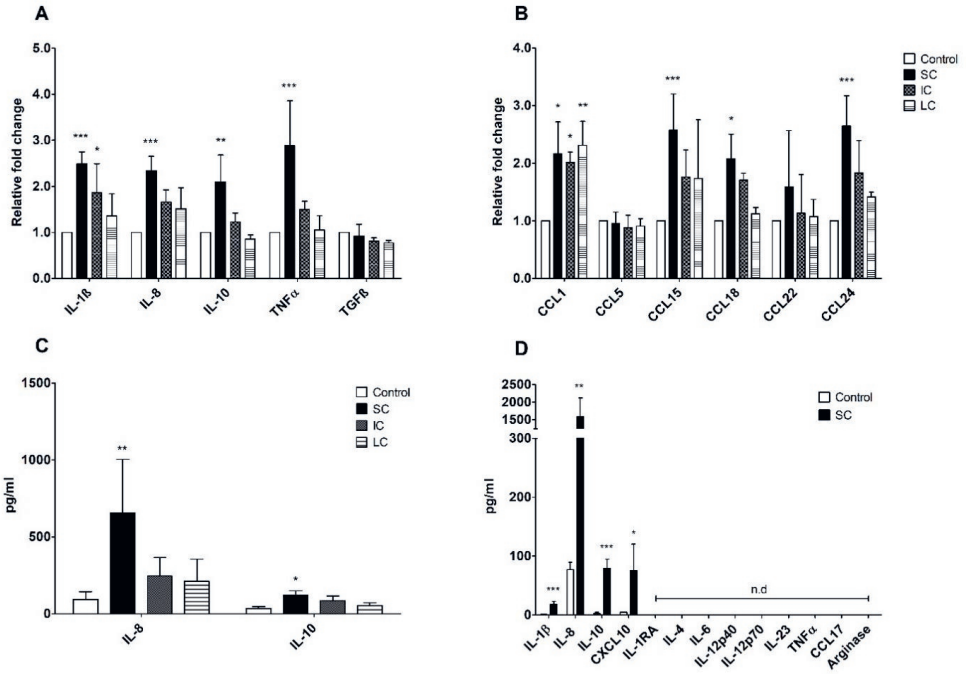

Figure 3: Transcription and secretion of signalling molecules by THP-1 macrophages following exposure to chitin fractions. THP-1 macrophages were exposed to medium (control) or to chitin fractions for 24 h after which the transcription of cytokines and chemokines was measured with qPCR (A, B), the secretion of IL-8 and IL-10 with ELISA (C) and the secretion of a panel of cytokines and chemokines with a bead-based multiplex assay to simultaneously detect 13 analytes using flow cytometry (D). Bar charts show the mean + SD of n=3 independent experiments. IC: intermediate chitin; LC: large chitin; n.d: not detected; SC: small chitin; Statistical analysis was performed by one way ANOVA with *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.5 THP-1 macrophage responses to small size chitin was mediated via clathrin-dependent endocytosis

Receptor binding and/or endocytosis are the most likely pathways leading to secretion of signalling molecules by THP-1 macrophages following exposure to chitin. To determine whether the chitin fractions were endocytosed, THP-1 macrophages were exposed to fluorescently labelled chitin fractions and analysed using flow cytometry. The results showed

an inverse correlation between chitin size and endocytosis efficiency (Figure 4A) with approximately one-third of the macrophages endocytosing SC (*i.e.* 37,7%) and a minor fraction endocytosing IC and LC (*i.e.* 13% and 1,7%, respectively).

The SC were further investigated for the receptors involved in the endocytosis. To examine this, TLR2, mannose receptor, dectin-1 and galectin-3, which are potentially involved in SC endocytosis, were blocked (Figure 4B). The results revealed that blocking either of these receptors significantly decreased the endocytosis of SC by approximately 20%, indicating that all tested receptors are involved in the endocytosis of SC.

Similar as for receptors, compounds can be endocytosed via different routes. To investigate which routes were used by THP-1 macrophages to endocytose SC, cytochalasin B (CB), nystatin (NYS) and chlorpromazine (CP) were used to specifically inhibit phagocytosis, caveolin-dependent endocytosis and clathrin-dependent endocytosis, respectively. The inhibitors did not affect cell viability which could have led to false negative results (data not shown). Cytochalasin B and nystatin did not affect SC endocytosis by THP-1 macrophages but chlorpromazine significantly inhibited the endocytosis by 74% (Figure 4C).

Finally, to verify that clathrin-dependent endocytosis was the dominant pathway for endocytosis of SC we analysed whether inhibiting clathrin-mediated endocytosis of SC by macrophages affected the cytokine production (Figure 4D). THP-1 macrophage pre-incubation with chlorpromazine before SC exposure significantly lowered the secretion of all tested signalling molecules.

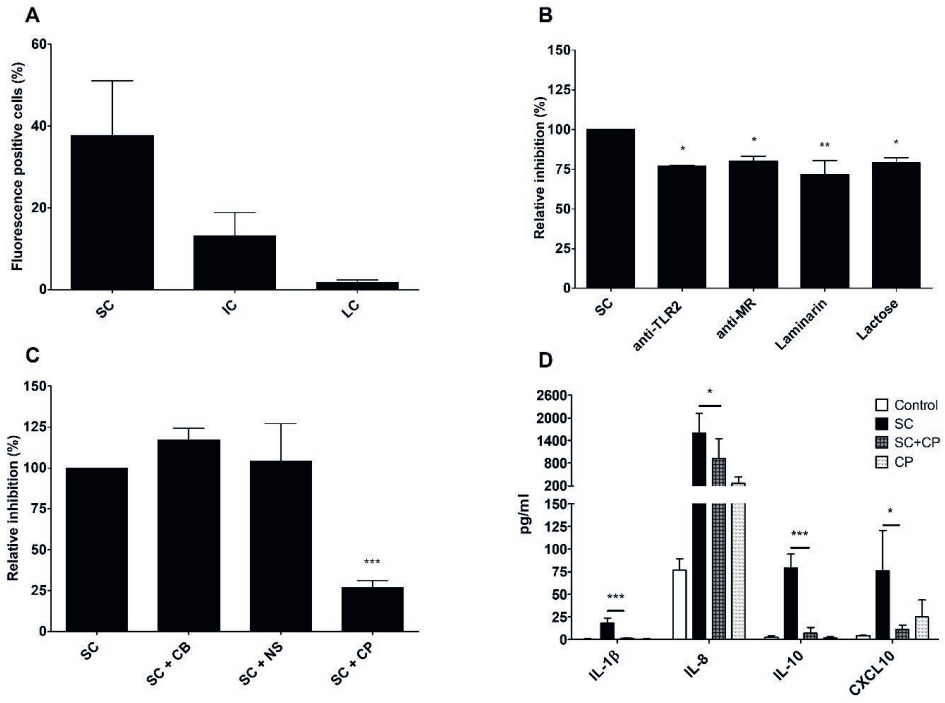

Figure 4: Endocytosis of chitin fractions by THP-1 macrophages. THP-1 macrophages were incubated for 24 h with FITC-labelled chitin fractions and analysed for: endocytosis (A); endocytosis of SC in the presence of receptor blocking agents (B) or endocytosis blocking agents (C). THP-1 macrophages incubated with SC, CP or SC + CP for 24 h were also analysed for cytokine secretion (D). Bar charts show the mean + SD of n=3 independent experiments. CB: cytochalasin B; CP: chlorpromazine; IC: intermediate chitin; LC: large chitin; MR: mannose receptor; NS: nystatin; SC: small chitin. Statistical analysis was performed by one way ANOVA with *: $p< 0.05$; **: $p< 0.01$; ***: $p< 0.001$.

4. Discussion

The present study represents a comprehensive evaluation of activity of chitin with clearly characterized physicochemical properties towards *in vitro* intestinal immune models and microbiota. We demonstrated that chitin with a DA of ~83%, regardless of size, does not affect the intestinal epithelial barrier integrity. Only LC was actively fermented by microbiota as

determined by SCFA levels, but without significantly changing total or relative abundance of different bacterial groups of the total microbiota. Furthermore, small sized chitin strongly activates THP-1 macrophages to secrete IL-8, IL-10, IL-1β and CXCL10 in a clathrin- and multi-receptor-dependent manner.

The gastrointestinal tract has two main and opposing functions. It selectively absorbs nutrients released from the food matrix through digestion or fermentation, but prevents translocation of microbiota that are critical for fermentation or harmful pathogens that can enter the body with the food [28]. This is achieved through maintaining intestinal barrier function and immune homeostasis. Two important tissues in this respect are the lamina propria which supports the immune system and the intestinal epithelium. The epithelium forms a physical barrier against pathogen entry and is composed mainly of absorptive intestinal epithelial cells (80%), but also mucus producing goblet cells, antimicrobial peptide secreting Paneth cells and antigen sampling microfold cells [29]. Many studies have investigated nutraceutical components for their capacity to strengthen the epithelial barrier against pathogens or reduce pathogen-induced damage. Inulin and oligofructose were found to improve the intestinal barrier function in pigs by increasing the depth of mucosal crypts and the density of intestinal epithelial cells [30]. Similarly, Nofrarias and colleagues revealed that long-term ingestion of resistant-starch enhanced the colonic mucosal integrity in pigs through decreasing apoptosis of colonocytes and colonic immune cells [31]. The underlying mechanisms for these beneficial effects of dietary fibres were attributed to their ability to alter the microbiota composition and microbiome metabolome. Intestinal microbiota ferment dietary fibres for growth which releases energy and metabolites such as SCFAs. SCFAs have important roles in maintaining intestinal health. Here, we demonstrated that chitin does not directly affect the integrity of small intestinal-like or colonic epithelial cells in a Caco-2 model, but large chitin significantly increased the production of acetate in an *in vitro* microbiota fermentation model (Figure 1 and 2). So far, descriptions of SCFA production upon chitin fermentation are limited to acetate, propionate and butyrate production upon chitin-glucan [32] or chitosan oligosaccharide fermentation (the deacetylated (<50%) form of chitin [33, 34]); or acetate, butyrate, propionate, isobutyrate, isovalerate and valerate production following whole insect fermentation [35]. In contrast to these experiments, our findings are based solely upon chitin more clearly demonstrating its specific prebiotic potential. So far, deacetylase activity has only been identified in fungi and bacteria [36], but if present in the human microbiome this could account for the observed acetate levels. However, acetate production is only observed following microbiota exposure to large chitin, and

deacetylation would also occur with intermediate or small chitin, making fermentation the more likely source of acetate. Microbiota composition analysis following 24 h of chitin incubation revealed no significant changes in diversity, richness or relative abundance of specific classes of the total microbiota. In line with our findings, in a similar *in vitro* fermentation setting Sasaki and colleagues found that the prebiotics Fibersol-2 and Dextran 40kDa increased the levels of acetate and propionate, without changing the microbiota composition or diversity [37]. Potentially, the incubation period (*i.e.* 24 h in our setting and 30 h in Sasaki and colleagues.) is sufficient to significantly changes metabolite concentrations, but not to significantly induce compositional changes. Taken together, our results show that chitin is not harmful to the intestinal epithelial barrier nor affects the gut microbiota composition and only to a limited extent supports production of SCFAs.

The second tissue of the intestinal immune barrier is the lamina propria. The lamina propria is home to a large collection of immune cells of which macrophages represent an important subset [38]. Gut macrophages are important for maintaining intestinal homeostasis as they regulate the inflammatory response to microbes that breach the epithelium, scavenge dead cells and their metabolites, and protect the mucosa against harmful pathogens [39, 40]. Of note, we did not observe any transport of SC to the basolateral medium in small-intestinal-like transwell culture (Figure S3). This makes the alternative routes of exposure of luminal content to macrophages, via microfold cells or by directly sampling the lumen, the most likely mechanism of macrophage interaction with chitin [41]. Microfold cells are specialized intestinal epithelial cells that are associated with Peyer's patches and transport luminal antigens or microbes to this Peyer's patch in which, amongst other immune cells, macrophages reside [42]. Alternatively, macrophages, but arguably also a subset of dendritic cells, can directly sample luminal antigens via sending their protrusions out into the lumen by means of the CX3CR1 receptor [41]. The interaction between chitin and macrophages has been extensively investigated, however, the response of macrophages to chitin varied in many studies [16]. The different responses of macrophages to chitin putatively resulted from variation in the physicochemical properties of chitin including size, degree of acetylation, source, and contamination [43]. Correlative analysis of these studies is hampered by incomplete physicochemical characterization of the chitin preparations. Here, we used chitin that we defined according to the above mentioned physicochemical parameters (Table 1) and separated on size as this was previously shown to be important for immunological responses [44]. Indeed, results showed that the immunomodulatory effect of chitin on THP-1 macrophages was inversely linked to its size.

Small sized chitin (SC) significantly increased the transcription of chemokine genes (CCL1, CCL15, CCL18 and CCL24) and cytokine genes (TNF-α, IL-10, IL-8 and IL-1β) and cytokine secretion (IL-8 and IL-10), which was less pronounced upon increasing chitin size (Figure 3). This is in line with previous studies in which murine peritoneal macrophages were exposed to SC (2-10 μm) and IC (40-70 μm) chitin resulting in increased production of TNFα by both and IL-10 by only SC [44]. To explore a broader spectrum of the impact of SC on THP-1 macrophage activation, we measured secretion of 11 other signaling molecules. The results showed that SC also induced the secretion of IL-1β and CXCL10 by THP-1 macrophages but not IL-1RA, IL-4, IL-6, IL-12p40, IL-12p70, IL-23, TNF-α, TARC, or arginase (Figure 3D). Notably, this is the first report of IL-8 and CXCL10 secretion by THP-1 macrophages upon exposure to SC. The secreted signaling molecules are linked to recruitment of innate immune cells such as monocytes/macrophages, neutrophils, and nature killer cells [45, 46]. Similar as for signaling molecule production, chitin size was also inversely related to endocytosis, with SC chitin being most efficiently endocytosed (Figure 4A). Chitin endocytosis therefore appeared to be at the basis of the responses by THP-1 macrophages. We inhibited internalization via phagocytosis, clathrin-dependent endocytosis or caveolin-dependent endocytosis and found that secretion of IL-1β, IL-8, IL-10 and CXCL10 were all mediated through clathrin-dependent endocytosis of SC (Figure 4C). In contrast, Da Silva and colleagues found that SC-mediated TNFα production was to some extent a result of phagocytosis and partially blocked by cytochalasin D and nocodazole [44]. However, they did not investigate the effect of inhibiting clathrin-coated endocytosis. In general, cells employ phagocytosis to engulf large particles (> 1 μm), and clathrin-mediated endocytosis to internalize small particles after binding to membrane receptors [47, 48]. It has also been demonstrated that clathrin-coated pits can be highjacked by bacteria up to 6 μm in length [49]. A similar mechanism might apply to the internalization of chitin particles. Finally, we demonstrated that the surface receptors TLR-2, dectin-1, mannose receptor, and galectin-3 receptors were involved in this process (Figure 4B), which was also in line with previous findings [18, 44, 50]. The present study focused on multiple *in vitro* models to investigate the effect of chitin particles on intestinal immunity and microbiota. The epithelial cells model and microbiota assays provide novel insight into potential interaction and suitability of chitin as dietary supplement. Using the THP-1 macrophage cell model we extended our understanding of the immunomodulatory potential of chitin. Together the results indicate that large-sized chitin could act as prebiotic and increase intestinal acetate levels, which has shown to beneficially impact the host energy and substrate metabolism [51]. Furthermore, shrimp-derived small sized chitin with a DA of ~83% does not

impede the intestinal epithelial barrier, but activates macrophages to produce pro-inflammatory signaling molecules and putatively recruit a wide array of innate immune cells. This activity could be beneficial to subjects suffering from parasitic infections [52] or allergic reactions [53] as small-size chitin interventions in murine studies demonstrated to relief symptoms in such models. In contrast, caution should be taken with regard to subjects suffering from autoimmunity [54] or IBD [55]. Taken together, the immune activating potency of chitin warrants further *in vivo* analysis and be tailored to the immune status of the subjects. Further (mechanistical) research and animal studies are required to better specify the nature of the immunomodulatory potential, but moreover unified methods and description of physicochemical parameters of chitin are required to effectively correlate findings.

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Conflict of interest statement

The authors report no conflict of interest.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Author contributions

L.D, C.G, and H.J.W conceived and designed the experiments. L.D, M.T, and R.A performed the experiments. L.D and R.A performed data analysis and interpretation. L.D and C.G wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary information

Figure S1: LPS titration on HEK-BLue™ human TLR4 cells to establish detection limits. HEK-Blue™ human TLR4 cells were incubated with titrated concentration of lipopolysaccharide (LPS) (0.0001 ng/ml to 100 ng/ml). SEAP-mediated conversion of Quanti-Blue, as marker for TLR4 activation, was detected by measuring absorbance at 655 nm. The line charts show the mean + SD of n=3 independent experiments.

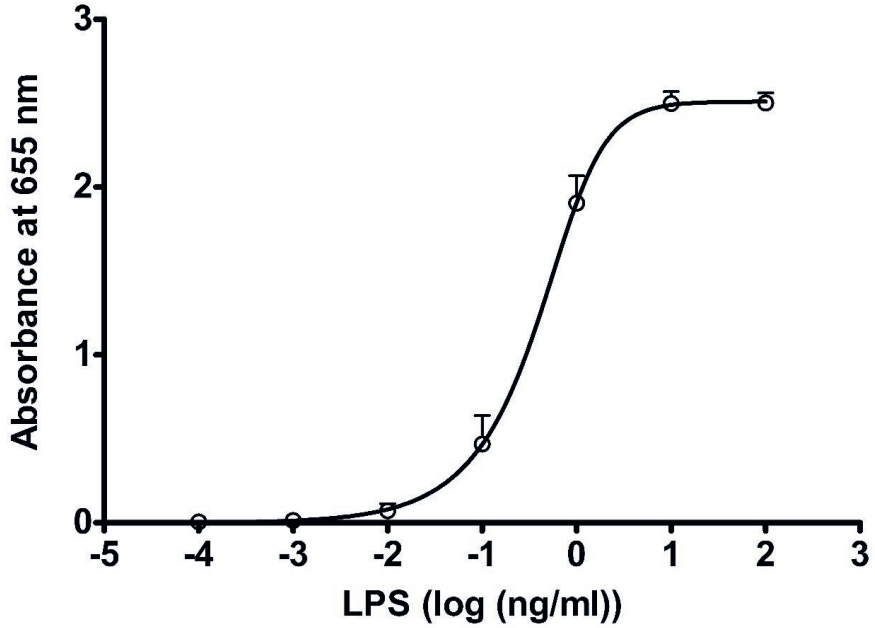


Figure S2: During 24 h incubation of microbiota with PBS the total number of species or relative abundance did not significantly change. Gut microbiota cultures were incubated for 24 h with PBS (control). The alpha diversity of the gut microbiota was determined and indicated as total OTU count (A) and Shannon index (B). The relative abundance of microbiota was tested and shown at a class level (D). Box plots shows the mean of 2 independent experiments using 2 different donors. Statistical analysis was performed by Kruskal-Wallis test and no significant differences were found. OTU: operational taxonomic unit.

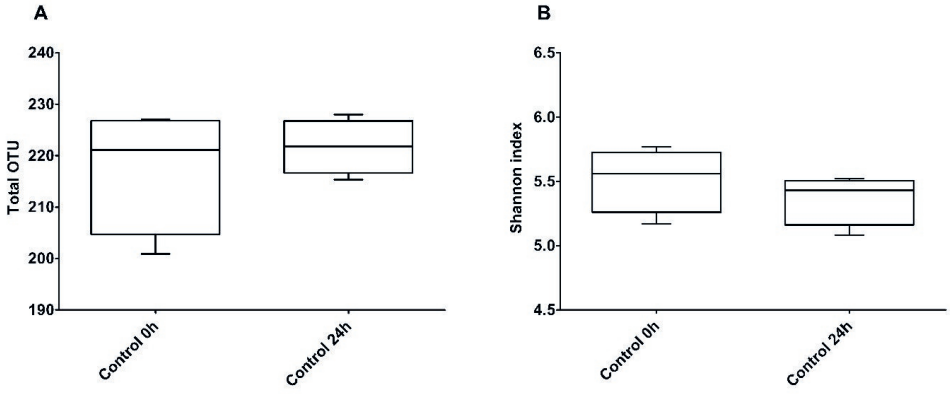
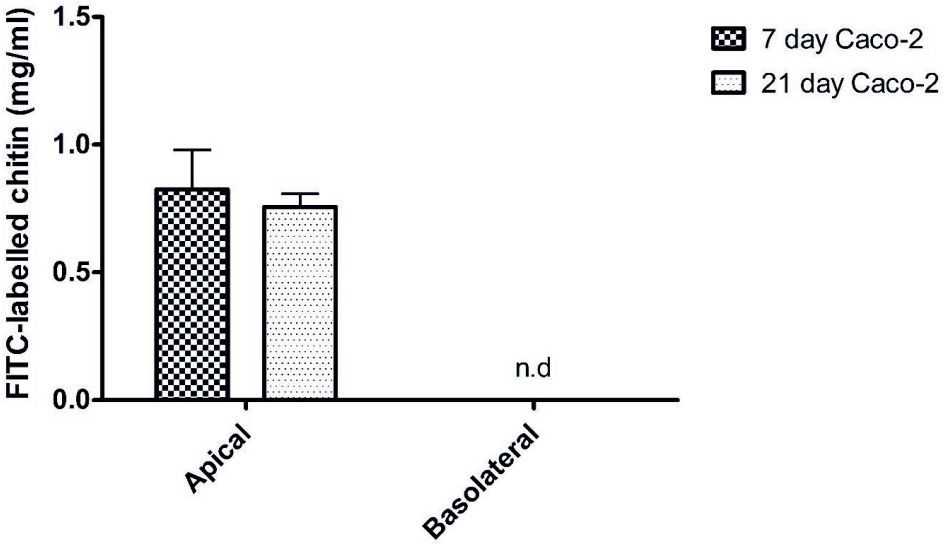


Figure S3: FITC-labelled chitin translocation over small- and large- intestinal epithelial-like Caco-2 cells models. Small- and large- intestinal epithelial-like Caco-2 cells (7 days and 21 day culture on transwell inserts respectively) were exposed to 1 mg/ml FITC-labelled chitin fractions for 6 h. The concentration of FITC-labelled chitin in both apical and basolateral compartment was measured after 6 h incubation. Bar charts show the mean + SD of n=2 independent experiments. FITC: fluorescein isothiocyanate; n.d: not detected.



Chapter 5

Beneficial health effects of chitin and chitosan

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Introduction

Chitin and chitosan have been recognised for their beneficial health effects since the eighties [1]. Over the past few decades numerous studies and several clinical trials have been performed which demonstrated that these compounds can reduce body weight and cardiovascular disease (CVD), improve wound healing, but also can modulate the immune system and demonstrate anti-fungal and anti-bacterial activity. In particular weight reduction and improvement of cardiovascular status are interesting targets as the prevalence of obesity and CVD is increasing. Both diseases are associated with various pathological disorders, including diabetes and hypertension and put a strain on healthcare costs and capacity. In general, lifestyle-based interventions such as the oral intake of chitin and/or chitosan are becoming increasingly popular as these are easily integrated in current treatments and improve the self-assertiveness of patients. Of interest, but beyond the scope of this chapter is the use of chitin-glucans as supplements in cosmetic [2, 3]. Several studies demonstrated that chitin-glucan reduced wrinkling and skin-aging suggesting an interaction between the chitin-glucan and cells of the epidermis. Although these effects may not be attributed to chitin alone, this does demonstrate the strong biological potential of chitin on cells of the human body.

In this chapter, scientific literature has been reviewed that demonstrated beneficial health effects of chitin and chitosan from an immunomodulatory point-of-view. First, we provide an overview of *in vitro* studies that offer in-depth mechanistic insights, followed by describing pre-clinical animal studies. Finally, we list various human intervention trials that most clearly demonstrate the beneficial health effects of chitin and chitosan. Furthermore, we purposely discriminate between data on chitin and chitosan as they are chemically distinct and therefore possibly demonstrate unique effects on health.

Immunomodulatory effects of chitin and chitosan as demonstrated with *in vitro* studies

Chitin and chitosan are not endogenous to mammals. In contrast, they can be found in various pathogens, such as *Candida albicans, Brugia malayi* and plasmodium [4, 5, 6] and hence may function as pathogen associated molecular patterns (PAMPs). The immune system has developed specific mechanisms and receptors, *i.e.* pattern recognition receptors (PRRs), to recognize and be activated by such PAMPs. A number of PRRs have been identified to bind to chitin and chitosan such as FIBCD1, NKR-P1, RegIIIγ, galactin-3, mannose receptor, dectin-1 and various toll-like receptors (TLRs) which are listed in Box 1 [7].

Box 1. Receptors that have been demonstrated to bind chitin or chitosan.

TLRs: Toll like receptors are expressed on the surface of multiple cells, including that of immune cells such as macrophages and dendritic cells. TLR2 and TLR4 are shown to bind to chitin or chitosan, and subsequently activate an innate immune response [8, 9]. In addition to TLRs expressed on the cell surface, TLR9, which is expressed in the endosomal compartments, was identified to be involved in chitin and chitosan mediated production of the anti-inflammatory cytokine IL-10 [10].

Dectin-1: this is a member of the C-type lectin receptors that is mainly involved in the recognition of fungal pathogens. It is also important for the immune response towards bacteria, viruses and parasites [11, 12] and reported to be able to recognize chitin.

Mannose receptor: MR is an endocytic receptor also belonging to the C-type lectin family. It is predominately expressed on the surface of macrophages and dendritic cells. Chitin can bind to the mannose receptor and activate the phagocytic process of immune cells [13].

NKR-P1: This again is a C-type lectin receptor that is primarily expressed on natural killer (NK) cells and T cells. This receptor was shown to possess a chitin oligomer binding domain which leads to the activation of NK cells [14].

FIBCD1: This is a transmembrane endocytic receptor that is expressed primarily by intestinal epithelial cells. It serves as an acetyl binding receptor to recognize chitin [15].

RegIIIγ: a secreted C-type lectin receptor which is expressed by intestinal epithelial cells. It can recognize *N*-acetyl-glucosamine oligomers [16].

Galectin-3: This is a member of the galectin family that is abundantly expressed on macrophages. This receptor was shown to have an *N*-acetyl-glucosamine binding domain [17].

NOD2: NOD2 is an intracellular chitin recognition receptors and is primarily expressed by macrophages and epithelial cells. It was reported to mediate chitin-induced IL-10 production [18].

Abbreviations: FIBCD1: Fibrinogen C Domain Containing 1; NKR-P1: Killer cell lectin-like receptor subfamily B, member 1; RegIIIγ: regenerating islet-derived protein 3 gamma; TLR: toll-like receptor; NOD2: nucleotide-binding oligomerization domain-like receptors.

Recognition and binding of chitin and chitosan by cell surface receptors results in downstream signalling, transcription factor activation, gene transcription and finally cellular activation. Cellular activation encompasses a number of biological responses of which the production and secretion of signalling molecules such as cytokines and chemokines are often investigated. These molecules can influence the direct environment and induce, for instance, an inflammatory condition in response to pathogenic recognition. Most studies that investigate immune responses upon recognition of chitin and chitosan have used innate-type immune cells and analysed cytokine secretion.

Although epithelial cells are not officially classified as immune cells, they are the first cells to come into contact with foreign material such as chitin and chitosan and therefore are the first cells to initiate cellular responses. In relation to a fungal lung inflammation in mice the responses of lung epithelial cells to chitin was studied [19]. This demonstrated that the epithelial cells respond to chitin with secretion of cytokines and chemokines such as interleukin-25 (IL-25), IL-33 and thymic stromal lymphopoietin (TSLP). These signalling molecules in turn activated lung-resident immune cells (*i.e.* innate lymphoid type 2 cells) to secrete IL-5 and IL-13 which induced migration of other immune cells (*i.e.* eosinophils and macrophages) to the site of chitin contact. The influx of eosinophils was corroborated in another lung infection model where increased exposure to chitin particles resulted in expression of eosinophil chemoattractant CCL11 and in addition revealed a T_h2 shift in T cell activation [20]. In another study focussing on fungal lung infections chitin was also shown to induce T_h2 skewing with increased production of IL-5, IL-13 and CCL5 [21]. The strength of the T_h2 responses were linked to Chit1 chitinase activity, but not AMCase, suggesting that smaller chitin fragments have an increased stimulatory effect. In addition, although dendritic cells were demonstrated to play an important role in T_h2 skewing, the initiating signal appears to originate from epithelial cells in the form of TSLP production.

Our bodies epithelial cell layer is also lined with immune cells for protection. A typical innate-type immune cell is the macrophage which expresses a number of receptors that can directly bind chitin (see Box 1). Chitin induced secretion of the anti-inflammatory cytokine IL-10 by macrophages. In contrast, chitin induced the secretion of the pro-inflammatory cytokines IL-6 and TNFα by peripheral blood mononuclear cells (PBMCs) which contain a large variety of immune cells, dominated by lymphocytes (*i.e.* T and B cells) [10]. A similar chitin preparation with a size of 1-10 μm was shown to induce IFNγ, IL-12 and TNFα secretion by an immune cell mixture isolated from the spleen [22]. Despite the differences in immune cell responses to

chitin, both studies demonstrated that the responses were dependent on chitin interaction with the mannose receptor, NOD2 and TLR9 and also that immune responses can be chitin concentration-dependent. Low concentrations of chitin induced secretion of a low level of TNFα whereas this exponentially increased upon stimulation with higher concentrations of chitin. In contrast, all chitin concentrations induced similar concentrations of IL-10.

Next to concentration, also the size of chitin was demonstrated to affect immune responses. Upon exposure to chitin fractions of <40 μm, 40-70 μm or 70-100 μm, macrophages showed different responses in cytokine production, receptor binding and downstream signalling pathway activation [23]. The smallest fraction induced both TNFα and IL-10 secretion, whereas the intermediate sized chitin only induced TNFα secretion and the large particles did not induce any cytokine production. The varying dependency of cytokine production on receptor binding adds to the complexity of immune responses and downstream signalling. TNFα secretion in response to small-sized chitin proved to be dependent on dectin-1 recognition with optional TLR2 and mannose receptor binding and rely on downstream NF-κB and partial Syk signalling. In contrast, TNFα secretion in response to intermediate sized chitin was dectin-1 and NF-κB dependent, but also fully TLR2 dependent and mannose receptor independent. The intermediate sized chitin fragments were also subject to a more in-depth study by which it was demonstrated that these also induced IL-17, IL-12 and IL-23 production in a TLR2- and MyD88-dependent manner [24]. Interestingly, IL-12, IL-23 and TNFα production all depended on the initial IL-17 production. Furthermore, intermediate sized chitin also induced, independently of IL-17, CCL5 and CXCL2.

Similar as for chitin, chitosan has been shown to exert pro- and anti-inflammatory effects on immune cells. Chitosan oligomers with a limited polymerisation degree of 3-8 units were shown to activate macrophages [9]. This particular macrophage cell-line (RAW 264.7 macrophages) demonstrated increased proliferation and production of pro-inflammatory mediators TNFα and nitric oxide. TLR4 was shown to be important for these responses as an anti-TLR4 antibody appeared to block direct interaction between chitosan and the macrophages and nitric oxide production. Small fragments of chitosan were also shown to be most potent in enhancing intra-epithelial lymphocyte (IEL) cytotoxicity against YAC-1 cells *ex vivo* [25]. As YAC-1 cells are in particular sensitive to NK cell mediated cytotoxicity, these results suggest that chitosan specifically enhanced NK cell activity in the IEL population. In addition to the direct immunomodulatory effects of chitosan, a number of studies investigated the modulatory effects of chitosan upon primary lipopolysaccharides (LPS)-stimulation. LPS-stimulated macrophages

demonstrated to increase IL-1β production when co-stimulated with chitosan, but not chitin [26]. This increase in IL-1β production is both concentration and size dependent with higher concentration and smaller sized chitosan demonstrating to induce a more potent IL-1β production. Indeed, chitosan <20 μm proved most potent and >100 μm almost functionally inert which is reminiscent of other reported chitin size-dependent effects. In this study the authors also demonstrate that chitosan depends on the NLRP3 inflammasome for intracellular signalling.

In a similar study treatment of LPS-stimulated macrophages with chitosan reduces the TNFα, IL-6 and NO production [27]. Unfortunately it is unclear whether these experiments were performed with similar small sized chitosan. Another study, in which again production of pro-inflammatory mediators following LPS-stimulation of macrophages were measured, demonstrated that chitosan negatively affected the production of nitric oxide, PGE2, TNFα, IL-6 and also IL-1β [28]. Here, the authors again investigated the effect of chitosan size and demonstrate that the inhibiting effects on these mediators was most strong for smaller particles. Still, direct comparison between studies remains difficult as in this study the size of chitosan is not described in degree of polymerisation or μm but in kDa. Also, the authors demonstrate that the inhibiting effects of chitosan are mediated by reducing intracellular MAP-kinase activation. Interestingly, Li and colleagues revealed that LPS-mediated cytokine production is regulated by the transcription factor NF-κB which in turn is activated by MAP-kinases [29]. Therefore it appears that chitosan is inhibiting LPS-mediated pro-inflammatory macrophage responses by reducing intracellular MAP-kinase activation, potentially via activation of TLR4.

Initial insights in cellular responses towards chitin and chitosan were acquired using *in vitro* studies and some *ex vivo* studies. These studies have demonstrated that there are a number of receptors that can recognize chitin and chitosan which lead to modulation of responses of a number of cell types. It is important to note that the immunomodulatory effects of chitin and chitosan are highly concentration and size dependent. The variation of the chitin and chitosan concentration and size leads to different immune responses. In *in vitro* studies, chitin concentration has a positive correlation with its immunomodulatory activity. In contrast, chitin or chitosan size was inversely linked with its immune activity, and particles with a size bigger than 100 μm are considered as immunomodulatory inert.

These *in vitro* studies were in part related to analysis of *in vivo* effects of chitin or chitosan towards fungal lung infection and tumour growth. The *in vitro* results corroborated the *in vivo*

data which was encouraging for the potential of translational research to estimate the potential health effects of chitin and chitosan, which is described in more detail in the following paragraphs. However, it should be noted that in *in vitro* studies LPS contamination is commonly present and can strongly interfere with proper interpretation of the immunomodulatory effects of chitin or chitosan. Especially the chitosan studies clearly demonstrate that the presence of LPS can strongly affect the net result of immune responses. Nevertheless, many studies did not determine the LPS content during the preparation of the chitin or chitosan products [24, 30]. The presence of LPS in chitin or chitosan samples does not interfere with readouts when using *in vivo* models which is another advantage beyond testing the full complexity of the body. Therefore, we will focus on beneficial health effects and validation of immunomodulatory effects of chitin or chitosan in *in vivo* studies following oral intake whilst also focussing on the used particle sizes and doses.

Beneficial health effects mediated by chitin and chitosan as demonstrated with animal studies

The next step in the analysis of immuno-modulatory effects of chitin and chitosan is to translate and validate *in vitro* findings to animal studies. Below we summarised animal studies in which chitin and chitosan demonstrated beneficial health effects. We have divided the various studies into their reported immune modulation (Table 1), anti-pathogenic (Table 2), and anti-tumour effects (Table 3).

Immune modulation

Cellular studies into modulation of inflammation by chitin and chitosan have been discussed in the above section. The described inflammation modulatory properties are essential for the prevention or treatment of some inflammation related diseases. A mouse model of ragweed allergy nicely demonstrates the potential of chitin to modulate immune responses [31]. In this model the mice undergo an 11 day treatment regime to induce a T_h2 dominated allergic response. Mice are sensitized to ragweed by two intraperitoneal (i.p.) injections containing alum as adjuvant and a subsequent intratracheal challenge with ragweed and were assessed for allergic parameters on day 13. Starting three days before initiating treatments and during the 13 days of

the trial an oral intervention is performed with saline solution as control or small particulate chitin as treatment (1-10 μm diameter), on average 6.5 microgram per mouse per day. The preventive treatment with chitin significantly ($p<0.0005$) reduced the serum IgE levels and ragweed specific IgE levels. In contrast, the ragweed specific IgG2a levels are significantly ($p<0.05$) increased. By using bronchoalveolar lavage (BAL) to harvest cells and cytokines from the lung it became evident that oral chitin intervention significantly reduced ($p<0.05$) migration of eosinophils and lymphocytes to the lung and drastically ($p<0.05$) altered cytokine profiles from an IL-4 to an IFNγ dominated type. Strikingly, not only a 13 day preventive administration of chitin drastically altered a T_h2 dominated immune response to a T_h1 dominated type, also a curative setting in which chitin was provided on days 12 and 13 significantly ($p<0.01$) decreased total serum IgE levels and immune cell lung infiltrates ($p<0.05$).

A similar curative allergy model was used to study the immunomodulatory effects of low molecular (LM) chitosan [32]. In this study mice were sensitised to ovalbumin (OVA) with a 12 day protocol using three i.p. injections with alum as adjuvant after which allergic responses were induced by intranasal administration of OVA. The intranasal administration was performed from day 13 to 27 and coincided with daily oral administration of LM-chitosan (16.5 mg/kg) or a saline solution as control. Analysis of the allergic responses were focussed on cytokine production. BAL fluids of mice treated with LM-chitosan demonstrated a significant ($p<0.001$) reduction in IL-4, IL-13 and TNFα levels. Furthermore, lung tissue also revealed a significant reduction of mRNA levels of IL-4, IL-5, IL-13 and TNFα. Interestingly, no change in IFNγ mRNA levels were observed. In comparison to the previous study no chitosan-mediated skewing of the immune response was observed (*i.e.* T_h2 towards T_h1), but LM-chitosan did demonstrate to induce a strong reduction in allergic responses.

Chitosan and chitin were directly compared as preventive treatment in a peanut allergy model [33]. This model is in essence similar to the above described models but with a different timeline and location of sensitization and challenge. The mice were provided with α-chitin, β-chitin or β-chitosan mixed in the food pellets at a concentration of 0.2% (w/w). The interventions resulted in a reduction of anaphylaxis when the sensitized mice were challenged with peanut. To investigate whether the chitin or chitosan indeed affected immune cell responses, splenocytes were isolated and *ex vivo* stimulated with peanut extracts. This revealed that splenocytes from mice treated with α-chitin, β-chitin or β-chitosan were less sensitive to the peanut extract and produced significantly ($p<0.05$) less IL-5, IL-13 and IL-10. Authors also

investigated serum peanut specific IgE levels which were lowered by α-chitin and β-chitosan, but not β-chitin. Within the group of β-chitin treated mice there was a strong division in mice with low and high IgE levels, indicating that β-chitin has the potency to lower allergic responses but is dependent on a parameter beyond the scope of this study.

Instead of re-directing immune responses towards a T_h1 type response, chitin has also demonstrated the potency to reduce the strength of immune responses. To investigate the potential of chitin to reduce the inflammation in inflammatory bowel disease (IBD) a mouse model was used in which ulcerative colitis was induced using dextran sulphate sodium (DSS) [34]. Regular chitin with an average diameter of 200 μm or specifically generated chitin nanofibrils with average lengths of 4 nm at a concentration of 0.1% (w/v) in tap water was provided as a seven day intervention before the DSS-mediated induction of ulcerative colitis. Analysis of colonic epithelium demonstrated a significant ($p<0.05$) reduction in activation of the NF-κB transcription factor and serum analysis revealed significantly ($p<0.05$) reduced levels of monocyte chemotactic protein 1 (MCP-1), which is the key chemokine for migration and infiltration of monocytes and macrophages. These effects, however, were only observed following preventive treatment with chitin nanofibrils and not with regular chitin. Based on the knowledge gained from *in vitro* analysis it appears that the chitin length in *in vivo* situations as well might be a crucial parameter to reduce intestinal inflammation.

Chitosan and in particular chitin have long been associated with allergenic response. Recent studies indicate that these correlations are mainly attributable to various proteins [35] and clinical trials have demonstrated the safety of use chitosan in bandages [36]. The general observation that chitin and chitosan prevent allergenic responses in the lung and shift T_h2 responses towards T_h1 substantiates this. In addition, chitin also demonstrated to lower intestinal immune responses. Both applications could potentially improve the quality of life for many patients.

Anti-pathogenic effects

Although chitin and chitosan have already been used as anti-microbial agents for decades, the underlying mechanism remains unclear, but it has been proposed to involve cell lysis, penetration of cytoplasmic membranes and chelation of cations [37]. These mechanisms might also result in fungicidal and bactericidal effects. Furthermore, chitin and chitosan also appear to possess anti-parasitic activity which can be correlated to improved macrophage phagocytosis

capacity as demonstrated in *in vitro* experiments and overall improve innate and adaptive immune responses.

A study performed already over three decades ago demonstrated the potential of chitin and chitosan to reduce infections in mice with the yeast *Candida albicans* (*C. albicans*) [38]. In this study mice were pre-treated with i.p. injections of chitin or chitosan six, four and two days before i.p. *C. albicans* injections. The pre-treatment with chitin, but even more so with chitosan, increased survival rates of the mice. These beneficial effects coincided with increased levels of polymorphonuclear leukocytes in blood (chitin) and peritoneal immune cells (chitosan). These immune isolates from chitin or chitosan treated mice demonstrated to possess significantly increased reactive oxygen species production and candidacidal activity. Similar results were obtained when chitosan was orally provided in a preventive setting to treat *Staphylococcus aureus* (*S. aureus*) infections [39]. After a seven day pre-treatment period *S. aureus* was injected i.p. and survival of the mice was monitored over a six day period. With the preventive strategy the survival of mice increased from 10% to 70-100% depending on the chitosan concentration. This preventive strategy also proved to be efficient when treating shrimp or fish with microbial infections. Shrimp were treated with an injection of chitin or chitosan and challenged the next day with a *Vibrio alginolyticus* injection [40]. The pre-treatment significantly ($p<0.05$) enhanced the survival of the shrimp during the six day follow-up. In addition, the immediate and significant ($p<0.05$) increase of leukocyte responses such as respiratory burst activity and phagocytic activity proved to be transient of nature and subsided after four days. Of note, again chitosan appeared to be more potent regarding the anti-microbial and immune-stimulatory than chitin. Also, a similar increase in leukocyte activity was observed when carp-derived leucocytes were directly *ex vivo* incubated with small chitin particles and demonstrated increased phagocytic activity [41]. This increase in phagocytic capacity was unfortunately not measured when carp were provided with diets enriched in chitosan or chitin (1% (w/w)) to treat *Aphanomyces invadans* (*A. invadans*) infection. Interestingly, in this study pre-treatment and continued additions of either chitin or chitosan reduced mortality in fish from 70% to almost the levels of uninfected fish four weeks post-infection. Similar as for shrimp, chitosan was slightly more effective in reducing the mortality in fish which was accompanied by an increased level of red and white blood cell counts, among which were monocytes, lymphocytes and neutrophils, which was already apparent at the first moment of measuring one week after infection.

Studies to investigate anti-parasitic activity of chitin and chitosan were mostly performed in a curative setting. In a model to test the effects against *Leishmania major* (*L. major*) infections mice were subcutaneously injected with the parasite and treated with local injections of small sized chitin or chitosan (<40 μm) for two weeks with two-day intervals [42]. The onset of lesions in the treated groups was delayed and ulcer diameter of the lesions was significantly smaller ($p<0.05$). Twelve weeks after infection the mice were sacrificed and analysis revealed a significantly ($p<0.05$) reduced parasitic load in the draining lymph nodes in treated mice. In relation to reducing the infections the authors investigated the potential of chitin and chitosan to induce production of cytokines by lymph node-derived immune cells. Only chitin incubation resulted in a significant ($p<0.001$) increase in both IL-10 and TNFα production. Although the production of these cytokines appears contradictive, an activation of T_h1 response which is stimulated by TNFα enhances macrophage activity and increased IL-10 production critically limits the harmful side-effects of inflammatory responses. In a previous *L. major* infection mice trial performed by the same group chitin was shown to also increase IFNγ and reduce IL-5 production [43]. These results were in line with an *in vitro* study, also performed by this group, which showed that chitin induced the increase of TNFα production in peritoneal macrophages after *L. major* infection [44]. Although these *ex vivo* cytokine productions do not directly correlate to a lowered burden of infection, it does appear to substantiate that chitin enhances the immune system's ability to combat *L. major*.

The treatment of the intestinal parasite *Eimeria papillata (E. papillata)* was only tested with chitosan. Mice were intestinally infected with *E. papillata* and subsequently treated with a daily dose of chitosan for five days. The infectious grade was determined at day five by the oocyte count in the faeces of the mice and was found to be significantly reduced ($p<0.01$) by chitosan treatment compared to non-treated mice [45]. In line with this finding the authors observed a significant reduced number of parasites per crypt ($p<0.01$) and a reversion of the decline in goblet cells in histological sections following chitosan treatment. Chitosan treatment also reversed other infection related immunological changes such as an increase in the level of oxidative stress, neutrophil infiltration and inflammatory cytokine gene transcription and a decrease in anti-inflammatory cytokine gene transcription. In contrast, *E. papillata* infection also reduced the levels of T cells in the villi and IgA levels in sera and the intestine but treatment with chitosan did not prevent this.

Interestingly, when compared head-to-head based on a reduction of the burden of infection and accompanying histological and immunological changes it appears that chitosan is more effective in reducing the burden of microbial infection whereas chitin appears more effective against parasites. For all these studies it remains unclear, however, whether these chitin- and chitosan-mediated effects are instigated by potentiating the immune system or by reducing the virulence of microbes and parasites and thereby lowering immune responses.

A single study has demonstrated effects of chitosan beyond restoring immune parameters towards non-infected controls. To treat the infection of an intestinal tapeworm (*Hymenolepis nana (H. nana)*) mice were pre-treated with a weekly dose of 500 µg chitosan for four weeks [46]. As observed in above described studies most parameters, histological and immune-related, that were affected by the *H. nana* infection were restored to or towards the levels found in non-treated mice by the pre-treatment with chitosan ($p<0.01$). Similar as to the above described study the authors also observed a reduced load in *H. nana* adults and eggs which all together again suggests that chitosan reduces the virulence of *H. nana*. However, in contrast to the above described study, mast cell concentrations were investigated. These were not affected by the *H. nana* infection but significantly increased by chitosan beyond levels found in both non-treated and infected mice. It therefore appears that chitosan pre-treatment increases mast cell number and, potentially, their activity which allows a quick and efficient anti-*H. nana* response and thereby preventing a full scale immune response resulting in histological damage and production of inflammatory cytokines.

So far, most studies using various microbes or parasites infecting either skin or intestine demonstrate that chitin and chitosan can indeed reduce these infections although the mechanism behind this remains elusive. By reducing the extent of the infection, chitin and chitosan most likely also reduce the inflammatory response which explains the lowered cytokine levels and immune cell recruitment. Mast cells appear to play a critical role as their presence is selectively increased by chitosan in the intestinal infection by *H. nana*. In contrast, the production of IL-5, which is related to mast cell activity in allergenic responses, is reduced by chitin in ex vivo immune cell stimulations, which demonstrates the necessity for further research into the anti-parasitic potential of chitin and chitosan.

Anti-tumour effects

The anti-tumour effects of chitin and chitosan are strongly related to their immunomodulatory effects. The final anti-tumour activity is performed by cytotoxic T cells. The activation of these T cells is mediated by antigen presenting cells such as macrophages and dendritic cells, which are strongly responsive to chitin and chitosan (see Box 1). So far, however, animal studies have focussed more on the effects on tumour growth and less on the underlying immunomodulatory effects. For instance, fully or partially de-acetylated chitosan has been tested for its anti-tumour property in a colon-26 tumour bearing murine model [47]. Mice were provided with a diet containing 1%, 2%, and 4% (w/w) of chitosan for 28 days before the tumour inoculation which was continued for another 14 days thereafter. This diet significantly ($p<0.05$) reduces tumour growth which correlated with increased apoptosis in the tumour cells. The authors also observed a significant increase ($p<0.01$) in serum IFNγ and IL-12p70 levels which could have activated macrophages leading to an anti-tumour effect.

Similarly, low molecular weight (MW) water soluble chitosan has also been tested for its anti-tumour effect in a sarcoma 180-bearing mice model [25]. Chitosan with various molecular weight (21 kDa, 46 kDa, 130 kDa) was orally administered to mice twice daily at 100 mg/kg or 300 mg/kg for 20 days, starting 12 hours after the transplantation of tumour cells. The results showed that chitosan significantly ($p<0.05$) decreases the tumour weight and also inhibits tumour growth in a size dependent manner. The lowest molecular weight chitosan demonstrated the strongest effects whereas the largest molecular weight chitosan proved ineffective in reducing tumour growth. Of interest, chitosan could not be detected in blood or spleen but was detected in the small intestine. Coincidently, intestinal epithelial lymphocytes (IELs) from mice that were orally administered low molecular chitosan, but again not high molecular chitosan, demonstrate significantly ($p<0.05$) enhanced *ex vivo* cytotoxicity against YAC-1 cells in a size dependent manner. Recently, a group from China confirmed that low molecular weight water soluble chitosan contains an anti-tumour activity and did so in a H22 liver tumour mice model [48]. The chitosan was orally administrated to mice at 100 or 200 mg/kg for 14 days, starting 12 hours after the inoculation of the tumour cells. The treatment revealed a significant concentration dependent ($p<0.05$) reduction in tumour growth and spleen weight compared to non-treated animals. At a cellular level, the number of leukocytes, lymphocytes, monocytes and also neutrophils were significantly ($p<0.05$) increased in the chitosan intervention group in comparison to the untreated group. In particular, the $CD4^+$ T cells, but not $CD8^+$ T cells, and NK cell concentrations were significantly ($p<0.05$) increased after chitosan intervention.

Finally, also TNFα and IL-2 serum levels, cytokines that are critical in the anti-tumour response, were significantly ($p<0.05$) increased upon chitosan treatment [48, 49].

These papers demonstrate that low molecular weight chitosan can modulate the immune system and induce pro-inflammatory anti-tumour activities in different tumour bearing murine models. In these studies it is more plausible that the observed immune cell counts and activities are a direct chitosan-derived effect, in contrast to the potential physical interference in anti-pathogen studies. Therefore, low molecular weight chitosan may be a promising adjuvant for anti-tumour therapies.

Table 1: Immune modulatory effects of chitin and chitosan

Products	Origin	Size/MW	DD	Product administration	NO. of subjects (experimental arm/control); subjects status	Summary of effects	References
Chitin microparticles	n.d	1-10 µm	n.d	6.5 mg/day for 16 days; Oral administration	6 or 7/6 or 7; allergic mice	↓ IL-4, IL-5, IL-10 ($p<0.05$) ; IgE levels ($p<0.0005$) ↑ IFNγ ($p<0.05$) ; IgG2a levels ($p<0.05$)	[31]
LM-chitosan	n.d	<1 kDa	98.5%	16.5 mg/kg twice a day for 14 days; Oral administration	10-16/10-16; asthmatic mice	↓TNFα, IL-4, IL-5, IL-13 ($p<0.001$)	[32]
Chitin microparticles	n.d	1,000 kDa	n.d	0.2% wt chitin contained diet for 6 weeks; oral administration	8/8; peanut allergic mice	↓IL-5, IL-10, IL-13 ($p<0.05$)	[33]
Chitosan microparticles	n.d	70 kDa	91%	0.2% wt chitin contained diet for 6 weeks; oral administration	8/8; peanut allergic mice	↓IL-5, IL-10, IL-13 ($p<0.05$) ↓ serum IgE ($p<0.05$)	[33]
Chitin nanofibrils	Crab	200 µm	n.d	1% (w/v) chitin for 7 days; Oral administration	5/5; IBD mice	↓ NF-κB staining area ($p<0.05$) ↓MCP-1 ($p<0.05$)	[34]

Abbreviations: DD: degree of de-acetylation; IBD: inflammatory bowel disease; IFN: interferon; Ig: immunoglobulin; IL: interleukin; LM: low molecular; MCP-1: monocyte chemotactic protein 1; MW, molecular weight; NF-κB: nuclear factor kappa light chain enhancer of activated B cells; n.d: not described; NK, natural killer.

Table 2: Anti-pathogenic effects of chitin and chitosan

Products	Origin	Size/MW	DD	Product administration	NO. of subjects (experimental arm/control); subjects status	Summary of effects	References
Chitin	n.d	n.d	n.d	50 mg/kg per day for 6 days at a two-day interval; intravenous or intraperitoneal administration	18 (i.p.) or 20 (i.v.)/18 or 20; *C.albicans* infected mice	↑Serum polymorphonuclear leukocytes ($p<0.01$) ↑Survival time	[38]
Chitosan	n.d	n.d	n.d	50 mg/kg per day for 6 days at a two-day interval; intravenous or intraperitoneal administration	18 (i.p.) or 20 (i.v.)/18 or 20; *C.albicans* infected mice	↑Active oxygen generating cells ($p<0.01$) ↑Survival time	[38]
Chitosan oligosaccharides	Crab	208 kDa	70-100%	0.5 or 1mg per day for 5 and 10 days; intraperitoneal injection 0.5, 1, 2 mg per day for 7days; oral administration	5/5; *S.aurens* infected mice	↑The number of monocytes ($p<0.05$) ↑IFNγ, IL-6 ($p<0.01$) ↑Survival time	[39]
Chitin	Crab	n.d	n.d	4 μg/g or 6 μg/g for once; injection	10/10; *V.alginolyticus* infected shrimp	↑THC, respiratory burst ($p<0.05$) ↑Phagocytosis ability ↑Survival time	[39]
Chitosan	Crab	n.d	n.d	2 μg/g or 4 μg/g for once; injection	10/10; *V.alginolyticus* infected shrimp	↑THC, respiratory burst ($p<0.05$) ↑Phagocytosis ability ↑Survival time	[40]

(continued)

Table 2: Continued

Products	Origin	Size/MW	DD	Product administration	NO. of subjects (experimental arm/control); subjects status	Summary of effects	References
Chitin	Prawn	n.d	26.7%	1% (wt) chitin contained diet for 15 days; oral administration	50/50; *A.invadans* infected fish	↑WBC, RBC, haematocrit, lymphocytes, monocytes, neutrophils ($p<0.05$) ↑Phagocytosis activity ↓Mortality	[41]
Chitosan	Prawn	n.d	58.5%	1% (wt) chitin contained diet for 15 days; oral administration	50/50; *A.invadans* infected fish	↑WBC, RBC, haematocrit, lymphocytes, monocytes, neutrophils ($p<0.05$) ↑Phagocytosis activity, ↓Mortality	[41]
Chitin microparticles	n.d	<40 µm	n.d	100 µg/µL for 2 weeks at a two-day interval; injected at the infected site	5/5; *L.major* infected mice	↓Parasitic load ($p<0.05$) ↓Lesion formation ($p=0.021$) ↑TNFα, IL-10 ($p<0.001$)	[42]
Chitosan microparticles	n.d	<40 µm	n.d	100 µg/µL for 2 weeks at a two-day interval; injected at the infected site	5/5; *L.major* infected mice	↓Parasitic load ($p< 0.05$) ↓Lesion formation ($p=0.039$) ↑TNFα, IL-10 (n.s)	[42]
Chitin microparticles	n.d	<44 µm	n.d	100 µg/µL for 2 weeks at a two-day interval; injected at the infected site	6/6; *L.major* infected mice	↓Lesion formation ($p=0.023$) ↓TNFα ($p=0.026$) ↑IFNγ/IL-5 ($p=0.023$) ↑IL-10	[43]

(continued)

Table 2: Continued

Products	Origin	Size/MW	DD	Product administration	NO. of subjects (experimental arm/control); subjects status	Summary of effects	References
Chitosan	n.d	n.d	n.d	250 mg/kg once a day for 5 days; oral administration	8/8; *E.papillata* infected mice	↓The number of parasite in developmental stage ($p<0.01$) ↓oocyst in faeces ($p<0.01$) ↓NO production; TNFα, TGFβ gene expression($p< 0.001$) ↑IL-4, IL-10 gene expression ($P<0.001$)	[45]
Chitosan particles	n.d	n.d	88.5%	500 µg once a week for 4 weeks; oral administration	10/10; *H.nana* infected mice	↓Gene expression of iNOS, IFNα, IFNγ, IL-9 and TNFα ($p< 0.001$) ↑The number of mucosal mast cells; Gene expression of IL-4 ($p<0.001$)	[46]

Abbreviations: DD: degree of de-acetylation; IFN: interferon ; IL: interleukin; iNOS: inducible nitric oxide synthase; i.p.: intraperitoneal injection; i.v.: intravenous injection; MW, molecular weight; NF-κB: nuclear factor kappa light chain enhancer of activated B cells; n.d: not described; NK, natural killer; NO: nitric oxide; n.s: not significant; RBC: red blood cells; THC: haemocyte count; TNF: tumour necrosis factor; TGF: transforming growth factor; WBC: white blood cells.

Table 3: Anti-tumour effects of chitin and chitosan

Products	Origin	Size/MW	DD	Product administration	NO. of subjects (experimental arm/control); subjects status	Summary of effects	References
Chitin and chitosan oligomers	Crab	n.d	n.d	1, 2, 4% (wt) chitin or chitosan contained diet for 28 days; oral administration	8/8; Colon-26 tumour bearing mice	↓Tumour weight ($p<0.05$) ↑IFNγ, IL-12p70 ($p<0.01$)	[47]
LM-chitosan	n.d	21, 46 and 130 kDa	n.d	100 mg/kg or 300 mg/kg twice daily for 20 days; oral administration	10/10; Sarcoma 180-bearing mice	↓Tumour weight and growth ($p<0.05$) ↑NK cells activity	[25]
LM-chitosan	Shrimp	30 kDa	95%	100 mg/kg or 200 mg/kg per day for 14 days; oral administration	10/10; Liver tumour H-22 bearing mice	↓Tumour weight (100 mg/kg) ($p<0.05$) ↓Tumour weight (200 mg/kg) ($p<0.01$) ↑Leukocytes, monocytes, neutrophils and lymphocytes ($p<0.05$) ↑The number of CD3/CD4 T cells, NK cells ($p<0.05$) ↑TNFα, IL-12 ($p<0.05$)	[48]

Abbreviations: CD3: cluster of differentiation 3; CD4: cluster of differentiation 4; DD: degree of de-acetylation; IFN: interferon; IL: interleukin; MW, molecular weight; n.d: not described; NK, natural killer; TNF: tumour necrosis facto

Beneficial health effects mediated by chitin and chitosan as demonstrated with clinical trials

Extensive *in vitro* and animal studies indicate the potential beneficial health effects mediated by chitin and chitosan. It is crucial to validate such observations in human clinical trials. Part of this concerns the development of treatment regimens, as especially the dose and duration may be completely different from that in animal studies. So far, human clinical studies investigating the beneficial health effects of chitin and chitosan have been limited in number and type of investigations. Studies have largely focussed on the effect of cholesterol reduction, weight loss and glucose control.

Cholesterol reduction and CVD preventive effects

Chitin has been investigated in clinical trials for its hypocholesterolemic effects in a mixture with glucan (chitin-glucan; Table 4). This insoluble chitin-glucan was tested in a single-blind pilot study for its effect on overweight in hypocholesterolaemic subjects. Subjects orally ingested 4.5 g/day of chitin-glucan before every main meal for a period of four weeks. After this intervention the results showed a decrease in oxidized low density lipoprotein (LDL) levels in blood which reduces the risk for CVD [50]. Another study with the same chitin-glucan was performed in a full randomized, double-blind and placebo-controlled setting, a longer follow up and a larger study population (n=68) [51]. This phase IV clinical study revealed similar results as the pilot study and demonstrated that chitin-glucan significantly lowers oxidized-LDL blood levels compared to the placebo group.

Chitosan has also been tested in clinical trials for its hypocholesterolemic potency. A pilot study was conducted by Rizzo and colleagues to investigate the effect of fungal chitosan on regulation of plasma lipid and lipoprotein in hypertriglyceridemia patients [52]. Subjects were orally administered 125 mg/day of chitosan tablets for a period of four months. This intervention resulted in a significant decrease in total cholesterol ($p=0.019$) and triglycerides (TG) levels ($p<0.001$) and a concomitant significant increase in high density lipoprotein (HDL) cholesterol ($p=0.016$) and LDL-2 particles ($p<0.0001$), a less atherogenic LDL particle. Total LDL levels did decrease although not significantly ($p>0.05$). The observed increase in HDL and lowering of LDL constitutes a beneficial change in plasma lipid composition and lowering the risk of CVD. The potential of chitosan to reduce the risk of CVD has also been tested in smokers who

are at higher risk of developing this disease [53]. The study setup included a higher dose of chitosan (1 g/day) for a shorter period (six weeks) which also resulted in a significant decrease in total cholesterol ($p<0.01$) and LDL cholesterol ($p<0.05$) levels.

These results for chitosan have led to a European Food Safety Authority (EFSA) claim on cholesterol lowering and chitosan is available as commercial product. So far, however, the mechanism behind chitosan's effect remains to be elucidated. Chitosan might bind bile acids in the intestine preventing their re-uptake and forcing enhanced cholesterol metabolism to generate bile acids [54]. Other potential mechanisms are the increased expression of LDL receptor in the liver which would result in enhanced uptake and breakdown of serum LDL or the inhibition of pancreatic lipase activity to reduce the digestion and uptake of dietary fats [55]. Depending on the mechanism, and whether the acetylation level is involved in this, a similar EFSA claim might be possible for chitin which clearly demonstrated its potency in clinical trials.

Other health effects

Next to reducing CVD risk chitosan was also tested for its potential to address other illnesses such as reducing oxidative stress (Table 5). Here, water soluble chitosan with a molecular weight of 20 kDa was administered to young and healthy subjects at a daily dose of 540 mg for 4 weeks in an open label study [56]. In line with the above described studies, again a significant increase in HDL ($p<0.05$) and (non-significant) reduction in LDL levels were observed along with a significant decrease in the atherogenic index ($p<0.05$; ratio between triglycerides and HDL-C levels). Oxidative stress levels in blood were determined by measuring the oxidized albumin ratio and the total plasma antioxidant capacity. Both parameters were significantly reduced ($p<0.05$) which suggested that chitosan has antioxidant properties and reduces oxidant stress. Perhaps unexpectedly, the authors also observed a significant ($p<0.05$) reduction in blood glucose levels following the chitosan intervention. This finding has been the primary read-out parameter another clinical trial that focussed on the effect of chitosan on glucose control [57]. In this randomized, double-blind, placebo controlled study pre-diabetic patients were administered with a daily dose of 1.5 g chitosan for 12 weeks, which is a considerably higher dose for a longer period compared to the previous study. Again a significant lowering of serum glucose levels ($p=0.03$) was observed. Also, the glycated haemoglobin levels (*i.e.* HbA1c) that are indicative of blood sugar levels over a prolonged period were significantly ($p=0.021$) reduced in the chitosan treated patients when compared to the placebo group. Surprisingly, no

change in blood insulin levels were observed which might have been expected with lowered blood glucose levels. Another study also designed as randomised, double-blind and placebo controlled analysed insulin sensitivity, rather than insulin levels, in obese subjects following a chitosan intervention [58]. Subjects orally ingested 2.25 g of chitosan (750 mg before every main meal) for a period of three months which are similar conditions as the previous study which did not yield a change in blood insulin level. As hypothesized by the authors, chitosan significantly increased insulin sensitivity ($p=0.43$). At the same time, subjects also lost a significant amount of body weight through the intake of chitosan ($p=0.027$) which was accompanied by a significant decrease in body mass index (BMI) ($p=0.028$) and waist circumference ($p=0.028$). These changes might relate again to the reduction in uptake of cholesterol and bile acids as described above since also triglycerides levels were found to be significantly reduced ($p=0.028$). This potential relationship was used as hypothesis to investigate the effect of chitosan on body weight reduction [59]. Again similar study conditions were used with a 90 day intervention period and daily 2.5 g intake of chitosan and a randomised, double-blind, placebo-controlled setting. Of note, this study included a larger amount of subjects (*i.e*. 96 instead of 12) which increased the significance and validity of the study. Again in this study chitosan proved to significantly ($p<0.0001$) reduce the body weight when compared to the placebo group and again corresponding measurements such as BMI, body fat and visceral fat were also significantly reduced ($p<0.001$). Taken together, these clinical studies demonstrate that chitosan has a clear effect on metabolism that is reflection in cholesterol regulation, body weight, anti-oxidant capacity and insulin sensitivity.

The cationic interactions that underlie the binding of bile acids by chitosan might also make chitosan a suitable bactericidal and fungicidal component [60], in addition to the other putative mechanisms as described above. The positive charge of the amino group can interact with anionic cell surface components, such as lipopolysaccharides of gram-negative bacteria. This interaction was shown to destabilize the integrity of the outer membrane which might sensitize bacteria but cannot directly be described as bactericidal activity. This aspect of chitosan was utilized in a number of studies investigating the beneficial effect on oral health [61-63]. Chitosan was added to mouth wash or chewing gum and indeed reduced the plaque index and related oral bacterial levels and even local inflammation.

In contrast to chitosan, only a single clinical trial was performed with chitin focussing on non-CVD end-points. This study (Table 5) did include the analysis of interleukin production, which are key mediators of immune cells. This study was designed as a randomized, double-blind,

placebo-controlled, crossover study with a two weeks washout period in between each intervention [64]. Healthy participants were randomized into two groups and received either intranasal chitin microparticles (1-20 μm) or placebo. Chitin microparticles demonstrated to significantly reduce local IL-4 and IL-6 levels but to increase leukotriene B4 levels and total white blood cell counts. These results are somewhat contradicting as IL-6 is also an important pro-inflammatory marker preventing a general conclusion on how chitin microparticles affect the intranasal immune system.

Taken together, it is not possible to draw firm conclusions on chitin-mediated beneficial health effects because of the limited number of clinical studies. Although the potential of chitin in CVD treatment has been demonstrated, it only been tested in healthy subjects with relatively low oxidized LDL. More studies are required to potentially acquire an EFSA status similar to chitosan. The clinical studies towards the effect of chitosan on cholesterol reduction, glucose control, and weigh loss were more comprehensive and clearly demonstrated beneficial health effects.

Table 4: Clinical trials demonstrating cholesterol reduction and CVD preventive effects by chitin and chitosan

Products	Origin	Size	DD	Product administration	Study design	No. of subjects (experimental arm/ placebo); subjects status	Summary of effects	References
Chitin-glucan	Fungi	n.d	n.d	4.5 g/day for 4 weeks; orally ingested	Single-blind pilot	20/10; slightly overweight and hypercholesteraemic male	↓Oxidized LDL ↓Oxidized glutathione ↑Anti-oxidant enzyme activity	[50]
Chitin-glucan	Fungi	n.d	n.d	4.5 g/day for 6 weeks; orally ingested	Randomized, double-blind, placebo-controlled	33/35; healthy subjects	↓Oxidized LDL ($p=0.035$)	[51]
Chitosan	Fungi	n.d	n.d	125 mg/day for 4 months; orally ingested	A pilot study	28/n.a; hypertriglyceridemia patients	↓Total cholesterol ($p=0.019$) ↓LDL cholesterol ($p>0.05$) ↓TG levels ($p<0.001$) ↑HDL-cholesterol ($p=0.016$) ↑LDL-2 particles ($p<0.0001$)	[52]
Chitosan	Crab	< 1 kDa	90 %	1 g/day for 6 weeks; orally ingested	Open trial	19/n.a; healthy male smokers and non-smokers	↓Total cholesterol ($p<0.01$) ↓LDL cholesterol ($p<0.05$)	[53]

Abbreviations: DD: degree of de-acetylation; HDL: high density lipoprotein; LDL: low density lipoprotein; n.a: not applicable; n.d: not described; TG: triglyceride

Table 5: Clinical trials demonstrating beneficial health effects of chitin and chitosan

Products	Origin	MW	DD	Product administration	Study design	No. of subjects (experimental arm/ placebo); subjects status	Summary of effects	References
WSC	n.d	20 kDa	95 %	540 mg/day for 4 weeks; orally ingested	Open label	10/n.a; healthy subjects	↓Plasma glucose ($p<0.05$) ↓Atherogenic index ($p<0.05$) ↑HDL cholesterol ($p<0.05$)	[56]
Chitosan	n.d	n.d	n.d	1.5 g/day for 12 weeks; orally ingested	Randomized, double-blind, placebo-controlled	25/26; prediabetic participants	↓Serum glucose control ($p=0.03$); ↓Plasma level of haemoglobin level ($p=0.023$) ↑Plasma adiponectin ($p=0.013$)	[57]
Chitosan	n.d	n.d	n.d	2.25 g/day for 3 months; orally ingested	Randomized, double-blind	6/6; obese subjects	↓Body weight ($p=0.027$) ↓TG level ($p=0.028$)	[58]
Chitosan	Fungi	n.d	n.d	2.5 g/day for 90 days; orally ingested	Single-blind, placebo controlled, randomized	64/32; obese subjects	↓Body weight ($p<0.0001$) ↓HbA1c level ($p=0.0343$)	[59]

(continued)

Table 5: Continued

Products	Origin	MW	DD	Product administration	Study design	No. of subjects (experimental arm/ placebo); subjects status	Summary of effects	References
Water soluble chitosan	Crab	3-5 kDa	70 %	37.5 or 150 mg/day for 6 weeks; Rinsed with mouth rinse solution	Randomized, double-blind, placebo controlled	12/n.a; healthy subjects	↓Dental plaque formation (*S. mutans*) ($p=0.014$)	[62]
LMW-WSC	n.d	5 kDa	n.d	20 mg chitosan solution 4 times a day for 2 weeks; immerse in chitosan solution	Randomized, single-blind	40/n.a; denture stomatitis patients	↓ Formation of erythematous surface area ($p<0.001$) ↓ Number of blastopores and mycelia of *C. albicans* (n.s)	[61]
Chitosan	n.d	n.d	n.d	2% w/v solubility in saliva; chewed 8 gums in total	Randomized, double-blind, placebo controlled	50/n.a; healthy subjects	↓ Amount of bacteria (*Streptococci*) ($p<0.05$)	[63]
Chitin	Prawn	1-20 μm	n.d	2 mg in total; intranasal administration	Randomized, double-blind, placebo-controlled	14/n.a; healthy participants	↓ IL-4;IL-6 ↑Leukotriene B4	[64]

Abbreviations: DD: degree of de-acetylation; HbA1c: haemoglobin A1c; HDL: high density lipoprotein; IL: interleukin; LDL: low density lipoprotein; LMW-WSC: low molecular weight water soluble chitosan; MW: molecular weight; n.a: not applicable; n.d: not described; n.s: not significant; TG: triglyceride.

Requirements to forward the field of study towards the beneficial health effects of chitin and chitosan

Modulation of immune response as induced by chitin or chitosan are dependent on their physical and chemical properties. Variations in the physical properties such as size and concentration, and purification methods and chemical properties including degree of de-acetylation levels and sources may induce different immune responses [65]. This has been illustrated by various in vitro studies.

Size-mediated variations of immune responses may result from different mechanisms of interaction and downstream signalling pathways. Specifically, small sized chitin (<10 μm) could be taken up by phagocytosis, leading to the induction of immune responses in a phagocytosis-dependent manner [22]. In contrast, cells are physically limited in absorbing particles bigger as 10 μm. These larger particles most likely affect cellular responses via receptor clustering. Such differences in interaction with cells likely result in various downstream signalling pathways, which consequently leads to different cellular responses.

Next to this, the degree of de-acetylation has been shown to be a critical factor to determine cellular responses to chitin and chitosan. Julianne and colleagues found that chitin with different DD can induce distinct cytokine production [66]. Fully acetylated chitin (100%) only induces TNFα production, but chitin with a DD of 39% induces both TNFα and IL-1β production by THP-1 macrophages. Moreover, chitin with a DD of 85% also induces the production of TNFα and IL-1β by THP-1 macrophages but at a lower level. Similarly, acetylation levels were also demonstrated to affect the amount of accumulated leukocytes at sites of inflammation [67].

Additionally, chitin or chitosan isolated from different sources can lead to distinct results. Human PBMCs exposed to chitin from crustaceans were found to produce TNFα and IL-6 [68]. Similarly sized chitin from fungi induces the production of IL-10 when presented to human PBMCs [10]. In fact, chitin isolates from sources from the same kingdom but different genus also show different immunogenicity. Chitin derived from *A. fumigatus* induces a six times higher IL-6 production than *C. albicans*-derived chitin in human PBMCs [65].

Chitin or chitosan may also induce different responses when they have been alternatively isolated from the same source, as purification methods during isolation may be differed in distinct studies. Interaction of residual proteins, fibres or secondary metabolites during extraction of chitin and chitosan can affect immune responses. Chitin extracted from shrimps and crabs are commonly commercially used, however, the purification steps during the

extraction process can be different [22, 23, 30]. For instance, it has been demonstrated that β-glucan may largely remained in chitin extracts if not specifically removed [65]. β-glucan is a well-known and well-described biological response modifier and capable of influencing immune cell phenotypes, production of cytokines and recruitment of immune cells [69].

Surprisingly, many of these key characteristics have not been described in many of the animal studies and clinical trials (see Tables). Although the impact of these characteristics has only more recently became evident this does not take away from the fact that study compound details should have been described. This, however, is a challenge of the growing field of studies towards beneficial health effects of food compounds or orally administered supplements such as protein, dietary fibers, etc. The lack of detailed information prevents a direct comparison of results and therefore hampers the analysis of contradictory results and the accumulation of knowledge to unlock the potential of chitin and chitosan.

Outlook on beneficial health effects of chitin and chitosan

The terms 'chitin' and 'chitosan' cover a wide range of molecules with varying molecular weights, acetylation levels and sources and purification methods. All these variables are demonstrated to impact immune responses. There is a large body of work describing the immunomodulatory effects of chitin and chitosan, but often lack of detailed description of the structural characteristics of the preparations under investigation, which hampers clear-cut conclusions of the potential of chitin and chitosan. As for other areas of food and nutrition research, we are only scraping the surface of knowledge of interaction with the immune system. Most studies focus on epithelial and macrophage responses which represents important parts of the innate immune system. Studies towards the adaptive immune system are more complicated and arguably more receptive to minor differences in chitin and chitosan structure. To ensure that work benefits the current body of knowledge on immunomodulatory effects of chitin and chitosan it is crucial that all parameters as indicated above are analysed and described.

Currently, the beneficial health effects of chitin and chitosan have already been commercialised in various products (*e.g.* Chitosan®, xtendlife™). The main functions of these products are weight loss and cholesterol reduction. The clinical trials and animal studies performed to

demonstrate other beneficial health effects demonstrate the potency of chitin and chitosan to be more broadly applied to improve health.

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Chapter 6

General discussion

Insects are considered as ideal additives for animal feed for decades as they are high in nutrients, have low environmental impact and are already part of the natural diet for many animals such as pigs, fish and chicken [1]. With the booming global population, an increased requirement of food is needed to feed this population. Therefore, the concept of applying insects as food for human has gained increasing attention. So far, there are more than 2000 insect species reported to be edible for human and the nutritional value is comparable to that of meat [2]. In addition to their nutritive value, insects are also characterised for having many beneficial health effects because they contain significant amounts of bioactive compounds such as proteins, fats and fibres. To help food companies to develop novel functional foods that can bring optimal health impacts for human, we intend to characterize the effects of insect derivatives on the intestinal health and the underlying mechanisms. This aim was achieved by investigating the antimicrobial properties of insect-derived products on the pathogenic bacterium *Clostridium perfringens* (**Chapter 2**), the protective impact on barrier function of intestinal epithelial cells (**Chapter 3**), the modulatory impact of chitin on the intestinal immune barrier by testing exposure to epithelial cells, microbiota and immune cells (**Chapter 4**), and is completed with reviewing current literature that focussed on beneficial health effect of chitin and chitosan (**Chapter 5**). The various aspects of the work described in these chapters are discussed in more depth in the following sections.

Using Caco-2 as an *in vitro* intestinal epithelial model

The Caco-2 cell line is a well characterized human colon adenocarcinoma derived cell line that has been extensively used in laboratories as an *in vitro* model to mimic the growth of intestinal epithelial cells [3]. In **Chapter 3 and 4**, Caco-2 cells were cultured for 21 days to mimic small intestinal epithelial cells. This *in vitro* intestinal model has been applied to examine the impact of insect-derived fractions or chitin on the barrier integrity as measured by transepithelial electrical resistance (TEER) changes and fluorescein isothiocyanate–dextran (FD4) transport (Figure 3.1), and the immunomodulatory response, reflected as gene transcription of small intestinal-like Caco-2 cells (Table 3.2). Expectedly, our data verified that differentiated Caco-2 cells express cell junctional system genes such as occludins (*i.e.* OCLN), tight junction proteins (*i.e.* TJP3), claudins (*i.e.* CLDN1) and epithelial cell adhesion molecules (*i.e.* EPCAM) (Table 3.1). The expression of junctional proteins by Caco-2 cells indicates that the barrier features of Caco-2 cells might be similar to the small intestinal epithelial cells *in vivo*. This

allows us to predict the impact of insect-derived fractions, chitin or other food bioactive compounds on the barrier integrity of the small intestinal epithelium *in vivo*. Next, we also studied the transcriptional response of Caco-2 cells to insect-derived fractions (**Chapter 3**) and chitins (**Chapter 4**) and an increased transcription of tight junction proteins genes (*i.e.* TJP3, CLDN1, OCLN, etc) were observed after exposure to digest of insect-derived fractions when compared with empty digest control (Table 3.1).

In addition to the physicochemical barrier function, intestinal epithelial cells are essential for regulating the intestinal immune response [4]. They express pattern-recognition receptors such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs) on the membrane to sense luminal content and produce signalling molecules such as IL-1β and TSLP to instruct immune cells in the lamina propria [4, 5]. Caco-2 cells share some immunological features with intestinal epithelial cells, such as expression of TLR1-4 on the cell membrane [6] and the ability to secrete IL-6, IL-8, TGF-β and thymic stromal lymphopoietin (TSLP) [3]. Therefore, the Caco-2 model allows us to study, at least partially, the immune-related response of intestinal epithelial cells to different samples. Furthermore, many studies have demonstrated that the differentiated Caco-2 cell monolayer is a reliable model for studying the absorption of drugs and other compounds after oral administration to humans as the permeability coefficients of differentiated Caco-2 cells have a high correlation with the absorption data in the human jejunum [3, 7, 8]. This may indicate that proteins, antimicrobial peptides in this case (**Chapter 3**), could also be absorbed at a similar rate by small intestinal epithelial cells *in vivo* and an enhanced transcription of junctional genes (*i.e.* CLDN1,2 and OCLN) observed in the *in vitro* results might also be observed in the *in vivo* environment.

Although Caco-2 cells express morphological and functional similarities with the human intestinal epithelium, variations are observed in gene expression between Caco-2 cells and human intestinal epithelial cells [9]. This can be explained by the presence of different cell types in Caco-2 transwell cultures and human intestinal epithelial cells. Intestinal epithelial cells contain goblet cells, Paneth cells, microfold (M) cells and mainly intestinal enterocytes (80%) [10]. However, the differentiated Caco-2 cells successfully mimic the growth of enterocytes or colonocytes but fail to mimic the growth of other cell types such as M cells [3]. M cells are a type of specialized cells that regulate the communication between luminal content and the immune cells in the lamina propria in the *in vivo* setting [11]. Lack of the simulation of M cells or other cell types in the Caco-2 cell model may limit this *in vitro* model to explore the transportation function of particulate samples. This limitation can be addressed by using a

co-culture model or intestinal epithelial organoid model. Specifically, researchers have established a co-culture model of B-cell lymphoma Raji cells (Raji B cells) with Caco-2 cells to more closely mimic the intestinal epithelium [12]. Caco-2 cells can acquire an M cell phenotype when co-cultured with Raji B cells, which could help to understand the transportation of nanoparticles by the intestinal epithelium. In addition to co-culture, a better mimic of intestinal epithelium could be achieved by employing an intestinal epithelial organoid model. Human intestinal epithelial organoids present a three-dimensional structure developed by using human tissue and this model can be differentiated into different intestinal epithelial cell subsets which allows to closely mimic the *in vivo* situation [13]. It allows to test the transportation of particulate samples by M cells and hormones secreted by secretory cells such as enteroendocrine cells [14]. Due to the 3D structure, however, using intestinal epithelial organoids encounters challenges such as imaging and accessing the central lumen [15].

Notably, selection of suitable *in vitro* intestinal models depends on the objective of research. The aim of **Chapter 3** and **4** is to investigate the barrier integrity changes and immunomodulatory response of Caco-2 cells to insect-derived fractions or chitin and to predict the response in an *in vivo* situation. As described above, the Caco-2 cell model is a valuable and qualified *in vitro* model to predict the changes in barrier integrity, immune-related response and protein and drug absorption. Comparing to co-culture and intestinal epithelial organoid models, the Caco-2 model is simpler and easier to perform and qualified enough to fulfil our research aims. Therefore, although the Caco-2 model has its limitations, the simplicity and reproducibility of this model makes it an indispensable *in vitro* intestinal model and maybe it performs better than other complex models such as co-culture and organoids under certain situations.

Protective effects on barrier integrity of the digest of insect-derived fractions

In **Chapter 3**, we have studied the impact of the digest of insect-derived fractions on the intestinal barrier damage induced by *Clostridium* secreted toxins. We found that BPD, BchPD and MPD can effectively attenuate the damage induced by *C. difficile* toxin-A (Figure 3.2). Moreover, preincubation of small intestinal-like Caco-2 cells with BPD, BchPD and MPD also mitigates the damage to the barrier induced by *C. difficile* toxin A (Figure 3.3). In addition to *C. difficile*, pathogenic bacteria such as enterotoxigenic *Escherichia coli, Staphylococcus aureus*, *C. difficile* and *C. perfringens* can secrete different toxins to induce damage onto the

small intestinal barrier [16-18]. To extend our knowledge on the preventive effects of insect-derived fractions on barrier damage by pathogens, we tested the impact of commercial *Staphylococcus aureus* α-haemolysin and *C. perfringens* ε-toxin on small intestinal-like Caco-2 cells (Figure 6.1). Even though these two commercial toxins were reported to disrupt the barrier integrity of Caco-2 cells [19, 20], our results showed that they did not effectively decrease the integrity of Caco-2 cells in our hands. Next, we attempted to acquire a mixture of enterotoxins synthesized by *C. perfringens* (NCTC 8239; an enterotoxin gene positive strain) directly through inducing stress following the protocol developed by Granum and colleagues [21]. However, the enterotoxin mixture again did not effectively damage the barrier of Caco-2 cells either (Figure 6.1). In contrast to our findings, *C. perfringens* secreted enterotoxins have been extensively reported to disrupt the barrier integrity of intestinal epithelial cells both *in vitro* and *in vivo* via targeting tight junction proteins such as claudins and occludins [22-24]. Nonetheless, the *C. difficile* toxin A did damage the Caco-2 barrier. RNA sequencing to study the transcriptional changes of cell junctional and proliferation genes of Caco-2 cells after exposure to digest of insect-derived fractions (Table 3.1 and Table S3.2) revealed the enhanced expression of tight junction and proliferation proteins including OCLN, TJP3, CLDN1, SLC20A1 and thus prevent or mitigate the damage to the intestinal barrier induced by *C. perfringens* enterotoxin. Hence, the impact of the digest of insect-derived fractions on the barrier disruption mediated by *C. perfringens* enterotoxin should be assessed in the future.

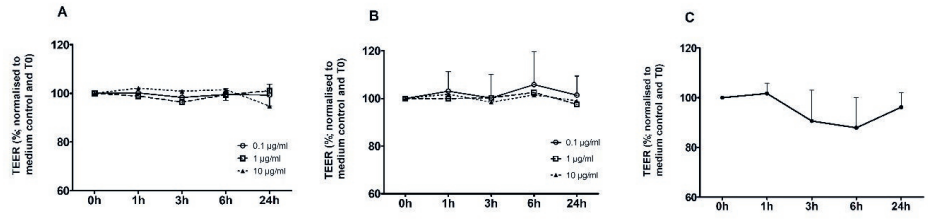

Figure 6.1: Impact of *S. aureus* α-haemolysin (A), *C. perfringens* ε-toxin (B) and *C. perfringens* enterotoxins (C) on the TEER value of Caco-2 cells.

Using THP-1 macrophages to study the immunomodulatory effects of bioactive compounds.

Gut macrophages play an essential role in the intestinal immune response. To investigate the response of gut macrophages to food bioactive compounds *in vitro*, different macrophage models have been applied in previous studies [25, 26]. In general, THP-1 macrophages and human primary macrophages are two of the most commonly used macrophage models. THP-1 macrophages are differentiated from THP-1 cells, a human monocytic cell line derived from acute monocytic leukaemia patient, by the stimulation with phorbol 12-myristate 13-acetate (PMA) [25]. THP-1 macrophages were used in **Chapter 4** to characterize the immunomodulatory effects of chitin fractions by evaluating the gene transcription (*i.e.* IL-1β and IL-8), cytokine secretion (*i.e.* IL-1β and IL-10) and endocytosis after exposure (Figure 4.3 and 4.4). It has been reported that THP-1 macrophages are a simplified model to substitute human primary macrophages to study the straightforward biological process such as endocytosis and polarization [27]. Also the selection of the most suitable *in vitro* macrophage system depends on the aim of research. Human primary macrophages are obtained from human blood, so they possibly better extrapolate the immunological readouts to *in vivo* intestinal macrophage function. The disadvantage is that the immune response of primary macrophages varies between donors. Alternatively, THP-1 macrophages are stable and easy to work with and the immune response would not be affected by donor phenotype. In **Chapter 4**, we aimed to investigate the immunomodulatory impact of chitin fractions on macrophages to predict the intestinal macrophage response *in vivo*. Even though both THP-1 macrophages and primary macrophages are qualified to allow us to study the immunomodulatory potentials of chitins, we should note that both these two macrophages have some differences with intestinal macrophages.

Intestinal macrophages are adapted to the antigen- and microbe-rich environment so they are strong in phagocytic and antimicrobial function but weak in releasing proinflammatory mediators when compared with other macrophages [28, 29]. However, both primary macrophages and THP-1 macrophages are sensitive to proinflammatory stimuli such as LPS. We have investigated the impact of LPS on the gene expression of THP-1 macrophages (Figure 6.1). The results revealed that LPS at low concentration (1 pg/ml) could already increase the relative fold changes of genes such as CXCL8 when compared to medium control. Therefore, the LPS content in samples should be carefully assessed and removed when using THP-1 macrophages as a model to predict the activity of intestinal macrophages *in vivo*. In **Chapter**

4, the LPS content in all chitin fractions has been measured and no LPS has been detected. The results are therefore solely attributed to the immunomodulatory effects of chitin fractions. Even though we observed that small chitin fractions induced secretion of proinflammatory cytokines and chemokines such as IL-1β, IL-8 and CXCL10 by THP-1 macrophages (Figure 4.3), this effect should be further verified *in vivo* studies due to the weak ability of gut macrophages to produce proinflammatory cytokines. In addition, in **Chapter 4**, IL-10 production by THP-1 macrophages was significantly increased after exposure to small chitin when compared with medium (Figure 4.3). IL-10 can be produced by intestinal macrophages in a steady state and this cytokine plays an essential role in the differentiation and maintenance of regulatory T cells in the intestinal lamina propria [29]. Hence, the IL-10 induction ability by small chitin fractions might contribute to the maintenance of intestinal immune homeostasis *in vivo*.

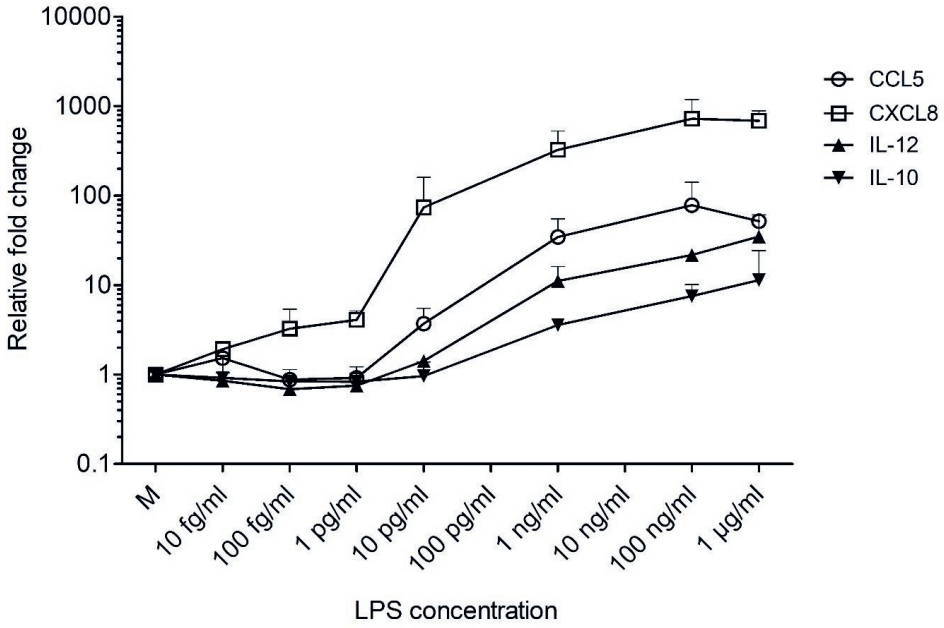

Figure 6.2: Gene expression of THP-1 macrophages following exposure to titrated amounts of LPS. THP-1 macrophages were stimulated with different concentration of LPS for 6h and the gene expression was analysed using qPCR. Line charts represent the mean + SD of 2 individual experiment.

Using co-culture models to predict the intestinal immune response to bioactive compounds *in vivo*.

To investigate the impact of food bioactive compounds on the intestinal health in *in vitro* studies, co-culture models have been applied in many studies [30, 31]. In particularly, the co-culture model should mimic both the intestinal epithelial layer and immune cells, and this has been achieved by co-culturing intestinal-like epithelial cells (*i.e.* Caco-2 or HT-29 cells) with immune cells (*i.e.* macrophages or dendritic cells). In **Chapter 3**, we have established a co-culture model to investigate the immunomodulatory impact of the digest of insect-derived fractions (Figure 3.7). The co-culture model was achieved by seeding Caco-2 cells on the apical side of the transwell inserts and loading THP-1 macrophages on the back of the inserts (Figure 6.3A). A similar co-culture setting has also been applied by co-culturing dendritic cells and Caco-2 cells to predict the intestinal immune response to enteropathogenic *E. coli* [31]. The benefit of our co-culture system is that it allows a more immediate interaction of THP-1 macrophages with luminal contents after these have been translocated into the basolateral side. Moreover, the signalling molecules such as TGF-β and TSLP that are secreted by Caco-2 cells could immediately be sensed by THP-1 macrophages, allowing for analysis of the crosstalk between these two types of cells.

In addition to our co-culture system, another simplified co-culture model has been used widely. The simplified model encompasses seeding of Caco-2 cells on the apical side of inserts and loading THP-1 macrophages at the bottom of the plates rather than the back of inserts [30] (Figure 6.3B). Comparing to the co-culture model used in **Chapter 3**, this model is easier to set-up and handle. However, the simplified model lacks the contact between Caco-2 cells and THP-1 macrophages. The crosstalk between intestinal epithelial cells and macrophages has been demonstrated to be important for maintaining the intestinal immune homeostasis [29]. In our model, direct cellular contact appears likely, but has the formally validated through confocal microscopy or immunohistochemistry. Moreover, the simplified setting would result in an underestimated immune response as the luminal content will be diluted by the basolateral medium before it interacts with immune cells at the bottom of the plate after translocation.

As described above, Caco-2 cells cannot take up particulate samples, for which Raji B cells were co-cultured with Caco-2 cells to acquire an M cell phenotype. To better predict the *in vivo* intestinal environment, we attempted to co-culture THP-1 and Caco-2 system (Figure 6.3A) with Raji B cells. However, the addition of Raji B cells to Caco-2 cells results in detachment

of THP-1 macrophages from the back of inserts (data not shown). In future studies, the coculture of Raji B cells with the simplified Caco-2 cells/ THP-1 macrophages system (Figure 6.3B) is worthwhile for being studied further if the luminal samples are particulate samples such as chitin or other fibres. Furthermore, in some co-culture models, the immune cells (*i.e.* dendritic cells and macrophages) might not be able to stretch their dendrites to sample luminal contents like *in vivo* [31], particulate samples then would need help from M cells to interact with underlying immune cells in the *in vitro* setting. Additionally, particulate samples would less likely be affected by the basolateral dilution factor as they will eventually sediment to the bottom if a sufficiently long period of incubation will be applied.

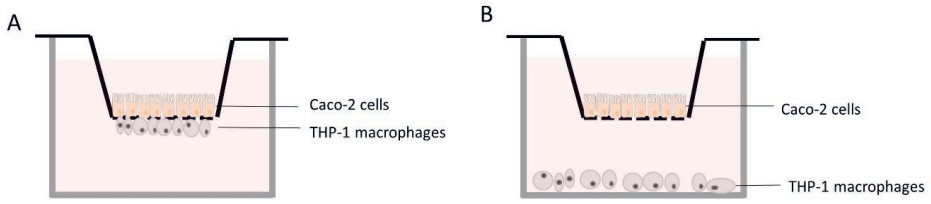

Figure 6.3: Different co-culture models of Caco-2 cells and THP-1 macrophages.

Using an *in vitro* fermentation model to study the gut microbiota response to bioactive compounds.

In vitro fermentation models range from simple batch cultures to multistage continuous flow models based on using different faecal inoculation techniques. Batch fermentation is one of the simplest *in vitro* fermentation systems allowing to study the species composition and functionality of gut microbiota [32]. Batch fermentation models have been extensively used in studies investigating the impact of dietary carbohydrates such as inulin and resistant starch on the fermentation by gut microbiota [33, 34]. The advantage of a batch fermentation system is that it is easy to set-up and useful for robust and quick fermentation studies. The disadvantage is that the environmental parameters such as pH cannot be properly controlled, because batch fermentation has commonly been performed in a closed vessel and SCFAs secretion could reduce the environmental pH during fermentation. To better mimic the intestinal fermentation, more advanced *in vitro* fermentation models such as multistage continuous culture and artificial digestive systems have been developed [32]. For example, the simulator of the human intestinal

microbial ecosystem (SHIME) is an artificial digestive system compromised of five consecutive fermentation vessels of which the first two vessels mimic processes occurring in resp. the stomach and the small intestine, and the following three vessels simulate the fermentation as taking place in the ascending, transversal and descending colon [35]. This artificial digestive system mimics the conditions in each digestion compartment, such as maintaining pH and pressure, allowing us to study the impact of carbohydrate samples on fermentation in each region. However, the downside, especially when performing a twin SHIME, is the lack of an identical control. Each vessel containing microbiota is inoculated separately, resulting in variations in microbiota composition (data not shown).

In **Chapter 2**, we performed a batch fermentation by using faecal-derived microbiota and exposed these to the digest of insect-derived fractions to study the microbiota compositional changes and SCFAs secretion. The results revealed that the digest of insect-derived fractions, in particular of black soldier fly larvae protein meal (BPD), chitin enriched black soldier fly larvae protein meal (BchPD) and mealworm larvae powder (MPD) increased the secretion of total SCFAs. BPD and BchPD also significantly ($p< 0.01$) increased butyrate acid production by gut microbiota (Figure 2.6). Consistent with our findings, an *in vivo* study also showed that black soldier fly larvae meal induced the secretion of SCFAs production and increased the abundance and diversity of gut microbiota in laying hens [36]. The author attributes these beneficial effects to the fermentation of chitin, which is the main fibre in insects. However, in **Chapter 4**, we have demonstrated that pure chitin derived from shrimp did not affect the gut microbiota composition with respect to the diversity and abundance, indicated by the total OTUs and Shannon index. In addition, the relative abundance of the microbiota did not significantly ($p< 0.05$) change after exposure to chitin fractions at a class level (Figure 4.3). This difference might be explained by the limited amount of chitinase in the human digestive tract when compared with chicken [37]. Chicken produce chitinase in both stomach and intestine, which can degrade chitin into dimers of N-acetyl-glucosamine and further to produce chitooligosaccharides [37]. It has been demonstrated that chitooligosaccharides are considered as prebiotics that can increase the diversity and abundance of gut microbiota, and the secretion of SCFAs in an *in vivo* study [38]. Despite the limited potential for fermentation in our batch fermentation model, large chitin fractions significantly ($p< 0.05$) induced secretion of acetic acid (Figure 4.3). Acetic acid is an essential energy source and the main substrate for the lipid biosynthesis [39]. In addition, it has been reported that acetic acid is beneficial for the prevention of colonic cancers due to its ability to inhibit proliferation and induce apoptosis of

cancer cells [40]. The ability of chitin to induce SCFAs production makes it a potential prebiotic to support intestinal health.

Due to social and ethical concerns, functionality studies in humans are difficult to perform. Clinical trials studying human gut microbiota are limited to faecal samples [32, 41]. To break through this limit, a more advanced drug delivery and monitor device called IntelliCap® system was developed [42]. It is an ingestible wireless endoscopy capsule that can sense pH, temperature and pressure and send visual images in the lumen. Even though this system was developed for diagnosing intestinal disorder and delivering drugs, it also provides the possibility to investigate the pH, pressure or morphological changes induced by bioactive compounds.

Taken together, using *in vitro* fermentation models is generally convenient and, when compared to a human trial, inexpensive, allowing us to quickly verify the research hypothesis. The selection of *in vitro, in vivo* or combination of *in vitro* and *in vivo* fermentation models depends on specific topics and the research purposes. In **Chapter 2 and 4**, the aim of our *in vitro* studies is to investigate the impact of digests of insect-derived fractions or chitin on the compositional changes and SCFAs production by microbiota to predict the impact of these samples on gut microbiota fermentation *in vivo*, and batch fermentation is qualified for fermentation studies.

Potential bactericidal effects of insects towards other pathogenic bacteria

In **Chapter 2**, we investigated the impact of the digest of insect-derived fractions on the growth of the pathogenic bacterium *C. perfringens*. We found that the digest of black soldier fly larvae protein meal (BPD) and chitin rich black soldier fly larvae protein meal (BchPD) significantly ($p< 0.05$) inhibited the growth of *C. perfringens* (Figure 2.1). The antimicrobial effect against *C. perfringens* has been related to the presence of antimicrobial peptides. However, most of digested proteins and peptides will be absorbed in the small intestine and very few peptides can reach the proximal colon [43]. Therefore, the antimicrobial effect of the digest of insect-derived fractions might not be effective for bacteria that only colonize in the colon, but possibly be effective against pathogenic bacteria such as enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* that can also colonize the small intestine and cause small intestinal bacterial overgrowth (SIBO) [44, 45]. Enterotoxigenic *E. coli* is a diverse group of pathogens that are responsible for most of the diarrheal illness in developing countries [44]. It has been

demonstrated that insect-derived antimicrobial peptides such as cecropin P1 can effectively inhibit the growth of enterotoxigenic *E. coli* [46, 47]. To identify the antimicrobial peptides in digest of insect-derived fractions, we performed a proteomic analysis by using Liquid Chromatography Tandem-mass Spectrometry (LC-MS/MS). The LC-MS/MS data was converted to mgf. format by MSconvert and interpreted by pNOVO3 *de-novo* search algorithm followed by matching the resulting peptide to the sequence of linking antimicrobial peptides (LAMP2) database. The peptides with identical sequence matched between pNOVO output and LAMP2 database were retained and only peptides with a maximum of peptide spectrum matching (PSM) score > 50 were listed in Table S2.2. To annotate these antimicrobial peptides, we searched the peptides with an identical match in the database of antimicrobial activity and structure of peptides (DBAASP). Peptides that can be found in DBAASP are presented in Table 6.1. Even though DBAASP database has recorded the biological function and target species of the peptides, the database might be built by compiling all references based on key words but without a detailed interpretation. Therefore, Table 6.1 was presented here but not in **Chapter 2** to only indicate the potential of the antimicrobial peptides in BPD and BchPD to inhibit the growth of various microbes.

According to Table 6.1, we found most of the peptides such as “LPLP”, “KPA”, “LLK” and “LWE” in BPD and/or BchPD have been recorded to inhibit the growth of both *E. coli* and *S. aureus*. Combining the antimicrobial effect on such a broad spectrum that indicated by this table and the strong anti-*C. perfringens* effect described in **Chapter 2**, studies to explore the microbicidal effects on a broader range of pathogenic bacteria that can colonize in small intestine are interested to be conducted in the future.

Table 6.1: Annotation of antimicrobial peptides in BPD and/or BchPD. The peptides were annotated by searching in DBAASP database. N.A: not applicable.

Sequence	**Target groups**	**Target compounds**	**Target Objects**
LPLP	Gram+, Gram-	N.A	*Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli*
KPA	Gram+, Gram-, Fungus	Lipid Bilayer	*Escherichia coli UB1005* *Staphylococcus aureus SAP 0017* *Candida albicans 105* *Propionibacterium acnes*

LLK	Gram+, Gram-	Lipid Bilayer	*Staphylococcus aureus USA 300* *Pseudomonas aeruginosa PAO1* *Escherichia coli ATCC 25922* *Klebsiella pneumoniae*
LWE	Gram+, Gram-, Mammalian Cell	Lipid Bilayer	*Staphylococcus aureus USA 300* *Pseudomonas aeruginosa PAO1* *Escherichia coli ATCC 25922* *Klebsiella pneumoniae*
EW	Gram+, Gram-, Fungus, Mammalian Cell	N.A	*Staphylococcus aureus USA 300* *Pseudomonas aeruginosa PAO1* *Escherichia coli ATCC 25922* *Klebsiella pneumoniae* *Candida albicans ATCC 10231*
FF	Gram+	Lipid Bilayer	*Staphylococcus aureus* *Streptococcus sanguis*
FW	Gram+, Gram-, Fungus	Lipid Bilayer	*Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli* *Klebsiella pneumoniae* *Candida albicans*
WE	Gram+, Gram-, Fungus, Mammalian Cell	Lipid Bilayer	*Staphylococcus aureus USA 300* *Pseudomonas aeruginosa PAO1* *Escherichia coli ATCC 25922* *Klebsiella pneumoniae* *Candida albicans ATCC 10231*
WL	Gram+, Gram-, Cancer, Fungus, Mammalian Cell	Lipid Bilayer	*Bacillus subtilis MTCC 2756* *Staphylococcus aureus MTCC 902* *Escherichia coli MTCC 2622* *Klebsiella pneumoniae MTCC 109* *Salmonella typhi MTCC 3216* *Aspergillus flavus MTCC 183*
YR	Gram+, Gram-	N.A	*Escherichia coli* *Salmonella enteritidis* *Staphylococcus aureus* *Listeria innocua*

Furthermore, our results showed that black soldier fly trilaurin, a triglyceride containing a significant amount of lauric acid, did not significantly inhibit the growth of *C. perfringens* (Figure 2.1). In contrast to our findings, lauric acid has been reported to effectively inhibit the growth of *C. perfringens* in an *in vitro* study [48]. This difference might be explained by the insufficient hydrolysis of trilaurin during the INFOGEST *in vitro* digestion which has yet to be optimised for lipase activity, and therefore inadequate release of lauric acid. In addition to *C. perfringens*, lauric acid has been reported to be effective against pathogenic bacteria such as *S. aureus*, which is a well-known bacterium causing food poisoning [49, 50]. The underlying mechanism was proposed to be associated to the ability of lauric acid to increase the

permeability of bacterial membranes, resulting in apoptosis of the bacterial cells [51]. Interestingly, in addition to inhibiting bacterial growth, lauric acid could also suppress the toxin secretion of *S. aureus* by disrupting the signal transduction that is responsible for the production of the exoprotein virulence factors [51, 52].

The combination of the antimicrobial peptides that are active against *S. aureus* in BPD and BchPD (Table 6.1) provide a potential for the digested insect fractions to inhibit also other pathogenic bacteria in addition to *C. perfringens* and thus to potentially support small intestinal health.

Potential beneficial effects of insects towards small intestinal bacteria overgrowth

Small intestinal bacterial overgrowth (SIBO) is defined as an excessive number of bacteria in the small intestine in particularly in the proximal jejunum [53]. In general, the aetiology of SIBO is associated with disorders including disruption of protective antimicrobial mechanisms such as immunodeficiency and achlorhydria [53]. A clinical study including 55 SIBO patients to analyse the bacterial population in the upper gut and the author has identified 114 micro-aerophilic strains and 117 anaerobes including the genus of *Streptococcus*, *Staphylococcus, E. coli* and *Lactobacillus* [54]. In **Chapter 2**, we have measured the gut microbiota compositional changes after exposure to the digest of insect-derived fractions. The results revealed that the abundance and diversity of the gut microbiota and relative abundance of *Streptococcus* and *Lactobacillus* were decreased after exposure to BPD and BchPD (Figure 2.4). This reduction was considered to be explained by the presence of antimicrobial peptides in BPD and BchPD. In line with our speculation, the small antimicrobial peptides such as "FF" presented in BPD and BchPD were also reported to have the ability to inhibit the growth of *Streptococcus* species (Table 6.1). Therefore, our results indicated that BPD and BchPD might be effective to inhibit the overgrowth of bacteria such as *Streptococcus* and *Lactobacillus* in the small intestine. Even though *Streptococcus* and *Lactobacillus* were found to be the main genus in the 55 SIBO patients, other bacteria such as *Clostridium* were also found to be present in their jejunal juice [54]. Hence, antibiotic treatment might not be an optimal choice although it is still being applied in the treatment of SIBO [53]. Since BPD and BchPD decreased the abundance of the major bacteria in SIBO patients and contain a strong potential to inhibit growth of pathogenic bacteria that cause infection in small intestine, it is worthwhile to perform studies to explore the potential of using insects to support the therapy for SIBO.

Potential applications for insect as functional food

In this thesis, we have investigated the health supportive impact of insects, in particularly black soldier fly and mealworm fractions. Our results have demonstrated that insect-derived fractions contain antimicrobial properties (**Chapter 2**), SCFAs-related effects (**Chapter 2**), and intestinal epithelium protective effects (**Chapter 3**). These beneficial effects indicate that they could be used as food or food additive ingredients to support the intestinal health in animals and humans.

For animals, insects have long been considered as a valuable nutritional source for feed, but health supportive impacts of insects were less reported. So far, studies on mealworm are limited to nutritional perspectives, but studies on black soldier fly have extensively described its antimicrobial effects [55, 56]. In particularly, black soldier fly antimicrobial peptides were reported to decrease the viability of *S. aureus* and *S. epidermidis* [56], its fat was found to inhibit the growth of *Pasteurella moultocida* and *Listeria monocytogenes* [55], and the digest of black soldier fly larvae was shown to suppress the growth of *C. perfringens* (**Chapter 2**). Although the antimicrobial effects of black soldier fly make it a promising antibiotic alternative to many pathogenic bacteria, the functional compounds that contribute to the antimicrobial effects of black soldier fly should be carefully considered when developing a commercial product and deciding the route of administration. For instance, an *in vitro* study from Park has demonstrated the antimicrobial effects of a peptide based on chemical isolation from immunized black soldier fly [56]. Therefore, the antimicrobial peptides could be developed as a therapeutic agent and should be administrated to animals by injection to keep the antimicrobial property in practice. They argue that other administrative routes, such as enteral, might abolish the antimicrobial effects by enzymatic hydrolysis, which could also apply to fats such as lauric acid. However, in **Chapter 2** we demonstrate that black soldier fly larvae can be applied through feed without losing anti-microbial activities. Moreover, we cannot exclude that the digestion process is key in releasing its antimicrobial properties. Hence, identification of compounds to which we can attribute the antimicrobial effects could contribute to the specific application of insects or insect-derived products. It is important to note, however, that digestion processes differ between animals, which can impact potential destruction or release of anti-microbial peptides.

Even though humans already consumed insects in prehistorical times, eating insects has still not been widely accepted in Western countries. It has been shown that consumption of

mealworm is, nutritionally, a realistic protein source for human consumption [57]. However, the health supportive effect of mealworm has rarely been investigated in clinical studies. Although we did not find a significant ($p > 0.05$) inhibitory effect of mealworm larvae fractions on the growth of *C. perfringens*, these can stimulate the secretion of SCFAs such as acetic acid and propionic acid (Figure 2.6). As described above, acetate can induce the apoptosis of colonic cancer cells and propionate was reported to lower lipids and serum cholesterol level and reduce the incidence of cancer [58]. Moreover, mealworm was also shown to mitigate the barrier damage induced by *C. difficile* toxin A, indicating a protective impact on small intestinal barrier function (Figure 3.2 and 3.3). Therefore, mealworm is a promising food source for humans from both nutritive and functional perspectives. In addition to mealworm, the health supportive effect of black soldier fly was also studied. Different from mealworm, black soldier fly could significantly ($p < 0.05$) inhibit the growth of *C. perfringens* and attenuate the barrier damage induced by *C. difficile* toxin A (Figure 2.1, 3.2 and 3.3). Furthermore, black soldier fly also induces the production of SCFAs including acetate, propionate and butyrate (Figure 2.6). Butyrate has been reported to be associated with many beneficial health effects, including anti-inflammatory and colonocyte-proliferation inducing effects [59, 60]. Even though the high nutritional and functional value of black soldier fly were broadly reported, it has been only used as feed for animals. Black soldier fly has not been defined as an edible food for human, which might be due to the microbial (*i.e. Salmonella* spp., *Enterobacteriaceae*) and heavy metal (*i.e.* copper and cadmium) contamination [61]. Hence, the removal of contaminations is indispensable for applying black soldier fly as food for human. Taken together, the nutritional value of mealworm and black soldier fly has been extensively reported in previous studies and potential health supportive effects of these two insects have been demonstrated in this thesis. Animal studies are recommended to be performed in the future to verify the value of these two insects as functional food.

Future perspectives

From the topics discussed in the above sections, it is clear that future studies are needed to further verify the intestinal health supportive impact of insect-derived fractions and chitin in *in vivo* or clinical studies. This is essential to gain an insight in the application of these two products as functional food for humans.

Investigation of the biological activities of *in vitro* digested insects helps to predict the biological effect in the *in vivo* situation when insects are administrated to animals or humans as food. However, the *in vitro* digestion protocol (INFOGEST) used in our thesis is not yet optimized for lipid digestion. The lipid digestion process is essential for investigation of the biological activity, in particularly antimicrobial effect, of insects as insect containing fatty acids which are characterized with microbiocidal properties [48, 55]. Therefore, optimization of the lipid digestion process of the INFOGEST protocol is necessary for studying the biological activity of lipid in the future.

After *in vitro* digestion, digested insect-derived fractions contain antimicrobial peptides, undigested fibre and other soluble and insoluble nutrients. To characterize the intestinal health supportive effect of different insect compounds, centrifugation should be performed on the digested insect samples and the supernatant and pellets should be separated to perform different studies. The supernatant that contains antimicrobial peptides and other soluble nutrients can be used to study the antimicrobial effects on other pathogenic bacteria that can colonize the small intestine to extend the bactericidal spectrum of insects in the future. The pellet will contain fibres that should be used to study their effect on fermentation by gut microbiota in future studies. This set-up prevents the interference of antimicrobial peptides on the growth of gut microbiota and allows to solely study the impact of fibre fermentation and thus better mimic the temporal and spatial activity of the different insect constituents in the gastrointestinal tract after consumption.

In addition, animal studies are indispensable for verifying the intestinal health supportive effects of insects. Administration of black soldier fly and mealworm to animals such as broiler chicken with or without *C. perfringens* challenge could help to understand the impact of insects on the growth performance and verify the anti-*C. perfringens* properties of insects showed in the *in vitro* studies. Such as study has been performed based on our findings and *ex vivo* analysis is ongoing. The results will help us to understand whether our *in vitro* translated to *in vivo* bioactivity.

Next, we have discussed the beneficial impact of chitin in **Chapter 4 and 5**. In addition to size, physicochemical properties such as the degree of acetylation, polymorphic forms (*i.e.* α, β or γ chitin) and source are closely linked to the biological effect of chitin. Even though many studies have reported the response of macrophages to chitin is different from that to chitosan, no studies have shown the relationship between the degree of acetylation of chitin and the response of

macrophages. This also applies in regard to fermentation [62, 63]. This might be a promising topic to be researched in the future as understanding this relationship allows us to help pharmaceutical companies to design chitin with specific physicochemical properties as an agent to support intestinal homeostasis through activating or suppressing immune responses and support microbial balance.

Conclusion

This thesis has indicated the supportive effect of chitin and insect fractions on intestinal health by a set of *in vitro* studies that show the impact on intestinal barrier protection and immune and microbiota modulation. The intestinal epithelial protective and antimicrobial effects of insect fractions suggest their use in replacement or reduction of the use of antibiotics to combat *C. perfringens* infection. The immunomodulatory effect, in particularly the ability to activate macrophages, of small chitin fractions make these possibly of help for patients who need immune support. Both chitin and insect fractions can induce the secretion of SCFAs by gut microbiota, indicating the possibility to modulate systemic health. Combining beneficial effects as shown in different chapters, chitin and insect fractions show potential to be developed as functional foods to support the intestinal health of humans. Therefore, verification of these *in vitro* results in *in vivo* studies is strongly recommended.

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Summary

In this thesis, we aimed to investigate the intestinal health supportive effect of insect and chitin fractions by using a set of *in vitro* models to provide a knowledge base for food companies to develop novel functional foods. Current knowledge on the impact of insects and chitin on intestinal health is limited. The *in vitro* assays we applied centered around the intestinal immune barrier using models to mimic the growth of intestinal epithelial cells, immune cells and microbiota.

In **Chapter 2**, the antimicrobial effect against *Clostridium perfringens* of the insect-derived fractions was studied. *C. perfringens* is a commensal that is present in the intestine of animals and humans in homeostasis. However, this pathogen can lead to lethal diseases when overgrowth occurs due to disruption of homeostasis. So far, application of antibiotics is the recommended treatment for *C. perfringens* induced diseases. The application of antibiotics comes with two major drawbacks being 1) non-specific killing of microbiota leading to a further disruption of intestinal balance and 2) the increasing occurrence of antibiotic resistance. Here, we studied the microbiocidal effect on *C. perfringens* of different black soldier fly- and mealworm-derived fractions by using a set of *in vitro* models. We exposed *C. perfringens* to insect-derived fractions that were digested based on the INFOGEST protocol. The results revealed that the digest of black soldier fly protein significantly ($p< 0.05$) inhibited the growth of *C. perfringens*. To unravel the underlying mechanism, a proteomic analysis was performed of the digested insect-derived fractions, revealing the presence of antimicrobial peptides. To examine whether the insect-derived fractions also impacted healthy microbiota 16S rRNA sequencing and HPLC analysis was performed following incubation to determine changes in the composition of the microbiota and in SCFA secretion, respectively. This showed a small but significant ($p< 0.05$) decrease of the abundance and diversity of microbiota as indicated by the reduction of total OTU and Shannon index. Moreover, a strong reduction in *Firmicutes* and increased abundance of *Proteobacteria* and *Klebsiella* were observed and these changes were related to the increased secretion of acetic, propionic and butyric acid. This reduction in healthy microbiota by the digest of insect-derived fractions might be explained by the microbicidal effect of antimicrobial peptides. However, *in vivo*, the antimicrobial peptides will be absorbed in the small intestine and this detrimental impact on healthy microbiota is expected to be limited or absent. Taken together, the effect of black soldier fly protein on the *in vitro* assays indicated that it is a possible alternative to antibiotics to combat *C. perfringens* infection.

In addition to *C. perfringens*, *C. difficile* is another commensal that can lead to intestinal diseases such as diarrhoea upon overgrowth. The pathogenesis of *C. difficile* is associated with the secretion of toxins which can disrupt the intestinal epithelial barrier. Therefore, in **Chapter 3**, we investigated whether insect fractions (*i.e.* black soldier fly- and mealworm-derived fractions), prepared with the adapted INFOGEST *in vitro* digestion protocol, can counteract the barrier damage of small intestinal-like Caco-2 cells induced by *C. difficile* toxin A. Incubation and pre-incubation with digested insect fractions significantly ($p < 0.05$) attenuated the decrease of the transepithelial electrical resistance (TEER) of Caco-2 cells induced by *C. difficile* toxin A when compared with the control group. To investigate the mechanism, we performed RNA sequencing of Caco-2 exposed to the digested insect-derived fractions. This revealed an increased transcription of cell junctional and proliferation protein genes, putatively explaining the protective effect of the insect fractions. Meanwhile, the transcription of immune signalling genes was also increased. To explore whether the increased transcription of immune related genes in Caco-2 cells could activate THP-1 macrophages and thus secret immune signalling molecules, a co-culture model of Caco-2 cells and THP-1 macrophages was exposed to the digested insect fractions. However, the insect-derived fractions did not significantly ($p > 0.05$) alter the basolateral IL-8 secretion of the co-culture model. To sum up, the digested black soldier fly- and mealworm-derived fractions can mitigate the Caco-2 barrier damage induced by *C. difficile* toxin A, most likely through supporting cell junctions and proliferation, indicating their potential to combat the pathogenesis of *C. difficile* infections.

Chitin is the most abundant polysaccharide in nature and it is available from a huge side-stream of insect and crustacean production. Even though there is a huge potential for chitin-rich side-streams from the food processing industry, limited cellular models were developed to test their impact on intestinal health. In **Chapter 4**, we investigated the impact of chitin fractions of various sizes (*i.e.* small, intermediate and large) on intestinal health by studying their immunomodulatory potency using THP-1 macrophages, their effect on intestinal epithelial cells using Caco-2 cells and their impact on colonic fermentation using faecal-derived microbiota. The results showed that chitin fractions with a degree of acetylation of 83% did not affect the integrity of Caco-2 cells as indicated by TEER, indicating that chitin fractions may not harm the intestinal epithelial cells. Large chitin fractions induced gut microbiota to increase secretion of acetic acid but did not alter the microbiota composition. Acetic acid is characterised for its ability to reduce the incidence of colonic cancer by inducing the apoptosis of cancer cells. In addition, small chitin fractions induced THP-1 macrophages to secrete IL-

1β, IL-8, IL-10 and CXCL10 in a multi-receptor and clathrin-mediated endocytosis dependent manner. Induction of these proinflammatory molecules by small chitin fractions suggest they could be used as immunostimulant to activate the immune system of subjects are in need of immune support. To conclude, both large chitin and small chitin fractions demonstrated specific characteristics that might be taken advantage of when applied in a tailored manner.

In addition to chitin, chitosan, a derivative of chitin, was extensively studied. In **Chapter 5**, we reviewed current scientific literature and discussed the beneficial effects of both chitin and chitosan. This review included an overview of the *in vitro* studies that provide the mechanistic insight, animal studies that verified *in vitro* results, and clinical studies that demonstrated that chitin and chitosan can prevent the occurrence of cardiovascular diseases by reducing the cholesterol level in blood. Moreover, chitosan has also been reported to have an antioxidant capacity and the ability to reduce body weight of overweight people. However, chitin and chitosan mediated beneficial health effects according to *in vitro* and pre-clinical results reveal contradictions, which hampers further clinical validation of chitin and chitosan. These contradictory biological data were due to the variation in, often poorly described, physicochemical properties of chitin or chitosan used in different studies. Therefore, a clear description of the physicochemical properties of chitin or chitosan will be needed to firmly demonstrate the unique health benefits.

The last chapter (**Chapter 6**) discusses the key findings of the different research papers. The advantages and disadvantages of the *in vitro* models used in this thesis were discussed. Based on the key results that are obtained from research chapters, we extend our knowledge to indicate the potential application of insect and chitin fractions. Finally, we conclude that this thesis has improved our understanding of the biological functionalities of insect derived and chitin fractions, regarding to their role in immunomodulation, intestinal epithelium protection and microbiota modulation. We believe there will be a critical role for both insect-derived and chitin fractions in the future food or food additives to support the intestinal or even systematic health of humans or animals.

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About the author

Curriculum vitae

Liyou Dong was born on September 5th 1991 in Zigong city, Sichuan Province, China. In 2010, he started his bachelor study in Animal and Plant Quarantine at Sichuan Agricultural University, China. In 2014, he obtained his bachelor's degree and moved to the Netherlands to start his master study in Medical Biotechnology at Wageningen University. During the master program, he studied the impact of dry heating on the immunogenicity of β-lactoglobulin as his master thesis, in which he found out his interests in food and health research. To follow his passion in research, he successfully obtained a PhD fellowship from China Scholarship Council (CSC) and worked as a PhD candidate at Food Biobased Research & Food Chemistry, Wageningen University, under the supervision of Prof. Dr. HJ (Harry) Wichers and Dr. CCFM (Coen) Govers. During his PhD, the author studied the intestinal health supportive effect of insects and derivatives by using various *in vitro* models. The results of his PhD research are presented in this thesis.

List of publications

Dong L, Wichers H J, Govers C (2019). Beneficial health effects of chitin and chitosan [J]. Chitin and Chitosan: Properties and Applications, 145-167.

Dong L, Ariëns R M C, Tomassen M M, Wichers H J, Govers C (2020). In Vitro Studies Toward the Use of Chitin as Nutraceutical: Impact on the Intestinal Epithelium, Macrophages, and Microbiota [J]. Molecular nutrition & food research, 64(23): 2000324.

T Veldkamp, **L Dong**, C. Govers, A. Paul (2021). Bioactive properties of insect products for monogastric animals - a review [J]. Journal of Insects as food and feed. Accepted (In press).

L Dong, M M Tomassen, R M C. Ariëns, E Oosterink, H J Wichers, T Veldkamp, J J Mes, C Govers (2021). *Clostridium difficile* toxin A-mediated Caco-2 barrier damage was attenuated by insect-derived fractions and corresponded to increased gene transcription of cell junctional and proliferation proteins. Submitted.

L Dong, R M C Ariëns, A H P America, A Paul, T Veldkamp, J J Mes, H J Wichers, C Govers (2021). *Clostridium perfringens* suppressing activity in black soldier fly protein preparations. Submitted.

Overview of completed training activities

Disciplined specific activities	
Courses	
Molecular Immunology	Aarhus University, Aarhus, Denmark, 2017
Vaccine Development	WIAS, Wageningen, 2018
Intestinal microbiome of humans and animals	VLAG, Wageningen, 2019
Symposiums	
Gut microbiome	VLAG, Wageningen, 2020
PUFA-Microbiota-Immune system	WUR, Wageningen, 2017
Integrating experimental and theoretical approaches in immunology	VLAG/WIAS, Wageningen, 2019
Opening the Science of Food	ILSI, Wageningen 2020
Conferences	
20th International Summer School on Immunology and Immune System: Genes, Receptors and Regulation [ab]	FEBS, Hvar, Croatia, 2019
BSI conference 2019 [b]	BSI, Liverpool, Unite Kingdom, 2020
NIZO Plant Protein Functionality	NIZO, Online, 2020
34th EFFoST International Conference	EFFoST, Online, 2020
NVVI annual meeting 2020 [b]	NVVI, Online, 2020
General courses and activities	
VLAG PhD week	VLAG, Wageningen, 2017
The Essentials of Scientific writing and presentation	WGS, Wageningen, 2017
Data management planning	WGS, Wageningen, 2017
Review a scientific paper	WGS, Wageningen, 2018
Teaching and supervising thesis students	WGS, Wageningen, 2019
Scientific writing	WGS, Wageningen, 2019
Efficient writing strategy	WGS, Wageningen, 2019
PhD workshop Carousel	WGS, Wageningen, 2019
WUR Career day	WUR, Wageningen, 2019-2020
Other activities	
Preparation of research proposal	FCH, Wageningen, 2017
PhD trip 2018 [ab]	FCH, Austria-Italy, 2018
Weekly group meetings	FCH/FBR Wageningen 2016-2020
Teaching obligations, courses	
Food related allergy and intolerance (FCH-20806)	FCH, Wageningen, 2017-2020

Abbreviations:

BSI: British Society Immunology

EFFoST: European Federation of Food Science and Technology

FBR: Chair group Food Biobased Research

FCH: Chair group Food Chemistry

ILSI: International Life Sciences Institute

NVVI: The Netherlands Dutch Society for Immunology

PUFA: Polyunsaturated fatty acid

VLAG: Graduate School Food Technology, Agrobiotechnology, Nutrition and Health Sciences

WGS: Wageningen Graduate School

WIAS: Graduate School Wageningen Institute of Animal Sciences

WUR: Wageningen University and Research

[a] poster presentation

[b] oral presentation

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