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In-vitro tools for broiler chickens and infants

Miriam J. Oost

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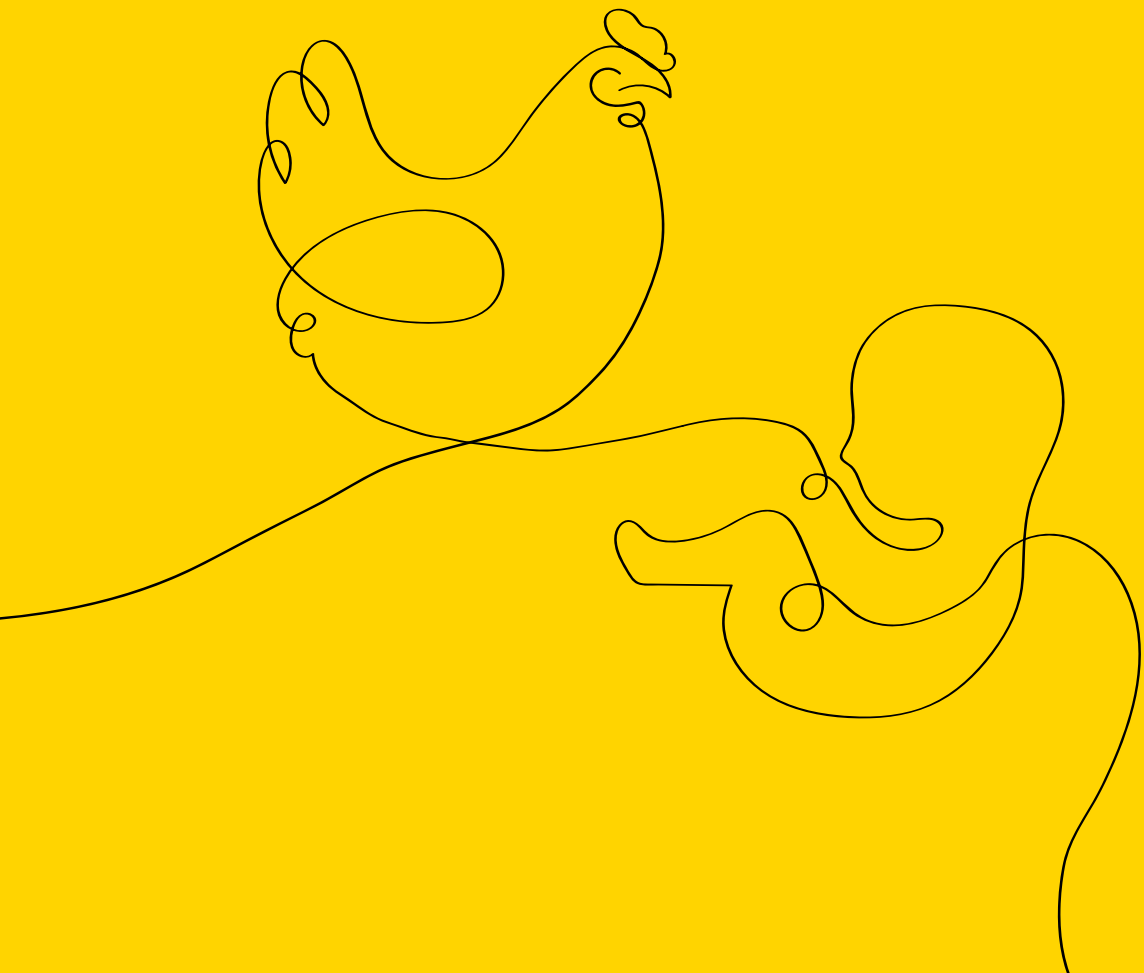
Chapter 1

General Introduction



1.1 Intestinal health

In order to sustain general health and well-being in both human beings and animals, the intestinal tract plays a crucial role (1-3). The intestine harbors about 10^{14} microorganisms in humans and an estimated 10^{13} in chickens, collectively called the microbiota, which has been found to influence a broad range of physiological processes including digestion, nutrient absorption, metabolism, and immune function (4-9). Colonization with microbial communities starts at birth or hatch, respectively, and the development of the intestinal microbiota is influenced by several factors, including genetics, mode of delivery for humans, diet, and environment (10-13). During the early postnatal period, the gut microbiota undergoes rapid and dynamic changes, with the establishment of a diverse and stable microbiota occurring by the end of the two to three years of life in humans, and by 3-5 weeks of age in broiler chickens (14-18). Thus, the first years of life are characterized by marked changes and high diversity in microbiota composition, but this is gradually leading to the development of a relatively stable adult microbiota (19). This composition can be influenced by many factors, including dietary ingredients, environment, use of antibiotics and other medications, and the presence of pathogens (13, 20). The microbiota is mostly able to restore its equilibrium after perturbations (21). If the equilibrium is severely disturbed, this leads to dysbiosis, which can lead to health problems, which for humans include inflammation of the intestine, low-grade systemic inflammation, and increased susceptibility to the development of chronic immune diseases (22-27). The shift in intestinal microbiota composition, resulting in the imbalance between beneficial and harmful bacteria in broiler chickens is associated with intestinal inflammation and shortening of small intestinal villi resulting in reduced nutrient absorption and less efficient growth of the animals (28). Dysbiosis in broiler chickens can be caused by multiple factors, such as coccidiosis, heat stress, and diets containing high amounts of protein and non-starch polysaccharides (29-32), which make the broiler chickens more susceptible to pathogenic bacterial colonization (33). Dysbiosis can also influence behavior and cognition in humans and chickens, through the gut-brain axis, which is the bidirectional communication between the gut (and its microbiota) and the brain (34, 35).

1.2 Bacterial composition and resilience

The healthy microbiota is very diverse in composition and can provide a barrier against pathogenic bacteria. The type and relative abundance of microorganisms found in the intestines can determine the homeostasis of the host. Some bacteria, like *Lactobacillus* and *Bifidobacterium*, provide health benefits for the host (humans and chickens) by producing vitamins and short-chain fatty acids (SCFAs) and are considered beneficial bacteria.

The bacterial density in infants is the highest in the colon and starts with facultative anaerobes, such as *Enterococcus* and *Escherichia*, which can consume the last traces of oxygen in the gut (36). Later in life, these are largely replaced by strict anaerobes. One of the most dominant genera is *Bifidobacterium*, which is becoming slowly less abundant upon aging (37). Also upon aging, the bacterial diversity increases, and the adult-like microbes consist of the dominant phyla *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia,* of which *Firmicutes* and *Bacteroidetes* represent ~90% of the human gut microbiota (38).

In chickens, the cecum has the highest microbial density and this is the place where active microbial fermentation and the production of SCFAs mostly take place (39, 40). Broiler chicken ceca are mainly colonized by anaerobic microorganisms. The most dominant bacterial phyla in broiler ceca between day 0 and 35 days of age are *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria (13)*, with *Firmicutes* having the highest relative abundance (41).

As elucidated above, the gut microbiota is constantly exposed to various stressors, which can alter its composition and function. Despite these challenges, the gut microbiota has shown remarkable resilience and adaptability in both humans and animals and can return to the initial stable state, which is schematically depicted in Fig. 1A, and B (42). When there is a continuous or a severe perturbation, e.g., a permanent change in diet, the composition of the microbiota can adapt to a new stable state (Fig. 1C). However, if resilience is failing and the microbiota cannot return to its original or another beneficial stable state, this can lead to increased susceptibility for intestinal infections, allergies, and inflammatory diseases (Fig. 1D) (43-46).

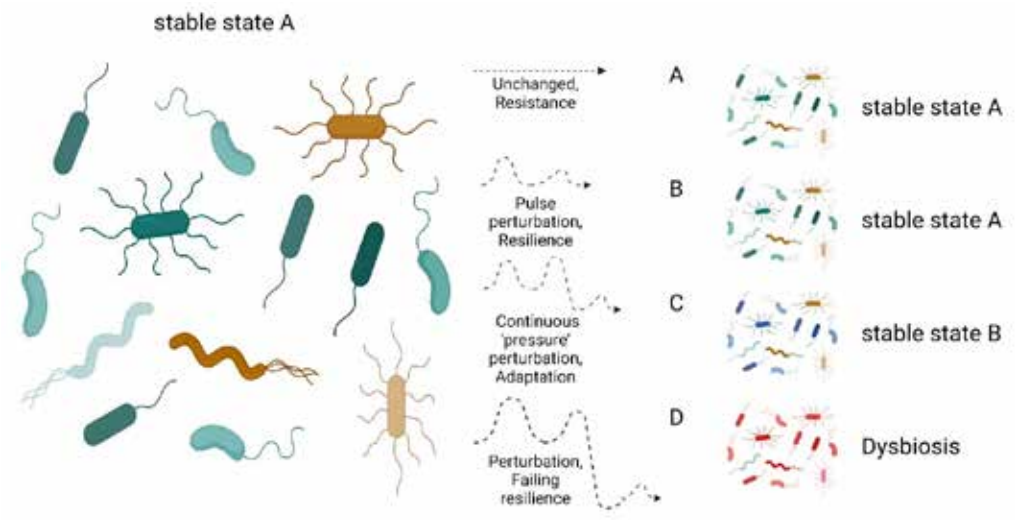


Figure 1 Conceptual elements that regulate the stability of the intestinal microbial ecosystem, adapted from Sommer et al. (42) The stable functioning of the intestinal microbial ecosystem relies on several key principles. **A.** A perfectly 'resistant' microbial community is capable of withstanding short-term disturbances without any noticeable changes in its composition, **B.** A resilient community can recover from short-term disruptions, such as mild inflammation, dietary changes, infection, returning to its normal function and composition (stable state A) after a lag phase or recovery. **C.** A long-term 'pressure' perturbation, such as a drastic change in diet, requires the microorganism community to adapt, leading to an alternative, stable, and beneficial state (stable state B) if the selection pressure is released. **D.** If the initial microbial community (stable state A) fails to show resilience to a perturbation, it may lead to a stable but detrimental state known as 'resilient dysbiosis' (stable state D), which is the threshold of no return. Created with BioRender.com.

1.3 Intestinal immune system

The intestinal tract acts as a line of defense against luminal pathogens, whilst also maintaining tolerance to commensal bacteria and dietary antigens. The first component of the firewall is the microbiological barrier. The commensal bacteria provide colonization resistance against pathogen colonization (6, 47, 48). The intestine contains a layer of mucus covering the epithelial layer. The mucus is part of the second line of defense and is built up of mucins that are secreted by goblet cells, which are part of the epithelial layer. The mucus layer regulates contact between the commensal bacteria and the epithelial cells (47, 49). The mucus layer also helps to maintain the integrity of the intestinal

epithelial barrier by increasing tight junction protein expression. Moreover, antimicrobial peptides are secreted in the mucus by Paneth cells and enterocytes (50). The third line of defense consists of intestinal epithelial cells, which form a continuous monolayer and are attached by adherens junctions. The epithelial cell layer assists the absorption of nutrients but also provides a physical barrier that prevents pathogen invasion and extra intestinal translocation of commensal microbes. In addition, the epithelial cells can also be considered as an integral part of the innate immune response. They possess pattern recognition receptors that detect microbe-associated molecular patterns and have the ability to produce cytokines and chemokines, which initiate an inflammatory response to combat pathogen infections. Moreover, intra epithelial lymphocytes are present in this epithelial cell layer. The lamina propria directly underneath the epithelial monolayer also contains immune cells that are part of the immunological defense mechanism of the intestinal barrier (51).

A balanced mucosal immune response between protective and regulatory immune responses is important, to avoid chronic inflammation of the intestine and barrier dysfunction (6, 52). The gut-associated lymphoid tissue (GALT), which is located in the intestinal mucosa, is an important site for immune cell development, differentiation, and activation, and contains an estimated 70% of all immune cells. The GALT includes Peyer's patches, lymphoid follicles, and intra-epithelial lymphocytes, which are responsible for recognizing and responding to pathogens that enter the intestine in mammals (53). In general, the chicken immune system is similar to that in mammals, however, chickens lack structured lymph nodes and GALT (54). Most of the chicken gut tissue is separated from the lumen by regular epithelium with intra-epithelial lymphocytes and lamina propria lymphocytes, and some specialized parts with unique epithelium, such as cecal tonsils, Peyer's patches, and Meckel's diverticulum (55, 56).

The GALT also harbors innate immune cells, such as dendritic cells, macrophages, and Natural Killer (NK) cells in both mammals and chickens. These cells recognize the pathogen/microbe-associated molecular patterns through pattern recognition receptors and can activate the adaptive immune system by the secretion of chemokines and cytokines. In the chicken intestine, the intraepithelial cells comprise of NK cells, γδ, and αβ T cells of which the NK and γδ T cells are part of the innate immune cells (56-58). The NK cells and γδ T cells are important players in the chickens and their activation increases the resistance of chickens to infection (59, 60).

Additionally, the beneficial gut microbiota can directly interact with the intestinal epithelium, by promoting the production of mucus and the maintenance of intestinal barrier function (6, 61). Moreover, bacteria can ferment dietary fibers in the intestine and produce SCFAs, which have immunomodulatory functions and can induce the differentiation and activity of regulatory T cells, which in its turn can induce tolerance development (62, 63). SCFAs exert their regulatory effect on epithelial cells, antigen-presenting cells, and T cells and mechanisms such as activation via protein coupled receptors, and metabolic regulation play a role in this (64). The metabolites influence many aspects, with for example facilitating tolerance in homeostasis by promoting regulatory T cells and stimulate an active immune response during pathogen invasion (64, 65).

1.4 Intestinal pathogens

Numerous studies have concentrated on how to establish a stable microbiota early in life and maintain its stability and function in the gut. During the development of the intestinal tract, there are many opportunities for pathogenic microorganisms to colonize the gut.

For example, overgrowth of *Clostridium perfringens*, leads to a decrease in microbial diversity. Moreover, *C. perfringens* can induce inflammation and compromise the integrity of the intestinal barrier, potentially leading to a leaky gut. Antibiotics can target the pathogens, but can also have unintended consequences on the intestinal microbiota. Antibiotic use can contribute to a disruption of the microbiota and increases susceptibility to enteric pathogens. For instance, in mammals, streptomycin treatment can deplete commensal (beneficial) bacteria that produce butyrate, leading to a decline in butyrate levels and an increase in epithelial oxygenation. This creates the ideal environment for the colonization of *Salmonella enterica* (66). Enteric diseases are also a significant concern in the poultry industry due to production losses, increased mortality, and a higher risk of contaminating poultry products for human consumption. This thesis focuses on *C. perfringens. C. perfringens* is a Gram-positive spore-forming anaerobic bacterium that can be found in the intestinal microbiome of broiler chickens and infants/humans, where it can cause diseases, and it is considered one of the most common pathogens in humans and chickens. It is also a common foodborne pathogen that can have a significant impact on human health and economy (67-69). The virulence of *C. perfringens* is largely dependent on its ability to produce toxins, which have been associated with various systemic and enteric diseases, including food poisoning and enterocolitis (70, 71).

1.4.1 Necrotizing enterocolitis in infants

As indicated earlier, proper colonization of the gut is important for the general health of the infant. The development of intestinal barrier function and the immune response are depending on proper colonization. Research has found the gut of preterm infants is immature and that, upon insult, barrier recovery of the small intestine is significantly reduced in neonates as compared to juveniles (72, 73), because of their inability to mount a robust reparative response (73). Neonatal barrier function can, for instance, be challenged by the birthing process alone, and especially in combination with the hospital environment, this results in microbial colonization with pathogenic microbes, which can prompt adverse, perinatal outcomes such as necrotizing enterocolitis (NEC). NEC is characterized by inflammation and inappropriate bacterial overgrowth throughout the small and large intestines and is a disease with high mortality rates (20-30%). NEC patients show an increase in the facultative anaerobic *Enterobacteriaceae,* more specifically the genus *Klebsiella,* and showed a decrease in members of *Firmicutes* (70, 72), which represents a delay in maturation of the intestinal microbiota. Another group of NEC patients showed an increase in the presence and abundance of *C. perfringens* in meconium compared to healthy infants (70, 71).

1.4.2 Necrotic enteritis in broiler chickens

In broiler chickens, necrotic enteritis (NE) is one of the most important enteric diseases, caused by *C. perfringens* and was first described by Parish (74). Numerous predisposing factors, such as high-protein diet, environmental stress, and coccidiosis (a common intestinal disease in broiler chickens caused by protozoal parasites of the genus *Eimeri*a), can directly alter the gut's physical properties by damaging the epithelial surface, inducing mucus production, or altering the gut microbiota, thereby increasing the risk for *C. perfringens* infection (30, 69). NE usually occurs in broiler chickens between 2-6 weeks after hatch and can be classified into clinical and subclinical forms (75). The clinical form is characterized by gaseous lesions, mucosa necrosis, and damage to the intestinal wall, which can lead to a sudden increase in flock mortality (76). The subclinical form is less clear but can cause significant economic losses in the

poultry industry, as chronic intestinal mucosal damage can result in poor digestion and absorption of feed, leading to reduced weight gain and increased feed conversion ratio (75, 77). Prevention of dysbiosis by reducing diet- and environmental related risk factors and *Eimeria* infections are essential for NE control. Historically, the broiler industry has used antibiotics and anticoccidial drugs to prevent, treat, and control NE, and to improve health and performance of chickens. However, strict regulations on the use of antimicrobials in livestock farming in the European Union and the USA, has stimulated the use of non-antibiotic alternatives with antimicrobial, anti-inflammatory, and immune-fitness-promoting properties (78). Therefore, in food producing animals, alternatives such as probiotics, prebiotics have been studied as alternatives for antibiotics. Several of these prebiotics are discussed in section 1.6.

1.5 High need for additional research

There has been a growing interest in developing interventions that can improve early-life intestinal health and also can be an alternative to antibiotic use, such as probiotics, prebiotics, and other dietary interventions, e.g. plant-bioactives (79). Moreover, it is important to minimize the use of animals in research as much as possible, but it is also relevant to evaluate the efficacy of interventions that can improve animal and human (intestinal) health and welfare before they can be applied in practice. For this thesis, we performed experiments for which animal trials were not needed. For example, we optimized the gut fermentation model TNO *in-vitro* model of the colon (TIM-2) (80) (resulting in the Chicken ALIMEntary tRact mOdel; CALIMERO-2, see section 1.7.1) and intestinal organoid culture models for chickens, and used the combination of the validated TIM-2 fermentation system and an intestinal co-culture cell model for humans, to evaluate the effects of dietary interventions on the gut microbiota. Such *in-vitro* experiments have several advantages over *in-vivo* studies. For example, *in-vitro* experiments can be conducted under controlled circumstances and are not affected by individual variation between animals, and allow for efficient pre-screening of interventions before moving to *in-vivo* experiments in animal trials or under field circumstances.

1.6 Non-digestible oligosaccharides

Prebiotics are defined by the International Scientific Association for Probiotics and Prebiotics as a substrate that is selectively utilized by host micro-organisms conferring a health benefit (81). Prebiotics are often Non-digestible oligosaccharides (NDOs), but not all NDOs can be specified as prebiotics. NDOs are a class of carbohydrates that are resistant to digestion by the host and absorption in the small intestine, however, they can be fermented by the gut microbiota in the colon (82). In recent years, prebiotics have gained attention for their potential health benefits, particularly in modulating the microbiome, immune system, and intestinal barrier function. They interact with the gut microbiota by promoting beneficial microbes, or inhibiting the adhesion of pathogenic bacteria to human epithelial cells by acting as a decoy receptor (83). Additionally, SCFAs that are produced by the gut microbiota upon the fermentation of NDOs can attenuate inflammation by improving barrier function and enhancing immune regulatory T cells in the intestine (62). Recent research has shown that adverse changes in the gut microbiota composition and function may result in intestinal barrier disruption, suggesting the crucial role of crosstalk between the gut microbiota and epithelial cells (84). Prebiotics, such as fructo-oligosaccharides, have been found to recover epithelial tight junction assembly and re-distribution to intercellular regions, strengthening barrier function, which is essential for preventing intestinal diseases (85). Moreover, research has revealed that NDOs may also have direct (microbe-independent) effects on the host, by acting as an immune modulator and increasing cytokine production of e.g. TNF-α and IL-1β (86, 87). In this thesis, we investigated a few selected NDOs, which will be highlighted in the following sections.

1.6.1 Galacto-oligosaccharides (GOS)

The prebiotic GOS is derived enzymatically from lactose, and consists of galactose and glucose monomers linked by β-glycosidic bonds. It reaches the large intestine and has the ability to modulate the intestinal microbiota, offering beneficial effects on intestinal barrier integrity and the mucosal immune system in mammals and broiler chickens (88-90). As one of the initial NDOs added to infant formulas, GOS is used to mimic the effect of the oligosaccharides found in breast milk (91, 92). Numerous studies have demonstrated that GOS stimulates the growth and activity of beneficial bacteria in the gut, such as *Bifidobacterium* and *Lactobacillus,* which produce acetate and, through cross-feeding, leads to the production of butyrate and conversion of lactate to propionate (93, 94). This is particularly important because butyrate and propionate can help maintain gut barrier function and reduce inflammation (95, 96).

Moreover, research indicates that GOS not only enhances intestinal barrier function *in-vitro* in the human immortalized cell line of human colorectal adenocarcinoma cells (Caco-2), but also *in-vivo* by promoting tight junction assembly after impairment (97, 98) Additionally, GOS exhibits other advantageous effects, including the inhibition of pathogen adherence, such as *Escherichia coli* and *Salmonella* (83, 99). Notably, GOS has been shown to enhance the growth rate and feed conversion of chickens (100). Overall, GOS stands as a versatile prebiotic, offering a wide range of benefits for gut health and immunity.

1.6.2 2'-Fucosyllactose (2'-FL)

2'-FL is a human milk oligosaccharide (HMO), and nowadays also allowed to be added to infant formula (101). 2'-FL reaches the large intestine, where it can have direct and indirect effects by stimulating gut microbiota fermentation processes. It has been shown to have an effect on the microbiota composition and can lower the risk of bacterial and viral infections in infants (102-104). By promoting the growth of *Bifidobacterium* and *Lactobacillus* in the gut, it results in production of microbial metabolites, in particular butyrate. Even though both *Bifidobacteria* and *Lactobacilli* do not produce butyrate, this increase can be explained by the cross-feeding of acetate and lactate to butyrate in the distal colon (105). The most dominant butyrate-producing bacterial species are *Faecalibacterium prausnitzii* and *Eubacterium rectale* (106). Moreover, 2'-FL can prevent the epithelial adhesion of intestinal pathogens, by acting as decoy-receptors through resembling the structure of certain host epithelial cell surface glycans (102, 107).

1.6.3 Mannan-oligosaccharides (MOS)

MOS are derived from yeast cell walls and contain mannose monomers linked by β-glycosidic bonds. Several studies have demonstrated the beneficial effects of MOS, and these vary from improving gut morphology to adjusting intestinal health or pathogen exclusion, for example *C. perfringens* (108, 109). Research with MOS has focused mostly on livestock, for example, broiler chickens. It has been shown to improve villus length and villus surface area, which is important for efficient feed absorption and can improve feed conversion ratio (108, 110-112). In addition, MOS can contribute to luminal barrier protection, by increasing mucin secretion and the number of goblet cells (113). MOS can also result in pathogen exclusion in the intestine by acting as a decoy receptor for type-1 fimbriae, which are used by some harmful bacteria such as *Salmonella*, thereby preventing them from adhering to the intestinal epithelium and facilitating their elimination (114, 115). Even during the prenatal period, before hatching, MOS can have a microbiota-independent effect *in-ovo* on gut morphology, functionality, and innate immunity. For example, an increase in mRNA expression of TLR4 and TLR2 on epithelial cells was observed, which is related to an enhanced epithelial barrier, and provides defense against pathogen invasion (108).

1.6.4 Pectins

Pectins cover a complex family of polysaccharides that are present in all plant cell walls. Pectins are mainly composed of homogalacturonan, rhamnogalacturonan I and II, xylogalacturonan, arabinan, and arabinogalactan type I and type II (116). These complex structures can provide health benefits. The chemical structure and the origin of the pectin are important herein. For example, the amount of methyl-esterification has an impact on the physical properties (117, 118). Pectins are fermented in the large intestine by the microbiota, which converts them into SCFAs (119). Just like other fibers, they can also have a microbiome-independent effect on health, such as interacting with immune receptors in the small intestine, and recent studies have shown that pectins can stimulate TLRs in the intestinal epithelial layer to induce beneficiary immune effects (120). Pectin with a low-degree of methyl-esterification can bind and inhibit TLR2 and specifically inhibits the pro-inflammatory TLR2-TLR1 pathway in mammals (121).

1.6.5 Isomalto/malto-polysaccharides (IMMP)

IMMP is derived from potato starch, and is slowly fermentable and shows potential prebiotic functions (122). IMMP are produced using a 4-6-α-glucanotransferase enzyme, which forms a linear chain of α-(1 →6) linked glucose residues that remain undigested in the upper gastrointestinal tract, and reach the large intestine (123, 124). In human *in-vitro* fermentation models and a mouse model, IMMP has demonstrated potential prebiotic function by inducing the relative abundance of *Bacteroides* and butyrate producers. Additionally, both studies found that IMMP could stimulate the proliferation and activity of *Bifidobacterium* and *Lactobacillus* (122, 125). So far, the effect of IMMP has only been investigated concerning the microbiota, and no direct effect of IMMP on the epithelial barrier or mucosal immune system have been studied.

1.7 Gut fermentation models

In the field of intestinal microbiology, validated *in-vitro* techniques offer an attractive alternative to animal experimentation for testing the efficacy of prebiotics. The most commonly used *in-vitro* models to study the fermentation of carbohydrates by bacterial populations are batch and continuous culture fermentation systems (80, 126). They vary from simple batch fermentations, which are mostly inoculated with selected species of bacteria or fecal slurry to which the carbohydrate of interest is added, to more complex continuous *in-vitro* fermentation models, which mimic the physiological situation better and are a useful tool for assessing treatment-related changes in microbiota metabolism and composition (127). An example of a continuous fermentation system is TIM-2 (128). This model is available for humans, but also for several animals, for example, pigs (129).

1.7.1 TNO *in-vitro* model of the colon (TIM-2)

TIM-2 is an innovative computer-controlled dynamic system designed to simulate the key features of the human large intestine (130). The system consists of glass compartments and has a flexible membrane inside, which makes it possible to mimic peristaltic movements (Fig. 2). The space between the glass compartment and the flexible membrane is filled with temperature-controlled water (37 ℃ for humans) which simulates the natural environment of the colon. To regulate the pH, sodium hydroxide is secreted to neutralize acids that are produced by the microbiota. By constantly flushing the system with nitrogen gas, an anaerobic environment is created. To maintain physiological concentrations of metabolites, fermentation products are removed through a dialysis system. The system is continuously fed with simulated ileal-efflux medium (SIEM), which simulates the fraction of the diet that reaches the colon and provides the required nutrients for the microbiota to thrive. Throughout the experiment, samples can be taken to study the effect of the food and feed/intervention (in this thesis: the applied

carbohydrates) on the microbiota composition and activity. The ability to test compounds using this system provides valuable insights into the effects of different substances on the microbiota, paving the way for future advances in gut health research.

In this thesis, the focus is on CALIMERO-2, which mimics the chicken cecum, which plays an important role in the gastrointestinal tract of the chicken, because of active microbial fermentation and the production of energy-rich SCFAs. CALIMERO-2 is derived from TIM-2, and can be used to evaluate the effects of feed additives on microbial composition and activity over time.

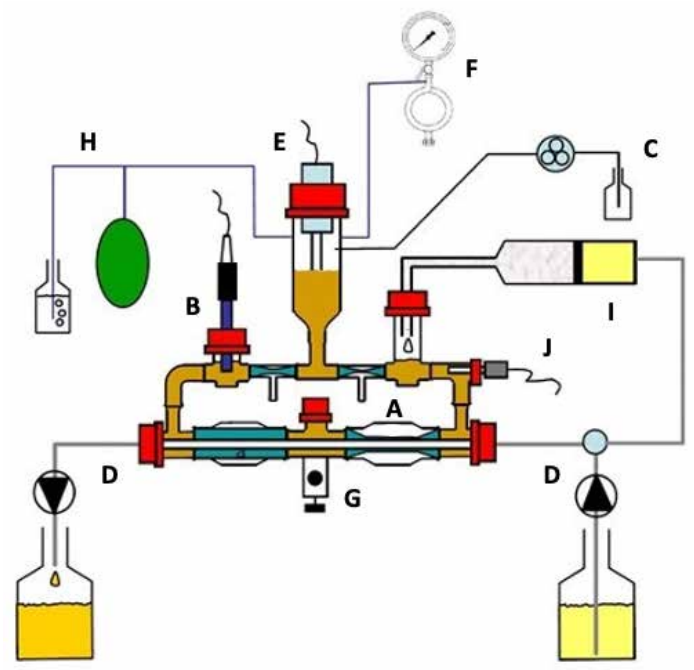


Figure 2. Schematic picture of TIM-2. **A** = peristaltic compartments with a dialysis membrane inside, containing fecal inoculum; **B** = pH sensor; **C** = NaOH secretion; **D** = dialysate liquid circuit with hollow fiber membrane; **E** = level sensor; **F** = N_2 gas inlet; **G** = sampling port; **H** = gas outlet; **I** = feeding syringe containing SIEM or test compound; **J** = temperature sensor (130).

1.8 Chicken intestinal organoids

Intestinal organoids serve as prominent examples of cellular models used extensively in current mammalian studies. These advanced epithelial cellular models were first introduced by Sato and colleagues (131). Intestinal organoids are self-organized three-dimensional tissue epithelial cultures, derived from stem cells, and represent the physiology of *in-vivo* situations better than monolayer epithelial cell lines. This is primarily due to the ability of stem cells to differentiate into various epithelial cell types found in the intestine. While limited studies describe intestinal organoids in chickens (132-136), the optimal culture conditions for chickens are still undergoing development.

1.9 Human Intestinal cell models

Cell models can be used to better understand the effect of prebiotics on the host cellular level. For example, the human immortalized cell line of human colorectal adenocarcinoma cells (Caco-2 cells) are widely used as a model for the intestinal epithelial barrier. During culturing, they undergo spontaneous differentiation, which results in polarization and the formation of tight junction proteins between epithelial cells. The cells can be used to test barrier integrity and epithelial permeability in response to (microbial) compounds (137-139). These cells can also be used in a co-culture with innate and adaptive immune cells, to study the epithelial barrier function more realistically, as well as the cross-talk between epithelial and immune cells.

1.10 Scope of this thesis

As outlined in the previous paragraphs, the gut microbiota is important in health and disease. The focus of this thesis was mainly on broiler chicken intestinal health, which is comparable in many ways to that of infants. Therefore, in addition to the studies using broiler chicken models, fermentation products of potential prebiotics and their effect on intestinal barrier integrity was also studied in infant cellular models. Moreover, to be able to efficiently study the potential of alternative treatments to improve intestinal health we investigated and optimized the use of a combination of multiple *in-vitro* models that can be used as a pre-screening pipeline in the future. It should be noted that the nomenclature of bacterial taxa is changing regularly. In this thesis (which started before the nomenclature changed), mostly the old taxa names are used. If necessary for a better understanding, both old and new taxa names are provided in the text of this thesis.

The aim of this thesis was to:

- Develop *in-vitro* tools to study the microbiome and cellular responses in broiler chickens upon interventions with dietary carbohydrates
- Investigate the prebiotic effects of IMMP and pectins on a) the microbial composition and b) its metabolic activity of a cecal microbiota of broiler chickens *in-vitro* in CALIMERO-2.
- Study the effect of fermentation products of IMMP and pectins on chicken intestinal organoids and NK cells.
- Analyze the effect of fermentation products, produced by an infant microbiota when provided with GOS and 2'-FL in TIM-2, on the barrier integrity and immune responses in a human *in-vitro* intestinal co-culture model.

Chapter 1 (this chapter) summarizes the importance of the development of a healthy, and resilient microbiome and the need for developing and optimizing *in-vitro* models. It also reflects on the use of (potential) prebiotics to correct a dysbiotic state, especially in young chickens and infants, which can

also benefit health and welfare of adult chickens and humans. **Chapter 2** describes the optimization of the CALIMERO-2 system, to study the composition of the microbiota of broiler chickens and their production of metabolites. To study the cellular responses of the intestine in chickens, we optimized the culture of chicken intestinal organoids, which is described in **Chapter 3**. The optimized CALIMERO-2 system was used to investigate the effects of IMMP and three different citrus pectins on the cecal microbiota composition and its metabolite production, the results of which are described in **Chapter 4.** Furthermore, in this thesis, we did not only focus on chickens but also investigated the effect of fermentation products of two prebiotics (GOS and 2'-FL) on the human intestinal barrier and immune response, which is described in **Chapter 5**. Finally, all major findings, limitations, and prospects are discussed in **Chapter 6.**

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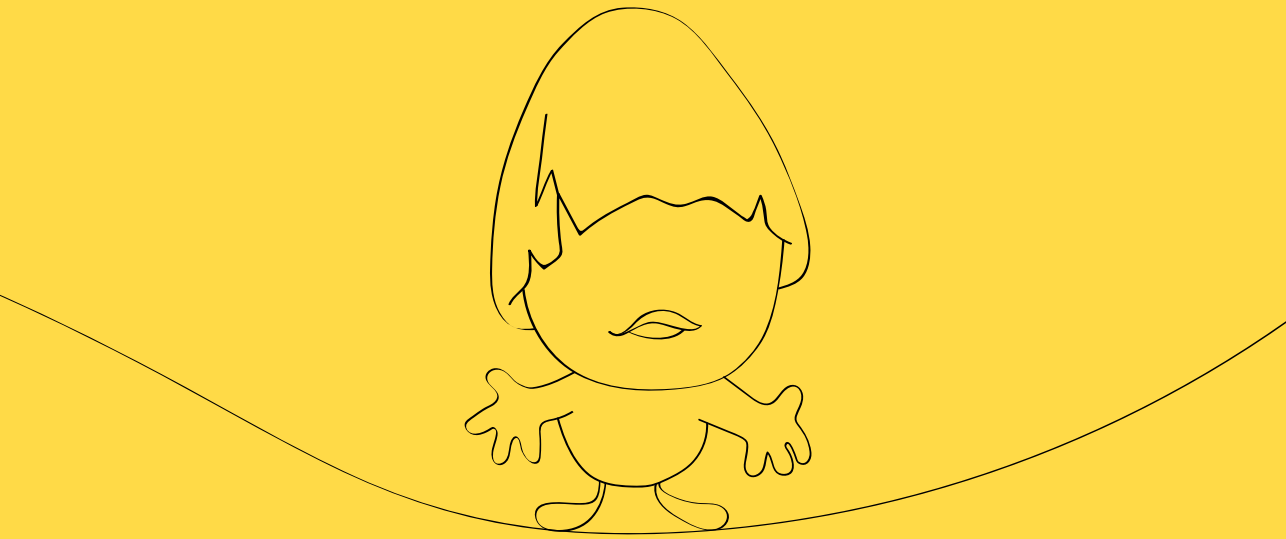
Chapter 2

Development of the *in-vitro* cecal Chicken ALIMEntary tRact mOdel-2 to study microbiota composition and function



Abstract

The digestive system of the chicken plays an important role in metabolism, immunity, and chicken health and production performance. The chicken ceca harbor a diverse microbial community and play a crucial role in the microbial fermentation and production of energy-rich short-chain fatty acids (SCFA). For humans, dogs, and piglets *in-vitro* digestive system models have been developed and are used to study the microbiota composition and metabolism after intervention studies. For chickens, most research on the cecal microbiota has been performed in *in-vivo* experiments or in static *in-vitro* models that may not accurately resemble the *in-vivo* situations. This paper introduces an optimized digestive system model that simulates the conditions in the ceca of the chicken, i.e., the Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2). The system is based on the well-validated TNO *in-vitro* model of the colon-2 (TIM-2) and is the first dynamic *in-vitro* digestion model for chicken's species. To validate this model, the pH, temperature, and different types of microbial feeding were compared and analyzed, to best mimic the conditions in the chicken ceca. The bacterial composition, as well as the metabolite production at 72 h, showed no significant difference between the different microbial feedings. Moreover, we compared the CALIMERO-2 digestive samples to the original inoculum and found some significant shifts in bacterial composition after the fermentation started. Over time the bacterial diversity increased and became more similar to the original inoculum. We can conclude that CALIMERO-2 is reproducible and can be used as a digestive system model for the chicken ceca, in which the microbial composition and activity can be maintained and shows similar results to the *in-vivo* cecum. CALIMERO-2 can be used to study effects on composition and activity of the chicken cecum microbiota in response to in-feed interventions.

2.1 Introduction

The digestive system of the chicken plays a pivotal role in metabolism, immunity, and therewith in the health and production performance (1-4). The gastrointestinal tract (GIT) of poultry differs from the GIT of mammals in many ways, including a shorter size relative to body length, and the size and role of the ceca (5, 6). The ceca play an important role in the GIT of poultry, because of active microbial fermentation and the production of energy-rich short-chain fatty acids (SCFAs) (7, 8). The avian ceca harbor a diverse microbial community that is dominated by strict anaerobic bacteria (9). Many factors can influence the microbial composition (10). Some perturbations can induce a shift in the intestinal microbiota composition and can lead, for instance, to the enteric disease necrotic enteritis (11, 12). Many studies have aimed to optimize the gut microbiota of mammals as well as that of chickens with dietary interventions. For example, previous studies have shown that prebiotics can stimulate the growth of beneficial endogenous microbes by providing nutrients to beneficial bacteria (13, 14), and can lead to better growth and health of the chickens (15). By stimulating beneficial bacteria, relative abundance of harmful bacteria like *Clostridium perfringens* can be reduced. Most of the current research on the microbiota in the chicken GIT has been performed in *in-vivo* experiments or field studies (16). *In-vivo* experiments have many downsides, including that these methods are invasive for the animals or in case of non-invasive methods like cloacal swabs, these might not completely represent the composition of the ceca (14, 17). Therefore, there is a need for *in-vitro* digestive system models to study the behavior of microbiota, with high predictive value for *in-vivo* animal trials, to gain in-depth knowledge of the effect and possible mechanisms of action of dietary interventions on the chicken cecal microbiota.

This paper introduces CALIMERO-2, which is an acronym for Chicken ALIMEntary tRact mOdel-2, based on the validated, dynamic, computer-controlled TNO intestinal model of the colon (TIM-2) (17). CALIMERO-2 mimics the cecum of a chicken and can be used to evaluate the effects of feed additives, and other compounds on the microbial composition and activity over time. Here, the dynamic digestive system model was developed and optimized for the chicken species. The effect of different types of microbial feeding was analyzed and compared, to best mimic the chickens' diet and support the growth of the chicken microbiota. Moreover, bacterial composition in the *in-vitro* digestive system model was compared to the original cecal inoculum, to investigate the resemblance with the *in-vivo* situation.

2.2 Materials and Methods

2.2.1 The Chicken ALIMEntary tRact mOdel-2

The Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2; Fig. 1) simulates the ceca of the chicken and is based on the same concept as TIM-2 described by Minekus et al., (18) and Venema (19). Briefly, CALIMERO-2 consists of four identical independent units that can be run in parallel. Each unit has four interconnected glass units, with a flexible wall inside. The volume of the lumen is approximately 150 ml. The temperature and the pH are regulated in the system to mimic the body temperature, which for broiler chickens is 41°C at a pH of 6.6 (20, 21). The temperature is regulated by pumping water into the space between the glass jacket and the flexible wall [Fig. 1 (J)]. Additionally, the water pressure is changed constantly to create peristaltic movements similar to those in the gut [Fig. 1 (A)]. The pH is constantly measured by pH electrodes [Fig. 1 (B)] in the system and maintained by adding 2M sodium hydroxide when necessary [Fig. 1 (C)]. By flushing the system with nitrogen gas [Fig. 1 (F)], the model is kept anaerobic. Moreover, the metabolites produced by the microbiota are continuously filtered out of the lumen by making use of a unique semi-permeable membrane that functions as a dialysis system [Fig. 1 (A)]. This dialysate is continuously collected and can be sampled for microbial metabolites [Fig. 1 (D)]. By making use of such a dialysis system, the physiological concentrations of, e.g., short-chain

fatty acids (SCFAs) are maintained and there is no accumulation of these small molecules, which would otherwise lead to inhibition or death of the microbiota within a matter of hours (19). The system was inoculated with a standardized anaerobic cecal microbiota of broiler chickens [Fig. 1 (G)], obtained as described below. Furthermore, the microbiota was fed with microbial feedings as described in subsequent sections [Fig. 1 (I)].

2.2.2 Collection of Cecal Samples and Standardization

The cecal content was obtained from slaughterhouse van der Linden Poultry products B.V. (Beringe, Netherlands), where broiler chickens (Ross 308) were brought from local chicken farms. The broiler chickens were fed a coccidiostat-free diet and were not treated with antibiotics the days before slaughter. The birds were stunned, debled and the ceca were removed within 1 h after killing and placed in sterile plastic bags containing an anaerocult® strip (AnaeroGenTM, Cambridge, United Kingdom) and transported on ice where the ceca were processed immediately after arrival, within 1 h after collection of the ceca. In the laboratory, the cecal content was removed and pooled under strictly anaerobic conditions in an anaerobic cabinet (Sheldon Lab –Bactron IV, Gomelius, OR, United States). A total amount of 945 g was 1:1 diluted with dialysis liquid (content per liter: 2.5 g $K_2HPO_4 \cdot 3H_2O$, 4.5 g NaCl, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.45 g $CaCl_2 \cdot 2H_2O$, 0.05 g ox bile, and 0.4 g cysteine hydrochloride, plus 1 ml of vitamin mixture [see next section]) and as a cryo-protective agent, 15% (w/v) glycerol was added. The cecal samples were aliquoted (35ml), snap-frozen in liquid nitrogen, and stored at −80°C.

2.2.3 Microbial Feeding

To compare microbial composition and activity in CALIMERO-2 to the *in-vivo* situation, different feeding types were tested, namely Standard Ileal Effluent Media (SIEM), which is standard for experiments with human microbiota (22), modified SIEM-I and modified SIEM-II (Supplementary Table 1). We tried to mimic the chickens' diet in the modified microbial feedings, by replacing arabinogalactan with soy-based arabinoxylan. Furthermore, the potato starch was replaced by wheat and maize starch, since the diet of broiler chickens is composed mainly of soy, maize, and wheat (23). Standard Ileal Effluent Media was prepared as described by De Souza et al., (24) with the following compounds (g L^{-1}): 9 citrus peel pectin, 9 beechwood xylan, 9 larch arabinogalactan, 9 potato amylopectin, 74.6 potato starch, 31.5 Tween 80, 43.7 casein, 0.7 ox-bile, 43.7 bactopepton, 4.7 $K_2HPO_4.3H_2O$, 0.009 $FeSO_4.7H_2O$, 8.4 NaCl, 0.8 $CaCl_2.2H_2O$, 0.7 $MgSO_4.7H_2O$, 0.02 hemin, and 0.3 cysteine·HCl, plus 1.5 mL of a vitamin mixture containing (mg L^{-1}): 1 menadione, 0.5 vitamin B12, 2 D-biotin, 10 pantothenate, 5 p-aminobenzoic, 4 thiamine, and 5 nicotinamide acid. The pH was adjusted to 6.6 to mimic the chicken ceca and 60 ml/day was administered. Modified SIEM-I was adjusted to mimic chicken feed by replacing citrus peel pectin and larch arabinogalactan with soybean rhamnogalacturonan (9) and raffinose (9). Furthermore, the potato starch component was replaced by 80% wheat and 20% maize starch. Modified SIEM-II had the same components as modified SIEM-I, with additionally 9 g L^{-1} arabinoxylan (Bioactor, Maastricht, Netherlands), oat beta-glucan, and konjac glucomannan and an adjusted composition of starch, namely 50% maize and 50% wheat. Standard SIEM and vitamin mix were purchased from Tritium microbiology (Eindhoven, Netherlands).

2.2.4 Experimental Setup

Two independent experiments in CALIMERO-2 were done, each using four independent fermentation units which were run simultaneously (Supplementary Fig. 1A). In each experiment, two fermentation units included SIEM as control and the other two fermentation units contained either Modified SIEM-I or the Modified SIEM-II. Each experiment started with inoculation of the system with 60 ml of the standardized cecal microbiota, to which 90 ml of pre-reduced dialysis liquid was added. In both

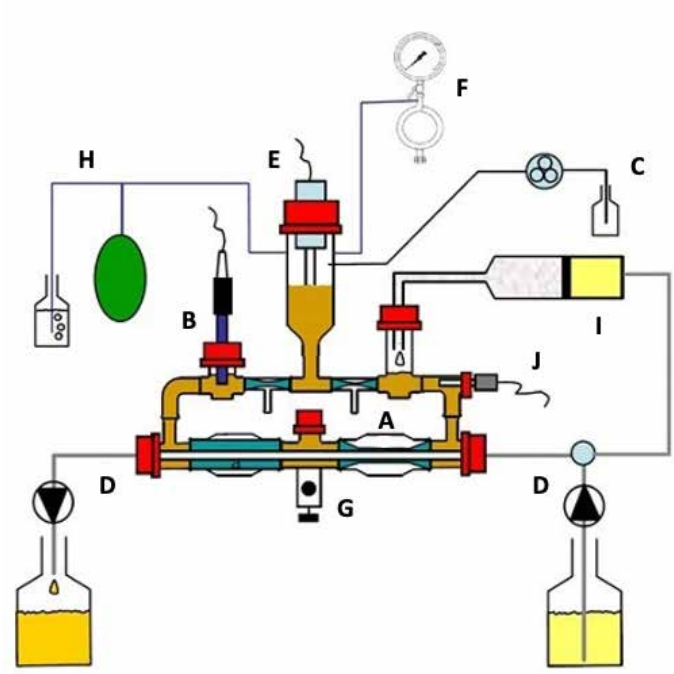


Figure 1. **Schematic representation of Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2).** A = Peristaltic compartments with a dialysis membrane inside; B = pH sensor; C = NaOH inlet; D = dialysate system; E = level sensor; F = gaseous N_2 inlet; G = sampling port; H = gas outlet; I = feeding syringe; J = temperature sensor.

experiments, the same batch of inoculum was used. There were five sample time points (t-16 h (time of inoculation), 0 h (after overnight adaptation), 24 h, 48 h, and 72 h) from lumen and dialysate to analyze the microbial composition (lumen) and metabolite composition (lumen and dialysate) over time (Supplementary Fig. 1B). All samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. After 24 and 48 h, a total volume of 25 ml of lumen sample was removed from the system to simulate passage of chyme to the chicken large intestine (22).

2.2.5 Microbial DNA Extraction

DNA was extracted from 250 µl of the lumen samples taken during the CALIMERO-2 experiments using 1000 µl InhibitEx buffer (Qiagen, Venlo, Netherlands). The sample was transferred to a Precyllus tube containing 0.5 mm microbeads and treated in a bead beater (Precellys 24, Bertin technologies, Montigny-le-Bretonneux, France) at a speed of 6000 Hz for 3 × 30 s, with cooling on ice between steps. Afterward, the sample was incubated at 95°C for 7 min and centrifuged (Rotina 420 R, Hettich Benelux B.V. Netherlands), at 13500 g for 1 min to pellet stool particles and cell wall fragments. From this point, the QIAamp DNA stool Mini kit (Qiagen) was used following the manufacturer's protocol from step 4 onward, with some adjustments. Briefly, 30 µl of proteinase K was added to a 1.5 ml microcentrifuge tube and 400 µl of the supernatant of the sample was added together with 400 µl of Buffer AL and vortexed before the sample was incubated at 70°C for 10 min. After incubation, 400 µl of ethanol (96%–100%) was added and the volume was transferred to a QIAamp spin column in two steps and centrifuged at 13500 g for 1 min. Next, 500 µl of AW1 buffer was added and centrifuged at 13500g for

1 min, followed by addition of 500 µl of AW2 buffer and centrifugation for 3 min. To elute the DNA, the QIAamp spin column was placed into a new microcentrifuge tube and 100 µl of ATE buffer was added, incubated for 3 min at room temperature, and centrifuged at 13500g for 1 min. To quantify the DNA concentration Qubit dsDNA HS Assay kit was used and the DNA was measured using a Qubit 3.0 Fluorometer (Invitrogen, Landsmeer, Netherlands) and stored at −20°C until further use.

2.2.6 Bacterial Composition

To study the composition of the bacteria during the experimental phase, the composition of the bacteria was evaluated by 16S rRNA gene sequencing using Illumina Miseq (Illumina, San Diego, CA, United States). 16S rRNA gene amplicon libraries of the V3-V4 region were generated following the 16S Metagenomic Sequencing Library preparation manual of Illumina Miseq systems using the Nextera XT kit, using a 2-step PCR. Briefly, in the first step, 10–25 ng genomic DNA was used as template for the first PCR with a total volume of 50 µl using the 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) primers appended with Illumina adaptor sequences. PCR products were purified (QIAquick PCR Purification Kit) and the size of the PCR products was checked on a Fragment analyzer (Advanced Analytical, Ankeny, United States) and quantified by fluorometric analysis (Qubit™ dsDNA HS Assay Kit). Purified PCR products were used for the second PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer and quantified, followed by equimolar multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. A mock community was run along with the samples to guarantee sequence quality.

2.2.7 Short-Chain Fatty Acids, Branched-Chain Fatty Acids, and Organic Acids Quantification in Lumen and Dialysate Samples

To quantify the SCFA (acetate, propionate, and butyrate), branched-chain fatty acids BCFA (iso-butyrate and iso-valerate), and other organic acids (succinate, formate, lactate, valerate, and caproate) in the samples from the lumen and dialysate, ion exclusion chromatography (IEC) was performed by Brightlabs (Venlo, Netherlands). Briefly, an 883 Ion Chromatograph was used (IC; Metrohm, Switzerland), with a Transgenomic IC Sep ICE-ION-300 column (30 cm length, 7.8 mm diameter, and 7 µm particles) and a MetroSep RP2 Guard. The mobile phase consisted of 1.5 mM aqueous sulfuric acid and the column had a flow rate of 0.4 ml min^{-1} and a temperature of 65°C. The organic acids were detected using suppressed conductivity detection. Samples were centrifuged at 13500 g for 10 min, and the clear supernatant was filtered through a 0.45 µm PFTE filter and diluted with mobile phase (for lumen 1:5, for dialysate 1:2). Ten µl were loaded on the column by an autosampler 730 (Metrohm). Molecules were eluted according to their pKa.

2.2.8 Bioinformatics Analysis

Microbiota bioinformatics was performed with QIIME2 2019.4 (25). Briefly, the raw sequencing data were demultiplexed, quality filtered, and denoised by using the q2-demux plugin and DADA2 (26). In the DADA2 step, the first 9 bases were trimmed off and for the forward reads there was a truncation at 290 base pairs and for the reverse reads, this was at 280 base pairs. Taxonomy was assigned using the SILVA 128 16Sr RNA gene reference database. Further analysis was continued with the packages *microbiome, vegan and phyloseq* after the qza files were converted to phyloseq object with the *qiime2R* package (27).

2.2.9 Statistical Methods

Shannon diversity, inverse Simpson, Gini-Simpson, Fisher, and coverage were calculated to define microbial alpha diversity for each sample by making use of the *phyloseq* and microbiome R packages.

Differences in alpha diversity were tested with a Kruskal-Wallis test, and pairwise comparisons were tested using a Wilcoxon rank-sum test and corrected for multiple testing with Benjamini-Hochberg in the open-source software package STAMP v2.1.3 (28). For the statistical analysis of the beta diversity for the different feeding types and comparison with the original inoculum, permutational multivariate analysis of variance (PERMANOVA) (29) was performed. Significance of the SCFA, BCFA and organic acid concentrations among different feeding groups were analyzed by Kruskal-Wallis Rank sum test followed by corrections for multiple testing with the Benjamini-Hochberg method in R. All analyses were done in R version 3.6.2 (30).

2.3 Results

2.3.1 Bacterial Alpha- and Beta Diversity

The bacterial composition of the lumen samples from runs with the different types of microbial feeding, i.e., SIEM, Modified SIEM-I, and Modified SIEM-II, which served as microbial growth medium, were analyzed and compared to the original inoculum, i.e., the pooled and standardized sample before inoculation into the system at time point -16 h. The different sampling time points (0 h, 24 h, 48 h, 72 h) during the fermentation runs in CALIMERO-2 and the original inoculum were also compared. For these samples, the Shannon diversity, to assess the bacterial alpha diversity within a community, was calculated. A significant difference was found between all the feeding types compared to the original inoculum based on the Shannon index (Fig. 2A; SIEM: $P < 0.0001$, Mod SIEM-I: $P < 0.001$, Mod SIEM-II: $P < 0.001$), indicating a lower diversity in the *in-vitro* digestive system samples, compared to the original inoculum. Between the different feeding types, no significant differences were found. The effect of the fermentation process on the alpha diversity over time was examined using the Shannon index, a significant difference was demonstrated between the different time points and the original inoculum at time point -16 h (Fig. 2B; 0 h: $P < 0.0001$, 24 h: $P < 0.0001$, 48 h: $P < 0.0001$ and 72 h: $P < 0.001$). There was also a significant difference between time points 48 h and 72 h ($P < 0.01$) (Fig. 2B). Other alpha diversity measures (Inverse Simpson, Gini-Simpson, Fisher, and coverage) are provided in Supplementary Figure 2 and showed the same trend as the Shannon index.

The beta diversity distance matrices weighted and unweighted UniFrac were examined to determine the variance between the different feeding groups. The unweighted UniFrac demonstrate an overlap of clusters of SIEM and modified SIEM-I. Modified SIEM-II and the original inoculum both show a shift in clusters compared to SIEM and modified SIEM-I ($P < 0.001$) (Fig. 2C). The principal coordinate plot of the weighted UniFrac show no clustering associated with the different feeding groups, whereas a significant difference was observed between the original inoculum and the different feeding groups ($P < 0.05$) (Fig. 2D).

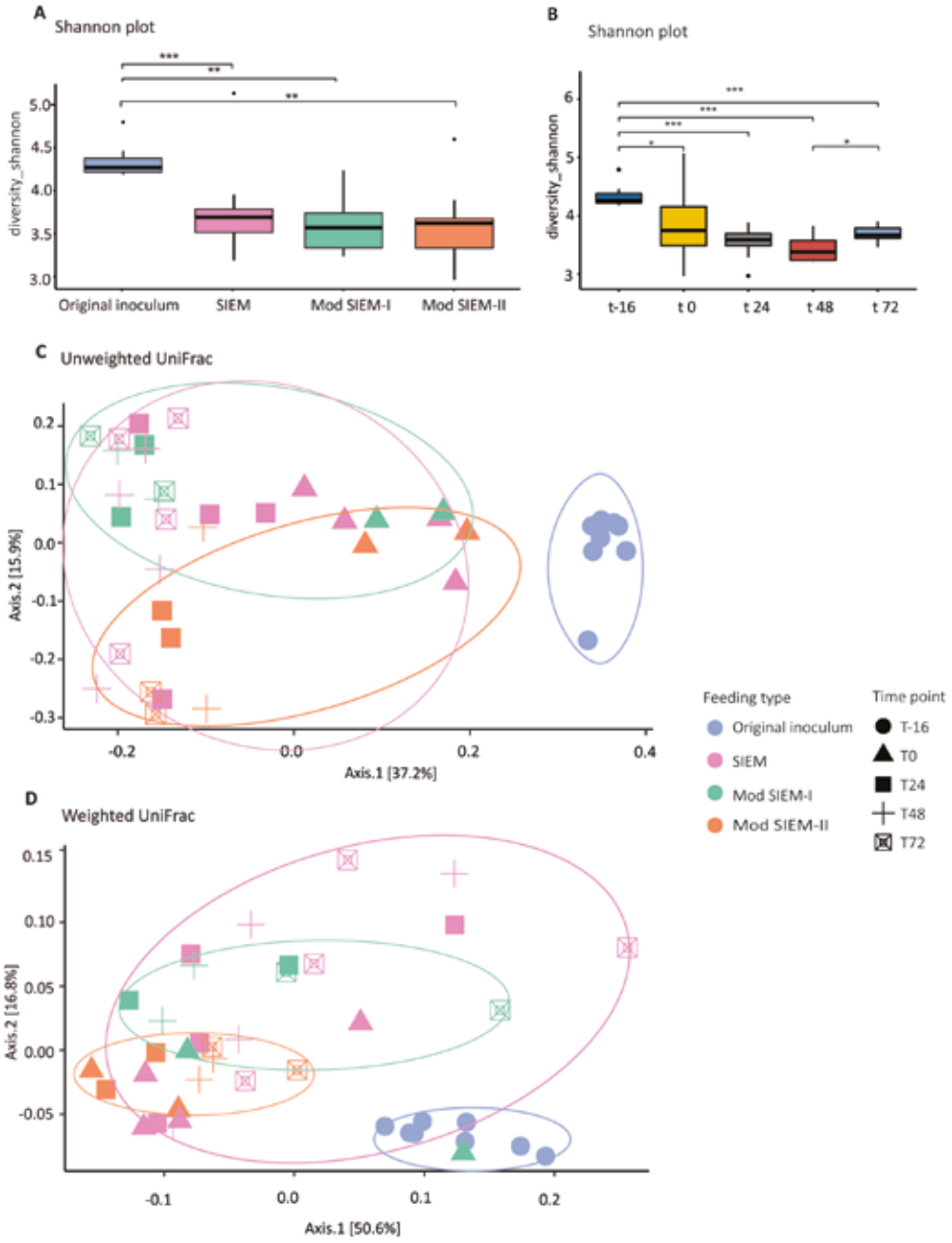


Figure 2. Bacterial diversity. A For the alpha diversity, Shannon indexes were calculated to verify the abundance and evenness of the species present in the of Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2) samples. Data are presented as mean (n = 2) ± sd. Significant difference is shown between original inoculum and SIEM (p < 0.001) and original inoculum and modified SIEM-I and II (p < 0.05). **B** Shannon index for the CALIMERO-2 samples taken at different timepoints. The beta diversity is represented as principal coordinates analysis (PCoA) using the **C** unweighted UniFrac or the **D** weighted UniFrac for the cecal microbiota of chickens form the CALIMERO-2 model.

2.3.2 Taxonomic Analysis

To assess the effect of feeding types on bacterial composition, the community was analyzed at the taxonomic rankings of phylum and family levels (Fig. S3A,B, respectively). The samples obtained with the different feeding types were compared to each other and the original inoculum. The taxonomic profiles at phylum level showed that the dominant populations were *Bacteroidetes* (56%), *Firmicutes* (35%), and *Proteobacteria* (7.6%) in all of the samples. The relative abundance (RA) of the phyla *Verrucomicrobia* ($P < 0.001$), *Cyanobacteria* ($P < 0.01$), and *Tenericutes* ($P < 0.01$) were significantly higher in abundance in the original inoculum samples, compared to the CALIMERO-2 samples (Fig. 3A). However, the bacterial composition was not significantly affected by the adjusted microbial feedings compared to the standard medium (SIEM). Moreover, the bacterial composition showed no significant differences between the different time points, within the feeding type groups. For SIEM there seem to be some individual differences, however, most of the samples within the group show similar profiles, and suggest that CALIMERO-2 can be seen as a reproducible system.

Within the phylum *Bacteroidetes*, the family *Porphyromonadaceae* was significantly higher in relative abundance in the original inoculum compared to the samples obtained from the CALIMERO-2 ($P < 0.01$). Moreover, the families *Clostridiales vadin BB60 group, Erysopelotrichaceae*, and *Veillonellaceae*, which belong to the phylum *Firmicutes*, were all significantly higher in the original inoculum, compared to the other samples ($P < 0.01$). Furthermore, *Campylobactereaceae* also showed a significant decrease in the CALIMERO-2 fermented samples, compared to the original inoculum ($P < 0.001$).

2.3.3 Production of Short-Chain Fatty Acids, Branched-Chain Fatty Acids, and Organic Acids

To characterize the fermentation concerning microbial activity, the cumulative total production of SCFA, BCFA, and other organic acids, representing the sum of metabolites that were present in the lumen and the dialysate, were measured over time and shown in Fig. 4. Overall, the production of SCFA was very similar between the tested microbial feedings and no significant difference was observed. For all the samples, the acetate production was the highest, followed by propionate and butyrate that showed the lowest cumulative production (Fig. S4A-C). The BCFA production showed also no significant difference between the different feeding types (Fig. S4D-F). When comparing the production of BCFA to SCFA, the amount BCFA produced was much lower than that of SCFA. The production of the other organic acids, succinate and caproate, was negligibly small, but the lactate, valerate and formate production was evident in time, but much lower than the SCFA production. The production over time of the other organic acids shows a delay in production compared to SCFA and BCFA. The other organic acid production started between 24 and 48 h (valerate) or even between 48 and 72 h of fermentation, whereas for SCFA and BCFA production is already evident at the start and 24 h of fermentation (Fig. S4G-I).

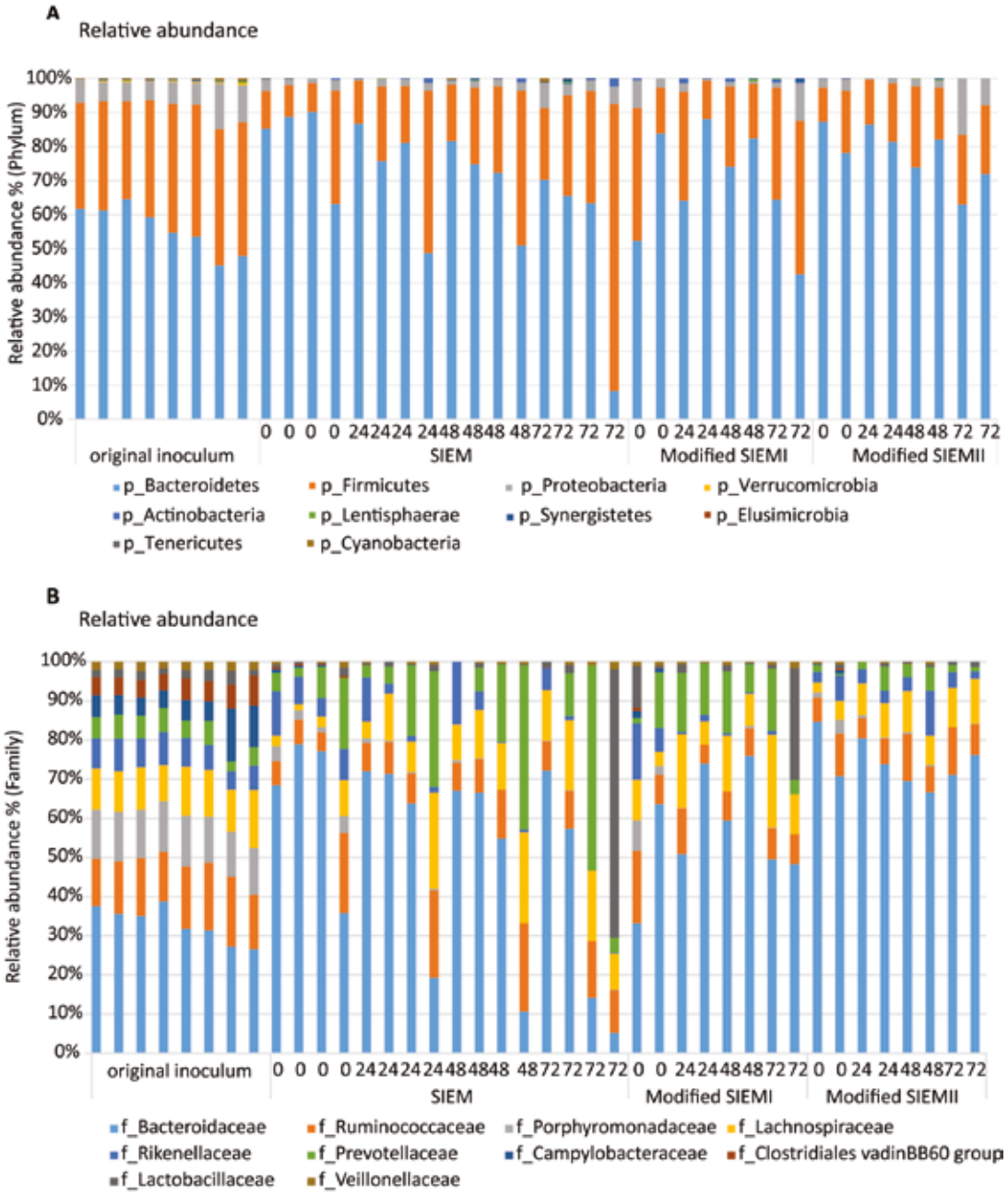


Figure 3. Bacterial composition. Relative abundance of **A** bacterial phyla and **B** families in Chicken ALIMEntary tRact mOdel-2 samples using different feeding type at different time points compared to the original inoculum.

2.4 Discussion

Prior work has documented that microbial composition can be influenced by many factors, for example by the addition of prebiotics to the diet. Testing the effect of different types of manipulations of the microbial composition, for instance with feed additives are important for further understanding of the modes of action and expected effects on improving animal intestinal health and performance (4, 12, 31, 32). Research on human and pig microbiota can be performed in *in-vitro* digestive system models, for example in the well-established and predictive TIM-2 and SLIM (Swine Large Intestinal Model) systems. These two models have been compared and validated to *in-vivo* conditions (18, 19, 22, 33-35). To study the effect of dietary substrates on the chicken gut microbiota, experiments have been performed *in-vivo*, or data were obtained from a static fermentation model or the SHIME model (Simulator of the Human Intestinal Microbial Ecosystem) (36, 37). So far, there is no validated, advanced dynamic *in-vitro* digestion system model known for chickens.

In this study, we established and optimized a dynamic *in-vitro* cecal digestive system model for chickens to mimic the *in-vivo* situation, which is based on the TIM-2 model. For the chicken model, we changed the pH and the body temperature, respectively, to 6.6 and 41°C (21). In addition, we compared two adjusted microbial feedings to the standard microbial feeding SIEM used for the human microbiota. In previous experiments with SLIM, we showed the need to optimize the composition of SIEM to allow the microbiota to stay close to the original pig inoculum (33). In our experiments here, we show that SIEM itself performs well in this system, and changing the medium composition of SIEM does not lead to a better representation of the microbiota composition.

To validate CALIMERO-2, the original inoculum was used to represent the *in-vivo* situation and CALIMERO-2 samples were compared to it. The taxonomic profile of the bacterial composition of the original inoculum showed that the phyla *Bacteroidetes*, together with *Firmicutes* and *Proteobacteria*, were most abundant, which is consistent with earlier research of Corrigan et al., (38) (39). In contrast to these previous studies, the relative abundance of the *Firmicutes* and *Bacteroidetes* relative to each other was reversed in our study. This different ratio of *Firmicutes* and *Bacteroidetes* might have been caused, amongst others, by differences in the type of feed the chickens received, housing conditions, the genetic background of the chickens, or different processing of the samples before analysis (38, 40, 41).

Although previous research suggests that diet affects bacterial composition and its metabolite production, the changes we applied did not result in significant differences between the feeding types in bacterial composition and metabolite production (33), indicating that the unmodified SIEM can be used in CALIMERO-2. To illustrate this, the taxonomic profile was examined. For all feeding types, *Bacteroidetes, Firmicutes*, and *Proteobacteria* remained most abundant over time, some of the other bacterial phyla, such as *Campylobacter*, were significantly decreased over time compared to the original inoculum. The reduction of these microaerophilic taxa might be caused by the lack of oxygen in the strict anaerobic environment in the system. Since the majority of cecal colonizers are strict anaerobes (42), we maintained strict anaerobic conditions in CALIMERO-2.

SCFA plays an important role in the health of the GIT and their production can be modulated by diet (43). To evaluate if there are changes in bacterial activities between the different microbial feedings the production of SCFA, BCFA, and other organic acids over time was measured. In this study, the ratio SCFA: BCFA was similar to earlier research of González-Ortiz and colleagues (44). In contrast, the ratio acetate, propionate, and butyrate was not in line with previous research. We found a lower butyrate concentration compared to propionate, whereas most studies show a reversed ratio (44-46).

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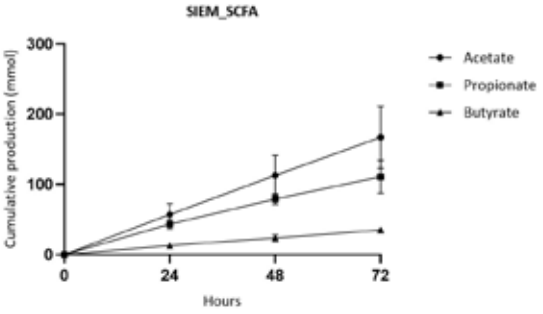
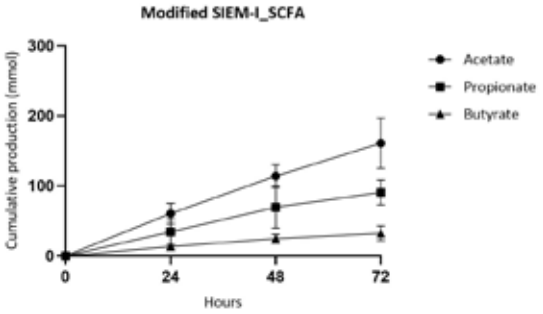
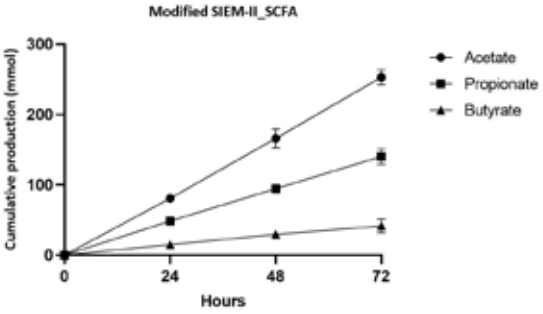

SIEM_SCFA
Cumulative production (mmol)
300
200
100
0
0
24
48
72
Hours
Acetate
Propionate
Butyrate

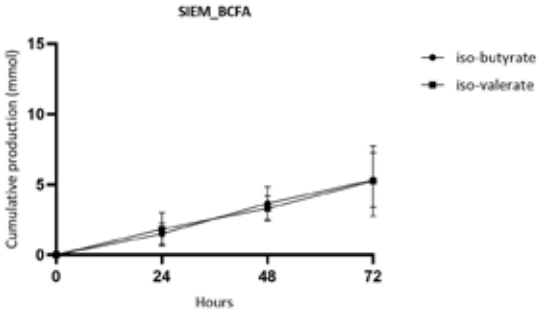
B


Modified SIEM-I_SCFA
Cumulative production (mmol)
300
200
100
0
0
24
48
72
Hours
Acetate
Propionate
Butyrate

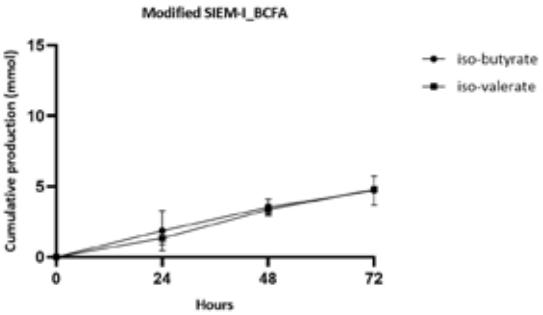
C


Modified SIEM-II_SCFA
Cumulative production (mmol)
300
200
100
0
0
24
48
72
Hours
Acetate
Propionate
Butyrate

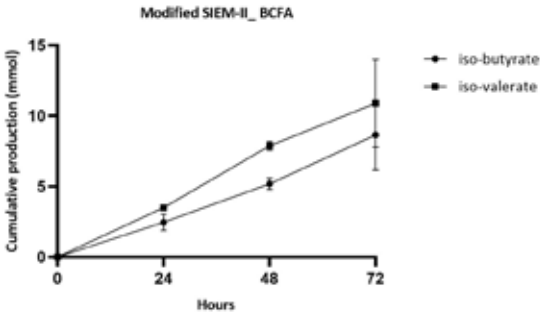
D

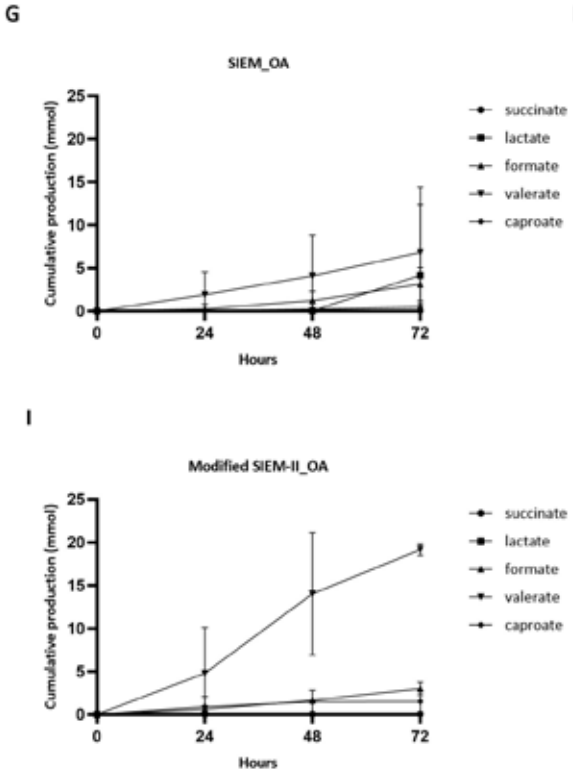

SIEM_BCFA
Cumulative production (mmol)
15
10
5
0
0
24
48
72
Hours
iso-butyrate
iso-valerate

E


Modified SIEM-I_BCFA
Cumulative production (mmol)
15
10
5
0
0
24
48
72
Hours
iso-butyrate
iso-valerate

F


Modified SIEM-II_ BCFA
Cumulative production (mmol)
15
10
5
0
0
24
48
72
Hours
iso-butyrate
iso-valerate



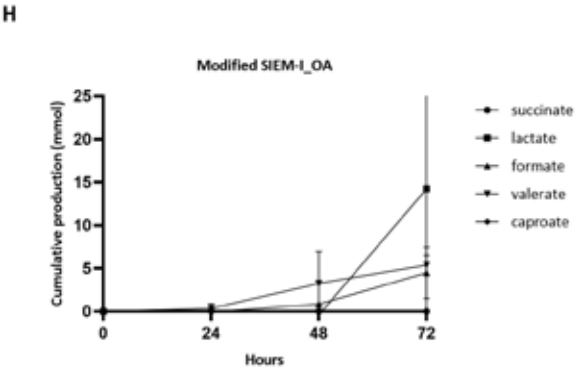


Figure 4. Production of microbial metabolites over time. Cumulative production (in mmol) over time of short chain fatty acids (SCFA), branched chain fatty acids (BCFA) and other organic acids (OA). SIEM **(A, D, G)**, modified SIEM-I **(B, E, H)** and, modified SIEM-II **(C, F, I)** Data are presented as mean (n = 2) ± sd.

This might be related to the age of the chickens or the type of breed of the chickens. Liao et al (47) showed an increase in SCFA and a smaller ratio between propionate and butyrate with increasing age of the chickens. Furthermore, in their research, they used Arbor Acres broiler chicks, whereas we studied Ross 308 broilers (40, 47). When comparing the BCFA and other organic acid production with the SCFA production, a much lower concentration was found, which corresponds with other studies (46, 48). The delay in the production of the other organic acids, especially for lactate, might have been because lactate was converted in propionate or butyrate before we could have measured the lactate concentration (19, 49, 50).

Both the taxonomic profiles as well as the metabolite production of the CALIMERO-2 samples showed some shifts compared to the *in-vivo* situation. These shifts can be related to the new environment the microbiota needs to adapt to, and some factors that are not present in the model, like a mucus layer (41). The changes in taxonomic profile are seen in most *in-vitro* systems (41, 51). Nevertheless, although we see a shift in taxonomic profiles in the beginning, the bacterial alpha diversity showed high similarity with the original inoculum after a longer period of fermentation. After an initial reduction after the start of the fermentation an increase in alpha diversity over time was observed and after fermentation for 72 h, the number of bacterial taxa had become more similar to the original inoculum.

For the metabolite production, we cannot simply compare our results to *in-vivo* experiments. During *in-vivo* experiments, we are limited to random sampling and the cumulative total production of SCFA cannot be measured, despite the possibility to euthanize animals and collect samples from multiple sites of the intestine. Static models are also restricted, because there is an accumulation of metabolites, which might severely influence the metabolic activity of the bacteria, due to inhibition of fermentation at high concentrations. With CALIMERO-2, where metabolites are removed through the dialysis system, we can therefore accurately measure the influence of feed additives on the production of SCFA, and other metabolites.

Another advantage of the CALIMERO-2 system is that experiments are reproducible, and variability usually observed *in-vivo* due to interindividual variability, is low because the cecal samples collected are pooled. There are multiple reasons to pool the cecal samples. Firstly, a practical reason, multiple need to be combined, to obtain a sufficient amount of volume to conduct a single experiment. Moreover, to get a good representation of the chicken population, pooling reduces variability between samples. In previous experiments with human microbiota, we observed that, with respect to carbohydrate fermentation, pooling and standardizing of the microbiota from several individuals led to the same microbial activity of the individual microbiota and the pooled inoculum despite differences in microbiota composition (52). This is due to the vast functional redundancy between microbial taxa, allowing different microorganisms to use the same substrate and produce identical metabolites (53). Standardizing the microbiota also allows numerous experiments (in our case close to 100) to be carried out with the same starting microbiota. Furthermore, the model is reproducible, since the system is computer controlled and is run under strict control. In this way, the environmental factors such as pH, temperature and food intake, are the same for each experiment and do not influence the microbiota. In the current study this allowed us to discover that different compositions of the SIEM media did not lead to differences in microbiota composition and activity. Also, the ability to change specific parameters in the system and the large amount of experiments that can be performed with CALIMERO-2 for pre-screening for efficacy of feed interventions, substantially reduces the number of *in-vivo* experiments needed for further validation before it can be commercially applied.

2.5 Conclusion

CALIMERO-2 can be used as a digestion system model for the chicken ceca, in which the microbial composition and activity can be maintained in a similar manner to the *in-vivo* cecum. Thus, the developed model allows measurements regarding modulation of composition and activity of the chicken cecum microbiota in response to, for instance, feed interventions. The standard growth medium SIEM can be used for experiments within this model.

In future work, CALIMERO-2 can be used to study the effect of several types of dietary substrates, or the effect of for example antibiotics, on the chicken cecal microbiota. In this paper we focused on the bacterial composition, however, the system can also be used to study the complete microbiota, for example the effect of dietary interventions on fungal composition. Furthermore, the digestion system model can also mimic an intestinal disease, like necrotic enteritis caused by the pathogen *Clostridium (C.) perfringens*. In addition to studying the microbiota, the samples obtained from CALIMERO-2 experiments, i.e., fecal waters, can be used for follow-up *in-vitro* experiments. *In-vitro* cell lines, co-cultures of intestinal cells with immune cells, or organoids mimicking the intestine can be exposed to the fecal waters, to further study effects of products aimed at modulating the gut microbiota on intestinal health.

Acknowledgments

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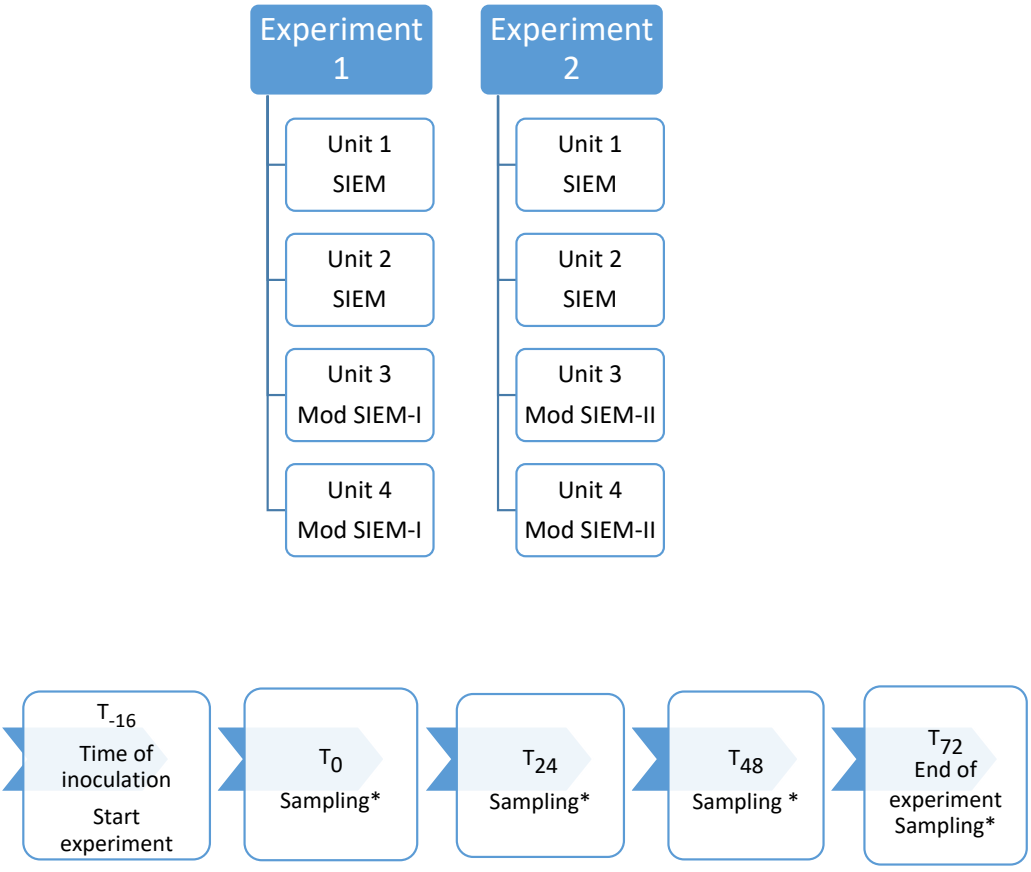
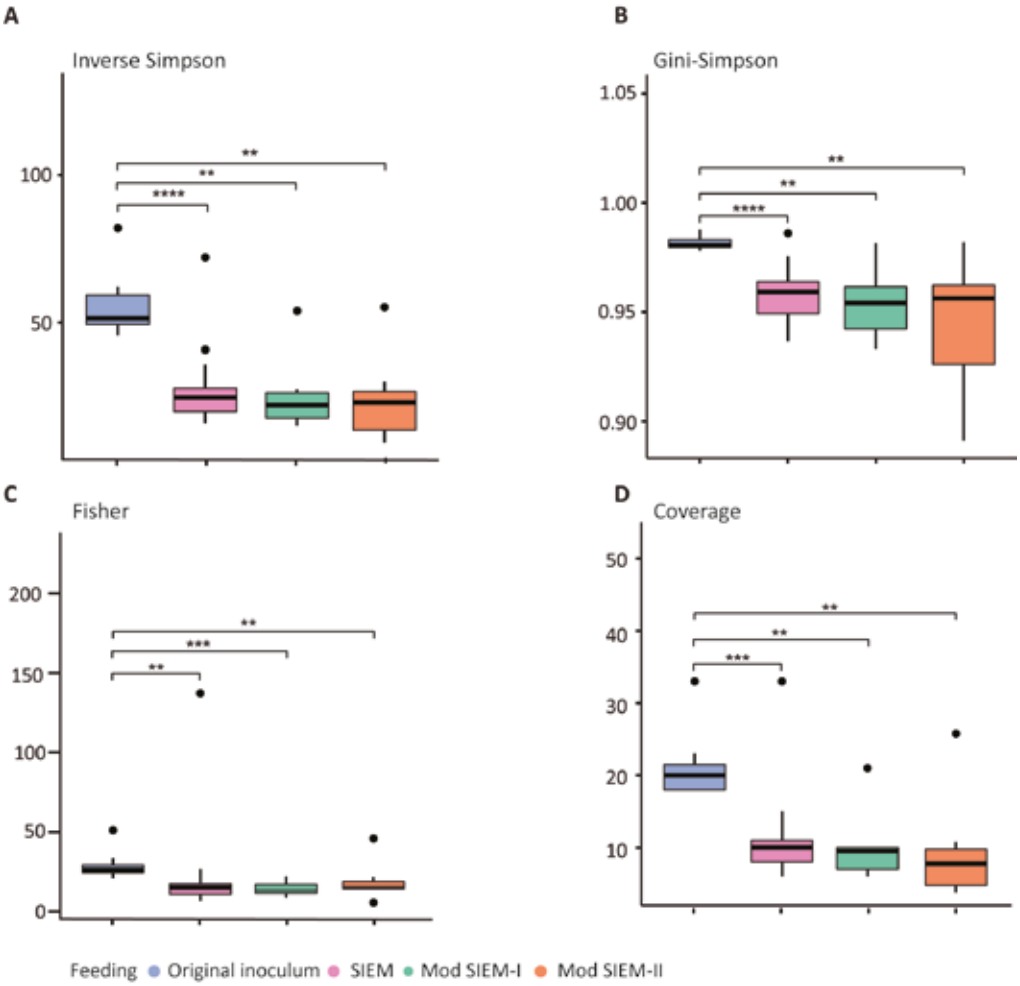
Supplementary files



Supplementary Figure 1. **Flow chart of experimental set up of CALIMERO-2. A**. Experimental set up for the different sample types, SIEM, Modified SIEM-I and Modified SIEM-II. **B**. The experimental set up over time. At the times indicated by * samples were taken from both the lumen and dialysate.



Supplementary Figure 2. Bacterial alpha diversity. Data are presented as mean (n = 2) + sd. (** P < 0.01 and *** P < 0.001) **A** Inverse Simpson **B** Gini-Simpsom **C** Fisher **D** coverage.

Table S1. Microbial feeding composition of SIEM, Modified SIEM-I and Modified SIEM-II

SIEM (CHO)	**Concentration**
citrus peel pectine	9 g/l
beechwood xylan	9 g/l
larch arabinogalactan	9 g/l
potato amylopectine	9 g/l
potato-starch	74.6 g/l

Modified SIEM-I (CHO)	**Concentration**
soybean rhamnogalacturonann	9 g/l
xylan	9 g/l
raffinose	9 g/l
amylopectine	9 g/l
maize-starch	14.92 g/l
wheat-starch	59.68 g/l

Modified SIEM-II (CHO)	**Concentration**
soybean rhamnogalacturonann	9 g/l
xylan	9 g/l
raffinose	9 g/l
amylopectine	9 g/l
maize-starch	37.3 g/l
wheat-starch	37.3 g/l
arabinoxylan	9 g/l
oat beta-glucan	9 g/l
konjac glucomannan	9 g/l

Table S2. Significant difference between bacterial phyla between groups

Bacterial phyla	p-value	corrected p-value
p_Verrucomicrobia*	0.000000424	0.00000466
p_Cyanobacteria*	0.000579	0.00318
p_Tenericutes*	0.00362	0.00996
p_Elusimicrobia	0.00356	0.013
p_Proteobacteria	0.013	0.028
p_Bacteroidetes	0.043	0.079
p_Firmicutes	0.056	0.088
p_Lentiphareae	0.138	0.189
p_Actinobacteria	0.261	0.319
p_Synergistetes	0.506	0.557

Table S3. Significant difference between bacterial families between groups

Bacterial family	p-value	corrected p-value
f_Campylobacteraceae	0.0000337	0.000825
f_Clostridiales vadinBB60 group	0.0000864	0.00141
f_Porphyromonadaceae	0.000183	0.0018
f_Veillonellaceae	0.000296	0.00242
f_Erysipelotrichaceae	0.000412	0.00288

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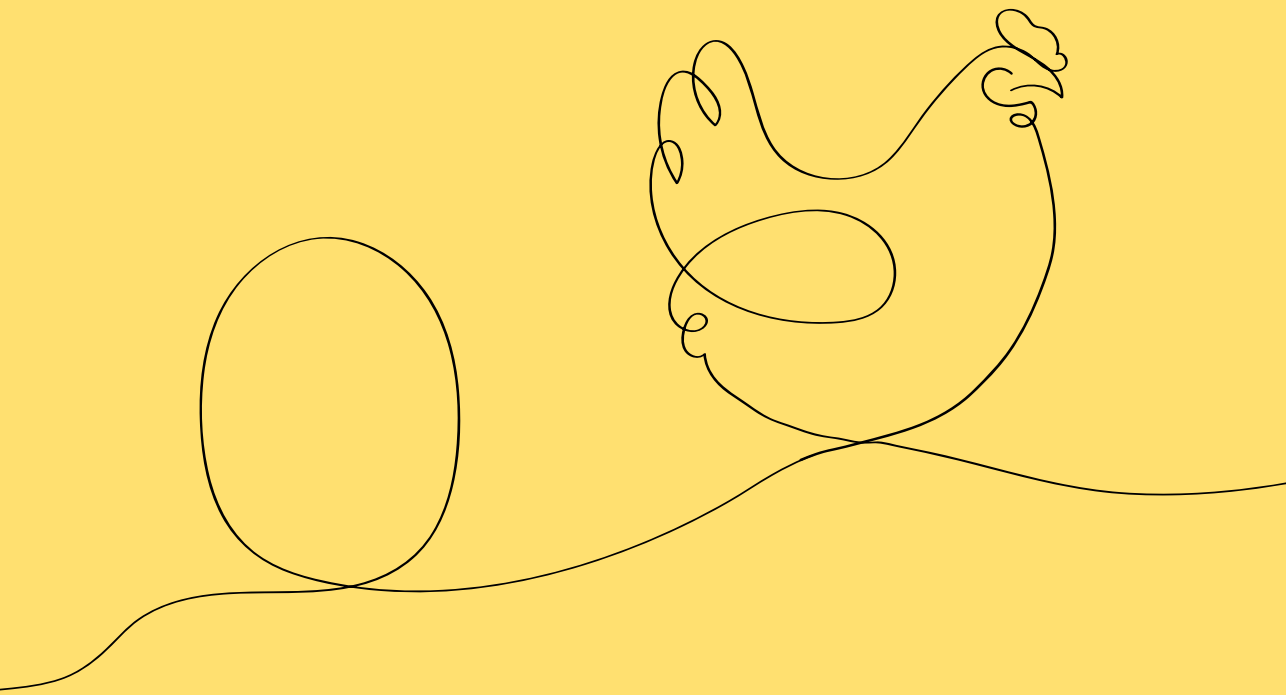
Chapter 3

Chicken-derived RSPO1 and WNT3 contribute to maintaining longevity of chicken intestinal organoid cultures



Abstract

Intestinal organoids are advanced cellular models, which are widely used in mammalian studies to mimic and study *in-vivo* intestinal function and host-pathogen interactions. Growth factors WNT3 and RSPO1 are crucial for the growth of intestinal organoids. Chicken intestinal organoids are currently cultured with mammalian Wnt3a and Rspo1, however, maintaining their longevity has shown to be challenging. Based on the limited homology between mammalian and avian RSPO1, we expect that chicken-derived factors are required for the organoid cultures. Isolated crypts from embryonic tissue of laying hens were growing in the presence of chicken WNT3 and RSPO1, whereas growth in the presence of mammalian Wnt3a and Rspo1 was limited. Moreover, the growth was increased by using Prostaglandin E2 (PGE_2) and a Forkhead box O1-inhibitor (FOXO1-inhibitor), allowing to culture these organoids for 15 passages. Furthermore, stem cells maintained their ability to differentiate into goblets, enterocytes and enteroendocrine cells in 2D structures. Overall, we show that chicken intestinal organoids can be cultured for multiple passages using chicken-derived WNT3 and RSPO1, PGE_2, and FOXO1-inhibitor.

3.1 Introduction

Poultry meat and eggs are low in production costs, are accepted in many cultures, and have a relatively low environmental impact compared to other sources of animal protein, because they produce significantly lower greenhouse gas emissions (1). This makes it an important and efficient source of animal protein and essential for feeding the growing world population (2). However, infectious disease control is a major challenge in poultry farming and antimicrobial drugs are often used to reduce the impact of intestinal infections. To reduce the contribution to risks of antimicrobial resistance to public health, strict regulations on antimicrobial use apply nowadays for livestock farming in the European Union (3). However, there is still a need to reduce and prevent intestinal diseases, which has led to a continuous search for alternatives with e.g. antimicrobial, anti-inflammatory, antioxidant, or immune-fitness promoting properties (4, 5).

The gastrointestinal system plays a pivotal role in health, metabolism, immunity, and production performance in chickens. There is, for instance, a close interaction between the commensal gut microbiota and the host immunity, contributing to an efficient innate immune response to potential pathogens (6, 7). With the intestine playing an important role in chicken's health, strategies to promote or maintain intestinal health are warranted. For example, feed additives, such as mannan-oligosaccharide, have been used to stimulate intestinal immune responsiveness (8, 9). Most studies on feed additives, that promote intestinal health, were performed *in-vivo* and require the use of experimental animals. Although these results are highly relevant to the field, *in-vitro* strategies to study intestinal health are needed in view of in-depth mechanistic research and in the context of replacing, reducing and refining (the 3R's) animal experimentation. Therefore, it is of great interest to have a near-physiological *in-vitro* model to study direct host-microbe interactions and the effects of food components on the epithelial cell barrier of the intestine.

Organoids are advanced cellular models, which have been widely used in mammalian studies since first introduced by Sato and colleagues (10). These self-organizing three-dimensional (3D) tissue cultures, derived from stem cells, represent the physiology of *in-vivo* situations better than monolayer cell lines because stem cells can differentiate into different epithelial cell types present in the intestine, e.g. enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. Most three-dimensional (3D) organoids have the apical site of the epithelium at the inside of the 3D structure, which makes it more difficult to access. By additionally growing intestinal organoids in a two-dimensional (2D) way, studies concerning apical stimulation, gut hormone secretion, and mucus production can be done, as well as absorption studies and co-culture studies with immune cells and bacteria (11).

For poultry, a limited number of studies on intestinal organoids have been reported (12-17). So far, most of the studies did not show growth of more than one passage, however, for our research we require cultures that maintain their stem cells and thus have longevity, resulting in multiple passaging of these chicken intestinal organoids. Extrapolating from mammalian intestinal organoids, it is suggested that Matrigel and the growth factors Wnt3 and Rspo1 are crucial for the formation, development, and maintenance of organoid cultures through activation of the WNT-pathway, which can be enhanced by the combination of a Glycogen synthase kinase-3β-inhibitor (CHIR99021) and a histone deacetylase inhibitor (valproic acid). Mammalian Wnt3 and Rspo1 have currently been used for chicken intestinal organoids, despite the difference in protein homology of these factors between chicken and mammalian origin. We expected that using mammalian-derived growth factors does not contribute to longevity of these cultures, and instead, require chicken-derived factors. Moreover, additional growth factors may be needed. Pierzchalska and colleagues showed that prostaglandin E2 (PGE_2) has a positive effect on the growth of chicken embryo intestinal organoids (18, 19). Another factor that seems promising is

the Forkhead box O1 (FOXO1) inhibitor AS1842856, because of its crosstalk with LGR5 signaling (20). FOXO1 suppresses the WNT/β-catenin pathway and is known to regulate maintenance of stemness in multiple systems, including the intestine (21). In this study, we show that chicken intestinal organoids can be cultured for multiple passages when using chicken-derived WNT3 and RSPO1, PGE_2 and a FOXO1-inhibitor.

3.2 Material and Methods

3.2.1 Medium and reagents

Components of the media formulations for different organoid experiments used in the study are listed in Supplementary Table1, and details can be found underneath.

3.2.1.1 Basic culture medium (BCM): Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) supplemented with 10% Fetal Calf Serum (FCS), 1% GlutaMAX, 2.5 ml, 1 mM sodium pyruvate, MEM Non-Essential Amino Acids, penicillin-streptomycin 10,000 U/mL (all Gibco, Life Technologies Limited, Paisley, UK).

3.2.1.2 Intermediate culture medium (ICM): Basic Culture medium, supplemented with CHIR99021 4.3 μM (Sigma-Aldrich, Saint Louis, MO, USA, SML 1046), Y27632, a selective ROCK1 inhibitor, 10 μM (Absource Diagnostics GmbH, München, Germany), valproic acid 0.78 mM (Sigma-Aldrich, Saint Louis, MO, USA, P6273), DMH-1, a selective inhibitor of activin receptor-like kinase 2 (ALK2, 1.3 μM (Sigma-Aldrich),

3.2.1.3 Chicken organoid culture medium (COCM): intermediate culture medium supplemented with FOXO1-inhibitor, AS1842856, 0.2 μM (Sigma-Aldrich), PGE_2 7.09 μM (Stemcell Technologies, Vancouver, Canada), chicken-specific WNT3 (1:5, in-house production; see below), and RSPO1 (1:5, in-house production).

3.2.1.4 Mouse organoid culture medium (MOCM): Basic culture medium, supplemented with 1:5 of supernatant of Rspo1 expressing Hek293t cells (Sigma-Aldrich; SCC111), 1:5 of supernatant of L Wnt-3A cells (ATCC, Molsheim, France; CRL-2647), and the BMP4 inhibitor DMH1 1.3 μM (Sigma-Aldrich; D8946)

3.2.2 In house production of chicken-specific growth factors WNT3/RSPO1

To produce chicken-specific growth factors WNT3 and RSPO1, we subcloned Chicken RSPO1-DYK (Genscript; OGa18707) and WNT3-DYK (Genscript; OGa25549D) cDNA clones into Tol2-containing E4 plasmid (Kind gift from Stefan Schulte-Merker (22)). Tol2 sequences allowed us to have stable genomic integration of RSPO1 and WNT3 cDNA in Hek293t cells upon Puromycin selection (Invitrogen; ant-pr-1). After selection, Hek293t -RSPO1 and -WNT3 were expanded in culture medium and when cells were 100% confluent for 2 days, supernatant was collected and centrifuged for 5 minutes at 295 x g. For organoid cultures, these supernatants were diluted 5 times in ICM.

3.2.3 Protein alignments

The protein sequences of WNT3 and RSPO1 of chicken and its orthologues of mouse and human were found using NCBI BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi). The following protein sequences were obtained from NCBI and used to make alignments: WNT3: NP_00107565.1 (Gallus gallus),

NP_033547.1 (Mus musculus), NP_110380.1 (Homo sapiens). RSPO1: NP_001305373.1 (Gallus gallus), NP_001033722.1(Mus musculus), NP_619624.2 (Homo sapiens).

3.2.4 Isolation of avian intestinal crypts and development of 3D intestinal organoids

Embryonic day 18 (ED18) Lohmann Brown chicken eggs (*Gallus gallus)* were obtained from the Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, the Netherlands. For each isolation, both small and large intestines were collected and pooled from three chicken embryos *post-mortem*, and repeated three times. This method was conducted in accordance with protocols approved by the Department of Biomolecular Health Sciences, Division of Infectious Diseases & Immunology of the Utrecht University. Although according to European legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) experiments with embryonated chicken eggs are not considered animal experiments. The study was carried out in compliance with the ARRIVE guidelines.

Immediately after collection, the intestines were placed in a petri dish containing 10 mL of 5 mM ice-cold phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA; Lonza, Basel, Switzerland). After washing with PBS-EDTA, the intestines were cut into small segments and placed into a 50 mL conical tube containing PBS-EDTA. To get a homogenous suspension of intestinal crypts, 2mL of PBS-EDTA containing intestinal segments was minced using a potter-elvehjem PTFE pestle and glass tube (Sigma-Aldrich). The intestinal crypts' suspension was collected in a 15 ml tube and centrifuged at 295 x g for 5 min at 4 ˚C. After centrifugation, the supernatant was discarded and crypt aggregates were resuspended in 10 mL PBS-EDTA and centrifuged again. This was repeated up to 5 times until supernatants free of floating particles. Next, the crypts' suspension was filtered through a 100-µm cell strainer (Corning, Amsterdam, the Netherlands) to get a uniform intestinal crypt solution. The filtrate was centrifuged at 295 x g for 5 min at 4 ˚C and supernatant was discarded. The pellet was resuspended in 800 µL of ice-cold Matrigel (Corning; 354234). Two drops of 25 µL intestinal crypts-matrigel suspension, containing 25-50 crypts, were added per well in a pre-warmed 12-well culture plate (Corning). Following polymerization of Matrigel, 2 mL of organoid media (as indicated) was added per well and the culture plate was placed at 41 ˚C, 5% CO_2, which is the optimal temperature to culture chicken cells, to mimic the body temperature of the chicken (Fig. 1). 3D intestinal organoids were passaged twice a week in a 1:3 ratio, by dissociating the organoids embedded in Matrigel drops in 1 mL fresh BCM through vigorous pipetting. After dissociation, the organoid solution was centrifuged at 295 x g for 5 min at 4˚C. Supernatant was discarded and the organoids pellet was suspended in ice-cold Matrigel for seeding to expand the 3D intestinal organoids culture.

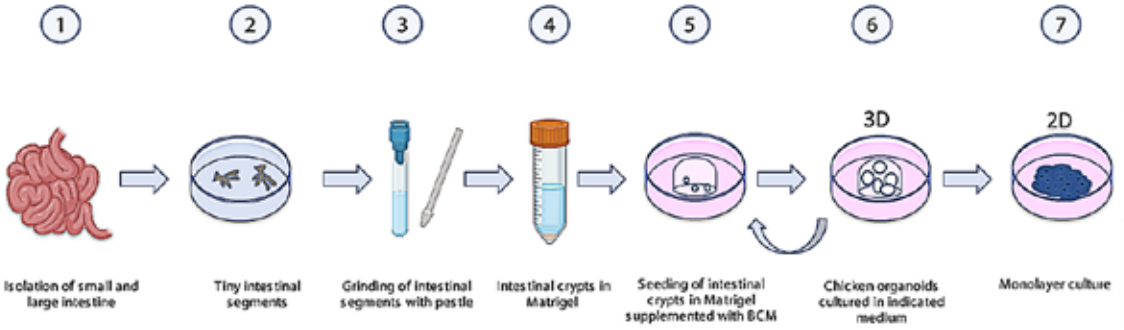


Figure 1. Schematic overview of the isolation and culture conditions of chicken intestinal organoid

1. Isolation of small and large intestines **2.** Intestinal tissue was cut into tiny segments and washed with PBS-EDTA. **3.** To obtain a homogenous suspension, segments were minced using a potter-elvehjem PTFE pestle and glass tube. **4.** After multiple centrifugation steps, the pellet was resuspended in ice-cold Matrigel **5.** and seeded in a culture plate. **6.** After polymerization of Matrigel, crypts were cultured with indicated media as defined in supplementary table 1. **7.** Monolayer cultures were obtained from three-dimensional cultures. Figure created with BioRender.com.

3.2.5 Isolation of mouse intestinal crypts

The mouse intestinal crypts were isolated from the ileum as reported previously (23). A female mouse C57/BL6, 6 months of age was sacrificed using carbon dioxide. The use of mouse intestine was approved by the local committee for care and use of animals at Utrecht University and all experiments were performed in accordance with the relevant guidelines and regulations. Only one surplus mouse was used for this study. The study was carried in compliance with the ARRIVE guidelines.

Villi were removed from the ileum, by scraping with a scalpel and the tissue was cut into small pieces, transferred to a tube with ice-cold PBS and washed three times with PBS (spun down at 295 × g for 5 min between every wash step). Crypts that were isolated from the villi were resuspended in Matrigel, containing 25-50 crypts/25 µL, and two drops of 25 µL suspension were added to each well. The organoids were kept in ICM for 3 days, and medium was subsequently replaced with MOCM to stimulate organoid formation. These organoids were passaged once a week in a 1:4 ratio, containing 25-50 organoids/well, by dissociation the organoids embedded in Matrigel drops in 1 ml fresh BCM through vigorous pipetting. After dissociation, the organoid solution was centrifuged at 335 x g for 5 min at 4°C. Supernatant was discarded and the organoids pellet was suspended in ice-cold Matrigel for seeding to expand the 3D intestinal organoid culture.

3.2.6 Conversion of 3D intestinal organoids to monolayer culture

To obtain 2D intestinal organoids, 3D intestinal organoids were used. Before seeding the monolayer chicken intestinal organoids, 12 mm sterile glass coverslips (Waldemar Knittel Glasbearbeitungs GmbH, Brunswick, Germany) were coated with Matrigel. This was done by placing the coverslips in a 24 well culture plate, and 40 µL of 2.5% ice-cold Matrigel solution in Dulbecco's Phosphate Buffered Saline with calcium and magnesium ($DPBS^{+/+}$) was added per well, followed by a 2h incubation at room temperature (RT). The Matrigel solution was removed by pipetting and the plate was air-dried for 20 min at RT.

To obtain a single-cell suspension, 4 wells of 3D intestinal organoids embedded in Matrigel were used to form 1 well of the monolayer organoids. The 3D intestinal organoids were dissociated mechanically by vigorous pipetting using a 200-µL pipet in cold BCM. After dissociation, the solution was centrifuged at 131 x g, 3 min at 4 ˚C. Supernatant was discarded and the pellet was resuspended in 200 µL 0.25% trypsin-EDTA (Gibco; 25200056) and incubated for 5 min at 37 ˚C. These steps were repeated until a single cell suspension was obtained (in general this required two steps), which was evaluated under a light microscope. The pellet containing the single-cell suspension was resuspended in 100 µL/well COCM and seeded on the matrigel-covered glass coverslips and an additional 600 µL of COCM was added per well and, subsequently, the culture plate was placed at 41 ˚C, 5% CO_2. Intestinal organoid monolayers that were formed on glass coverslips reached almost 70% coverage within 2 days.

3.2.7 Immunohistochemical staining

To identify various chicken intestinal epithelial cell lineages; glass coverslips containing the monolayer cultures were fixed with 200 µL of $DPBS^{+/+}$ and 4% paraformaldehyde (PFA) solution for 1 h at RT. Later on, monolayer cultures were washed with 1 mL of $DPBS^{-/-}$ and 10 mM glycine (Merck Millipore, Burlington, MA, USA) solution to quench the fixative. Following fixation, the monolayer cultures were blocked in 300 µL of blocking buffer consisting of $DPBS^{-/-}$, 0.05% Tween-20 (Sigma-Aldrich), and 2% bovine serum albumin (Sigma-Aldrich) for 2 h at 4 ˚C. Coverslips were then stained with monolayer faced down on parafilm containing 25 µL of blocking buffer containing primary antibodies, as mentioned in Table 1, overnight at 4 ˚C. The next day, monolayer cultures were stained with secondary antibodies as mentioned in Table 1, for 1 h at RT protected from light. In addition, nuclear staining was performed by incubating the glass coverslips for 5 min in 300 µL blocking buffer containing 10 µg/mL 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at RT. The samples were washed three times with 1 mL washing buffer containing $DPBS^{-/-}$, and 0.05% Tween-20 in between the staining steps. The last washing step after nuclear staining was done with 1 mL distilled water and then the samples were mounted on Polysine® microscope slides (Menzel Glaser, Braunschweig, Germany) containing FluorSave mounting reagent (Merck Millipore).

To determine the cross-reactivity of primary antibodies we performed staining of cryosections of embryonic day 18 (ED18) chicken intestine. The small and large intestines of ED18 chicken embryos were embedded in Tissue-Tek® (Sakura Finetek, Alphen aan den Rijn, the Netherlands) and frozen using liquid nitrogen. Cryosections (4 µm) of the ED18 intestine were obtained using a microtome and placed on glass slides (Superfrost/plus, Germany). ED18 intestinal cryosections were first fixed with 4% PFA, permeabilized with Triton X-100 for 10 min at RT, and blocked in blocking buffer for 2 h. Intestinal sections were then incubated with primary antibodies as mentioned in Table 1 in humified chamber at 4 ˚C overnight. The next day, secondary staining was performed followed by nuclear staining with DAPI. In between the staining steps, intestinal sections were washed with washing buffer.

Intestinal tissues sections and monolayer cultures were imaged using Leica TCS SPE-II microscope (Leica, Amsterdam, the Netherlands) using 20x, 40x, 63x, or 100x ACS apo cs oil objective, and images were analyzed with FIJI software (NIH, version ImageJ 1.52r). The spheroid size were measured manually.

Table 1. Characteristic of the used antibodies

Antibody	Concentration	Species cross-reactivity	Target site	Host/Isotype
Anti-Sox9 AB5535 Sigma-Aldrich	1:500	Human, Mouse, Rat, Chicken	Sox9, a transcription factor and recognized marker for stem/ progenitor cells	Rabbit
Anti-chromogranin-A Immunostar 20086	1:500	Buffalo, Chicken, Cow, Dolphin, Human, Mouse	Enteroendocrine cells	Rabbit
Anti-occludin Thermofisher 71-1500	1:100	Baboon, Bacteria, Chicken, Dog, Human, Mouse, Rabbit, Rat	Tight junction membrane	Rabbit
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:1000	Rabbit	Gamma Immunoglobins Heavy and Light chains	Donkey/IgG

3.2.8 Periodic acid-Schiff (PAS) staining

Mucus-producing goblet cells in monolayer culture were identified by Periodic acid-Schiff (PAS) staining method. Monolayer cultures were fixed with hemacolor solution-1 (Sigma-Aldrich) for 1 h and incubated with 0.5% PAS (Sigma-Aldrich) for 5 min at RT, followed by washing with distilled water three times and staining with Schiff's reagent (Sigma-Aldrich) for 40 min. The samples were then counterstained with hematoxylin (Sigma-Aldrich) for 1 min at RT and mounted on FluorSave reagent with monolayer faced down on Polysine® microscope slides. Glass slides were analyzed under Olympus BX41 microscope (Olympus, Leiderdorp, The Netherlands) using 20x and 40x plan phase objectives.

3.2.9 Immunoblot analysis

Cell lysates were dissolved in RIPA Lysis and Extraction buffer (Thermo-Scientific, Landsmeer, The Netherlands, 89900) and Halt Phosphatase Inhibitor Cocktail (Thermo-Scientific, 78420) was added to the samples. Proteins (30 µg/well) were separated by SDS-PAGE using pre-cast gel 10% Tris/Gly (Bio-Rad, Lunteren, the Netherlands). After transfer to PVDF membrane (0.22 µm), blots were probed with anti-flag HRP 1:500 (Invitrogen), or β-actin (#4967) and visualized using horseradish peroxidase-labeled anti-rabbit antibodies and chemiluminescence on a Bio-rad ChemiDoc XRS + system. The experiments were repeated 3 times.

3.2.10 Dot blot analysis

Supernatant from Wnt3 and RSPO1 producing Hek293t cells were collected and 5 µl of the supernatant was spotted on nitrocellulose membrane and dried for 30 minutes. Blocking of non-specific sites were done by incubating in 5% BSA in TBS-T for 1h at RT. Next, anti-flag HRP (1:500) was added, for 60 min at RT and subsequently rinsed with TBS-T (5x) and visualized using chemiluminescence on a Bio-rad ChemiDoc XRS + system. The experiments were repeated 3 times.

3.2.11 Real-Time Quantitative PCR

The gene expression level of various passages of 3D and 2D intestinal organoids was determined by Real-Time quantitative PCR (RT-qPCR). Two wells of 3D and 2D intestinal organoids culture were harvested from Matrigel using cold Advanced DMEM/F12 medium by vigorous pipetting and centrifuged at 295 x g, 5 min at 4 ˚C. The pellet was lysed in RLT buffer (Qiagen, Venlo, the Netherlands) supplemented with β-mercaptoethanol (Sigma-Aldrich). Total RNA was extracted using RNeasy mini kit (Qiagen, 74106) according to manufacturer's instructions and RNA was quantified using NanoDrop spectrophotometer (Isogen, de Meern, The Netherlands).

Reverse transcription of 0.5 µg of total RNA was done using iScript cDNA synthesis kit (Bio-Rad, 170-8891) as per manufacturer's instructions and cDNA was diluted 1:1 ratio in milli-Q. RT-qPCR assays were performed using CFX384 real-time PCR detection system (Bio-Rad). Primer LGR5 (Forward: CCCACTGCTATCAGGACACTAAC; Reverse: GAGGCACCATTTAAAGTCAGAG) was used as a marker for stem cells. Normalization of LGR5 was done using GAPDH (Forward: GTGGTGCTAAGCGTGTTATC; Reverse: GCATGGACAGTGGTCATAAG) as the housekeeping gene. 5 µL of cDNA template was added per well consisting of 20 µL reaction mixture using 12.5 µL SYBR Green Supermix, 5.5 µL of distilled water, 1 µL (400 nM) of forward and reserve primers. PCR thermal cycle conditions used were as follows: an initial 5 min step at 95 ˚C, followed by 40 cycles of 92 ˚C for 10 s, 55˚C for 10 s, and 72 ˚C for 30 s. This was followed by cycle 3 consisting of 95 ˚C for 1 min, and 65 ˚C for 2 min.

3.2.12 Microarray.

The microarray was performed as described previously (24). Briefly, One hundred nanogram of RNA was used for Whole Transcript cDNA synthesis (Affymetrix, inc., Santa Clara, USA). Hybridization, washing and scanning of Affymetrix GeneChip Mouse Gene 1.1 ST arrays was carried out according to standard Affymetrix protocols. All arrays of the small intestine were hybridized in one experiment. Arrays were normalized using the Robust Multiarray Average method (25, 26). Probe sets were assigned to the unique gene identifiers Entrez IDs. The probes on the Mouse Gene 1.1 ST arrays represent 21,213 Entrez IDs. Array data were analyzed using an in-house, on-line system (27).

3.2.13 Data analysis

Significant differences in the size of organoids when culturing with different supplements, and the mRNA levels of LGR5 between multiple passage numbers, were analyzed by the Kruskal-Wallis test, followed by the Dunn's multiple comparisons test in Graphpad Prism (version 9.3.1, San Diego, CA, USA). A P-value < 0.05 as considered significant.

3.3 Results

3.3.1 Chicken-derived growth factors WNT3 and RSPO1 are preferred for the maintenance of chicken intestinal organoids

Alignments of WNT3 and RSPO1 between human, mouse, and chicken shows that chicken WNT3 is 96% identical to mouse and human Wnt3 (Fig. 2A). Moreover, chicken RSPO1 is only 65% identical to mouse Rspo1 and 68% identical to human RSPO1 (Fig. 2B). This indicates that in chickens, both growth factors are different from the mammalian orthologue, therefore, urging us to produce chicken-specific WNT3 and RSPO1.

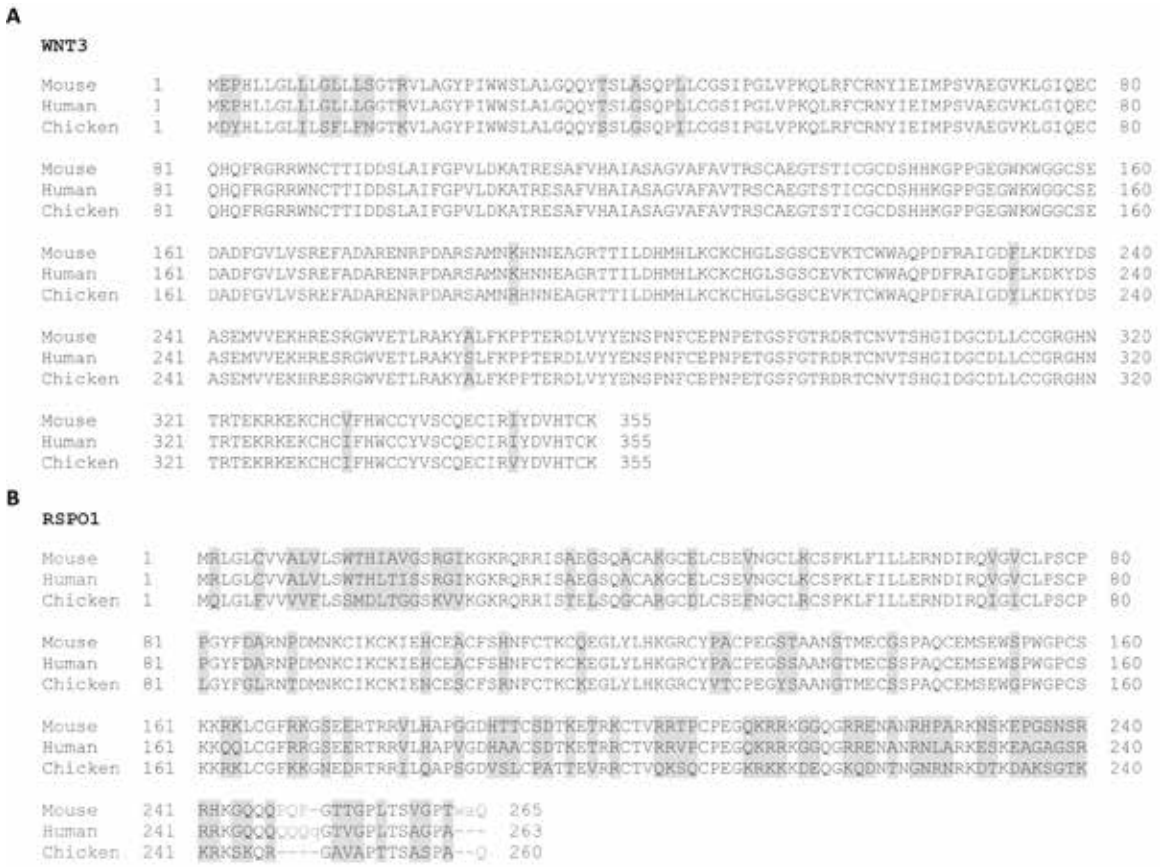


Figure 2. Sequence alignments of (A) WNT3 protein for mouse, human and chickens. Chicken vs mouse and chicken vs human are 96% identical (human vs mouse: 98%). **(B)** Alignment of RSPO1 for mouse, human and chicken. Chicken and mouse is 65% identical and chicken and human is 68% (human and mouse: 89%). Non-consensus amino acids (grey).

Hek293t cells expressing chicken WNT3 and RSPO1 were developed to obtain these growth factors for organoid cultures. Immunoblot analysis of a cell lysate of transfected cells showed a single protein of approximately 28 kD for both growth factors when stained with their cognate antibodies (Fig. 3a; full-length blot Fig. S1). Moreover, by making use of a dot blot, we showed that WNT3 and RSPO1

are successfully secreted in the supernatant (Fig. S2). The effect of chicken-derived WNT3 and RSPO1 were compared to the mouse-derived growth factors on cultures of chicken intestinal organoids and murine intestinal organoids (Fig. 3B-C). To this end, chicken intestinal organoids and murine intestinal organoids were cultured in ICM, supplemented with murine-derived Wnt3 and Rspo1 (MOCM) or with chicken-derived WNT3 and RSPO1. After 1 week, chicken intestinal organoids cultured in ICM supplemented with chicken-derived WNT3 and RSPO1 showed growth of spheroid-like structures, which increased in number (Fig. 3C (III, IV)), whereas only dark structures were observed when culturing chicken organoids in MOCM (Fig. 3B (III, IV)). When mouse organoids were cultured in MOCM, the growth and formation of lobular structures were observed (Fig. 3B (I, II)). Culturing mouse intestinal organoids in ICM supplemented with chicken-derived WNT3 and RSPO1 resulted in dark structures, without lobular structures (Fig. 3C (I, II)). Altogether, these data indicate that chicken organoids indeed require chicken-specific growth factors RSPO1 and WNT3 for optimal growth. Furthermore, we observed that, in contrast to mouse intestinal organoids, chicken intestinal organoids grow as spheroids rather than lobular structures (Fig. 3C (III, IV)).

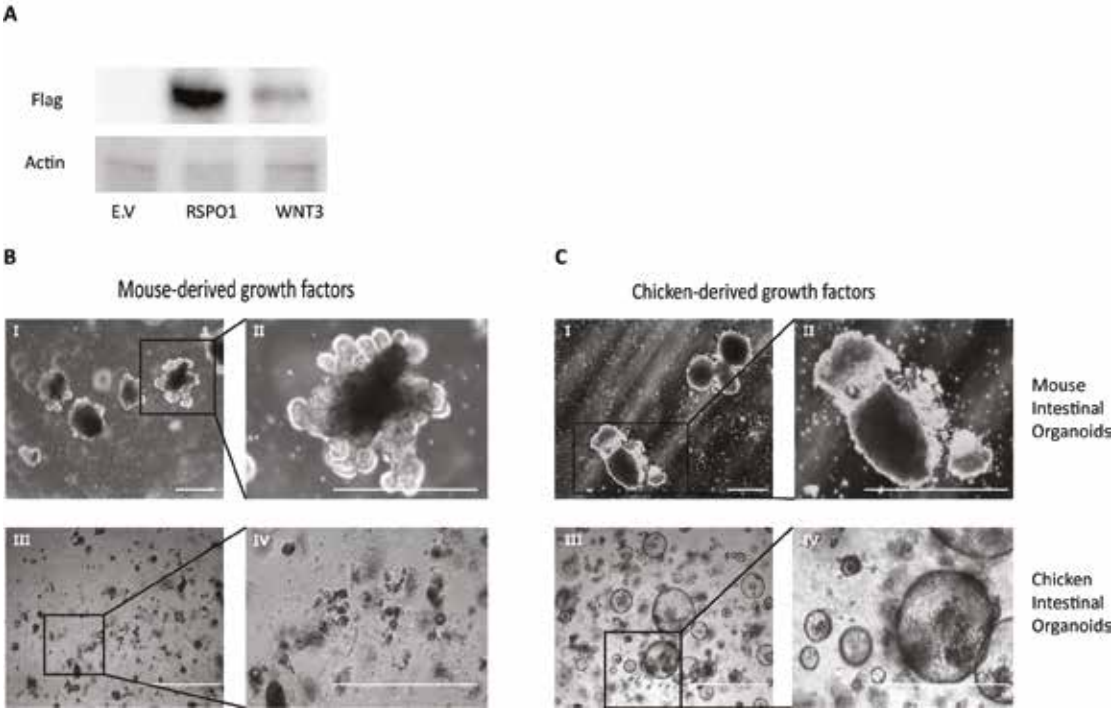


Figure 3. Growth factors RSPO1 and WNT3, from murine or chicken origin, are important for establishing intestinal organoid growth. A Immunoblot analysis of chicken RSPO1 and chicken WNT3 protein expression in transfected Hek293t cells. Actin was used as the protein loading control and anti-flag HRP was used to visualize RSPO1 and WNT3. Empty Vector (E.V.) is taken along as a control. **B** Brightfield images of mouse intestinal organoids **(I, II)** and chicken intestinal organoids **(III, IV)** at passage 2 (5 days after passage). Cultured in Matrigel with ICM, supplemented with Rspo1 and Wnt3 of murine origin, **C** or supplemented with chicken-derived RSPO1 and WNT3. Images and immunoblot are representative of three independent cultures. Scale bar 200 µm.

3.3.2 Defining optimal culture conditions for chicken intestinal organoids

Next, we evaluated the effect of additional components, such as PGE_2 and the FOXO1-inhibitor AS1842856 on the growth of chicken intestinal organoids. It is known from literature that PGE_2 has a positive effect on the growth of chicken intestinal organoids and FOXO1 plays a role in stemness (21, 28). We therefore cultured freshly isolated embryonal chicken intestinal organoids in ICM with chicken-derived WNT3 and RSPO1 (Fig. 4A, E, I), either supplemented with the FOXO1-inhibitor AS1842856 at a concentration of 0.2 µM (Fig. 4B, F, J) or PGE_2 at a concentration of 2.5 µg/ml (Fig. 4C, G, K) or both. The growth of organoids was monitored for 3 days. The addition of the FOXO1-inhibitor resulted in similar organoid growth and structures when compared to the ICM with chicken-derived WNT3 and RSPO1 cultures (Fig 4M). Interestingly, supplementation of PGE_2 resulted in more and larger spheroids (Fig 4M). Moreover, when PGE_2 was combined with the FOXO1-inhibitor, we observed an increase in spheroid diameter, compared to PGE_2-only exposed cultures.

3.3.3 The LGR5+ intestinal stem cells stay stable during multiple passages

To our knowledge, there are no publications that show the same efficient growth of embryonal chicken intestinal organoids, compared to mammalian species, for longer than 21 days. Therefore, we tested the longevity of the embryonic chicken intestinal organoid cultures. Organoid cultures were passaged every 3-4 days and the growth in size and number of the organoid culture was monitored over time (Fig. 5A). Up until 10 passages (approximately 5 weeks), we observed fast-growing spheroids, however, later cultures had reduced growth and after 15 passages this resulted in a low passage efficiency.

Organoid cultures are highly dependent on LGR5-expressing stem cells and thus the observed growth suggests that stem cells are present in these cultures (Fig. 5A). To confirm the presence of these stem cells, we analyzed the mRNA expression of the stem cell marker *LGR5* by RT-qPCR for the first ten passages and observed similar expression levels of *LGR5* in all the passage numbers tested (Fig. 5B). This suggests that the stem cell population stayed constant over time.

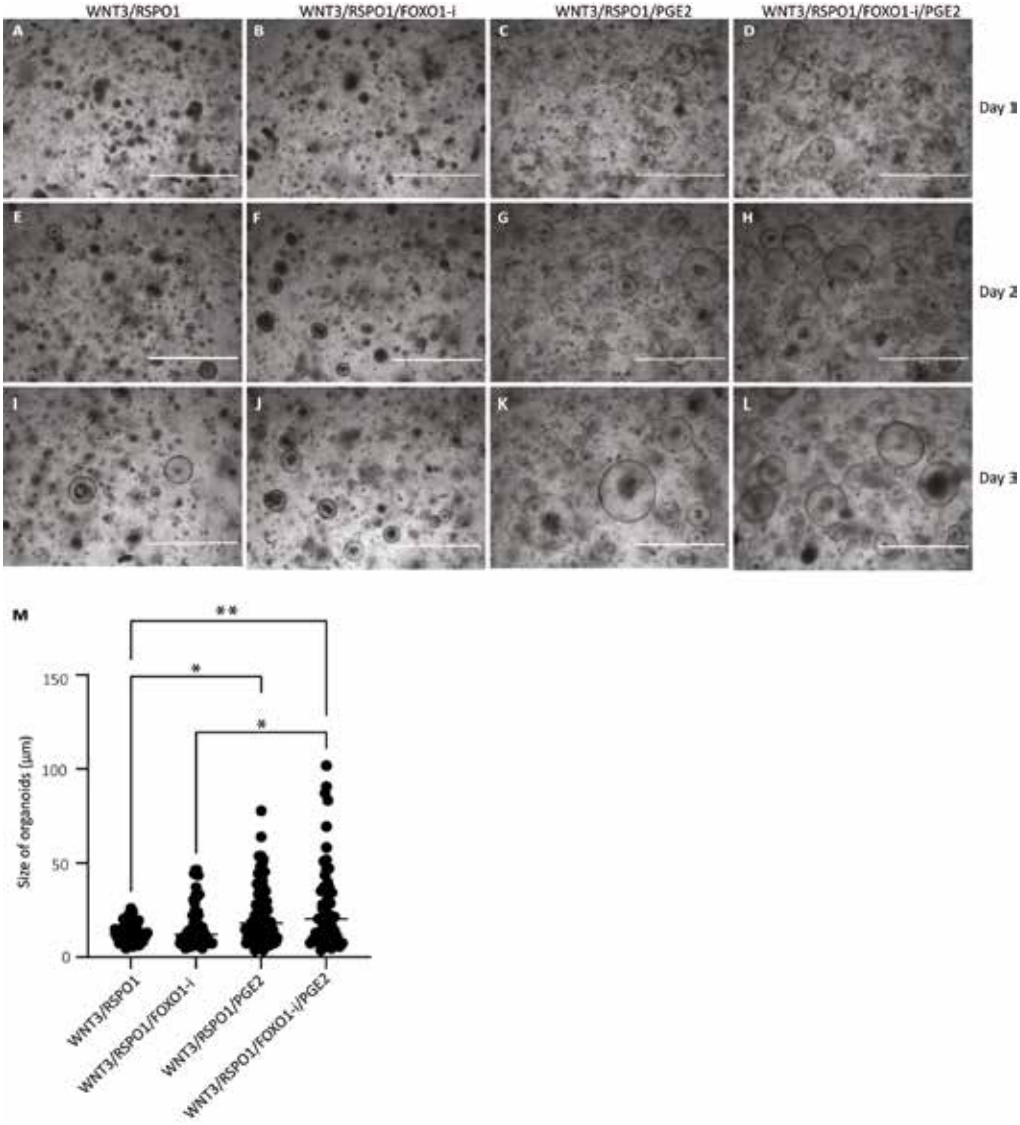


Figure 4. Supplementary compounds for organoid growth. Brightfield images of embryonic chicken organoid cultured in Matrigel with BCM of passage 1, 1, 2 and 3 days after splitting. (**A, E, I)** with additional RSPO1/WNT3 of chicken origin. (**B, F, J)** with additional RSPO1/WNT3 of chicken origin and 0.2 µM Forkhead box O1-inhibitor (FOXO1-i). (**C, G, K)** with additional RSPO1/WNT3 and 2.5 µg/ml prostaglandin E2 (PGE2).(**D, H, L)** with all 4 supplemented growth factors (WNT3/RSPO1/PGE2/FoxO1-i). Images are representative of three independent cultures. Scale bar: 1000 µm. **(M)** Diameter of organoids when culturing with different supplements, were measured manually and plotted in GraphPad Prism. Four outliers were excluded after performing an outlier test in GraphPad Prism. Statistics were performed using a Kruskal-Wallis test followed by Dunn's multiple comparisons test. *p<0.05 ** p<0.01

3.3.4 Cellular diversity of chicken intestinal organoids is comparable to intestinal tissue of chicken

For mouse and human intestinal organoids, it has been nicely established that the LGR5+ stem cells can differentiate into other intestinal cells, such as goblet cells, enteroendocrine cells, and enterocytes (29, 30). To investigate the cellular diversity of the embryonal chicken intestinal organoids we performed 3D cultures as well as 2D cultures. The 2D cultures allow us to study the differentiation of stem cells into enterocytes and functional enteroendocrine cells since enterocytes do not grow well in Matrigel, which is also supported by mRNA expression of specific enterocyte genes in murine intestinal organoids (Fig. S3) (11, 23). Subsequently, immunohistochemistry and PAS cell staining were performed on these 3D and 2D cultures (Fig. 6). First, to confirm that the antibodies are cross-reactive with chickens, embryonic intestinal tissue was used as a control and showed that the antibodies are functional in chicken tissue (Fig. S4). To identify stem cells and transit-amplifying cells, both 2D and 3D cultures were stained for SOX9. This resulted in a nuclear staining for both 3D and 2D (Fig. 6A, B, Fig. S5). Anti-Chromogranin-A was used as a marker for enteroendocrine cells and allowed us to detect this specific cell type in 3D as well as in 2D culture (Fig. 6C, D). Goblet cells can produce mucins that can be stained using PAS reagents (31). We observed a few PAS-positive cells in 2D chicken organoids, indicating that our cultures contain goblet cells (Fig. 6H). Subsequently, the presence of epithelial barrier proteins, such as CTNNB1 (β-catenin) and OCLN (Occludin), was determined by immunohistochemistry. B-catenin is present at cell-cell junctions in 3D as well as in 2D. Occludin is shown to be present in the 2D chicken intestinal organoids (Fig. 6E, F, G). Overall, this data shows that the different cell-types that have been described for mammalian intestinal organoids are also present in embryonal chicken intestinal organoids when cultured in COCM, indicating that these chicken organoids maintained their capacity to differentiate into the different intestinal specific cell types.

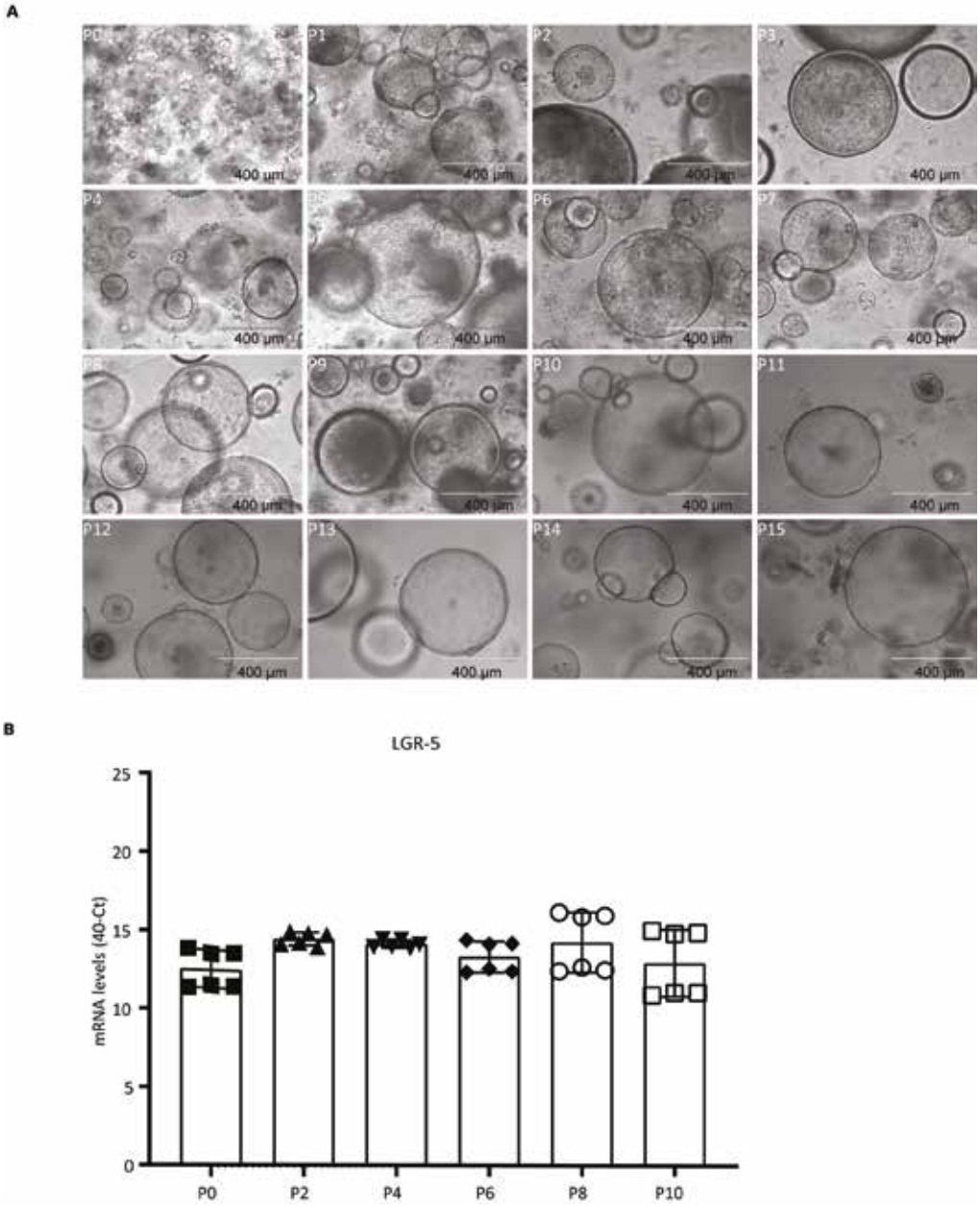


Figure 5. Longevity of the chicken intestinal organoids. A Brightfield images of chicken intestinal organoids cultured in chicken organoid culture medium. All pictures were taken 2 days after passaging. Scale bar: 400 µm. **B** mRNA levels of LGR5 in 3D embryonic chicken intestinal organoids, expressed as 40-Ct values with GAPDH as a reference gene. Two independent isolations were performed in technical triplicates. The error bars represent the standard deviation of the mean.

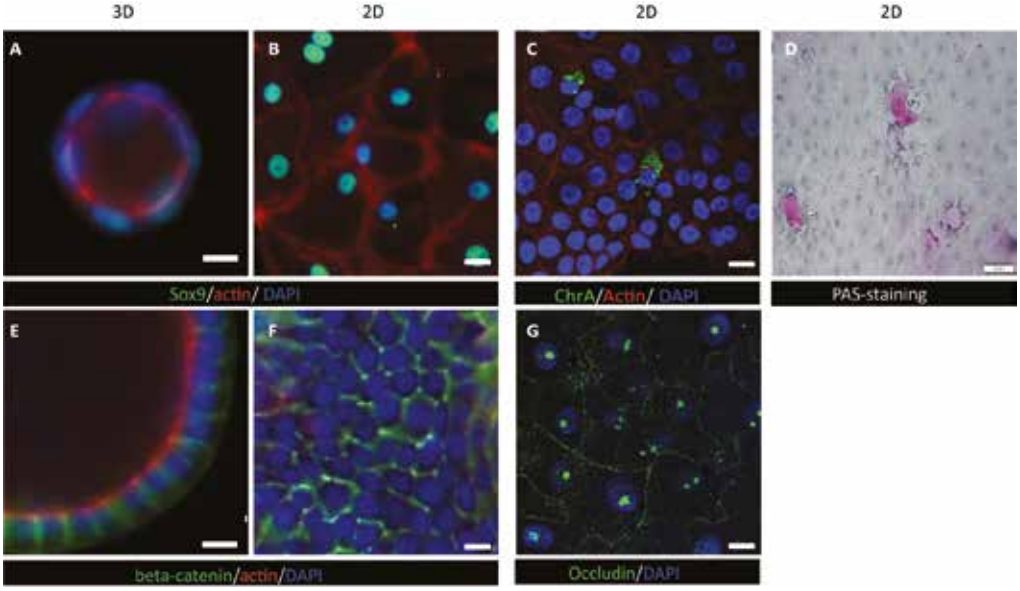


Figure 6. Identification of different cell structures in 3D chicken intestinal organoid and the monolayers. Immunohistochemistry images of 3D and 2D structures of chicken intestinal organoids, processed 2 days after seeding. **A, B** Progenitor cells are visualized with anti-Sox9. **C, D** Enteroendocrine cells are visualized by Chromogranin A. **E-G** Tight junctions are visualized with beta-catenin or occludin **H** Mucus-containing goblet cells are visualized using Periodic Acid Schiff (PAS)-positive cell staining of 2D chicken intestinal organoids. The actin filaments are visualized with rhodamine phalloidin (red), and nuclei are stained with DAPI (blue) Scale bar: 400 µm.

3.4 Discussion

In this study, we aimed to optimize the culture of chicken intestinal organoids and to extend their longevity (i.e. number of passages) compared to what has been reported so far (12-14, 16). We show that by using growth factors of chicken origin, instead of using the commercially available mammalian growth factors WNT3 and RSPO1, cultures could be passaged up to 15 times. Moreover, we show that the growth and longevity of these cultures can be enhanced by addition of PGE_2 and by inhibiting FOXO1. We also demonstrate that these organoids can be grown in 2D as well as 3D and have the capacity to differentiate into intestinal specific cell types, such as goblets, endocrine cells and enterocytes, which offers a broad application of chicken organoids.

It is known that the WNT, NOTCH, and BMP signalling pathways play a pivotal role in the proliferation and differentiation of intestinal stem cells (10, 32-34). Wnt3 and Rspo1 are used in mammalian intestinal organoid cultures (10, 29). Apart from the addition of Wnt3 and Rspo1, the small molecules CHIR99021 and valproic acid support the maintenance of self-renewal of intestinal stem cells and growth of organoids in mice by enhancing the expression of WNT-target genes (35), and also improved growth of chicken intestinal organoids (17). However, for chicken intestinal organoids, culturing with mammalian Wnt3a and Rspo1 did not result in successful long-term organoid cultures (Fig. 3) (12, 13, 16, 19). Powell et al., did show multiple passages, however, they observed that growth of chicken organoids is far less efficient than it is observed for mammalian organoids (15). In addition, Zhao et al. have shown that chicken intestinal organoids were not viable for longer than 3 weeks when using the mammalian Wnt3a and Rspo1 (36). Therefore, we investigated the effect of the species-specific WNT3 and RSPO1 and observed that chicken intestinal organoids benefit from the medium supplemented with chicken-derived WNT3 and RSPO1. Our results indicate that COCM allows growth of chicken intestinal organoids which can be maintained for more than 15 passages. The benefit of culturing for multiple passages is that we can produce faster and more robust outcomes and it is more sustainable compared to short-term cultures, concerning lab animals.

To further optimize the culture conditions, additional effects of PGE_2 and inhibition of FOXO1 were studied. PGE_2 evidently supports the growth of chicken intestinal organoids, compared to ICM with Chicken-derived WNT3 and RSPO1 (Fig 4). These findings extend those of Pierzchalska and colleagues, confirming a more defined and more rigid form of chicken intestinal organoids (14, 19). Although Pierzchalska already showed spheroid-forming chicken intestinal organoids, they were not capable of long-term cultures. By adding the FOXO1-inhibitor, we found an increase in number and size of intestinal organoids, suggesting that by inhibiting FOXO1, we can enhance the growth of organoids. This is in line with the research of Choi et al., where it was demonstrated that there is negative crosstalk between LGR5+ stem cells and FOXO1, in which FOXO1 inhibits the self-renewal capacity of gastric cancer cells (20). So, inhibiting FOXO1 would contribute to the activation of self-renewal.

Our embryonal chicken intestinal organoids grow as spheroids instead of the lobular structures, which are mostly observed in human and mouse intestinal organoids (10, 29). However, in contrast to most mammalian organoid papers (10), we do not isolate crypts from adult chickens, but rather from embryonal chickens. When we isolate crypts from adult chickens, they also show spheroid structures, however, it was not possible to split these organoids beyond passage 1 (data not shown). A recent paper from Mustata and colleagues showed that embryonal crypts from mouse intestines are also growing as spheroids, similar to the structures that we have observed in our cultures, suggesting that the spheroid-like growth might be due to their embryonic nature. Moreover, they showed that mouse embryonal spheroids turn into adult lobular organoids within 10 passages. If embryonal organoids

are destined to become adults *in-vitro*, then this might explain why we lose growth after 15 passages, as we already indicated that the current COCM is not suitable for growth of adult chicken organoids.

A unique feature of organoids, compared to most cell lines, is their capacity to form different cell types which all have their specific function in the intestine, such as barrier function, hormone secretion and absorption and metabolism of nutrients. By staining for specific markers we show that most of these cells are present, especially in 2D, thus reflecting the same plasticity of these long-term cultures as has been published for mammalian organoids (37). However, we also stained for progenitors by using SOX9 as a marker. It was against our expectation that there were also many SOX9 positive cells in 2D cultures, where there is limited matrigel present that is normally required to maintain stemness and thus proliferation. A possible explanation for this might be the presence of CHIR99021 and VA that enhance the WNT-pathway.

Organoids can be an important tool for the reduction of animal experiments and allow for efficient testing of multiple potentially health-promoting feed additives or pharmaceuticals. The ability to grow chicken intestinal organoids for more passages reduces the need for test animals and increases the possibility of more mechanistic research of pathways involved in intestinal health, metabolism, absorption, barrier function and host-microbe interactions. To study these interactions more in-depth, organoid cultures can be expanded by co-culturing them with, for instance, immune cells and fibroblasts or microorganisms.

Acknowledgments

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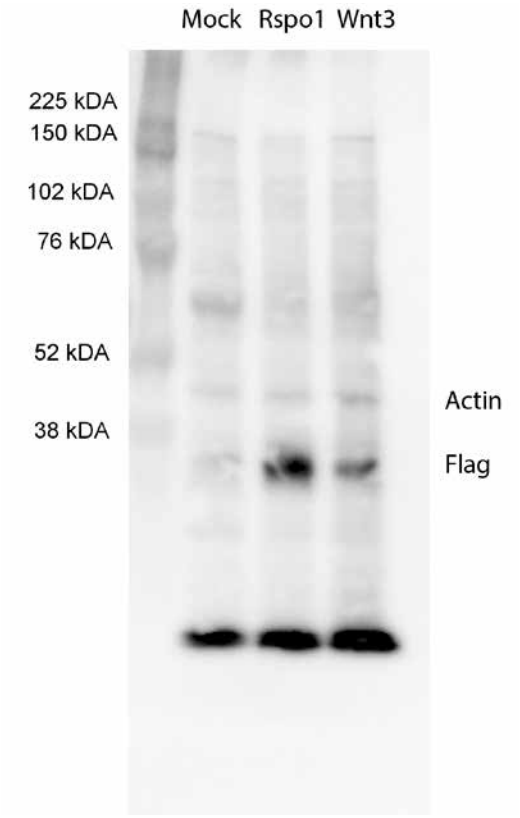
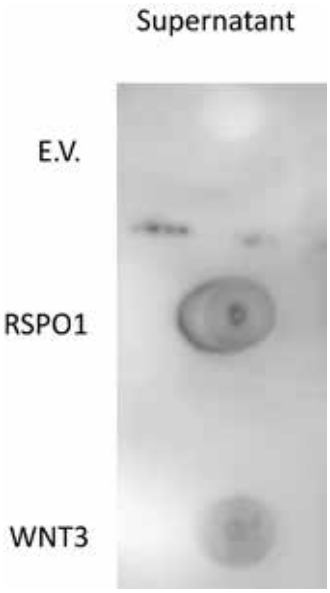
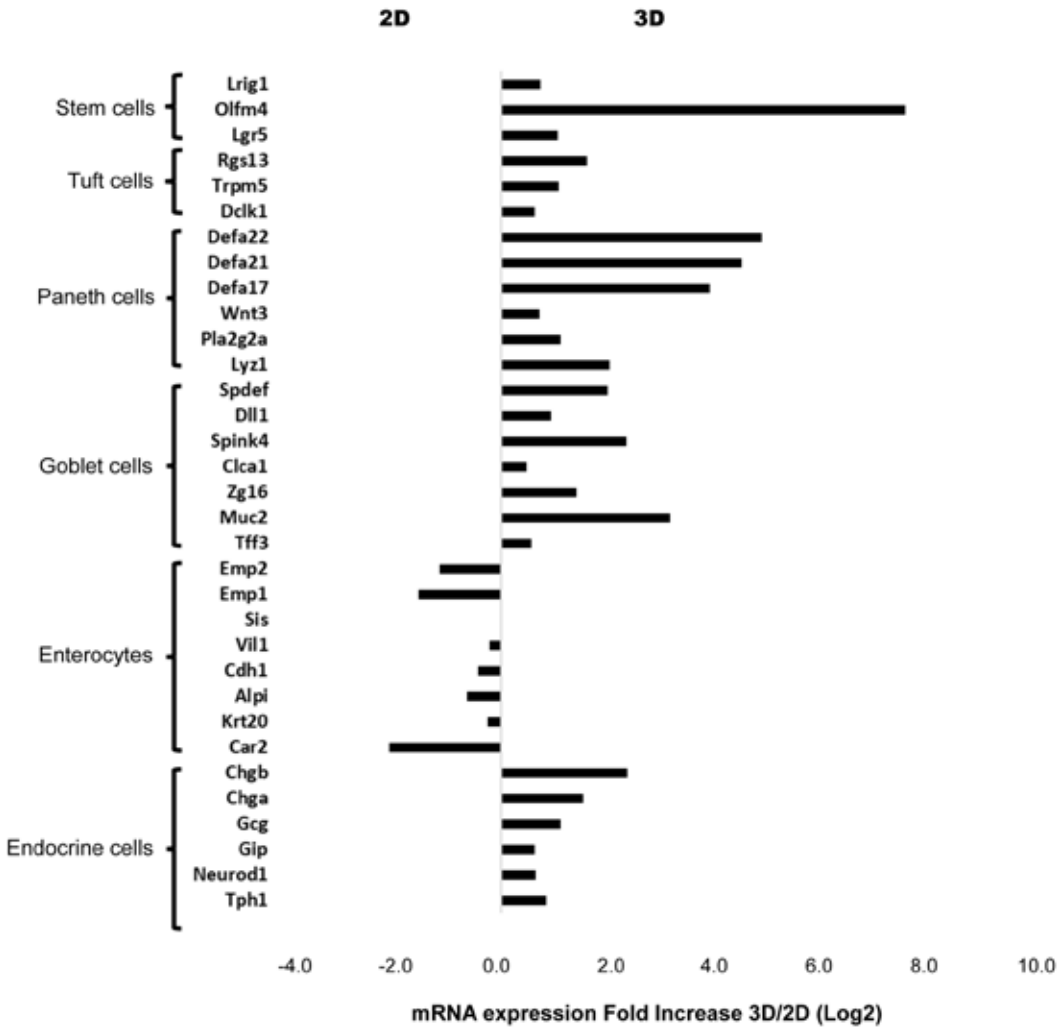
Supplementary files



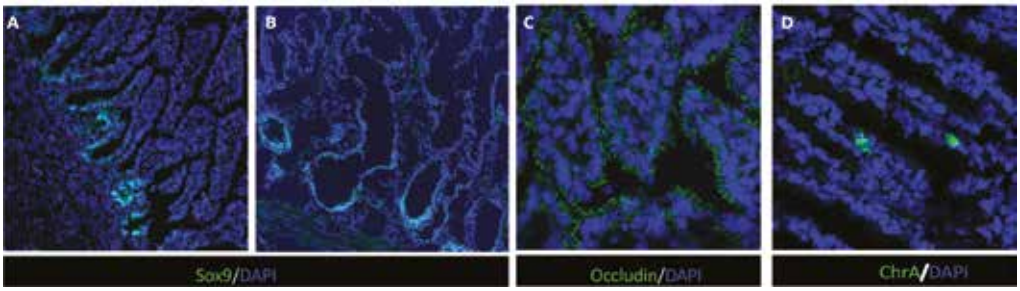
Supplementary Figure 1. Immunoblot of chicken r-spondin 1 (RSPO1) and chicken WNT3 protein expression in transfected HEK293T cells.



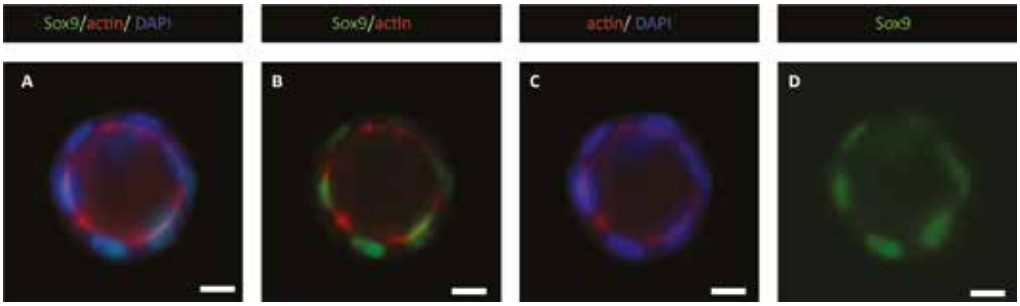
Supplementary Figure 2. Dot-blot of chicken RSPO1 and chicken WNT3 protein expression in supernatant of transfected HEK293T cells. Empty vector (E.V.), chicken r-spondin 1 (RSPO1) and chicken WNT3 are detected by anti-flag-HRP.



Supplementary Figure 3. mRNA expression of cell-specific genes expressed in 2D and 3D mouse intestinal organoids.



Supplementary Figure 4. Confirming functionality of antibodies in chicken tissue. Immunohistochemistry images of embryonic intestinal tissues stained with antibodies mentioned in Table 1. **A** Progenitor cells are visualized with anti-Sox9 in embryonic tissue (magnification 20 x) **B** and in adult intestinal tissue **C** tight juntions are visualized with anti-occludin (magnification 63 x) **D** Enteroendocrine cells are visualized by anti-chromogranin A. Nuclei are stained with DAPI (blue) (magnification 63 x)



Supplementary figure 5. **Immunohistochemistry images of SOX9 in 3D structures of chicken intestinal organoids processed 2 days after seeding**. Progenitor cells are visualized with anti-Sox9. **A** Sox9/actin and DAPI staining **B** Sox9/actin staining **C** actin/ DAPI staining **D.** Sox9 staining. Scale bar: 400 µm.

Table S1. Medium and reagents. Overview of all the reagents in Basic culture medium (BCM), Intermediate culture medium (ICM), Mouse organoid culture medium (MOCM) and Chicken organoid culture medium (COCM).

Reagent	BCM	ICM	MOCM	COCM
DMEM/F12	100%	100%	100%	100%
10% FCS	10%	10%	10%	10%
Glutamax	1%	1%	1%	1%
sodium pyruvate	1mM	1mM	1mM	1mM
Non-Essential Amino Acids	1mM	1mM	1mM	1mM
Penicillin-streptomycin	10,000 U/mL	10,000 U/mL	10,000 U/mL	10,000 U/mL
CHIRR99021		4.3 µM	4.3 µM	4.3 µM
Y27632		10 µM	10 µM	10 µM
Valproic acid		0.78 mM	0.78 mM	0.78 mM
DMH-1		1.3 µM	1.3 µM	1.3 µM
Mouse-specific Wnt3a			1:10	
Mouse-specific Rspo1			1:10	
FOXO1-inhibitor AS1842856				0.2 µM
Prostaglandin E2				7.09 µM
Chicken-specific WNT3				1:5
Chicken-specific RSPO1				1:5

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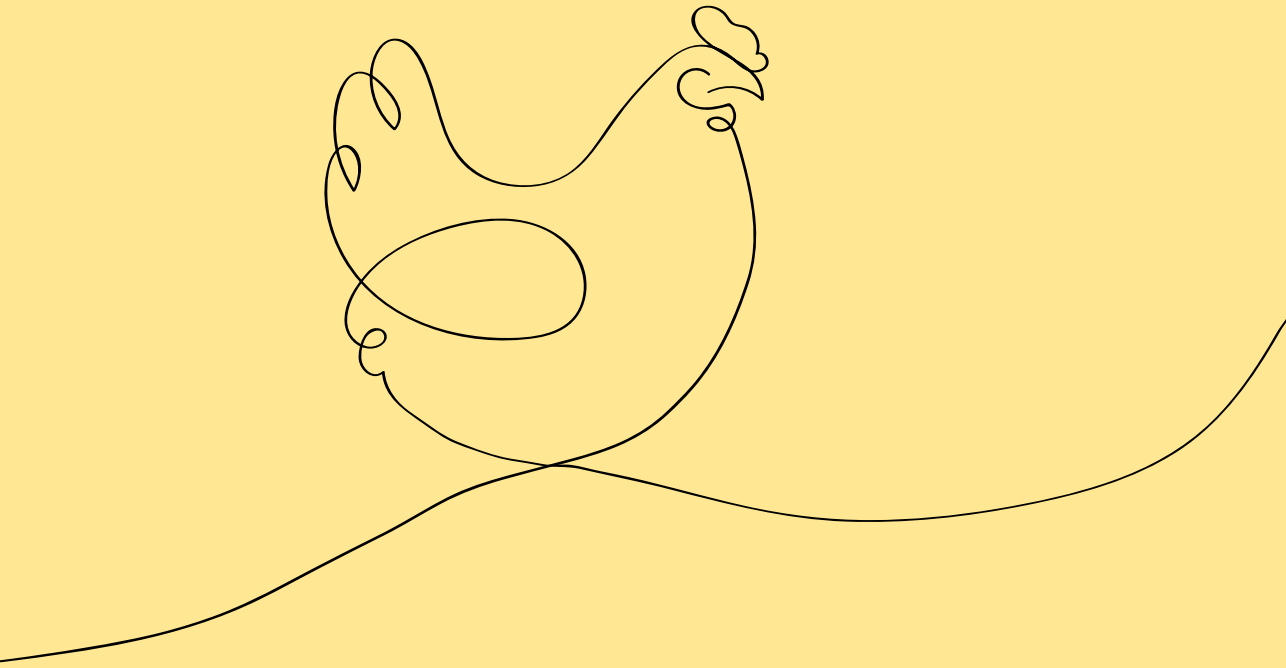
* Contributed equally to this work

Keywords: microbiota, cecum, in-vitro model, broilers, CALIMERO-2, IMMP, pectins, prebiotics

Submitted

Chapter 4

Characteristics of carbohydrates determine the shape of the gut microbiota in a chicken cecal *in-vitro* model



Abstract

The intestinal microbiota is crucial for maintaining intestinal and overall animal health. Coccidiosis, a common intestinal disease caused by *Eimeria* protozoa, poses a significant economic burden on poultry farming. It increases the risk of necrotic enteritis, an inflammatory intestinal disease caused by pathogenic *Clostridium perfringens (C. perfringens)* strains, which disrupt the gut microbiota. To promote and sustain a stable state of the intestinal microbiota, the addition of certain carbohydrates to the feed can be beneficial for the health of chickens. We compared the effects on microbiota composition and metabolites production during fermentation of isomalto/malto-polysaccharides (IMMP) and three different pectins (P), against a positive control, mannan-oligosaccharide (MOS), using the fermentation system Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2). CALIMERO-2 mimics fermentation in healthy ceca, and by spiking the model with *C. perfringens*, we aimed to mimic fermentation in diseased chicken ceca. Structural analysis revealed minor differences in monosaccharide composition and molecular weight among the pectins. Notably, P1 displayed a low degree of methyl-esterification (DM=26), while P2 and P3 had DM 63. Beta-diversity analysis demonstrated significant similarities between P2 and P3. *Bacteroidetes* was the dominant phylum, except for SIEM and MOS., where *Firmicutes* prevailed. Beneficial bacteria particularly *Lactobacillus,* remained stable across all samples. P1 exhibited significantly lower total short chain fatty acids (SCFAs) production compared to SIEM, IMMP, and P3, while IMMP closely resembled MOS, particularly in terms of butyrate level. This study advances our comprehension of the fermentability and structural impact of diverse carbohydrates on the broiler gut microbiota. Our findings underscore the potential of IMMP and pectins to promote intestinal health in poultry, warranting further investigations to optimize its inclusion in chicken feed.

4.1 Introduction

The chicken gastrointestinal tract is colonized by approximately 10^{13} bacteria, and the densest and most diverse population is found in the cecum (1-3). The intestinal microbiota plays a crucial role in maintaining intestinal and animal health, and intestinal homeostasis. Dysbiosis of the intestinal microbiota has been linked to many enteric diseases in humans and animals (4). In broiler chickens, the intestinal disease coccidiosis caused by the protozoa *Eimeria*, is one of the most common diseases with large economic significance (5). Damage to the intestinal mucosa, caused by coccidiosis, predisposes for colonization and proliferation of *Clostridium perfringens (C. perfringens)*. Pathogenic *C. perfringens* strains can cause necrotic enteritis (NE), an intestinal inflammatory disease that causes a shift in microbiota composition (6-9). Subclinical and clinical NE also results in economic losses in the poultry industry, estimated to be 6 billion US dollars worldwide [3]. Antimicrobial drugs were commonly used for the treatment and prevention of NE. However, since the use of antibiotics has been linked to the development of drug-resistant bacteria, regulations to reduce antimicrobial use in livestock have increased the need for alternatives to control and prevent NE in broiler chickens (10).

Currently, there is much interest in maintaining or restoring a stable state of the intestinal microbiota, by adding potential prebiotics to chicken feed. Prebiotics are defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health" (11). The potential prebiotic effect of non-digestible carbohydrates is largely unstudied, especially in broilers. Non-digestible carbohydrates, such as mannan-oligosaccharides (MOS) have been reported to have several health benefits, for example, by competing for attachment sites with pathogens like *C. perfringens* preventing their colonization in the colon (12). Moreover, they can increase the population of commensal bacteria and thereby restore the dysbiotic microbiota, caused by *C. perfringens* (13-16). MOS, as fermentable dietary fiber, can also increase the total short-chain fatty acids (SCFAs) concentrations in the cecum which have been shown to improve intestinal morphology (17). Pectins are also known for their beneficial effects on the intestinal microbiota and for their metabolites upon fermentation in the cecum (18, 19). Pectins are quite diverse in their structural characteristics and their functionalities and can contribute to a variety of health benefits (20). Isomalto/malto-polysaccharides (IMMP), are soluble dietary fibers derived from potato starch and are produced by modification of starch with 4,6-α-glucanotransferease enzyme and consist of α-(1→4), and α-(1→6) linked glucoses (21-23). IMMP has been shown to have potential prebiotic effects in mammals, by significantly increasing the relative abundance of *Bifidobacterium* and *Lactobacillus* (24).

This study evaluated three selected citrus pectins and IMMP for their prebiotic effect on the microbiota composition and its produced metabolites in a healthy and diseased chicken *in-vitro* model. We used the Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2) (25), in which we mimicked *C. perfringens* overgrowth, to simulate NE, as the diseased model.

4.2 Material and methods

4.2.1 Intervention carbohydrates

In total, six interventions were used for the fermentation, of which SIEM and MOS served as controls. MOS, from *Saccharomyces cerevisiae*, was provided by Nutrition Sciences N.V., Drongen, Belgium. The other four carbohydrates consisted of Isomalto/Malto-Polysaccharide-87 (IMMP), obtained from potato starch (provided by Royal Avebe, Veendam, the Netherlands), and three different orange pectins, called P1, a low methyl-esterified pectin, P2 and P3, highly methyl-esterified pectins (provided by Nutrition Sciences N.V.). P1 was formed from the de-esterification of P2 by the supplier.

4.2.2 Collection of cecal samples and standardization

The cecal contents of broiler chickens (Ross 308) were obtained from slaughterhouse van der Linden Poultry Products B.V. (Beringe, the Netherlands), as previously reported (25). Briefly, the days before slaughter, the broiler chickens were fed a coccidiostat-free diet and were not treated with antibiotics. The cecal content was removed, cooled, transported under anaerobic conditions, and pooled under strictly anaerobic conditions in an anaerobic cabinet (Sheldon Lab- Bactron IV, Gomelius, OR, USA). A total amount of 945 g was 1:1 diluted with dialysis liquid [see section "Medium and reagents"] and 15% (w/v, final concentration) glycerol was added as a cryo-protective agent. The cecal samples were aliquoted (35 mL), snap-frozen in liquid nitrogen, and stored at -80°C.

4.2.3 Chicken ALIMEntary tRact mOdel-2

The Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2, Fig. S1) mimics the chicken ceca and has been described before (25). Shortly, this dynamic *in-vitro* system closely mimics the *in-vivo* situation by regulating the temperature (41°C), pH (6.6), and anaerobic environment in the chicken ceca. Moreover, the metabolites produced by the microbiota are continuously removed by dialysis of the lumen by making use of a semi-permeable membrane that functions as a dialysis system, simulating the uptake by the chicken's intestinal cells. The system was inoculated with the standardized anaerobic cecal microbiota of broiler chickens, obtained as described above, and the microbiota was fed with several carbohydrates as described in the next section.

4.2.4 Experimental setup

In total twelve duplicate experiments in CALIMERO-2 were performed. During each experiment, four independent fermentation units were run simultaneously in parallel. Simulated ileal-efflux medium (SIEM; (26)) was taken along as a control medium and MOS served as a positive control. The experiments started with the inoculation of 60 mL of the standardized anaerobic cecal microbiota, to which 90 mL of pre-reduced dialysis liquid was added (Fig 1). After inoculation, an adaptation period of 16 h followed, for the microbiota to adapt to the system while being fed with SIEM (2.5 mL/h). Thereafter, the SIEM feeding stopped for 3 h, in which the microbiota fermented the remaining carbohydrates from SIEM (starvation period). After starvation, instead of the standard carbohydrates in SIEM (25) the intervention carbohydrates were added to SIEM, and these different media were subsequently continuously supplemented for 72 h with a constant flow (2.5 mL/h). There were four sample time points, 0 h (after starvation, and just before supplementation with the intervention carbohydrates), 24 h, 48 h, and 72 h, from the lumen and dialysate to analyze the microbial composition (lumen) and metabolite composition (lumen and dialysate) over time. All samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. After 24 and 48 h, a total volume of 25 mL of lumen sample was removed from the system to simulate the passage of chyme and replaced with pre-reduced dialysis liquid to keep a constant volume. To mimic *C. perfringens* infections, all runs were also performed with *C. perfringens* spiked into the system [see section 2.5].

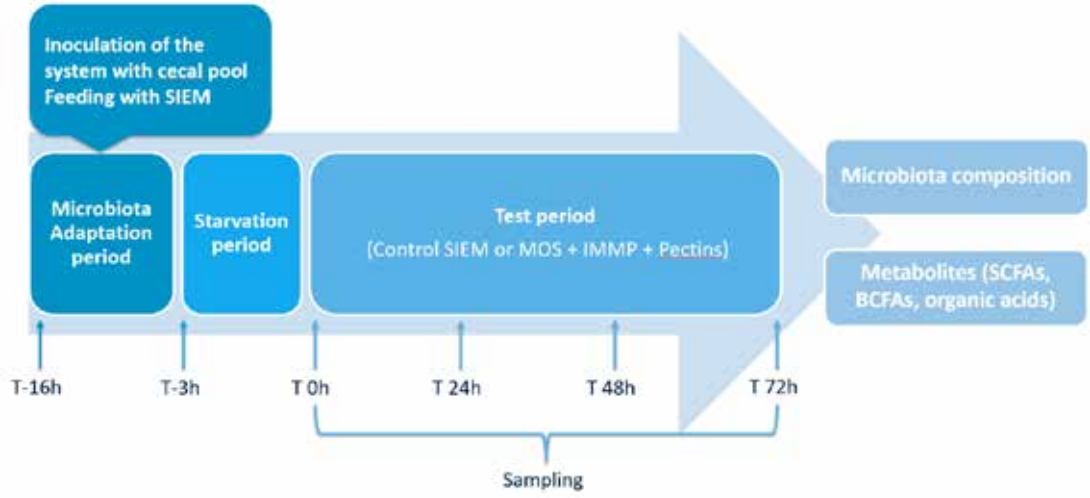


Figure 1. Flow chart of the experimental setup of CALIMERO-2. Experiments started with inoculation of the system, with or without spiking of *Clostridium perfringens*, and were followed by an adaptation period of 16 h, in which the system was fed with SIEM. After the adaptation, a period of 3 h of starvation followed, in which the system is not fed. At time point 0, test products; IsoMalto/MaltoPolysaccharide (IMMP), three pectins, Mannan-oligosaccharide (MOS), and SIEM were added to the system (7.5 g/day) and every 24 h samples were collected from the lumen and the dialysate.

4.2.5 Strain and preparation of *Clostridium perfringens*

In this study a toxin type A, *netB*-positive *Clostridium perfringens* strain (GD-Animal health, Deventer, the Netherlands) was used. To prepare the inoculum for spiking CALIMERO-2, *C. perfringens* was cultured on liver broth agar base (Biotrading, Mijdrecht, the Netherlands) followed by 48 h anaerobic incubation at 37 °C. Subsequently, a single colony was transferred to 10 mL liver broth medium, and CALIMERO-2 was inoculated with 1 mL of *C. perfringens* at $1.5*10^8$ colony-forming units (CFU)/mL to mimic *C. perfringens*-infected chickens.

4.2.6 Medium and reagents

4.2.6.1 Dialysis liquid

Dialysis liquid consisted of the following compounds (content gram per liter): 2.5 $K_2HPO_4 \cdot 3H_2O$, 4.5 NaCl, 0.005 $FeSO_4 \cdot 7H_2O$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.45 $CaCl_2 \cdot 2H_2O$, 0.05 ox bile (Sigma, Zwijndrecht, the Netherlands), and 0.4 cysteine.HCl, plus 1 mL of vitamin mixture containing (mg per liter): 1 menadione, 0.5 vitamin B12, 2 D-biotin, 10 pantothenate, 5 *p*-aminobenzoic acid, 4 thiamine, and 5 nicotinamide acid.

4.2.6.2 Standard Ileal Efflux Medium

SIEM was prepared as described by De Souza *et al.* (26) with the following components (g per liter): 9 citrus peel pectin, 9 beechwood xylan, 9 larch arabinogalactan, 9 potato amylopectin, 74.6 potato starch, 31.5 Tween 80, 43.7 casein, 0.7 ox-bile, 43.7 bactopepton, 4.7 $K_2HPO_4.3H_2O$, 0.009 $FeSO_4.7H_2O$, 8.4 NaCl, 0.8 $CaCl_2.2H_2O$, 0.7 $MgSO_4.7H_2O$, 0.02 hemin, and 0.3 cysteine.HCl, plus 1.5 mL of the vitamin mixture. The pH was adjusted to 6.6 to mimic the chicken ceca and 60 mL/day was administered. Standard carbohydrates in SIEM were replaced with 7.5 g of MOS, IMMP, or pectins.

4.2.7 Analysis of the carbohydrates

4.2.7.1 Analysis of IMMP

The IMMP used in this study was named after its percentage of total α-(1→6) glucosyl linkages. The total α-(1→6) linked glucosyl content, consisting of both α-(1→6) and α-(1→4) linked glucosyl residues, and was determined by hydrogen-1 nuclear magnetic resonance (1H NMR) spectroscopy, with the methodology and results already published previously (21). IMMP-87 (87% α-(1→6) linkages) originates from potato starch (Royal Avebe,) modified with *L. reuteri* 121 GTFB 4,6-α-glucanotransferase and pullulanase (23).

4.2.7.2 Characterization of pectins

Neutral monosaccharide composition was determined after Seaman hydrolysis and derivatization into alditol acetates. Briefly, samples were pre-treated for 1 hour at 30 ˚C with 72% (w/w) H_2SO_4, and further submitted to acid hydrolysis with 1 M H_2SO_4 (3h, 100 ˚C). The released neutral sugars were derivatized into their alditol acetates to be analyzed by gas chromatography (GC) (27). Inositol was used as an internal standard. The acidic monosaccharide, galacturonic Acid (GalA) was analyzed spectrophotometrically using the *m*-hydroxydiphenyl autoanalyser (28, 29). Saponification of pectins was performed (NaOH 0.1 M; 1 h at 4 ˚C, followed by 23 h at room temperature) to measure the degree of methyl-esterification (DM). The methanol released by samples was analyzed by head-space GC and DM was calculated (30).

4.2.7.3 Enzymatic hydrolysis of Pectins

Hydrolysis of pectin samples was conducted as follows: samples were dissolved in 50 mM sodium acetate buffer (pH 5.2), incubated with agitation at 40 ˚C with the addition of a pectin lyase (PL, EC 4.2.2.10; ID: 1043) from *Aspergillus niger* for 6 h, next *endo*-polygalacturonase (endo-PG, EC 3.2.1.15; ID 1027) from *Kluyveromyces fragilis* and incubated for another 18 h. After incubation, enzymes were inactivated at 100 ˚C for 10 min. Pectin digests were centrifuged and supernatant were analyzed by a described in the sections below (20).

4.2.7.4 High-performance size exclusion chromatography (HPSEC))

HPSEC (Ultimate 3000 system - Dionex, Sunnyvale, CA, USA) was used to analyze pectins before and after enzymatic hydrolysis for their molecular weights (M_w). Four TSK-Gel super AW columns were used in the following order: guard column (6 mm ID × 40 mm) and columns 4000, 3000, and 2500 SuperAW (6 mm × 150 mm) (Tosoh Bioscience, Tokyo, Japan) at 55 ˚C. As eluent, filtered (0.4 µm) 0.2 M $NaNO_3$ was used at a flow rate of 0.6 mL/min. Elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan) and pectin standards were used to estimate M_w distribution (20).

4.2.8 Microbial DNA extraction

DNA was extracted from 250 µl of the lumen samples taken during the CALIMERO-2 experiments as described before using the 2x300 bp protocol (25). Briefly, 1000 µl InhibitEx buffer (Qiagen, Venlo, the Netherlands) was added and samples were transferred to Precellys tubes containing 0.5 mm microbeads and treated in a bead beater (Precellys 24, Bertin Technologies, Montigny le-Bretonneux, France). Thereafter, the samples were incubated at 95°C for 7 min and centrifuged at 13500 x g for 1 min to pellet stool particles and cell wall fragments. From this point on, the QIAamp DNA stool Mini kit (Qiagen) was used following the manufacturer's protocol from step 4 onwards.

4.2.9 Bacterial composition of the cecal microbiota

The composition of the cecal bacteria was evaluated by 16S rRNA gene sequencing using Illumina Miseq (Illumina, San Diego, CA, United States) as described before (25). 16S rRNA gene amplicon libraries of the V3-V4 region were generated following the 16S Metagenomic Sequencing Library

preparation manual of Illumina Miseq systems using the Nextera XT kit, using a 2-step PCR. A mock community was run along with the samples to guarantee sequence quality.

4.2.10 Short-Chain Fatty Acids, Branched-Chain Fatty Acids, and Organic Acids Quantification in Lumen and Dialysate samples

SCFAs, branched-chain fatty acids (BCFAs), and organic acids were quantified through ion exclusion chromatography by Brightlabs (Venlo, the Netherlands). Briefly, an 883 Ion Chromatograph was used (IC; Metrohm, Switzerland), with a Transgenomic IC Sep ICE-ION-300 column (30 cm length, 7.8 mm diameter, and 7 mm particles) and a MetroSep RP2 Guard column. The mobile phase consisted of 1.5 mM aqueous sulfuric acid and the column had a flow rate of 24 mL/h and a temperature of 65°C. The acids were detected using suppressed conductivity detection. Samples were centrifuged at 13500 g for 10 min, and the clear supernatant was filtered through a 0.45 mm PFTE filter and diluted with mobile phase (for lumen 1:5, for dialysate 1:2). Ten µl were loaded on the column by an autosampler 730 (Metrohm). Molecules were eluted according to their pKa.

4.2.11 Bioinformatics analysis

Microbiota bioinformatics was performed with QIIME2 2019.4 (31). Shortly, the raw sequencing data were demultiplexed, quality filtered, and denoised by using the q2-demux plugin and DADA2 (32). In the DADA2 step, the first 9 bases were trimmed off and for the forward reads there was an additional truncation at 290 base pairs and for the reverse reads, this was at 280 base pairs. Taxonomy was assigned using the SILVA 128 16S rRNA gene reference database. Further analysis was conducted with R (version 4.2.0) within R-studio, with packages microViz (33), microbiome (34), and phyloseq (35) for analysis and visualization of microbiome sequencing data. Microbial alpha-diversity was determined using observed number of OTUs and the Shannon index. Beta-diversity was determined using weighted and unweighted UniFrac distance metrics. Differences in taxonomic profiles were analyzed using Statistical Analysis Metagenomic Profiles (STAMP) software v.2.1.3 with Kruskal-Wallis test, followed by a Tukey-Kramer post-hoc test (36). P values were corrected using the Benjamini-Hochberg method and a corrected P value < 0.05 was considered significant. Statistical significance of alpha-diversity was assessed with Kruskal-Wallis test, followed by a Dunns' post-hoc test. For the statistical analysis of the beta-diversity for the different carbohydrates and comparison with the controls permutational multivariate analysis of variance (PERMANOVA) (37) was performed. The statistical analysis of the remaining data was performed using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, USA). The metabolites were assessed with two-way ANOVA analysis, followed by Bonferroni's post-hoc test with selected pairs. For the relative abundance, phylum, and genus levels statistical significance was determined, using multivariate analysis for the different types of carbohydrates and a Welch's t-test for the two groups healthy and diseased (the latter for the samples spiked with *C. perfringens*).

4.3 Results

4.3.1 Composition and structural properties of Pectins and IMMP

The three different pectins and IMMP were analyzed regarding their monosaccharide composition, molecular weight distribution, and DM (only pectins) (table 1). The pectins are mainly composed of Galacturonic Acid (GalA), typically present in homogalacturonan (HG) type pectins, with slight differences in neutral sugar content. The molecular weight (M*w*) distribution among the samples ranged from 115 kDa to 136 kDa. The DM, defined as the percentage of methyl-esters distributed within GalA residues over the HG backbone, was 63% for pectins P2 and P3, and 26% for pectin P1.

Besides the similarities in sugar content and M*w*, pectins P2 and P3 are featured as highly methyl-esterified (HMP > 50% DM) and pectin P1 is low methyl-esterified (LMP < 50% DM).

Table 1. Structural characteristics of pectins and isomalto/malto-polysaccharide (IMMP)

Chemical features of Pectins and IMMP

Sample	**Rha**[a]	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**GalA**[b]	**Total**	**Mw**	**DM**
				mol%				(w/w%)[c]	(kDa)[d]	(%)[e]
P1	0.7	1.7	0.2	0.6	7.0	0.4	89.4	85.0	115	26
P2	1.0	2.2	0.2	0.9	7.7	0.7	87.4	76.9	131	63
P3	0.8	3.5	0.1	1.1	3.7	0.6	90.2	78.7	136	63
IMMP						100		95	10	-

[a] Rha: Rhamnose; Ara: Arabinose; Xyl: xylose; Man: Mannose; Gal: Galactose; Glc: Glucose; GalA: Galacturonic Acid.

[b] Determined spectrophotometrically using the m-hydroxydiphenyl automated skalar method.

[c] Total carbohydrate content anhydrous in w/w%.

[d] Average molecular weight (Mw) determined by HPSEC based on the pectin standards.

Average Mw for IMMP was determined by Multi-angle light scattering detector.

[e] Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample. Pectin presenting DM lower than 50% is considered low methyl-esterified and DM above 50% is considered highly methyl-esterified.

To have a better understanding of the methyl-esterification pattern over the pectin backbone, the analysis of distribution of non-methyl-esterified GalA residues over the pectin backbone is useful. As a first step, the pectins were enzymatically degraded with pure, and well-defined enzymes polygalacturonase (endo-PG) and pectin lyase (PL) and analyzed by HPSEC-RI (20). PL can cleave glycosidic linkages in vicinal methyl-esterified GalA units by introducing a double bond, while endo-PG requires four consecutive non-esterified GalA units to act. Figure 2 shows the degradation pattern of the pectins. The three different pectins demonstrated rather similar M*w* before PL and Endo-PG digestion. After digestion, pectins were degraded into oligomers. Figure 2 shows that the HM pectins P2 and P3 had a very similar degradation pattern indicating a similar methyl ester level and distribution. For the LM pectin P1, the peak shape in the oligomer region (12.3 – 14.5 min) reflects the present of different oligomeric degradation products and shows indeed a different methyl ester level and distribution (20).

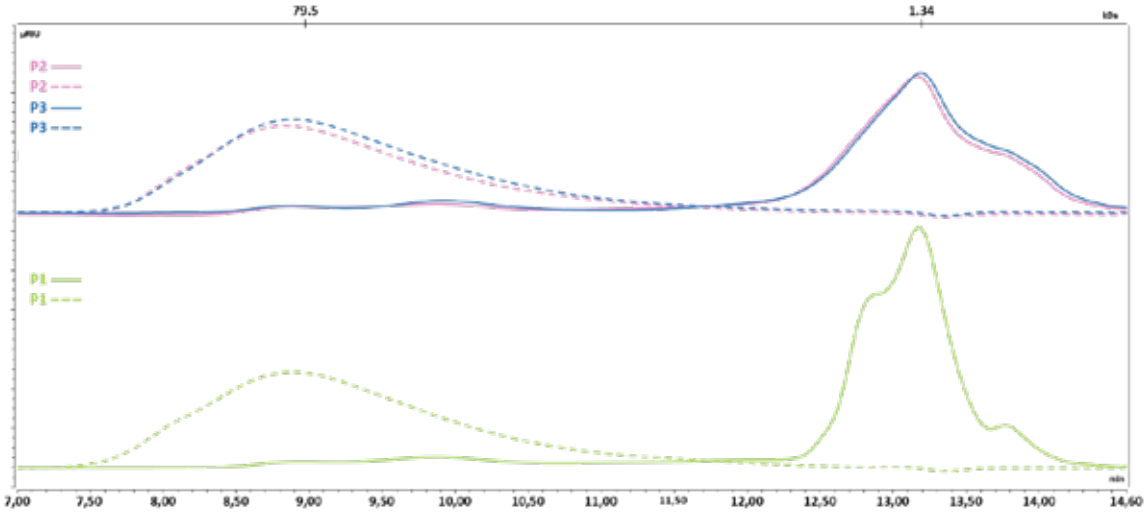


Figure 2. HPSEC elution pattern of pectins P1, P2 and P3 before (dots) and after (straight line) pectin lyase and endo-polygalacturonase digestion. Molecular weight is indicated as kDa.

4.3.2 Alpha- and beta-diversity of the cecal microbiota in CALIMERO-2

The effect of the different carbohydrates on microbial diversity was assessed by comparing alpha- and beta-diversity between the three different pectins and IMMP, and to the controls SIEM and MOS. Also, the diversity for the diseased model samples from CALIMERO-2, in which necrotic enteritis was mimicked by spiking in *C. perfringens*, was compared to the healthy model (Fig. 3). For the alpha-diversity, both the Shannon index (Fig. 3A) and the observed Operational Taxonomic Units (OTUs) were calculated for the fermented carbohydrates every 24 h up to 72 h (Fig. 3B). The Shannon index and the observed OTUs indicate a significant decrease in microbiota for SIEM and MOS in the *C. perfringens* inoculated model, compared to the corresponding healthy model (Fig. 3A-B). This difference between samples from the healthy and diseased models was not observed for the other substrates. When comparing the different types of carbohydrates in the healthy groups, the Shannon index shows that SIEM results in more diverse microbiota communities compared to P1 ($P < 0.05$). Also, MOS produced more diverse microbiota communities compared to IMMP ($P < 0.05$), P1 ($P < 0.001$), P2 ($P < 0.05$), and P3 ($P < 0.01$). Based on the observed OTUs, a similar pattern was found, but here SIEM showed higher observed OTUs compared to P2 instead of P1.

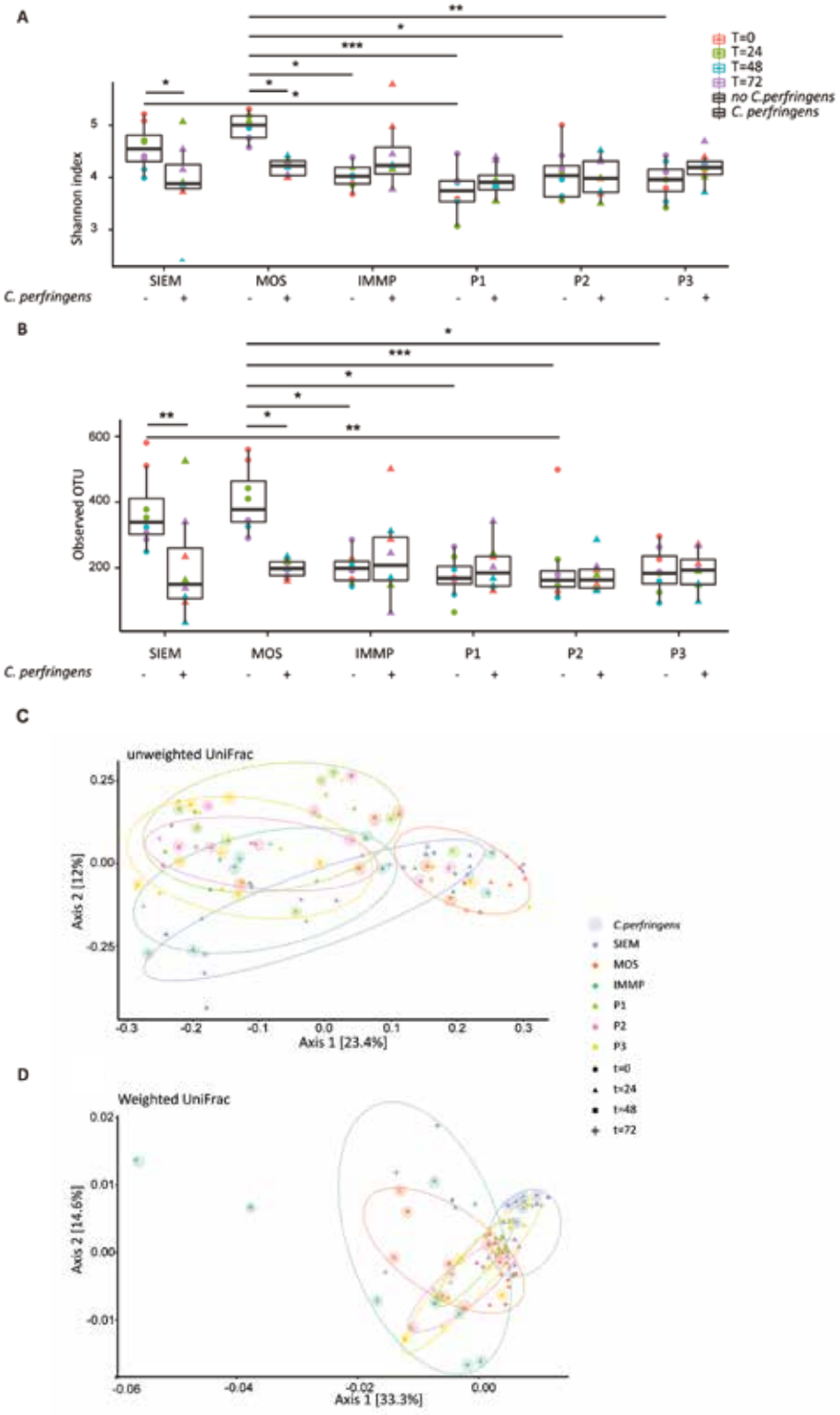

A
T=0
T=24
T=48
T=72
no C.perfringens
C. perfringens
Shannon index
SIEM
MOS
IMMP
P1
P2
P3
C. perfringens
B
Observed OTU
C
unweighted UniFrac
Axis 2 [12%]
Axis 1 [23.4%]
C.perfringens
SIEM
MOS
IMMP
P1
P2
P3
t=0
t=24
t=48
t=72
D
Weighted UniFrac
Axis 2 [14.6%]
Axis 1 [33.3%]

Figure 3. Bacterial diversity for the alpha-diversity, **A** Shannon indices, and **B** observed operational taxonomic units (OTUs) were calculated to determine the abundance and evenness of the species present in the Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2) samples. Points are colored by the time points samples were taken. SIEM = Simulated ileal-efflux medium, MOS = mannan oligosaccharides, IMMP = isomalto/malto-polysaccharide, P = pectin. – represent the healthy model, and + the diseased model, in which *Clostridium perfringens* was added. Data are presented as mean (n=2) ± SD. (* P < 0.05; ** P < 0.01; *** P < 0.001). The beta-diversity is represented as principal coordinate analysis (PcoA) using the **C** unweighted UniFrac and **D** weighted UniFrac for the cecal microbiota of chickens from the CALIMERO-2 model. Points are colored by carbohydrate intervention, and the shape of points represent different time points samples were taken. Samples in which *Clostridium perfringens* was added, are marked with a grey sphere.

The similarity in community structure between samples was studied with the beta-diversity metrics unweighted and weighted UniFrac measures. Principal coordinate analysis (PcoA) of unweighted UniFrac demonstrates an overlap of the two pectins P2 and P3. A significant difference in beta-diversity is observed for MOS compared to IMMP, P2, and P3 (P values, see Table 2, Fig. 3C). PcoA of the weighted UniFrac also shows an overlap of P2 and P3, indicating the high similarity of these substrates. P1 shows a significant difference compared to MOS, IMMP, and P3 (Table 2; Fig. 3D).

Table 2. P values of PERMANOVA on unweighted and weighted UniFrac. * P < 0.05. SIEM = Simulated ileal-efflux medium, MOS = mannan oligosaccharides, IMMP = isomalto/malto-polysaccharide, P = pectin.

Unweighted UniFrac

	SIEM	**MOS**	**IMMP**	**P1**	**P2**	**P3**
SIEM	-	0.120	0.779	0.522	0.626	0.993
MOS	-	-	0.008*	0.084*	0.022*	0.002*
IMMP	-	-	-	0.434	0.610	0.484
P1	-	-	-	-	0.753	0.178
P2	-	-	-	-	-	0.232
P3	-	-	-	-	-	-

Weighted Unifrac

	SIEM	**MOS**	**IMMP**	**P1**	**P2**	**P3**
SIEM	-	0.640	0.090	0.142	0.650	0.380
MOS	-	-	0.110	0.027*	0.336	0.583
IMMP	-	-	-	0.020*	0.056	0.180
P1	-	-	-	-	0.377	0.015*
P2	-	-	-	-	-	0.191
P3	-	-	-	-	-	-

4.3.3 Changes in composition at the phylum and genus taxonomic levels

The microbiota composition was determined at time point 0 and after 72 h of fermentation in CALIMERO-2 in which the microbiota was exposed to carbohydrates. Phylum and genus levels, showed small changes in microbiota between the different experimental groups after 72 h fermentation (Fig. 4A). *Bacteroidetes* was the most abundant phylum for most of the groups, except for SIEM and MOS in the healthy model, in which *Firmicutes* was dominant. Within the phylum *Firmicutes*, the genera *Lachnospiraceae UCG-010*, *Anaerofilum*, and *Intestinimonas* were significantly higher in the healthy, compared to the diseased samples ($P < 0.05$; Fig. S2). At genus level, *Bacteroides* was significantly lower in the MOS and SIEM fermentations compared to *Bacteroides* for the other carbohydrates in the healthy model after 72 h fermentation (Fig. 4C). Moreover, for MOS in the diseased model *Bacteroides* was also higher compared to MOS in the healthy model (Fig. 4C). The genus *Lachnoclostridium* was significantly higher in the MOS samples compared to the other substrates (Fig. 4D). The pectins P1 and P2 showed an increase of the genus *Akkermansia* compared to SIEM and MOS in the healthy model (Fig. 4E). *Akkermansia* also increased in response to the addition of pectin P1 in the diseased model. *Lactobacillus* levels were maintained for all carbohydrates, except for MOS, that demonstrated a significant increase of *Lactobacillus* in the healthy model (Fig. 4F). *Bifidobacterium* showed the same relative abundance for all carbohydrates, only for SIEM in the diseased model there was a significant decrease (Fig. 4G).

4.3.4 Cecal production of SCFAs in CALIMERO-2

Acetate, propionate, and butyrate are the three main SCFAs produced during the *in-vitro* fermentation of carbohydrates. Lactate and succinate, the intermediate products of carbohydrate fermentation, were present in very low concentrations compared to SCFA concentrations in the samples. The total SCFAs is the sum of acetate, propionate, and butyrate. The addition of *C. perfringens* did not affect metabolite production, compared to the healthy model (Fig S3). Figure 5A shows the total SCFAs over time for the different intervention carbohydrates. Fermentation of P1 yielded a lower amount of total SCFAs when compared to SIEM, IMMP, and P3 after 72 h of fermentation ($P < 0.05$). When comparing the carbohydrates on the separated SCFAs, acetate showed a significantly higher cumulative production after 72 h of fermentation for P3 ($P < 0.01$) and IMMP ($P < 0.05$) compared to MOS. P2 did not show any significant difference. P3 also lead to significantly higher acetate compared to P1 (Fig. 5B). Butyrate was also significantly increased under SIEM and MOS fermentation at 48 h and 72 h, compared to the three pectins (Fig. 5C). After 72 h of fermentation, this was also shown for IMMP compared to P1. Propionate showed significantly higher production after 48 h of SIEM compared to MOS ($P < 0.01$) (Fig. 5D). Propionate production was also higher for SIEM compared to P1, P2, and P3 ($P < 0.001$). Propionate demonstrated similar production on IMMP and SIEM, and they were both significantly higher compared to MOS ($P < 0.05$) and P1, P2 and P3 ($P < 0.001$). After 72 h also MOS produced more propionate compared to P1 ($P < 0.05$). Both lactate and succinate did show a significant difference at 24 h and 48 h for SIEM compared to the pectins and IMMP, which vanished after 72 h (Fig. 6). The negative cumulative production indicates that these are converted into the other SCFA (primarily propionate and butyrate). The amount of iso-butyrate, one of the BCFAs, was significantly higher upon providing SIEM and MOS, compared to the three pectins at 48 h and 72 h. For the other measured BCFA, iso-valerate, MOS had the highest cumulative amount, and this was significantly different from the other four carbohydrates at 48 h, and after 72 h also significantly higher compared to SIEM. SIEM only showed a significant increase compared to P1 and P2 at time points 48 and 72 h.

4.4 Discussion

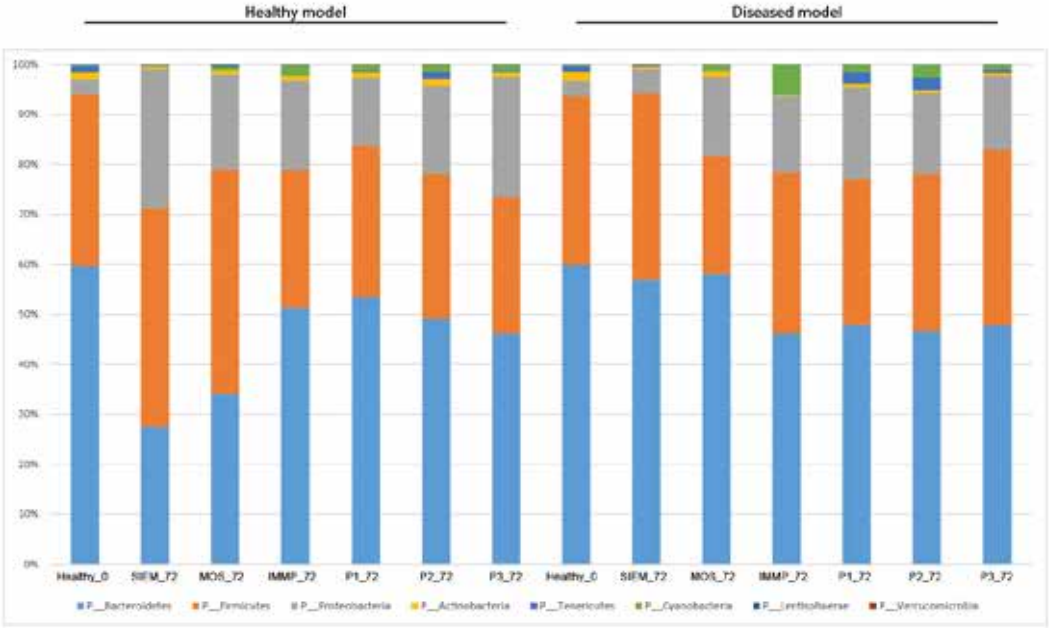
In this study, we aimed to investigate the potential prebiotic effect of three citrus pectins and the ten times smaller polymer IMMP. To this end, it is crucial to know the chemical features of these carbohydrates to increase understanding of the correlation between structure and their fermentability. We first analyzed and compared the monosaccharide composition, molecular weight distribution, and DM among the three pectins. Even though there are similarities, these pectins distinctively affected the gut microbiota. The different monosaccharides, DM, and glycosidic linkages present in the pectin backbone, each pose a different challenge to their fermentation by microorganisms. The molecular machinery needed for fermentation is structure-specific, thus the complex and diverse structure of pectins may require many steps for enzymatic catalysis for pectin degradation and SCFA production (38). Less complex carbohydrates and with lower molecular weight, such as IMMP and MOS, may be degraded in less enzymatic steps by a broad range of bacteria due to the common molecular machinery (39). Consequently, both the different pectins as well as oligosaccharides can distinctively affect the gut microbiota.

Pectins P1 and P2 were quite similar regarding their monosaccharide composition, due to de-esterification of P2 to form P1. Albeit P3 has a higher percentage of arabinose and a lower percentage of galactose when compared to P1 and P2. P3 has a significantly higher cumulative production of acetate compared to P1 and also has a higher propionate concentration compared to the other two pectins after fermentation. The higher percentage of arabinose has been linked to an increase of acetate and propionate producers in earlier research (40).

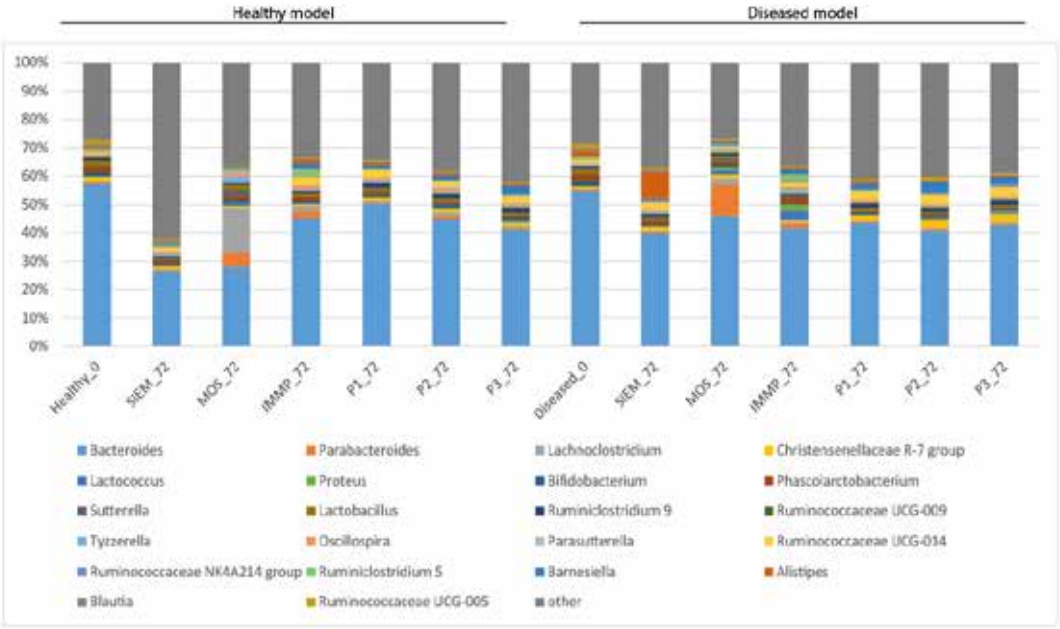
We compared the effects of the pectins and IMMP on bacterial composition and metabolite production. In these comparisons we also took along a fermentation control SIEM and the prebiotic compound MOS, the latter of which has been studied widely for its beneficial effects on intestinal health in chickens. Firstly, SIEM and MOS induced an increase in gut microbiota diversity and also promoted a higher number of observed OTUs in the healthy model. However, SIEM and MOS were not able to prevent the loss of microbes in numbers in the diseased model. This reduction of the alpha-diversity (species richness) of the cecal microbiota can be linked to the addition *C. perfringens*, which normally causes these shifts in the intestinal microbiota, and reduces the alpha-diversity within the samples (4). This might take longer than 72 h of fermentation, which explains the initial decrease of the alpha-diversity. The control SIEM is rich in several carbohydrates, such as arabinogalactan and xylan, and might have promoted growth of a wider range of different bacteria. The diversity in both the healthy and the diseased group is lower for the pectins and IMMP compared to SIEM. For pectin, this could be related to the fact that the gut microbiota might need to adapt to a pectin-degrading microbiota, thus fermentation can be slower, due to the lack of bacterial enzymes to degrade pectins (39). For IMMP the lower diversity compared to SIEM and MOS might be related to the delayed and slow-fermentation behavior compared to other prebiotics, because of the presence of the α 1,6 glycosidic linkages in IMMP (24, 41).

Beta-diversity (community structure) was significantly different for MOS compared to IMMP, P2, and P3, which can be explained by the changes in microbiota composition towards a microbiota that can degrade the complex structures of the pectins and IMMP. P1, however, showed significant differences compared to MOS, IMMP, and P3, which could be related to its low methyl-esterified feature. The microbiota composition was determined, and small differences between the experimental carbohydrates were shown at the phylum and genus levels. MOS is known for creating a diverse gut microbiota, by supporting the growth of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, and decreasing the presence of pathogens such as *C. perfringens* (12-16, 42, 43). *Lactobacillus* was also increased in CALIMERO-2 in the MOS-treated samples. However, when *C. perfringens* was added to the

A


Healthy model
Diseased model
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Healthy_0
SIEM_72
MOS_72
IMMP_72
P1_72
P2_72
P3_72
Healthy_0
SIEM_72
MOS_72
IMMP_72
P1_72
P2_72
P3_72
P__Bacteroidetes
P__Firmicutes
P__Proteobacteria
P__Actinobacteria
P__Tenericutes
P__Cyanobacteria
P__Verrucomicrobia

B


Healthy model
Diseased model
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Healthy_0
SIEM_72
MOS_72
IMMP_72
P1_72
P2_72
P3_72
Diseased_0
SIEM_72
MOS_72
IMMP_72
P1_72
P2_72
P3_72
Bacteroides
Parabacteroides
Lachnoclostridium
Christensenellaceae R-7 group
Lactococcus
Proteus
Bifidobacterium
Phascolarctobacterium
Sutterella
Lactobacillus
Ruminiclostridium 9
Ruminococcaceae UCG-009
Tyzzerella
Oscillospira
Parasutterella
Ruminococcaceae UCG-014
Ruminococcaceae NK4A214 group
Ruminiclostridium 5
Barnesiella
Alistipes
Blautia
Ruminococcaceae UCG-005
other

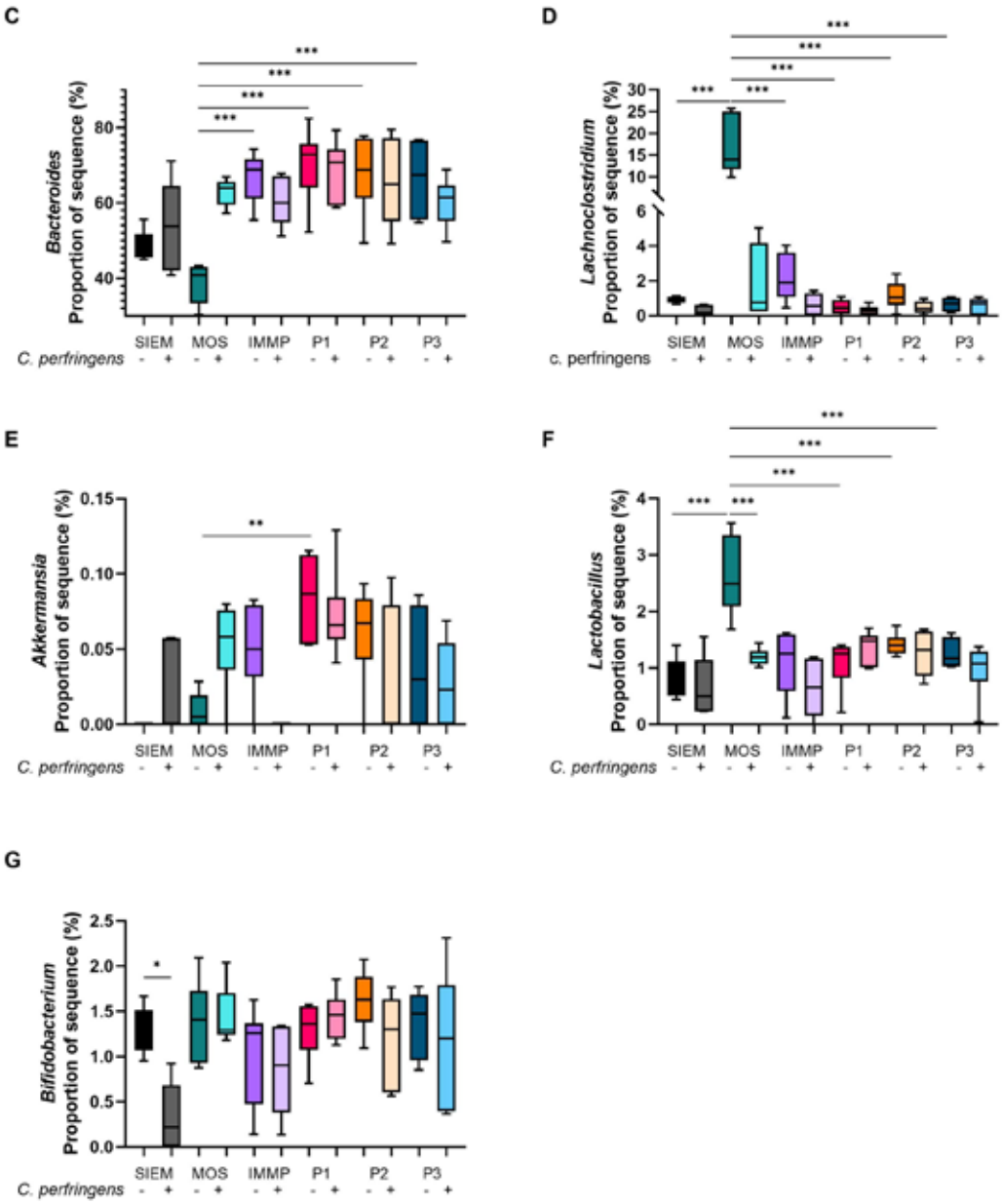


Figure 4. Bacterial composition. Relative abundance of bacterial **A** phyla and **B** genera in Chicken ALIMEntary tRact mOdel-2 samples in which different carbohydrates were added. **C-G** Relative abundance of genera, *Bacteroides*, *Lachnoclostridium*, *Akkermansia*, *Lactobacillus* and *Bifidobacterium*, that showed significant differences between the intervention carbohydrates. SIEM = Simulated ileal-efflux medium, MOS = mannan oligosaccharides, IMMP = isomalto/malto-polysaccharide, P = pectin. – *C. perfringens* represent the healthy model, and + *C. perfringens* the diseased model, in which the pathogen was added. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$

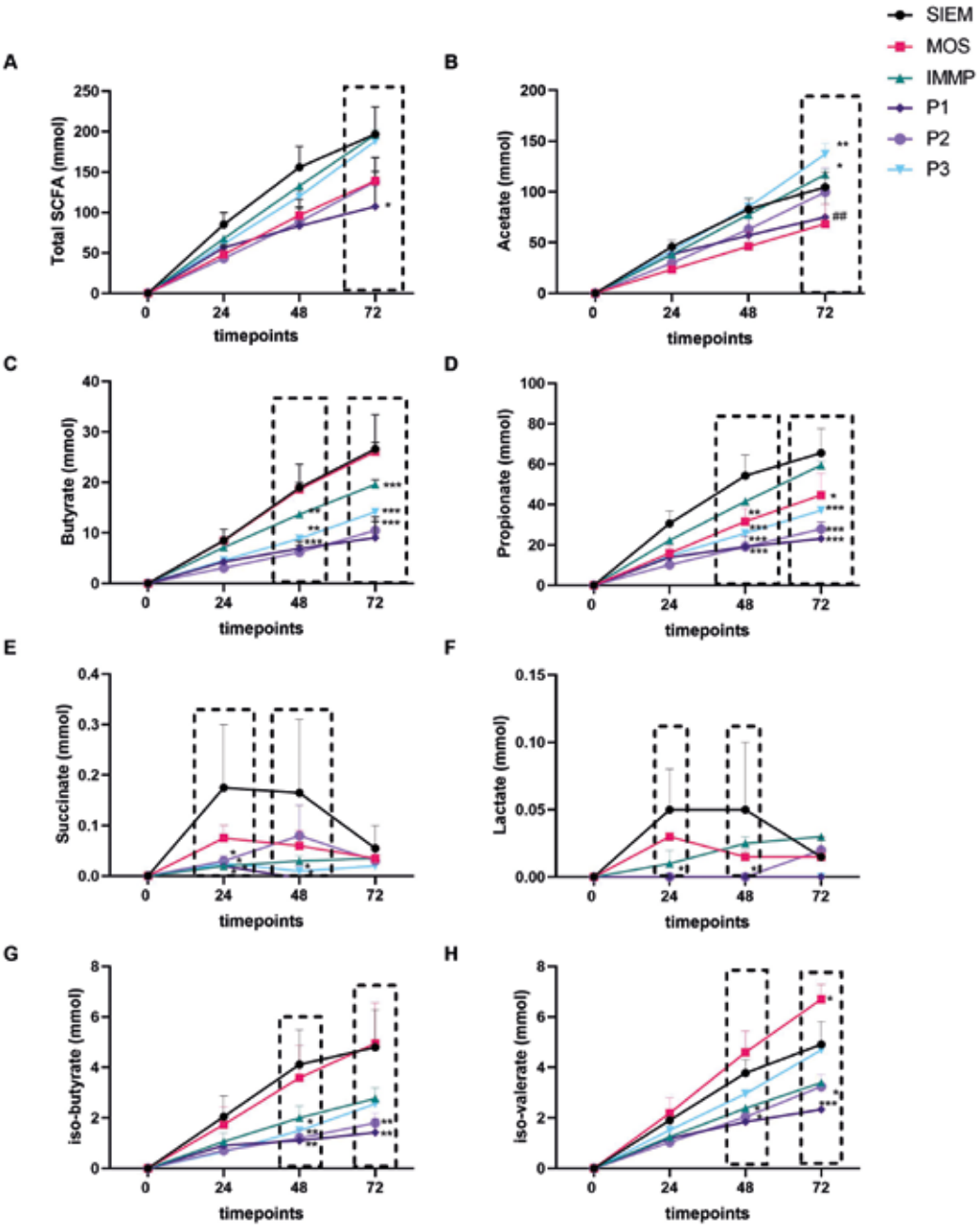


Figure 5. Cumulative short chain fatty (SCFAs) and branched chain fatty acid levels mmol for the different carbohydrates produced during *in-vitro* fermentation in the Chicken ALIMEntary tRact mOdel-2. Total SCFAs are the sum of acetate, propionate, and butyrate. Error bars represent the SEM, n=2. The color of the line represents the carbohydrate interventions. Significant difference are shown in the marked area and those marked with * are compared to MOS, and marked with # are compared to SIEM. (P < 0.05, ** P < 0.01, *** P < 0.001)

system, Lactobacillus was significantly decreased in the MOS group. Bifidobacterium was present in all the samples. An observation that stood out at the phylum level, was that all samples, except the MOS and SIEM in the healthy model, had Bacteroidetes as the most abundant bacteria, whereas Firmicutes was dominant in MOS and SIEM in the healthy model. This is in line with previous research, wherein MOS also promoted *Firmicutes* population in the chicken cecal microbiota (44).

The genus Akkermansia, which is associated with gut health in humans, was increased by P1 and P2 compared to SIEM and MOS in the healthy model. However, whether Akkermansia has the same beneficial effects *in-vivo* in chickens, is still under debate. For example, it has been shown to have a protective effect on the intestinal barrier, but it has also been linked to a higher number of necrotic enteritis cases, and significant overgrowth and colonization of *C. perfringens* (45, 46). Additionally, it is also interesting that Akkermansia survives in CALIMERO-2, with the lack of mucus in the system.

The fermentation of non-digestible carbohydrates by anaerobic bacteria in the gut yields SCFAs, and these metabolites are related to health benefits to the host. In broilers, SCFAs production is related to protection against pathogens by building a balanced gut community, and improvements of gut immunity, gut barrier, and mucin secretion and may enhance broiler production performance (47). In our research, we showed that different non-digestible carbohydrates promote different cumulative production of metabolites. For instance, fermentation of P1, which is a low DM pectin, provided the lowest amounts of total SCFAs, organic acids, and BCFAs. It raises the question of whether this outcome was due to a slower fermentation rate of the pectin or if it requires more time for the gut microbiota to adapt to this particular type of pectin. Similarly, Tian et al. (41, 48), tested LMP and HMP, and also found quite different fermentation patterns for each pectin.

Comparing the three pectins, it was observed that P1 lead the formation of low levels of SCFAs when compared to P2 and P3. P2 exhibited intermediate levels of fermentation, and P3 yielded the highest amounts of metabolites, which could indicate more extensive fermentation. The compositional variations of the three pectins likely contribute to the divergent fermentation patterns observed. Interestingly, the values of total SCFAs, organic acids, and BCFAs were found to be very similar between the SIEM, IMMP and P3, with IMMP and P3 exhibiting even greater similarity. This suggest that the fermentation patterns associated with IMMP and P3 might share common metabolic pathways. However, it is worth noting that the gut microbiota was differently modulated by these two substrates, once more demonstrating that the fermentation metabolites' similarity in amounts does not necessarily reflect identical microbial community responses.

The results of our study revealed that succinate and lactate were not dominantly present in the samples, also not by the fermentation of IMMP. Gu et al. reported that IMMP-94 and IMMP-96 predominately produce the intermediate SCFA, succinate, next to the SCFAs in a batch fermentation model using human inoculum. Our results showed that succinate and lactate were mainly converted to SCFAs, which might have happened faster because the microbiota of chickens is different compared to the human inoculum. Moreover, this might also be related to the decrease in pH in their model, compared to CALIMERO-2, in which the pH was constantly regulated (24).

In conclusion, our results enhance our understanding of the correlation between carbohydrate structure and fermentability, emphasizing that the complexity of carbohydrates leads to contrasting outcomes on the gut microbiota and the production of metabolites. Although carbohydrates, especially IMMP affect the relative abundance of bacteria and the total SCFAs production, future research is needed to determine if IMMP or the pectins are beneficial for chicken gut health.

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We want to thank Royal GD Deventer, the Netherlands, for providing the *Clostridium perfringens* strain in this research. We would like to thank Natalia Hutnik for her support with the pectin analysis, and Rob van Dinter, Jessica Verhoeven and Sanne Verbruggen for their technical support with the CALIMERO-2 experiments and 16S rRNA sequencing.

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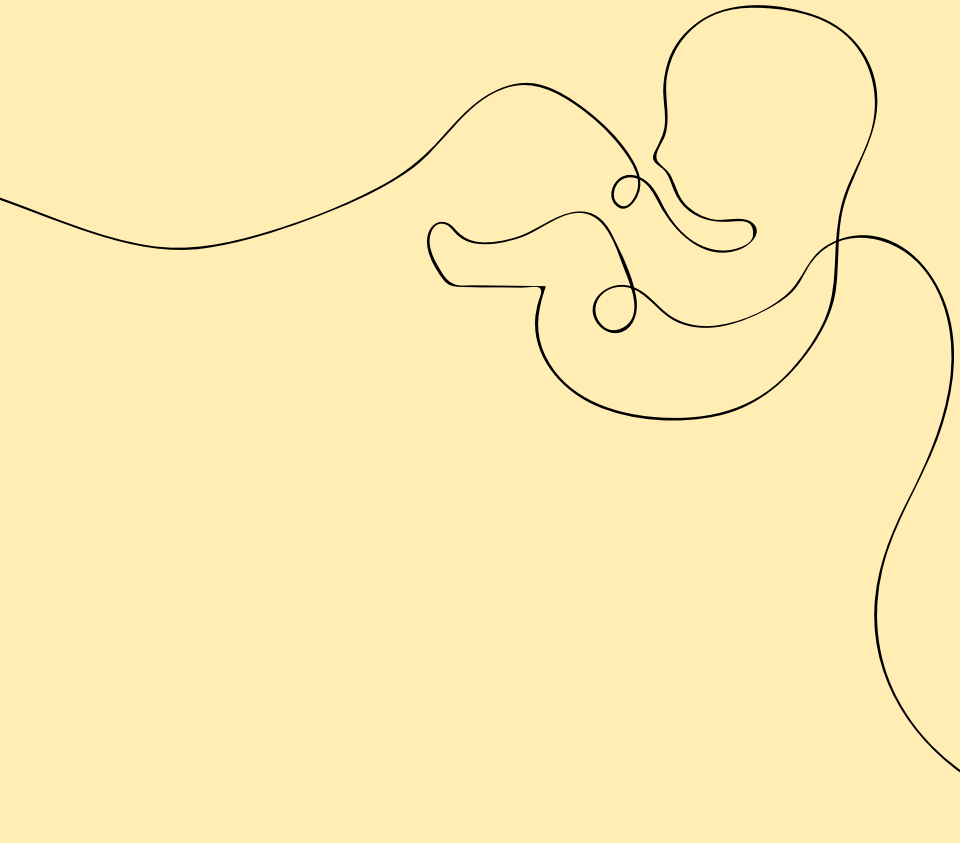
Keywords: prebiotics, fermentation, short chain fatty acids, *in-vitro* models for digestion, and intestinal mucosa

Chapter 5

Prebiotic-derived fermentation products affect the epithelial barrier integrity and immune activation in a human co-culture model of epithelial and peripheral blood mononuclear cells



Abstract

The development of the gastrointestinal microbiota starts at birth, and many factors can influence the microbiota composition in these early days. The intestinal microbiota influences various physiological processes, including nutrient absorption, metabolism, and immune function. The short chain fatty acids that are produced after the fermentation of non-digestible oligosaccharides (NDOs) by the microbiota, play an important role in intestinal health, and modulation of the immune response, and are proven to enhance intestinal barrier function. The prebiotics, 2′-fucosyllactose (2′-FL) and Galacto-oligosaccharides (GOS) have been shown to improve host defense and promote intestinal barrier function in young infants. While previous research has focused on the effect of NDOs on the microbiota or microbiota-independent effects on intestinal cells, we aimed to investigate the effect of complete microbial fermentation products of 2′-FL or GOS. Samples with prebiotic fermentation products, obtained from an *in-vitro* large intestine fermentation model (TIM-2) and containing short chain fatty acids and other metabolites, were collected. Intestinal epithelial cells (Caco-2) in co-culture with basolateral non-activated and αCD3/CD28-activated peripheral blood mononuclear cells were used to study the effect of the fermentation products of 2′-FL and GOS on barrier function and immune response. Apical exposure to the fermentation products of 2′-FL did not improve barrier integrity nor modified the response of underlying immune cells compared to the control fermentation product. In contrast, GOS fermentation products enhanced epithelial permeability, which was associated with an increase in interferon-γ release by underlying immune cells. Both control and 2′-FL and GOS fermentation products further increased inflammatory interleukin-1β (IL-1β) release of activated immune cells. The fermentation products all contained substantial, but similar amounts of lipopolysaccharide (LPS). LPS-filtered samples or the addition of an anti-TLR4 antibody, which blocks the binding of LPS to the receptor, did not result in a more beneficial effect on the barrier integrity or immune responses, ruling out that LPS caused the inflammatory response. Since the control fermentation product itself already tended to enhance epithelial permeability, while at the same time increasing IL-1β release of underlying activated immune cells, this suggests that other components or metabolites present in the control fermentation product may interfere with the experimental outcome in this model. These results highlight the complexity of the interactions between complete *in-vitro* large intestine-derived fermentation products in this *in-vitro* co-culture model combining intestinal epithelial cells and immune cells.

5.1 Introduction

After birth, the development of the gastrointestinal microbiota starts. Many factors can influence the microbiota composition in these early days in infants, like the type of delivery and whether they are being breastfed or formula-fed (1, 2). The intestinal microbiota can influence many physiological processes including digestion, nutrient absorption, metabolism, and immune function (3, 4). Gut microbial degradation of nutrients can generate bioactive metabolites that bind target receptors, activate signaling cascades, and modulate several metabolic pathways with local and systemic effects (5). Short chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, are the most abundant bacterial metabolites in the gut and are mainly produced after the fermentation of non-digestible carbohydrates (6). These SCFAs, especially butyrate, can contribute to intestinal health, by enhancing intestinal barrier function, and modulating immune responses (7, 8).

In recent years, there has been extensive research on the benefits of non-digestible oligosaccharides (NDOs) and SCFAs derived from their fermentation on young infants' intestinal health and beyond. Examples of NDOs are 2'-fucosyllactose (2'-FL) and galacto-oligosaccharides (GOS), which have been shown to improve host defense by modulating immunity and promoting intestinal barrier function (9-12). Thereby, they reduce the impact of for example gut inflammation or the development of allergy (13). Moreover, they can also have a prebiotic effect, by promoting the increase of beneficial bacteria like *Bifidobacterium* and *Lactobacillus* and the production of SCFAs (14-17). SCFAs in general, and more specifically butyrate, are also known to have many beneficial effects on intestinal health, by for example regulating paracellular permeability through their effect on the expression of tight junction proteins (18). Moreover, SCFAs have been shown to have anti-inflammatory effects (19). This has been supported in a study in which intestinal epithelial cells were activated with the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), and SCFAs prevented cytokine-induced CXCL10 release, which is an inflammatory chemokine that mediates immune responses through activation and recruitment of leukocytes (20, 21).

The focus so far has been on the effects of NDOs on microbiota or the microbiota-independent effects of NDOs that act directly on for example intestinal cells. However, the effects of the complete microbiota–derived fermentation products of 2'-FL or GOS on intestinal cells are unknown. Here, we aimed to study this. Luminal samples, referred to here as fermentation products, were collected from the *in-vitro* large intestine fermentation system, known as the TNO intestinal model- 2 (TIM-2), after 72 h of fermentation of NDOs using feces of healthy 3-month-old babies. These fermentation products from TIM-2 contained SCFAs and other microbial metabolites that are produced during the fermentation of NDOs. Our objective was to study the effect of these fermentation products on the epithelial barrier and inflammatory-induced barrier disruption in a co-culture model of intestinal epithelial cells and activated peripheral blood mononuclear cells (Caco-2/PBMCs) (22).

5.2 Methods

5.2.1 Prebiotics

Aequival 2'-FL powder and purified Vivinal GOS were provided by FrieslandCampina Ingredients (Amersfoort, the Netherlands). Aequival 2'-FL powder is a high-purity human milk oligosaccharide (94% 2'-FL on dry matter basis). Purified Vivinal GOS contained 1.7% monomers, 6.6% dimers (allo-lactose, lactose, and lactulose), and 91.7 % GOS on a dry matter basis.

5.2.2 Fecal sample collection and standardization

Infant fecal samples of 3-month-old babies, that were part of the Baby Carbs study, were collected as described by Endika et al. (23). Briefly, parents of participating babies collected fecal samples daily, consecutively for 1 week, from diapers using a sterile spoon (Sampling Systems, Coleshill, UK) and kept the faces anaerobically in a sterile 50 mL collection tube with a BD GasPak EX anaerobe generating pouch (BD Diagnostics, Sparks, MD, USA). The fecal samples were stored in the home refrigerator (±4 ℃) for a maximum of 72 h before the collection by one of the researchers for transport to the lab. For transport to the lab, the samples were put in an insulated bag containing frozen cooling elements, and upon arrival processed in the laboratory on the same day. At the laboratory, the fecal samples from individual infants were pooled in a sterile 50 mL tube (CELLSTAR™ CELLreactor™ tube, Greiner Bio-One, Alphen aan den Rijn, the Netherlands) and weighed inside an anaerobic chamber (Bactron300-2, Sheldon Manufacturing, OR, USA; 96% N_2, 4% H_2). The fecal samples were diluted in a sterile pre-reduced dialysis solution (pH 6.5) containing 2.5 g/L KH_2PO_4, 4.5 g/L NaCl, 0.005 g/L $FeSO_4.7H_2O$, 0.05 g/L ox bile, 0.04 g/L Cysteine-HCl, and glycerol (final concentration of 10%, v/v). The fecal slurries (25% w/v) were mixed using a vortex, then aliquoted into sterile 10 mL vials (La-Pha-Pack, Langerwehe, Germany). The vials were sealed with a sterile butyl rubber stopper with a crimp cap. The sealed fecal slurries were taken out from the anaerobic chamber and immediately snap-frozen in liquid nitrogen before storage at −80 °C.

5.2.3 TIM-2: fermentation of the prebiotics

The TNO *in-vitro* model of the colon (TIM-2) is a dynamic *in-vitro* model of the large intestine (24). TIM-2 was inoculated with the dense infant fecal slurry [described above]. Body temperature, pH in the lumen, mixing, and transport of the intestinal contents, and absorption of water and metabolites (through dialysis) were simulated as described in detail before (23). Two independent experiments in TIM-2 were done, each using four independent fermentation units, which were run simultaneously.

The feeding medium for TIM-2 used was simulated ileal efflux medium for infants (i-SIEM) (25). The i-SIEM consists of the following components (per L): 24.0 g lactose (Merck, Darmstadt, Germany), 6 g tryptone (Oxoid, Basingstoke, UK), 6 g lactalbumin hydrolysate (Merck), 0.8 g ox bile (Merck), 15 g porcine gastric mucin (partially purified type III; Sigma-Aldrich, St. Louis, MO, USA), 0.6 g urea (Thermo Fisher Scientific, Waltham, MA, USA), 0.4 g cysteine HCl (Merck), 10 mL antifoam B emulsion (Sigma-Aldrich), salt solution (Tritium Mikrobiologie B.V., the Netherlands) containing 4.5 g, NaCl, 2.5 g $K_2HPO_4.3H_2O$, 0.45 g $CaCl_2.2H_2O$, 0.005 $FeSO_4.7H_2O$, 0.01 g hemin, and 1 mL vitamin solution (Tritium Mikrobiologie B.V.) containing (per L) 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg D-pantothenate, 5 mg p-aminobenzoic acid, 4 mg thiamine HCl and 5 mg nicotinamide adenine dinucleotide. The prebiotic, either 2′-FL or GOS, was supplemented in the concentrated i-SIEM feeding medium at a concentration of 30 g/L, which was further diluted in the colon model. This corresponds to a prebiotic supplementation of 1.8 g/day.

In each experiment, two fermentation units included i-SIEM as control and the other two fermentation units included i-SIEM with added 2′-FL or GOS. Each experiment started with inoculation of the system with 60 mL of the standardized fecal slurry [section 5.2.2], to which 30 mL of pre-reduced dialysis liquid was added. Lumen samples were taken at the end of the TIM-2 experiment, i.e., after 72h, which are named fermentation products of i-SIEM (F_i-SIEM), 2′-FL (F_2′-FL), and GOS (F_GOS). The samples were snap-frozen in liquid nitrogen and stored at -80℃ until further analysis. For more details on the experimental set-up of the fermentation experiment, see Endika et al. (23).

5.2.3.2 Analysis of microbial metabolites

The metabolites, acetate, propionate, butyrate, formate, iso-butyrate and iso-valerate, and succinate produced present in fermentation products were analyzed by making use of High-Performance Liquid Chromatography (HPLC; Shimadzu LC-2030C Plus, Shimadzu Europa GmbH, Duisburg, Germany). Before HPLC analysis, the protein present in the samples was removed, by Carrez clarification step, based on the method described by Selak et al. (26). In short, the samples were diluted twice in an equal volume of 0.1 M Carrez A reagent containing $K_4Fe(CN)_6.3H_2O$ (Merck) and 0.2 M Carrez B reagent containing $ZnSO_4.7H_2O$ (VWR International). The mixture was centrifuged for 5 min at 21,000 g and the clear supernatant was further used for the HPLC analysis.

A SUGAR SH1821 column ((SHODEX, Showa Denko, Tokyo, Japan) and a refractive index detector (RID-20A, Shimadzu Europa GmbH) with a cell temperature of 40 °C, were used for the HPLC. The column was operated at 45 °C with a flow rate of 1 mL/min, using 0.01N H_2SO_4 as eluent. The autosampler mixed 10 µL of external standard or collected supernatant with 10 µL of 0.01N H_2SO_4, and 10 µL of this mixture was injected for analysis. The data was processed using Chromeleon™ CDS software version 7 (Thermo Fisher Scientific).

5.2.4 Cell culture

5.2.4.1 Intestinal epithelial cell culture

Human epithelial colorectal adenocarcinoma cells (Caco-2, ATTC, HTB-38, Manassas, VA, USA; passages 33-49) were used as intestinal epithelial cells. Caco-2 cells were maintained in a 25 cm^2 flask (Greiner Bio-One,) containing high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 10% Fetal Calf Serum (FCS) (Sigma-Aldrich), 1% penicillin/streptomycin (pen/strep) (100 U/mL; 100µg/mL) (Sigma-Aldrich), 1% non-essential amino acids (Gibco) and 2mM L-Glutamine (Gibco). The cells were incubated at 37℃, 5% CO_2, and the medium was refreshed every 2-3 days. The cells were passaged when they reached a confluence of 70-90%.

5.2.4.2 Peripheral Blood Mononuclear Cell Purification

Human PBMCs were isolated from buffy coats from healthy donors who had given informed consent for the use of their blood for research purposes (Dutch blood bank, Amsterdam, the Netherlands) by density gradient centrifugation using pre-filled Leucosep™ tubes (Greiner Bio-One). Tubes were centrifuged for 13 min at 1000 x g, and the isolated lymphocyte fraction was washed with phosphate-buffered saline (PBS; Lonza, Basel, Switzerland) supplemented with 2% FCS and the remaining erythrocytes were lysed using lysis buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg saline-ethylenediaminetetraacetic acid (Na_2EDTA) in 500 mL demi-water, sterile filtered, pH=7.4) for 5 min on ice. The isolated PBMC fraction was resuspended in RPMI-1640 (Gibco, Invitrogen, Carlsbad, CA, USA) with 10% FCS and 1% pen/strep.

5.2.5 Preparation of fermentation products for cell culture experiments

Lumen samples obtained from TIM-2 were centrifuged at 220x g, 5 min at 4°C. The supernatant was collected and pooled per carbohydrate source and diluted 1:1 with culture medium of the Caco-2 cells. After dilution, the supernatant was filtered through a 0.45-µm, 28mm strainer (Corning, Amsterdam, the Netherlands). The samples were stored at -80°C, until further use. The samples were before use in total 8 times diluted, to prevent toxicity [data not shown]. All values presented here, are based on the 8 times dilution of the samples.

5.2.6 Lipopolysaccharide concentration determination

To measure Lipopolysaccharide (LPS) in the fermentation products obtained from TIM-2 (8 times diluted), the Endosafe®- Portable Test System (Charles River Laboratories, Charleston, SC, USA) with matching Test Cartridges, with sensitivity 0.01-1 EU/mL were used, following manufacturers' guidelines.

5.2.7 Endotoxin removal

Pierce™ High Capacity Endotoxin Removal Spin Columns, with 1 mL capacity (ThermoFisher Scientific) was used to remove LPS from the fermentation products obtained from TIM-2 (8 times diluted). The manufacturer's protocol was used.

5.2.8 Co-culture of intestinal cells and Peripheral blood mononuclear cells

Three weeks before the experiment, the Caco-2 cells were seeded in a 12-transwell system (pore size: 0.4 µm) (Costar, Corning Incorporated, Corning, NY, USA). The cells were incubated at 37 ℃ and 5% CO_2. The cells were grown on the transwell inserts for an additional 2-3 weeks post confluency to ensure differentiation into fully polarized enterocytes that formed functional barrier properties, as described by Hubatsch and colleagues (27). Before the start of the experiment, the culture medium was changed to RPMI-1640 (Gibco) supplemented with 2.5% FCS and 1% pen-strep. In the basolateral compartment, PBMCs were seeded (2 x 10^6 cells/mL) and either activated or not after seeding with αCD3/ αCD28 (clone CLB-T3/2 and clone CLB-CD28, both 1:10.000, Sanquin, Nijmegen, the Netherlands), to mimic an inflammation (22). In the apical compartment, 8 times diluted fermentation products were added. To determine if the presence of LPS influenced the experiments, LPS filtered samples, 20 µg/mL of the neutralizing CD284 monoclonal antibody (anti-TLR4; ThermoFisher Scientific), and as a positive control, 20 µg/mL LPS [close to LPS present in the fermentation sample] were added apically. At 24 h the 50% of basolateral medium was stored at -20℃ until further analysis, and the wells were replenished with fresh medium. At t=48h the experiment was completed and the basolateral medium was stored at -20℃ until further analysis. See Figure 1 for a schematic overview of the co-culture.

5.2.9 Epithelial barrier function: Transepithelial Electrical Resistance (TEER) measurement

The integrity of the intestinal epithelial monolayer was assessed by measuring TEER using a Millicell-ERS volt meter(Merck Millipore, Burlington, MA, USA) as described by Korsten et al. (22) at t=0, t=24, and t=48 h. Data are shown as percentages compared to baseline TEER at t=0 h.

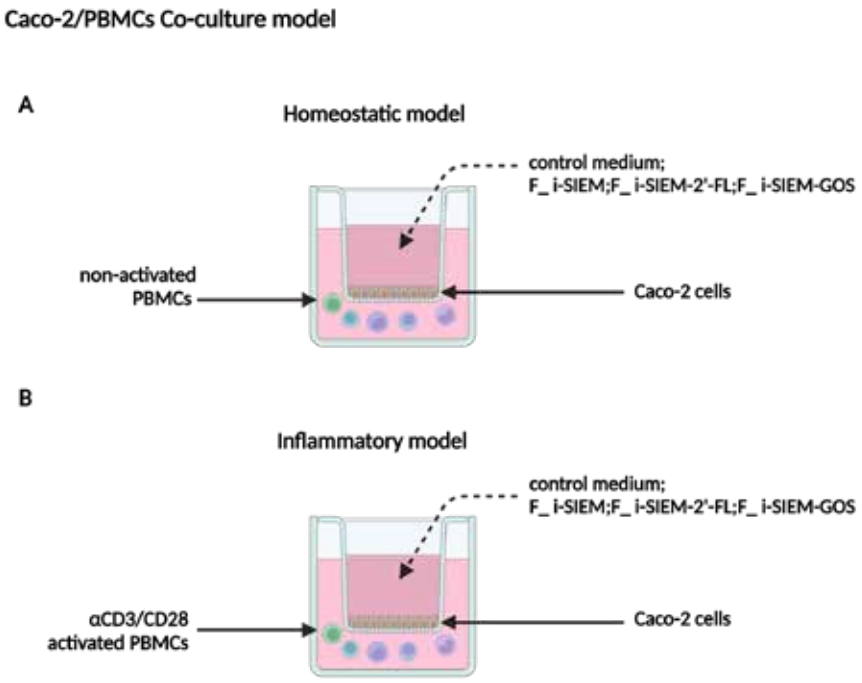


Figure 1. Schematic overview of the co-culture experiment. Caco-2 cells were grown in a 12-well transwell insert for 3 weeks post-confluence until full differentiation and tight barrier formation. On the basolateral side, PBMCs were added either non-activated (**A**, homeostatic model) or α-CD3/CD28 activated (**B**, inflammatory model). Apically, Caco-2 cells were exposed to the TIM-2 derived fermentation products of 2'-fucosyllactose (F_2'-FL), galacto-oligosacchardes (F_GOS), or simulated ileal efflux medium for infants (F_i-SIEM) (8 times dilution), and as a control growth medium. After 24 h of incubation, the basolateral supernatant was collected to analyze the cytokine production and refreshed. Figure created with BioRender.com.

5.2.10 Epithelial barrier function: permeability assay

The intestinal permeability was measured by making use of 4kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich). Before assessing permeability, the cells were three times washed with PBS and the medium was replaced with DMEM without phenol red (Gibco). The cells were incubated with the new medium for 1h before FITC-dextran was added to the apical side of the transwell system. The cells were incubated with 1.6 mg/mL FITC-dextran for 30 min and 1h before samples were taken on the basolateral side. Fluorescence was measured at Excitation = 492 nm and Emission = 518 nm on a microplate reader (GloMax Discover, Promega Corporation, Madison, WI, USA).

5.2.11 Enzyme-linked Immunosorbent Assay

Cytokine secretion was analyzed in the supernatant of samples taken from the basolateral side of the Caco-2/PBMCs co-cultures. Commercially available kits were used to determine IFN-γ, TNF-α, IL-1β, and IL-10 (all from ThermoFisher Scientific), according to manufacturer instructions.

5.2.13 Real-Time Quantitative PCR

Gene expression of select cytokines produced by the Caco-2 cells after permeability measurements were determined by Real-Time quantitative PCR (RT-qPCR). First, the RNA was isolated from the Caco-2 cells, using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. cDNA was generated from 500 ng RNA using iScript cDNA synthesis Kit (Biorad, Hercules, CA, USA). Next, RT-qPCR was performed using SYBR Green (Biorad). Primers used were, TLR4 (Biorad: Unique Assay Id: qHsaCED0037607), IL-8 (Biorad: Unique Assay ID: qUsaCED0046633), TNF-α (ThermoFisher; FW: CCTGCTGCACTTTGGAGTGA, Rev: GAGGGTTTGCTACAACATGGG), GAPDH (Biorad: Unique Assay ID: qHsaCED0038674). Relative mRNA expression was calculated as 100 x $2^{Ct\ reference - Ct\ gene\ of\ interest}$ (28).

5.2.14 Viability assay

After 48 h, after the FITC-dextran assay, the cell viability of the Caco-2 cells was determined using a WST-1 assay (Roche) according to the manufacturer's protocol. In short, WST was diluted in culture medium without phenol red (1:10 dilution) and added to each well for 30 min at 37°C in the presence of 5% CO_2. Subsequently, 100 µL of each well was transferred to a clear 96-well plate and absorbance was measured at 450 nm with a microplate reader (GloMax Discover). Data are shown as percentages compared to control cells.

5.2.15 Statistical analysis

Results are presented as means (± SEM) and for the produced metabolites (±SD) and statistical analysis was performed using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA, USA). The statistical significance of normally distributed data was assessed with the repeated measures one-way ANOVA analysis, followed by Bonferroni's post hoc test with selected pairs. Non-parametric analysis was performed for the non-normally distributed data, followed by Dunn's post hoc test. The correlation between the TNF-α production and TLR4 mRNA expression was determined by making use of the Spearman method. Results were considered statistically significant when P was < 0.05.

5.3 Results

5.3.1 TIM-2 fermentation products and their effect on the viability of the epithelial cells

2′-FL, GOS, or i-SIEM, the latter as a control, were added to the TIM-2 system, in which they were fermented by the bacteria present in the microbiota, originating from 3-month-old infants. The concentrations of the SCFAs, branched-chain fatty acids (BCFAs), and metabolites in the samples are presented in Table 1. The fermentation products F_2′-FL, and F_GOS, contained more total SCFAs (acetate, propionate, and butyrate) compared to F_i-SIEM. Acetate was higher for F_2′-FL and GOS compared to F_i-SIEM, and butyrate and propionate showed a similar inclining pattern. The metabolite formate was not detected for F_i-SIEM and F-2′-FL, but was increased in F_GOS, but still found at relatively low concentrations. Valerate also showed relatively low concentrations and was increased in F_2′-FL when compared to F_i-SIEM. The BCFA iso-butyrate and iso-valerate were relatively low and no differences were observed for i-SIEM and the NDO groups. To determine if the fermentation products were not toxic for the epithelial cells, the viability of the Caco-2 cells was measured at the end of the experiment and showed no difference between the medium control and F_i-SIEM, F_2′-FL, and F_GOS (Fig S1).

Table 1. The concentration of metabolites in mM present in the eight times diluted samples. Data represented from n=2 ± SD. Simulated ileal efflux medium for infants (F_i-SIEM), 2'-fucosyllactose (F_2'-FL), galacto-oligosacchardes (F_GOS)

	Total SCFAs	Acetate	Propionate	Butyrate
F_i-SIEM	4.79 ±0.05	3.21 ± 0.03	0.53 ± 0.03	1.04 ± 0.06
F_2'-FL	8.03 ±1.52	5.40 ± 0.26	0.93 ± 0.17	1.70 ± 0.18
F_GOS	9.62 ±0.68	6.80 ± 0.60	1.07 ± 0.29	1.72 ±0.37

	Formate	Valerate	Iso-butyrate	Iso-valerate
F_i-SIEM	0 ± 0	0.29 ± 0.03	0.10 ± 0.00	0.21 ± 0.03
F_2'-FL	0 ± 0	0.47 ± 0.00	0.08 ± 0.00	0.15 ± 0.04
F_GOS	0.46 ± 0.07	0.34 ± 0.00	0.10 ± 0.00	0.16 ± 0.03

5.3.2 Effects of the NDO-derived fermentation products on the intestinal barrier function in the co-culture model.

To investigate the effect of the control i-SIEM fermentation product and fermentation product of 2'-FL and GOS on the intestinal barrier function, these were tested in an 8 times dilution, in the co-culture of Caco-2 cells with PBMCs, and the trans-epithelial electrical resistance (TEER) was measured. The TEER values of Caco-2 cells, irrespective of exposure to culture medium or F_i-SIEM, F_2-FL, and F_GOS, showed a decline over time, as presented in Fig. 2A. After 48 h, the decrease in resistance in the inflammatory model (Caco-2 with activated immune cells) was not more pronounced than the decrease in resistance in the homeostatic model (Caco-2 with non-activated immune cells) for the medium control (Fig. 2B). F_ 2'-FL was found to enhance TEER in the inflammatory model after 48 hours of exposure, compared to the corresponding medium control (Fig. 2B). F_GOS and F_i-SIEM did not affect the TEER. Next, we evaluated the permeability of the intestinal cells after 48 h of exposure. There was no elevated permeability in the homeostatic model after exposure to the fermentation products, however in the inflammatory model there was an increase in FITC-dextran after 30 min and 1 h, albeit this was only found in the presence of the fermentation products (Fig. 2 C & D). In the inflammatory model, after 30 min, the control F_i-SIEM and F_GOS showed a significant increase in FITC_dextran concentration at the basolateral side compared to the medium control. F_2'-FL also showed an increase compared to the medium control, but this was not significant (Fig. 2C). In the inflammatory model after a 1 h incubation with FITC-dextran, F_i-SIEM, F_2'-FL, and F_GOS all increased the basolateral FICT-dextran concentration, but only for F_GOS this effect was significant (Fig. 2D), with a > 10-fold increase compared to the medium control.

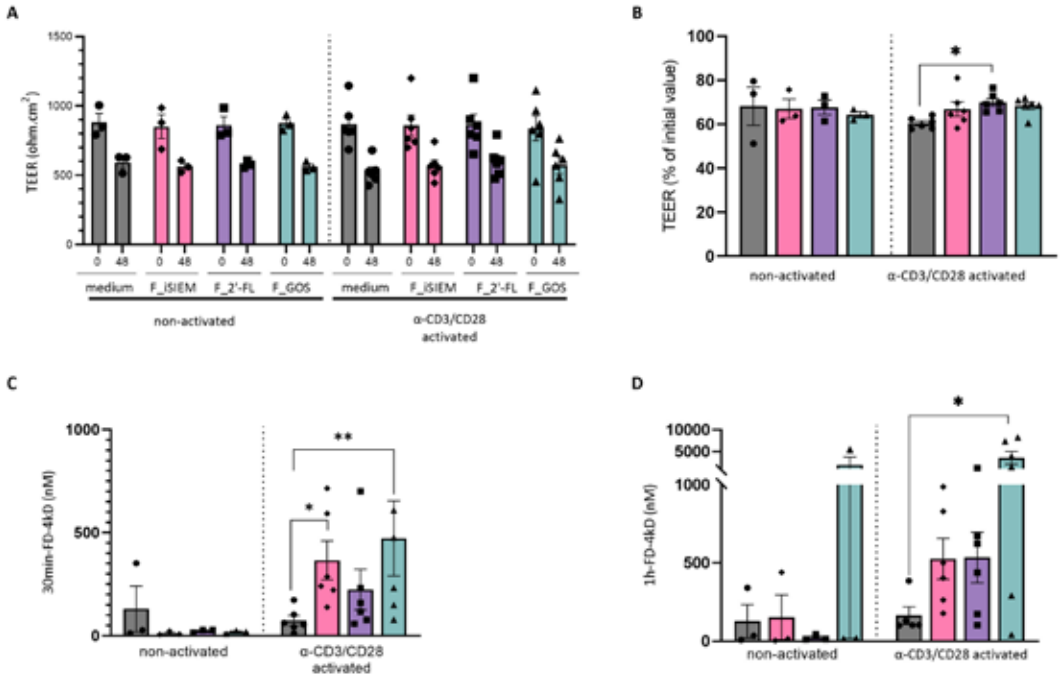


Figure 2. The effect of the fermented products of i-SIEM (F_i-SIEM, pink), 2'-fucosyllactose (F_2'-FL, purple), and galacto-oligosaccharides (F_GOS, green) after 48 h on the trans-epithelial electrical resistance and the functional permeability of Caco-2 cells. Caco-2 cells were treated with 8x diluted fermented products and control medium (grey) and basolateral the PBMCs were activated, or not with α-CD3/CD28. (A) trans-epithelial electrical resistance (TEER) absolute values at time points 0, and 48 (B) TEER presented as percentage of the initial value (C) Fluorescein isothiocyanate (FITC)-dextran 4kD (FD-4kD) permeability assay was performed after 48 h of exposure, and Caco-2 cells were incubated for 30 min or (D) 1 h, before concentration (nM) measurement at the basolateral side. All data is represented as mean ± SEM. The dotted line divides the non-activated PBMCs (n=3) and the activated PBMCs (n=6) which were analyzed separately. * $P < 0.05$; ** $P < 0.01$.

5.3.3 Basolateral cytokine release after exposure of epithelial cells to the TIM-2-derived fermentation products

To investigate the impact of epithelial cells apically exposed to bacterially fermented oligosaccharides on basolateral immune cell activation, we measured various cytokines in the basolateral compartment. Neither F_i-SIEM, F_2'-FL nor F_GOS affected basolateral cytokine production in the homeostatic model (Fig. 3). In the inflammatory model, TNF-α, IFN-γ, IL-10, and IL-1β all showed increased concentrations compared to the homeostatic model in the medium condition (Fig. 3). F_i-SIEM, F_2'-FL nor F_GOS did not affect TNF-α concentration at 24 h and 48 h, nor IFN-γ or IL-10 at 48 h compared to medium control (Fig. 3 A, E-G). However, at 24 h F_GOS increased IFN-γ and IL-1β while lowering IL-10 compared to medium control (Fig. 3 B-D). Also, F_i-SIEM and F_2'-FL enhanced IL-1β both at 24 h and 48 h compared to medium controls (Fig. 3 D, H).

5.3.4 Presence of Lipopolysaccharide in the fermentation products

To determine whether the observed effect of fermentation products on IL-1β and intestinal cell permeability might be due to LPS, we measured the LPS concentration in the TIM-2-derived fermentation samples (Table 2). The LPS concentration in F_i-SIEM was higher than in F_2'-FL and F_GOS. To remove a significant portion of LPS, we used endotoxin removal spin columns, which indeed resulted in a reduced concentration of LPS in all TIM-2 fermentation samples (Table 2). Filtered and unfiltered TIM-2 fermentation samples were used in the Caco-2/PBMCs co-culture model for further studies.

Table 2. Lipopolysaccharides (LPS) concentrations in the original 8 times diluted fermented product samples and after the LPS was removed, by making use of endotoxin removal spin columns. Simulated ileal efflux medium for infants (F_i-SIEM) 2'-fucosyllactose (F_2'-FL), galacto-oligosacchardes (F_GOS).

	LPS concentration (µg/mL)	
	Original	**After filtration**
F_i-SIEM	12.725	0.475
F_2'-FL	8.025	1.96
F_GOS	8.35	3.97

5.3.5 Relative mRNA expression of epithelial cells

The expression of TLR4 mRNA in Caco-2 cells was measured to verify the presence of the receptor in this cell line. TLR4 is a pattern recognition receptor and is most well-known for binding LPS (29). Next to studying the expression of this receptor, to check if the LPS present in the sample would be able to activate the epithelial cells, TNF-α and IL-8 mRNA expressions were measured. TNF-α is linked to epithelial activation by LPS and IL-8 is known to be produced as one of the key pro-inflammatory cytokines activated by LPS in the epithelial cells (30). The results indicate that Caco-2 cells express TLR4 mRNA in the inflammatory model, in combination with the fermentation products, however, the expression levels were rather low (Fig. 4A). However, TNF-α mRNA expression was significantly increased in Caco-2 cells under inflammatory conditions showing a similar pattern compared to TLR4 expression. Notably, there was a significant increase in TNF-α mRNA expression already for F_i-SIEM as well as F_2'-FL compared to control medium, while F_GOS did not exhibit a significant effect (Fig. 4B). Furthermore, IL-8 mRNA expression was present in both the homeostatic and inflammatory models. In the inflammatory model, all fermentation products tended to increase in expression of IL-8 (Fig. 4C). Finally, we found no correlation between epithelial TNF-α mRNA expression and the TNF-α production as measured in the basolateral compartment. This suggests that even though epithelial cells indeed are activated by the control fermentation product i-SIEM, the TNF-α concentration as measured during the co-culture was mainly produced by the activated PBMCs (Fig. 4D).

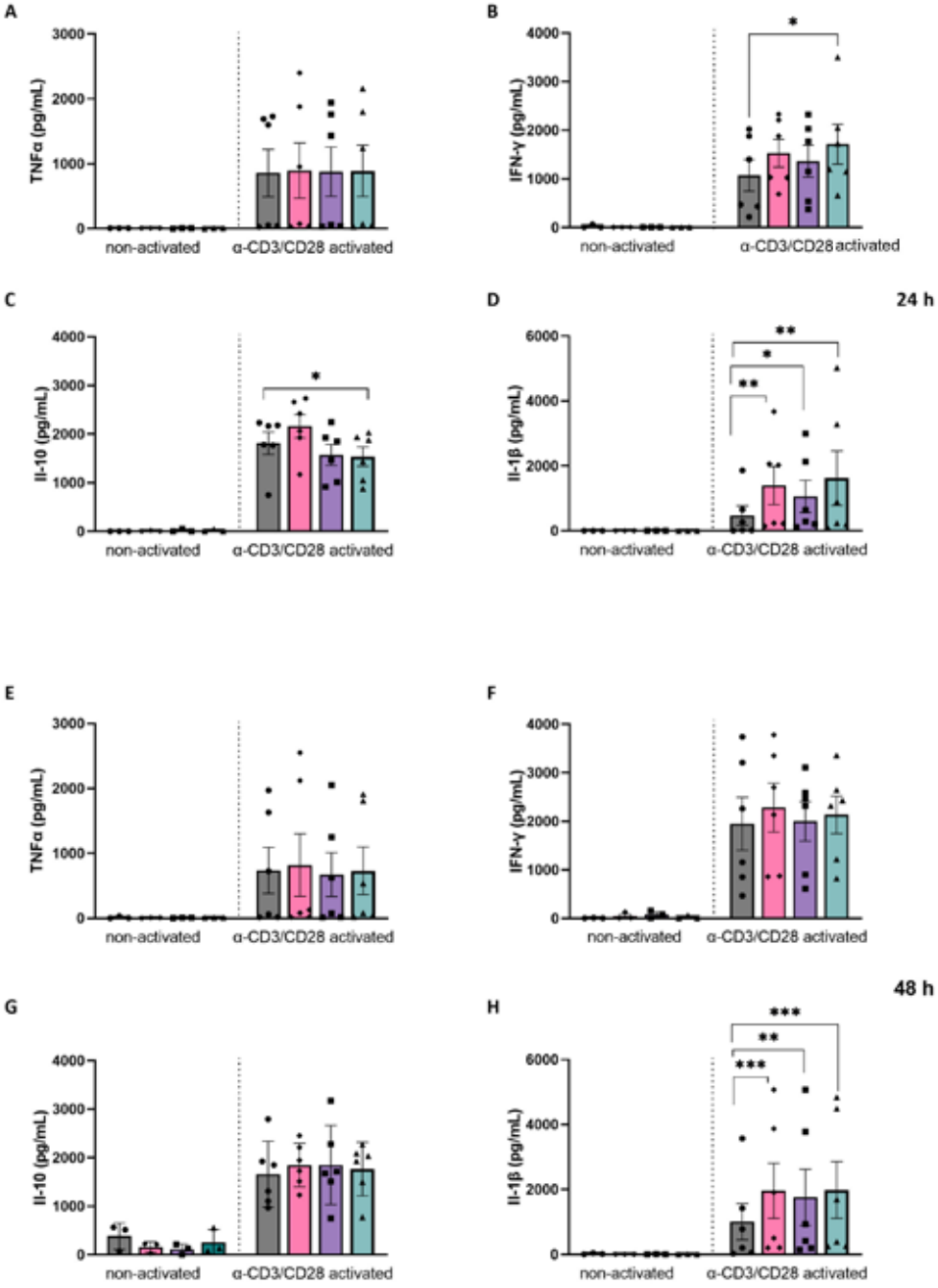


Figure 3. Cytokine production in a Caco-2/PBMC co-culture model. Caco-2 cells were exposed to control medium (grey), and fermented products of i-SIEM (F_i-SIEM, pink), 2'-fucosyllactose (F_2'-FL, purple), and galacto-oligosaccharides (F_GOS, green) for 24 h and 48 h. On the basolateral side, PBMCs were added, non-activated, or activated with α-CD3/CD28 for 48 h. After 24 h (**A-D**) and 48 h (**E-H**) incubation, TNF-α (**A, E**), IFN-γ (**B, F**), IL-10 (**C, G**), and IL-1β (**D, H**) were measured. Data are presented as mean ± SEM of n=3 and n=6 independent PBMC donors. The conditions with activated PBMCs were analyzed separately as represented by the dotted line. Medium served as a control of the system and F_i-SIEM served as a control of the fermentation products. * P < 0.05, ** P < 0.01, *** P < 0.001.

5.3.6 Role of LPS in the effects of TIM-2 fermentation products on epithelial integrity in the inflammatory model

To determine whether LPS is the cause of the observed effects of the fermentation products, we measured epithelial permeability and basolateral cytokine production with or without a neutralizing antibody against TLR4 (α-TLR4), and with filtered fermentation product samples. As a positive and negative control, medium with the addition of 20 μg/mL LPS or medium with α-TLR4 were used, respectively. The TEER-assessed epithelial resistance results showed no increase after exposure to control medium with LPS. The filtered fermentation samples or the addition of the neutralizing antibody against TLR4 to the fermentation products did not affect the drop in TEER of the Caco-2 cells (Fig. 5A, B). To assess permeability, FITC-dextran concentrations in the basolateral compartment were measured after 30 min and 1 h of exposure to fermentation products. Both F_SIEM, F_2'-FL, or F_GOS tended to increase permeability compared to medium controls at 30 min and/or 60 min but this did not gain significance (Fig. 5C, D). The high-dose LPS control however did show a trend to an increase in permeability. Filtering out LPS or blocking the TLR4 receptor did not lower the permeability as shown using F_i-SIEM or F_2'-FL or F_GOS when compared to medium controls. These results suggest that the effects observed by TIM-2 fermentation products were not explained by LPS contamination in Caco-2 cells.

5.3.7 Role of LPS in the effects of TIM-2 fermentation products on basolateral cytokine response

We measured the basolateral levels of TNF-α, IL-10, IFN-γ, and IL-1β in response to LPS added to the medium, fermentation products, without or with α-TLR4 or filtered fermentation products. As shown in Fig. 6 there is only a significant increase in IFN-γ as a result of the addition of LPS to the control medium, which is not shown for the other cytokines (Fig. 6). For TNF-α, there is no significant difference between the fermentation products (either filtered or not or with α-TLR4) and medium control after 24 h and 48 h exposure (Fig. 6A, E). No significant differences compared to medium control were found for IFN-γ and IL-10 measured at 24 h and 48 h for all the treatments (Fig. 6B, C, F, G). For the IL-1β concentration, there was no significant difference observed when LPS was added to the control medium, but the pattern was similar to the rise in IFN-γ (Fig. 6D). Thus high LPS content in the control fermentation products may have contributed to the increased inflammatory response in the inflammatory model. However, the increase of IL-1β for F_i-SIEM and F_GOS compared to medium control was observed similarly to previous experiments and was not reduced when LPS was removed nor when TLR-4 was blocked. This suggests that in the fermentation product factors other than LPS are responsible for enhancing the inflammatory response in activated PBMCs.

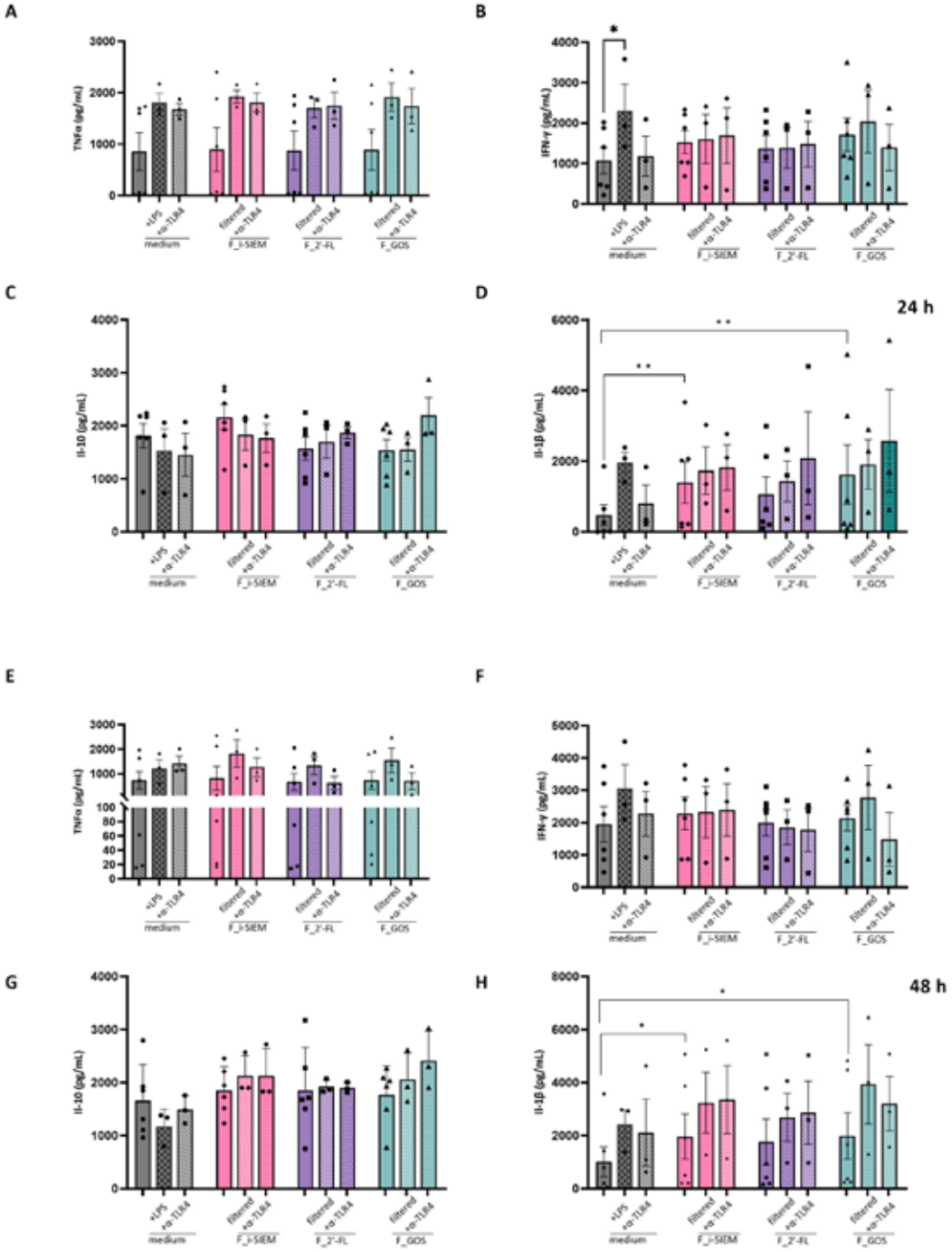


Figure 4. mRNA expression of TLR4 (A), TNF-α (B), IL-8 (C) relative to reference gene GAPDH. n=3 for non-activated and n=6 for activated PBMCs and isolations were performed in technical triplicates. Medium control (grey) , simulated ileal efflux medium for infants (F_i-SIEM, pink), 2'-fucosyllactose (F_2'-FL, purple), galacto-oligosacchardes (F_GOS, green). Data is represented as the mean ± SEM. (D) The correlation between TNF-α concentration measured on the basolateral side and TNF-α expression in the Caco-2 cells was tested using Spearman's correlation test. * $P < 0.05$.

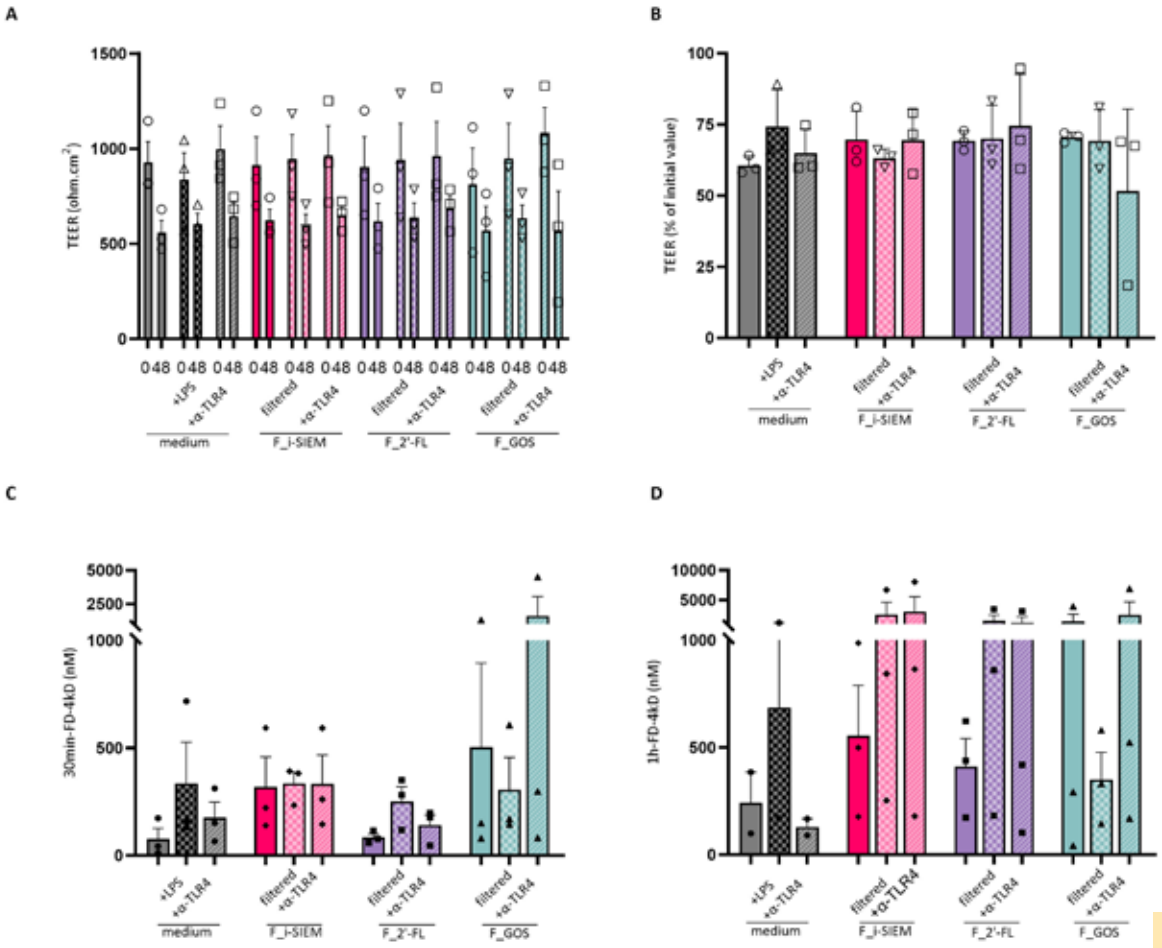
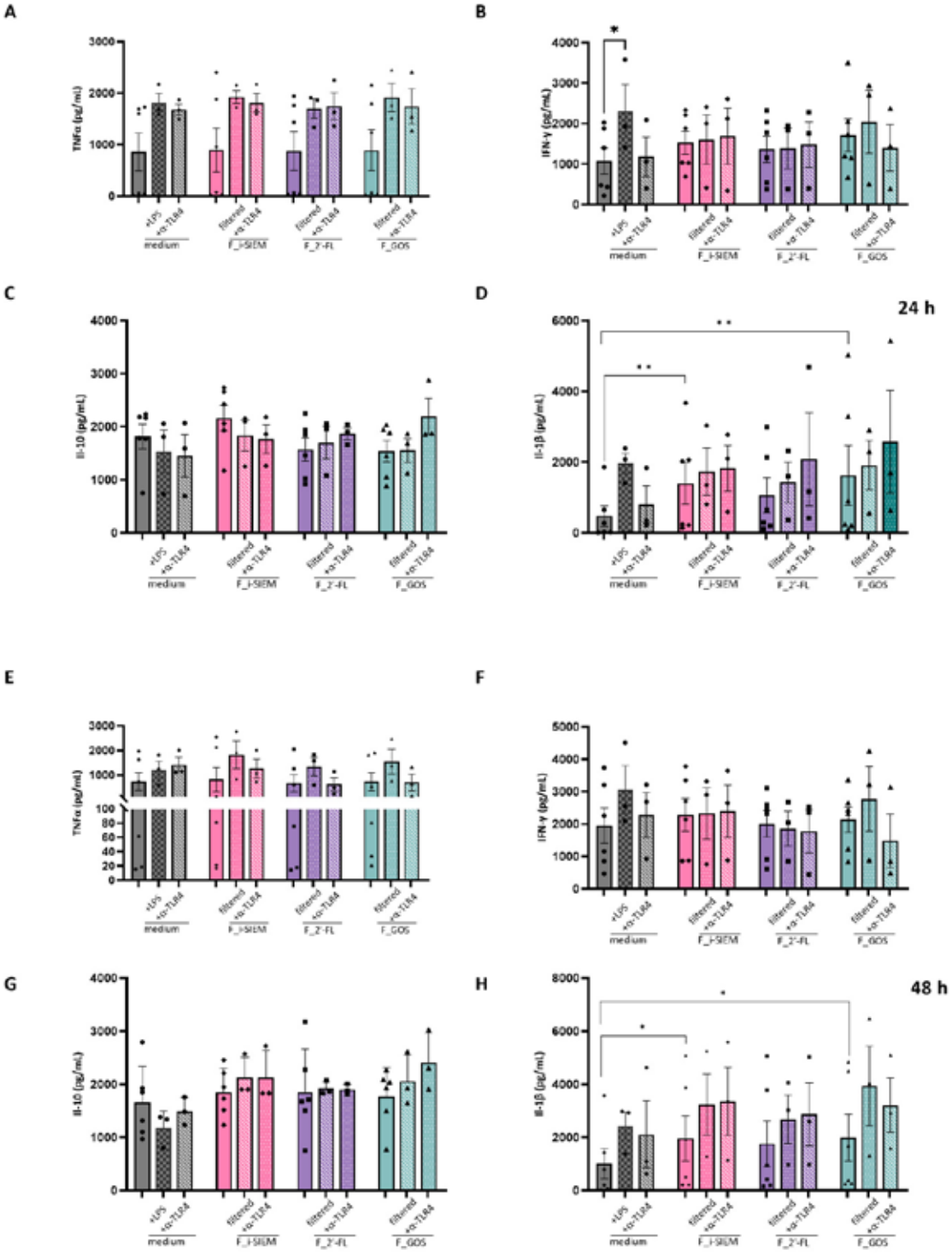


Figure 5. The effect of the fermentation products of i-SIEM (F_i-SIEM, pink), 2′-fucosyllactose (F_2′-FL, purple), and galacto-oligosaccharides (F_GOS, green), with and without filtering out lipopolysaccharide (LPS) or with the addition of the neutralizing antibody for Toll-like receptor-4 (TLR4) after 24 h and 48 h on the resistance and the functional permeability of Caco-2 cells. Caco-2 cells were treated with 8 times diluted fermented products and medium control (grey), and medium with addition of LPS (20µg/ml), and basolateral the PBMCs activated with α-CD3/CD28. **A** trans-epithelial electrical resistance (TEER) absolute values at time points 0, and 48; **B** TEER presented as percentage of the initial value; **C** Fluorescein isothiocyanate (FITC)-dextran 4kD (FD-4kD) permeability assay was performed after 48 h of exposure, and Caco-2 cells were incubated for 30 min or **D** 1 h, before concentration (nM) measurement at the basolateral side. All data is represented as mean ± SEM, n=3.


A
TNFα (pg/ml)
3000
2000
1000
0
+LPS
+α-TLR4
medium
filtered
+α-TLR4
F_I-SIEM
filtered
+α-TLR4
F_2'-FL
filtered
+α-TLR4
F_GOS
B
IFN-γ (pg/mL)
4000
3000
2000
1000
0
*
C
Il-10 (pg/mL)
4000
3000
2000
1000
0
D
24 h
Il-1β (pg/mL)
6000
4000
2000
0
* *
* *
E
TNFα (pg/ml)
3000
2000
1000
100
80
60
40
20
0
F
IFN-γ (pg/mL)
5000
4000
3000
2000
1000
0
G
Il-10 (pg/mL)
4000
3000
2000
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0
H
48 h
Il-1β (pg/mL)
8000
6000
4000
2000
0
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Figure 6. Cytokine production in a Caco-2/PBMC co-culture model. Caco-2 cells were exposed to fermented products of i-SIEM (F_i-SIEM, pink), 2′-fucosyllactose (F_2′-FL, purple), and galacto-oligosaccharides (F_GOS, green) and medium control (grey), and medium with addition of LPS (20µg/ml), with and without filtering out lipopolysaccharide (LPS) or with the addition of the neutralizing antibody for Toll-like receptor-4 (TLR4), for 24 h and 48 h. On the basolateral side, PBMCs were added, non-activated, or activated with α-CD3/CD28 for 48 h. After 24 h (**A-D**) and 48 h (**E-H**) incubation, TNF-α (**A, E**), IFN-γ (**B, F**), IL-10 (**C, G**), and IL-1β (**D, H**) were measured. Data are presented as mean ± SEM of n=3 and n=6 independent PBMC donors. The conditions with activated PBMCs were analyzed separately as represented by the dotted line. Medium served as a control of the system and F_i-SIEM served as a control of the fermentation products. * $P < 0.05$, ** $P < 0.01$

5.4 Discussion

The objective of this study was to investigate the impact of NDO-derived fermentation products from TIM-2 experiments, containing the metabolites from fermented i-SIEM (control), or 2′-FL in i-SIEM or GOS in i-SIEM, on the barrier function of epithelial cells and immune activation in an *in-vitro* co-culture model of epithelial cells and PMBCs under both homeostatic and inflammatory conditions. To investigate this, we utilized a co-culture system comprising Caco-2 cells, which form a polarized monolayer and represent the intestinal epithelial barrier, with immune cells present in the lamina propria, for which PBMCs were used. Initially, we measured the metabolites generated through the fermentation of 2′-FL and GOS in the TIM-2 fermentation model. The fermentation of 2′-FL and GOS resulted in an overall increase in the production of SCFAs, which led to almost a doubling in concentration of both butyrate, propionate, and acetate compared to the control i-SIEM with acetate providing the highest concentration. These findings were consistent with previous batch fermentation studies reporting elevated levels of total SCFAs and acetate upon exposure to these prebiotics (31, 32). The viability of Caco-2 cells co-cultured with PBMCs was not affected by the fermentation products.

After measuring the presence of select metabolites in the fermentation product and their non-toxic nature for the epithelial cells, we examined their effect on the epithelial barrier integrity. SCFAs are known to regulate paracellular permeability by influencing the expression of tight junction proteins, such as zonula occludens-1 (33). Therefore, it was hypothesized that the fermentation products containing SCFAs would have a beneficial effect on epithelial resistance and permeability. In the co-culture model, the activated PBMCs did not lower the epithelial resistance nor increase leakage of 4 kD dextrans. However, apical exposure to the control fermentation product i-SIEM or F_GOS to epithelial cells co-cultured with activated PBMCs did result in increased leakage of 4 kD dextrans. These results indicate that NDO-derived fermentation products of GOS, and to a lesser extent already that of the control i-SIEM, in TIM-2 samples disturb epithelial integrity in the inflammatory model. F_2′-FL however, did show beneficial effects in the inflammatory model by enhancing TEER and maintaining the permeability comparable to control. Natividad et al. also investigated the effects of fermented media of 2′-FL from another fermentation model, on Caco-2 cells, and did not find a protective effect when measuring TEER, however, they did show a decreased translocation of dextran to the basolateral compartment compared to the control (34). Besides the fermentation products, it is known that SCFAs such as butyrate can protect against inflammatory-induced barrier disruption and lower inflammatory cytokine release by activated PBMCs (22). In the latter studies, 2-8 mM butyrate was the most effective. The fermentation products studied here contained 8-10 mM SCFAs. The end concentration of particularly butyrate and propionate may have been too low to show the barrier-protective and anti-inflammatory effects.

Next, we assessed the impact of fermentation products on the immune system in the epithelial cell/PBMC co-culture model. Under homeostatic conditions, exposure of epithelial cells to fermentation products of i-SIEM (control), or i-SIEM with added 2′-FL, or GOS did not significantly affect the basolateral cytokine production by PBMCs. However, the inflammatory model showed an increase in cytokine levels compared to the homeostatic conditions in the medium conditions, despite no difference in TEER. In the inflammatory model, exposure to the fermentation products of 2′-FL and GOS did not result in significant differences in the production of the pro-inflammatory cytokine TNF-α compared to the F_i-SIEM and the medium control. Previous studies showed that direct exposure to GOS and fructo-oligosaccharides (FOS) in an inflammatory model decreased TNF-α levels (35). Additionally, exposure of intestinal epithelial cells to 0.5-1% 2′-FL showed a decrease in basolateral TNF-α concentration in a different co-culture model (36). Collectively, the direct effects of apical exposure to NDOs observed in other studies on the basolateral pro-inflammatory cytokines were not evident after fermentation of these prebiotics. It should be noted that our co-culture model differed from the previous studies in terms of intestinal cell type (Caco-2 cells instead of HT-29 cells).

Moreover, apical exposure of F_GOS to epithelial cells resulted in an increase in basolateral IFN-γ levels compared to the medium control in the inflammatory model after 24 h, and downregulated IL-10 production by active PBMCs, which was not observed after 48 h. This may have contributed to the rise in permeability shown by the F_GOS since IFN-γ is known to disrupt epithelial barrier properties (37), while IL-10 may be protective (38). Another pro-inflammatory cytokine known to contribute to barrier disruption is IL-1β (39). The levels of IL-1β in the fermentation product exposed conditions were all elevated compared to medium controls. This effect was already evident using the control F_i-SIEM. In a co-culture of Caco-2/PBMCs, Korsten et al. found LPS to increase IL-1β secretion by PBMCs (22). Considering the increased IL-1β observed with F_i-SIEM, we measured the LPS concentration in all the samples and found a substantial amount present. Apical exposure to the fermentation products did not affect the TLR4 mRNA expression in epithelial cells in the inflammatory model. However, apical exposure to these fermentation products did enhance epithelial IL-8 and TNF-α mRNA expression in the inflammatory model. Therefore, the presence of LPS in the samples was hypothesized to be responsible for the changes in epithelial integrity and the enhanced pro-inflammatory response of activated PBMCs in the co-culture model exposed to F_i SIEM. Indeed, apical exposure to LPS in the inflammatory model did not affect epithelial integrity but did induce increased IFN-γ and IL-1β production by activated PBMCs. However, when removing LPS from F_i-SIEM or the 2′-FL or GOS fermentation products did not lower the IL-1β levels in these cultures, nor did blocking of the TLR4 receptor. In conclusion, LPS in the fermentation products obtained from the TIM-2 system, including the F_i-SIEM control, did not appear to be responsible for the observed effects on the barrier permeability or rise in basolateral IL-1β production by activated PBMCs. I-SIEM itself may have a disruptive effect, potentially masking the true effect of the fermentation products.

Taken together, these results highlight the complexity of the interactions between complete *in-vitro* large intestine-derived fermentation products in this *in-vitro* co-culture model combining intestinal epithelial cells and immune cells.

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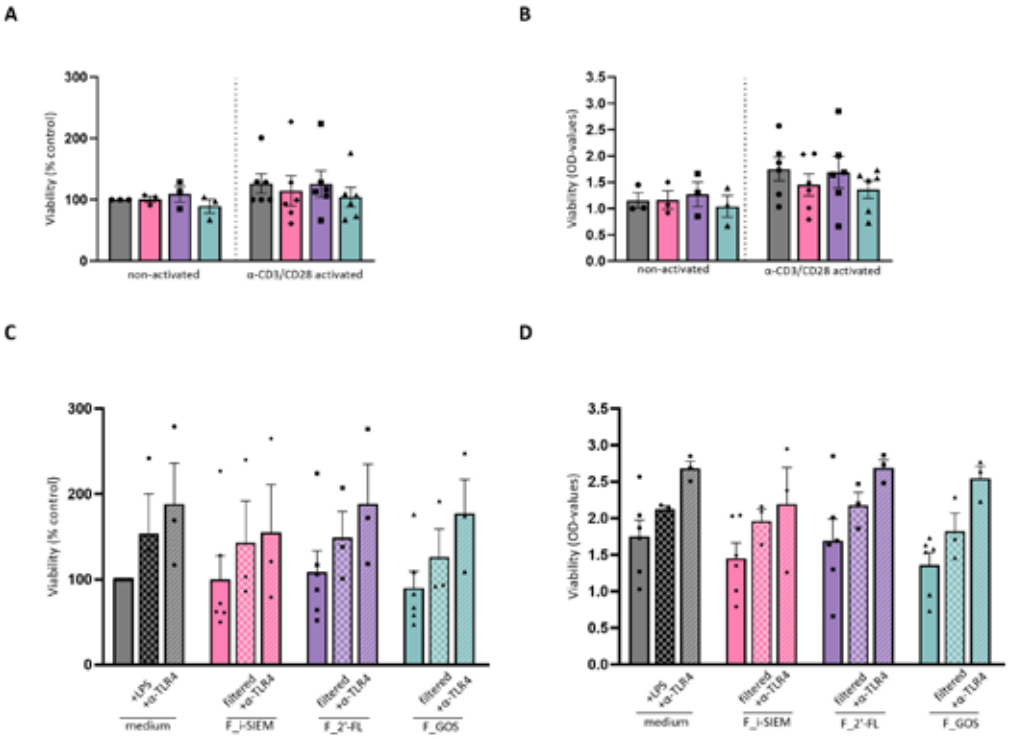
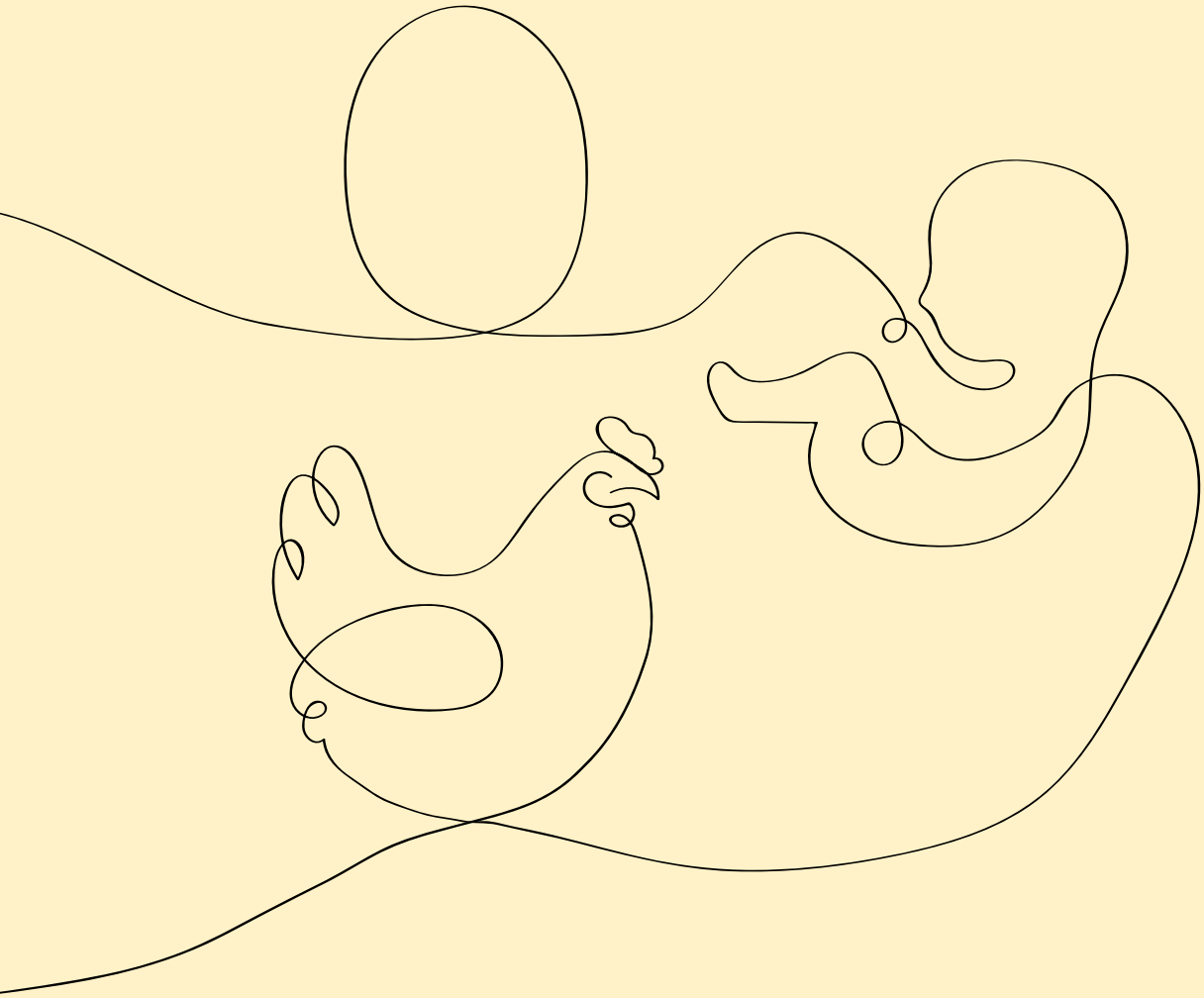


Supplementary figure 1. Viability by means of WTS-1 assay after exposure with 8 times diluted fermentation products of iSIEM (F_iSIEMm pink), 2'-fucosyllactose (F_2'-FL, purple) and galacto-oligosaccharide (F_GOS, green) in the homeostatic and inflammatory (α-CD3/CD28-actived PBMC) model. A. Viability expressed as percentage of control. B. Viability expressed as mean OD-values. Viability expressed as percentage of control (C) or mean OD-values (D) in the inflammatory model in which fermentation samples were filtered or treated with neutralizing anti-TNFα antibody, and LPS was added to the control medium, as a positive control. N=5

Chapter 6

General Discussion



In recent years, there has been a growing interest in developing interventions to improve early-life intestinal health in humans and animals. The intestinal tract, particularly the intestinal microbiota, plays a crucial role shaping the immune system and in regulating overall health. Intestinal microbiota is closely associated with early-life but also with long-term health and well-being of individuals and animals, as well as production (1-4). One way to modulate the microbiota, or increase its resilience, is by diet or specific dietary additives, such as pre-and probiotics (5). To understand their impact on intestinal health, extensive research has been conducted on prebiotics, probiotics, and other dietary interventions. Before implementing these interventions in practice, it is essential to thoroughly evaluate their effectiveness in enhancing intestinal health for both animals and humans. For this purpose, having efficient and predictive pre-screening tools before proceeding to *in-vivo* experiments in animal trials or under field circumstances or human clinical trials is valuable, as this protects and reduces animals used for biomedical research.

6.1 Motivation and aim of this thesis

The main objective of this thesis was to investigate the potential prebiotic effects of carbohydrates on intestinal health in broiler chickens and infants. We used sophisticated fermentation models, intestinal organoids, and co-culture models to achieve this. To study the microbiota and the metabolites produced during fermentation, the TNO *in-vitro* model of the colon (TIM-2) was used for humans/infants. Based on TIM-2 we developed the Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2) to screen and assess the ability of selected carbohydrates to modulate gut microbial communities and promote the production of beneficial metabolites in chickens. Additionally, we optimized the growth and longevity of chicken intestinal organoids by incorporating chicken-specific growth factors.

The research described in this thesis was performed within the public-private partnership program "CarboBiotics", coordinated by the Carbohydrate Competence Center (CCC, www.cccresearch.nl). In a parallel PhD thesis project by Adil Ijaz (Department Biomolecular Health Sciences, Division infectious Diseases and Immunology, Utrecht University), the chicken intestinal organoids were used to study cellular and immune responses to the selected carbohydrates and metabolites produced in the CALIMERO-2 system.

We aimed to translate knowledge obtained from broiler studies to humans. The fermentation products of the selected carbohydrates were obtained from research performed by Martha Endika, who was also part of the CCC consortium and focused on the role of prebiotics in the recovery of antibiotic-perturbed infant gut microbiota. We examined the effect of these fermentation products, on the integrity of the intestinal barrier and immune response through human cell culture experiments. For this purpose, we used a co-culture system of intestinal epithelial cells (Caco-2 cells) and immune cells (peripheral blood mononuclear cells, PBMCs) mimicking the human intestinal mucosal immune system (6).

This chapter provides a summary of the main findings, discusses their implication in relation to earlier research, and presents the concluding remarks and future recommendations for the work presented in this thesis.

6.2 Methodological approaches

A pipeline of *in-vitro* tools was developed, in which microbiological, epithelial, and immunological mechanisms can be unraveled. TIM-2 and CALIMERO-2, chicken intestinal organoids, and co-culture model of human intestinal epithelial cells and PBMCs are of great significance for testing/screening

functional dietary components, such as prebiotics and probiotics, or for evaluating other ingredients/ additives for human and animal intestinal health. This section provides an overview of the *in-vitro* tools developed and used in this thesis, their applications, and their strengths and limitations.

6.2.1 TNO *in-vitro* model of the colon

The TIM-2 system is a validated, dynamic computer-controlled fermentation model, which mimics the human colon. TIM-2 provides valuable insights into the effects of different substances on the microbiota composition and its metabolic activity. When conducting early developmental research in *in-vitro* models, the challenge is to mimic the dynamic and multifactorial nature of biological systems. The strength of TIM-2 is that it is a dynamic model, where removal of the produced metabolites during the fermentation by dialysis prevents metabolite build-up, which would otherwise inhibit or even kill the microbiota present in the system. Moreover, TIM-2 is a great alternative to clinical studies, since it is difficult to follow the fermentation process in infants, due to the inaccessibility of the proximal colon.

In this thesis, TIM-2 was used to mimic the infant's large intestine, by adding a fecal slurry of 3-month-old infants and mimicking the infant's diet, through the medium composition called *simulated ileal-efflux medium for infants* (i-SIEM). i_SIEM, mimics the composition of components that reach the colon from the terminal ileum through the ileal-cecal valve (7). In a separate collaborative project, Endika et al. investigated the effect of 2'-fucosyllactose (2'-FL) and galacto-oligosaccharides (GOS) on the resilience of infant gut microbiota to amoxicillin/clavulanate-induced changes in microbiota composition and activity, using TIM-2. 2'-FL or GOS were added to the system, and the fermentation process over time was closely followed. They found that 2'-FL and GOS promoted recovery of microbiota after antibiotics use (7). Lumen samples, obtained from these TIM-2 experiments containing the microbial fermentation products produced during the 3-day fermentation process, were further used in the human co-culture model, which is described in **Chapter 5**. We only studied the lumen samples of these TIM-2 experiments of the experiments without the antibiotics treatment. Addition of 2'-FL and GOS to infant microbiota in TIM-2 resulted in an enhanced production of short chain fatty acids (SCFAs) compared to the control. Van den Abbeele et al., who investigated the effect of 2'FL on intestinal microbiota of 3-month-old infants in another dynamic fermentation model, also observed an increase in total SCFA production induced by 2'-FL (8). Although the TIM-2 system has proven to be valuable to assess effects of GOS and 2'-FL on infant intestinal microbiota, TIM-2 also has some general limitations. Firstly, due to the variations in oxygen level, especially in infants, the intestinal tract is first colonized with facultative anaerobes, whereas adult microbiota is composed of strict anaerobes (9-11). TIM-2 is kept strictly anaerobic, and even though facultative anaerobic bacteria can survive and grow under both aerobic and anaerobic conditions, facultative anaerobes can be less prevalent in the TIM-2 system compared to the infant's colon. Secondly, the model does not have epithelial or immune cells, which in a real-life situation are in continuous interplay with the microbiota (12). In section 6.2.4 this is discussed in more detail. Since the technical limitations are similar for CALIMERO-2, and this model was mostly used in this thesis, some of the more general limitations are discussed in section 6.2.2.

6.2.2 Development of a fermentation model for chickens

The Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2), which is an *in-vitro* cecum fermentation model for chickens, was developed based on the TIM-2 system as described in **Chapter 2**. By adjusting multiple parameters, compared to TIM-2, this model effectively replicates the conditions of the chicken cecum, including pH (6.6) and system temperature (41°C). In comparing the bacterial composition of the original inoculum collected from chicken ceca with microbial samples taken from CALIMERO-2 every 24 h for 3 days, we observed significant shifts in bacterial diversity for all groups shortly after the fermentation started. However, over time, the bacterial diversity increased and became more

similar to the original inoculum. Previous studies on optimizing the TIM-2 model for piglets, known as SLIM (Swine *in-vitro* Large Intestinal Model), demonstrated the relevance of changing substrate types (compared to humans) to better mimic the swine gut (13). Similarly, we compared several medium compositions that simulate the components that reach the cecum, to simulate the chickens' diet and found that the medium composition had minimal impact on the outcomes for CALIMERO-2. Therefore, we decided to use the simulated ileal-efflux medium (SIEM) which is also used in TIM-2. Based on these results, we can conclude that CALIMERO-2 serves as a robust and cost-effective system for studying the effects of feed ingredients/additives on the chicken intestinal microbiota composition and the subsequent production of microbial metabolites. Like the TIM-2 system, CALIMERO-2 can be used as a pre-screening tool, contributing to the reduction of the use of laboratory animals. Furthermore, experiments are computer-controlled, and a standardized inoculum is used that allows for a large number of experiments, ensuring high reproducibility and comparability between runs, since the same microbiota is used in all of these experiments. This is deemed appropriate, because the microbiota in a chicken flock has a lot of overlap in microbiota composition, because of the low genetic variation between chickens (14). Moreover, previous research has shown that the majority of operational taxonomic units (OTUs) are shared among pooled inoculum and individual microbiota samples (15, 16).

Besides these strengths, it is important to note that also CALIMERO-2 has certain limitations. The *in-vitro* model lacks the complexity of the *in-vivo* situation, such as the absence of the intestinal mucus layer and cellular layer. Additionally, CALIMERO-2 cannot fully replicate the interactions that occur within living organisms, such as immune responses or the impact of host factors on microbial behavior. A study by Tran et al. (17) showed that the addition of mucins to an *in-vitro* batch pig fermentation model affected microbial genera and fermentation patterns. Moreover, the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) also incorporates a mucous environment to promote colonization of specific microbes similar to the *in-vivo* situation. This model can also be connected to a host-microbiota-interaction module, consisting of a double layer mimicking a luminal compartment and the epithelial layer (18). This model was developed to mimic the human gastrointestinal tract, but not for the chicken. In the future, the effect of the addition of mucins can be studied in CALIMERO-2. Similar to many other *in-vitro* models, CALIMERO-2 cannot fully replicate the interactions that occur within living organisms, such as the host immune responses or the host-microbiota interactions. *In-vivo* studies, under experimental or field circumstances will help to study these interactions. However, also *in-vivo* studies have many limitations. The microbiota composition can be affected by many, often hidden, host and environmental factors, including breed, age, housing and feed, that can make the interpretation and comparisons of results between *in-vivo* studies especially difficult (19). The development and improvement of gut microbe-intestinal organoid systems, incorporating more elements that reflect the complexity of the *in-vivo* system, can also contribute to better interpretation.

We should also acknowledge the limitation in the taxonomic classification below genus level in the follow-up analysis, as the V3-V4 region of 16S rRNA gene is highly similar or identical across multiple bacterial species (20). To allow the interventions to reach the full potential and to study the impact not only on the short-term, the fermentation period in an *in-vitro* system should not be too short. Therefore, a longer fermentation period might be needed, to study the long-term impact. Compared to simple batch fermentation, the TIM-2 and CALIMERO-2 systems can already study the fermentation for a longer period, since metabolites are filtered out of the system, and cannot reach a toxic level. Other dynamic models, such as M-SHIME for human studies, and PolyFermS, which is also developed for chicken, can be used to study a longer period, up to 48 h and respectively 38 days (18, 21)

6.2.3 Development of chicken intestinal organoids for *in-vitro* testing

Another *in-vitro* tool that was developed and described in **Chapter 3**, is a chicken intestinal organoid model that mimics the chickens' intestinal structure and function. This cellular model can be a valuable tool for pre-screening of intestinal health promoting interventions. This three-dimensional cell culture system, obtained from a culture of intestinal stem cells, provides a realistic representation of the intestinal epithelium, with all epithelial cell types present in the chicken gut. It enabled us to take the first step to assess the effects of compounds (carbohydrates or microbial metabolites) on intestinal health and functionality. While several attempts have been made by poultry researchers to develop such an *in-vitro* model, only a few groups have succeeded in isolating and growing chicken intestinal organoids (22-26). One of the limiting factors was the longevity of the culture. In **Chapter 3**, we describe how we established chicken intestinal organoids using cells from embryonic day 18 chicken eggs. The intestines were collected and used to isolate intestinal crypts, which were then cultured in a specialized medium containing prostaglandin E2 and Forkhead box O1-inhibitor (FOXO1-inhibitor). This medium proved effective in supporting the growth and differentiation of the organoids. Based on our experience with the development of mouse-derived organoids, we found that growth factors Wnt family member 3A (WNT3A) and R-spondin 1 (RSPO1) were crucial for the maintenance and differentiation of stem cells. Considering the limited homology between mammalian and avian RPSO1, we discovered that chicken-derived factor, in combination with avian WNT3, were necessary for maintaining the chicken organoid cultures. In addition to the three-dimensional culture, we also succeeded in growing organoids in a two-dimensional layer, which exhibited similar characteristics to the three-dimensional organoids. One of our future challenges is to grow them in a way that forms a tight monolayer, allowing for intestinal barrier integrity experiments.

The current chicken intestinal organoid model has proven to be a valuable *in-vitro* tool for assessing the impact of potential prebiotics on intestinal epithelium functionality. This has been done with the selected carbohydrates and will be done with the microbial metabolites obtained from CALIMERO-2 experiments, in a parallel project from the CCC consortium by Adil Ijaz (data not yet published). Furthermore, in the future, the chicken intestinal organoids can be used in a co-culture with immune cells and micro-organisms to mimic the host-microbe interactions, which is currently in progress. For humans, Puschhof and colleagues managed to develop such an intestinal organoid co-culture with microbes, demonstrating its value to study host-microbe interaction (27). This technique is also under consideration for chicken intestinal organoids.

Chicken intestinal organoids are constantly under development. We established growth and maintenance with our protocol and can grow them in a two-dimensional manner. Additionally, Nash et al. developed chicken intestinal organoids that grow inside-out, which have similar benefits as the two-dimensional culture. Furthermore, their culture also includes leukocytes, which the authors refer to as a natural epithelial-leukocyte co-culture enteroid model (26). In the future, if the cultures of Nash et al. and ours can be combined, a long-term culture, with the cells responsible for the immune response present would be ideal. Moreover, as already brought up in section 6.2.2, it is hard to cover the complexity of the *in-vivo* situation in *in-vitro* models. Despite the fact that the intestinal organoids are already an improvement compared to single (often immortalized or cancerous) cell lines, due to the presence of multiple intestinal cell types and especially when co-cultures with immune cells can be developed as well, they still lack a vascular and nervous system. These limitations can be addressed by employing static intestinal organoids within an "intestine-on-a-chip" model, which is a microfluidic device enabling controlled flow of fluids and nutrients to replicate the environment of intestinal cells, including peristaltic movements and the presence of intestinal microbes. This design is intended to

mimic the dynamic aspects of te intestine. In contrast, the M-SHIME, connected to a host-microbiota-interaction module, employs enterocytes instead of intestinal organoids.

6.2.4 Co-culture model: Caco-2 cells and PBMCs for evaluating prebiotic effects

To investigate the indirect effects of 2'-FL and GOS on a host-cellular level, we utilized a human co-culture system consisting of a human immortalized cell line of human Caco-2 cells and PBMCs (6) (**Chapter 5). The widely used Caco-2 cells** (28), a human colonic epithelial cell line, were seeded on the apical side of a Transwell insert, while PBMCs were added to the basolateral compartment. This co-culture system provides valuable insights into intercellular communication. The combination of PBMCs with Caco-2 cells offers the advantage of studying the intestinal barrier function and mucosal immune response, allowing to explore the cross-talk between epithelium and immune system.

Through use of the co-culture model, we investigated the potential of microbial metabolites, produced by a 3-month-old infant microbiota upon fermentation of selected carbohydrates in TIM-2, to modulate barrier integrity and the mucosal immune response. In this model, the inflammatory environment was mimicked by stimulating PBMCs with αCD3/αCD28, which represent the mucosal immune cells (6). αCD3/αCD28 are monoclonal antibodies that bind to T-cell receptors and co-stimulatory molecules (29). We found that activation of PBMCs was needed to promote the production of the cytokines.

The Caco-2 cells can differentiate into fully polarized enterocytes that form functional barrier properties (28, 30). Therefore, they are great assets for mechanistic studies and studying colonic barrier integrity. However, they lack goblet cells that produce mucus, which in the intestine form a thick layer. Mucus can provide nutrients for the commensal bacteria present in the intestine and plays a role in the physical barrier protecting the epithelial cell surface (31, 32). This is something that should be taken into account when interpreting the results. This can be covered by using a co-culture of Caco-2 and the mucus-producing HT-29 in a 9:1 ratio. This strategy has been previously shown to result in a tight intestinal barrier, enhanced mucus production, and increased expression of mucus-related genes (33). This modified co-culture system allows better replicating the physiological conditions of the intestine and more comprehensively studying interaction with commensal bacteria. moreover, organoid-derived intestinal co-culture models have gained recognition as a convenient tool for studying physiological events and exploring interaction between different cell populations, as well as investigating intestinal barrier integrity (34).

In this thesis, the validated co-culture model with Caco-2/PBMCs was used. When unraveling the indirect effects on a cellular level, human intestinal organoids can be used to get better-predicted values.

6.3 Carbohydrates and their effect on microbiota, intestinal barrier function and mucosal immune response

Human milk oligosaccharides and various non-digestible carbohydrates have demonstrated significant prebiotic functions, and thereby health benefits (35-40). They affect the gut microbiota, by having a beneficial effect on the resilience and stability of the gut microbiota in humans and animals (41-43). Prebiotics are defined as a substrate that is selectively utilized by host micro-organisms conferring a health benefit (44). Additionally, these carbohydrates can have a beneficial effect on intestinal

permeability and influence proteins involved in intestinal barrier integrity, such as claudins and occludins (45, 46). Notably, two specific compounds, 2'-FL and GOS, have gained recognition for their health benefits (47-50). Previous studies have demonstrated the direct impact of the human milk oligosaccharide, 2'-FL on the interaction between bacteria and epithelial cells, and protection against infection and inflammation (51-53). The indigestible GOS is fermented in the colon and can also contribute to various health-related functions, including increased SCFAs production and support for the growth of beneficial microbes, like *Lactobacillus* (38, 48, 54, 55).

Furthermore, studies have investigated the microbiota-independent effect of these non-digestible carbohydrates, revealing their impact on human health. GOS, for instance, has been found to directly influence intestinal epithelial cells and interact with immune cells, displaying a protective role in inflammatory environments by preserving the tight junction network and modulating the inflammatory response (56, 57).

Research has also focused on mixtures of non-digestible carbohydrates, particularly the combination of GOS and FOS, which closely mimics the structural diversity of human milk oligosaccharides. This combination has been extensively studied for its immunomodulatory and prebiotic effects and is already used as a supplement in infant formula (58). Additionally, the effects of the combination of 2'-FL together with GOS/FOS have been investigated, revealing distinct immune-related cytokine activation compared to single treatment (59, 60). Therefore, exploring combination treatments instead of relying solely on individual treatments may hold promising avenues for future research.

In this thesis, the microbial fermentation products of 2'-FL and GOS were further investigated, as well as the impact of these metabolites on the intestinal cells and immune responses.

In chickens, mannan-oligosaccharide (MOS) is a prebiotic that has shown to have intestinal health promoting effects, and was therefore used as a positive control in the study in **Chapter 4** (61, 62). In this thesis, we investigated the effects of the novel carbohydrate Isomalto/malto-polysaccharide (IMMP), which is of potato source, and three different citrus pectins concerning chicken health. Besides their health benefits, IMMP and pectins are also sustainable sources of carbohydrates. IMMP is derived from starch via enzymatic modification and has shown prebiotic potential in humans and mice (63-65). IMMP can escape digestion in the upper gastrointestinal tract because of the presence of α-(1→ 6) segments, and therefore can be used as a carbon source by microbiota in the colon (66). Moreover, during *in-vitro* fermentation of IMMP, an increase of SCFA, lactate, and succinate production was observed, and an increase in the genera *Bifidobacterium* and *Lactobacillus* was found (63). The pectins have not been studied in relation with the microbiota before.

6.3.1 Testing potential prebiotic effect of the three citrus pectins and IMMP in the *in-vitro* model CALIMERO-2: the importance of carbohydrate characteristics

The main findings of **Chapter 4** were that the chemical features of the carbohydrates are of great importance for their functionality and fermentability. Pectin polysaccharides have a complex composition, and the structure is highly diverse and is built up of four main structural elements, homogalacturonan, rhamnogalacturonan I and II, and xylogalacturonan (67). The monosaccharides [building blocks of the pectins] can be acetylated or methylated (68). The way the pectin molecule is built up also influences the behavior of the molecule (69, 70). We found in the *in-vitro system CALIMERO-2, that ev*en though there are slight differences between the different pectins in, for example structural elements, including amount of galactose, and degree of methylation, this can already have a great

impact on the modulation of microbiota diversity or metabolite production. For example, pectins P2 and P3 were both highly methyl-esterified and showed an overlap in modulation of beta-diversity of the microbiota. The galactose percentage however differed between P2 and P3, which might explain why only P3 was significantly different in beta-diversity compared to the low methyl-esterified pectin P1. The fact that the structural properties of pectins are of great importance for its effect on the microbiota composition, has also been found in previous research (69, 71, 72). It is imperative that the characterization of carbohydrates is considered in microbiota research, because it might clarify the differences induced in relative abundance and diversity of intestinal microbiota, or metabolite production. The potato-derived polysaccharide IMMP exhibited significant difference in both alpha- and beta-diversity compared to the positive control MOS. The difference in alpha-diversity suggests variation in the diversity and evenness of species within each group. The significant dissimilarity in beta-diversity indicates distinct bacterial communities between IMMP and MOS, implying that they have different species compositions. Interestingly, the values of total SCFAs, organic acids, and BCFAs were found to be very similar between the SIEM, IMMP and P3, with IMMP and P3 exhibiting even greater similarity. This suggest that the fermentation patterns associated with IMMP and P3 might share common metabolic pathways. However, it is worth noting that the gut microbiota was differently modulated by these two substrates, once more demonstrating that the fermentation metabolites' similarity in amounts does not necessarily reflect identical microbial community responses. IMMP showed a similar concentration of total SCFA's compared to MOS. The increase in total SCFA production was also found in the human studies in which IMMP was investigated (63). However, it should be noted that there may be an optimal level and proportion of SCFAs in chickens' intestine that benefit intestinal health. Excessive amounts of SCFAs, mainly acetate and butyrate, can lead to enteritis in chicken production (73).

In addition to the investigation of the carbohydrates, the pathogen *Clostridium perfringens* was added to CALIMERO-2, to mimic *C. perfringens* overgrowth, seen in chickens with necrotic enteritis (NE) (74). A reduction of the Shannon index of the cecal microbiota was shown in the diseased model for SIEM and MOS, which can be linked to the addition of *C. perfringens* since this pathogen can cause a shift, and reduces the alpha-diversity in intestinal microbiota. However, these effects between the healthy and diseased model were not observed in the IMMP and pectins group. This might be, because IMMP and the pectins already had an interaction in the first 24 h of the fermentation. It should be noted, however, that we could not confirm presence of the pathogen in the 16S rRNA sequencing data in the diseased model. This was expected, as the V3-V4 region of the 16S rRNA gene (used in this research) is highly similar or identical across multiple species (20), and therefore limits the taxonomic classification below the genus level. For species-level analysis, a high-throughput sequencing-based approach is needed that targets the complete 16S rRNA gene, or whole-genome shotgun sequencing should be performed. Since the pathogen *C. perfringens* could not be traced back due to technical limitations, and because there are no significant differences in diversity and metabolites produced for the other carbohydrates, except the SIEM and MOS samples, it is not possible to conclude whether the carbohydrates can have an impact on this specific taxon involved in the disease NE. In a healthy situation, we do see a promising effect of IMMP on the metabolites, by increasing the production of acetate, butyrate and propionate during the fermentation process, but this should be studied further.

6.3.2 The effect of fermentation products of 2'-FL and GOS on barrier integrity and immune responses

Besides effects of non-digestible oligosaccharides (NDOs) on microbiota, previous studies also looked into the microbiota-independent effects of NDOs, i.e. those that act directly on for example intestinal and/or immune cells (56, 57). To our knowledge, there has been no research on fermentation products

and their effect on cellular responses in a human co-culture model using epithelial cells and PBMC. In **Chapter 5,** the fermentation products of the prebiotics 2′-FL and GOS were examined on the Caco-2/PBMC co-culture model, to unravel the mechanism by which these metabolites affect the epithelial barrier and mucosal immune response. Firstly, we determined whether the fermentation products were toxic to the epithelial cells because of the bacterial components that might be present (not only the metabolites themselves, but also e.g., endotoxins like lipopolysaccharide [LPS]), which was not the case. Fermentation of 2′-FL and GOS in TIM-2 resulted in enhanced production of SCFAs compared to the control, especially that of acetate. SCFAs can increase the resistance of intestinal epithelial cells against inflammation-induced barrier disruption (6), as was also shown by the fermentation products of 2′-FL here. However, this was not apparent from the fermentation products of GOS. At the same time, the permeability of the intestinal cells was increased by the fermentation product of GOS (together with the fermentation control i-SIEM). Our results with GOS fermentation products are not comparable with results presented in a previous study, where the exposure of GOS to Caco-2 cells induced a protective effect (56). In addition, fermentation products of the control i-SIEM, 2′-FL and GOS induced an IL-1β response basolaterally. The fermentation products contained substantial amounts of LPS. LPS-filtered samples and the addition of an anti-TLR4 antibody did not change the effect of the fermentation products of 2′-FL and GOS on the barrier integrity or basolateral immune responses. It is a challenge to compare the data obtained in this study, with previous studies, since the composition of the fermentation products is partly unknown, as we only measured SCFAs. These results highlight the complexity of the interactions between complete *in-vitro* large intestine-derived fermentation products in this *in-vitro* co-culture model combining intestinal epithelial cells and immune cells. Future research should focus on the mechanisms behind the immune activation and permeability of the intestinal epithelial cells in relation to these fermentation products.

6.4 Future perspectives

TIM-2 has been validated and is in use for >20 years, and is predictive for human clinical studies. This makes the fermentation system a great tool for pre-screening for the efficacy of food interventions but also reduces the number of *in-vivo* experiments needed for further validation before the food interventions can be commercially applied. As mentioned, TIM-2 is already widely used, and CALIMERO-2 is likely to follow the same path to assess the effects of feed intervention on chicken intestinal microbiota. As a validated and trustworthy system, it can provide a substantial contribution to (mechanistic) poultry research and the development of health-promoting additives.

Even though this thesis only highlighted the use of CALIMERO-2 and TIM-2 for bacterial composition, it can also be implemented for a broader use, e.g. studying the virome, fungome and mycobiome. The use of the fermentation models for this kind of research is under development. Maas et al. focused on the link between bacterial composition and the virome and mycobiome, since the intestine harbors all these communities and there might be a relevant interplay between them (75, 76). Furthermore, the fermentation models should be developed further, for mimicking diseases or mimicking a state of dysbiosis. An effort has been made in this thesis, to mimic *C. perfringens* overgrowth, however, the inoculation of this pathogen should be further studied, by for example taking more samples earlier in the fermentation process, to follow the survival and multiplication of the specific bacteria using more in depth sequencing techniques. To cause dysbiosis in the fermentation models, Endika et al. added the antibiotic mixture amoxicillin/clavulanate to the system, which resulted in changes in the microbiota composition (7). However, it would be important to investigate the long-term impact of the antibiotic treatment, for this reason, the fermentation process should be extended.

In the broader context of poultry nutrition, the utilization of agricultural co-products in feed has gained popularity as a means to reduce production costs, and minimize the use of antimicrobials. Dietary fibers, for instance, have emerged as crucial modulators of the gut microbiome, immune responses, and promoting intestinal integrity. In the context of examining the effects of carbohydrates in relation to feed additives, it is noteworthy that studies involving the use of oligosaccharides as feed additives have yielded varying results. Nevertheless, there is substantial potential, especially for oligosaccharides such as MOS (58, 77). Some of these oligosaccharides have already made their way into the market and established a record of accomplishment of success, while others, especially newer variants, warrant further research(2, 5, 78).

It is essential to emphasize that research efforts should extend beyond *in-vitro* and *in-vivo* investigations of the effect and mechanisms of action. To truly replace antibiotics and other antimicrobials or chemicals, the applicability of these additives to be used as alternatives must be rigorously evaluated in the field, including assessing their impact on health, production performance and the quality of the animal product. Demonstrated efficacy is obviously a prerequisite for further development, but cost-effectiveness and practical applicability, including scalability and ease of incorporation into feed, are equally vital considerations for the ultimate success of these dietary interventions.

In this thesis, we already investigated the fermentation products of 2'FL and GOS derived from TIM-2 on a co-culture with intestinal epithelial cells and immune cells. In addition, it would be interesting to unravel the composition of the fermentation products, to further investigate and to explain the increase of the basolateral cytokine IL-1β and their observed lack of enhancing the barrier function of the intestinal cells. Therefore, the mechanisms behind the results are important. Moreover, the fermentation products of the pectins and IMMP could also be investigated on chicken intestinal organoids.

It is imperative that the chicken intestinal organoids will reach the same popularity as the intestinal organoids of mouse and human origin. Further research is needed to improve the two-dimensional culture so that in the future, these can properly be used for barrier function and epithelium-immune cell cross-talk experiments. Moreover, the inside-out culture of Nash et al.(26) could be further optimized, and exposure studies can be performed with these three-dimensional structures.

Moving forward, there are several avenues for future research based on the findings of this study, such as the enhancement of the SCFA by IMMP, and the induction of *Bacteroides* by the pectins and *Akkermansia* by Pectin 1. Therefore, the effect of the carbohydrates investigated in this thesis on the chicken intestinal organoids (with and without immune cells in co-culture) and the subsequently induced cellular responses are currently under evaluation. Next, these potential prebiotics can be tested *in-vivo*, in small trials, to validate their effects on gut health and explore their potential therapeutic applications.

Overall, the work of this thesis presents an *in-vitro* pipeline combining sophisticated fermentation models for chicken and man with intestinal models (organoids [chicken] or co-culture cell model [man] to investigate the potential prebiotic effect of carbohydrates. This *in-vitro* test pipeline for intestinal health is compliant to the principle of the "Three Rs": replacement, reduction and refinement; is more predictive than e.g. *in-vivo* mouse models, and can also be implemented for other mammals.

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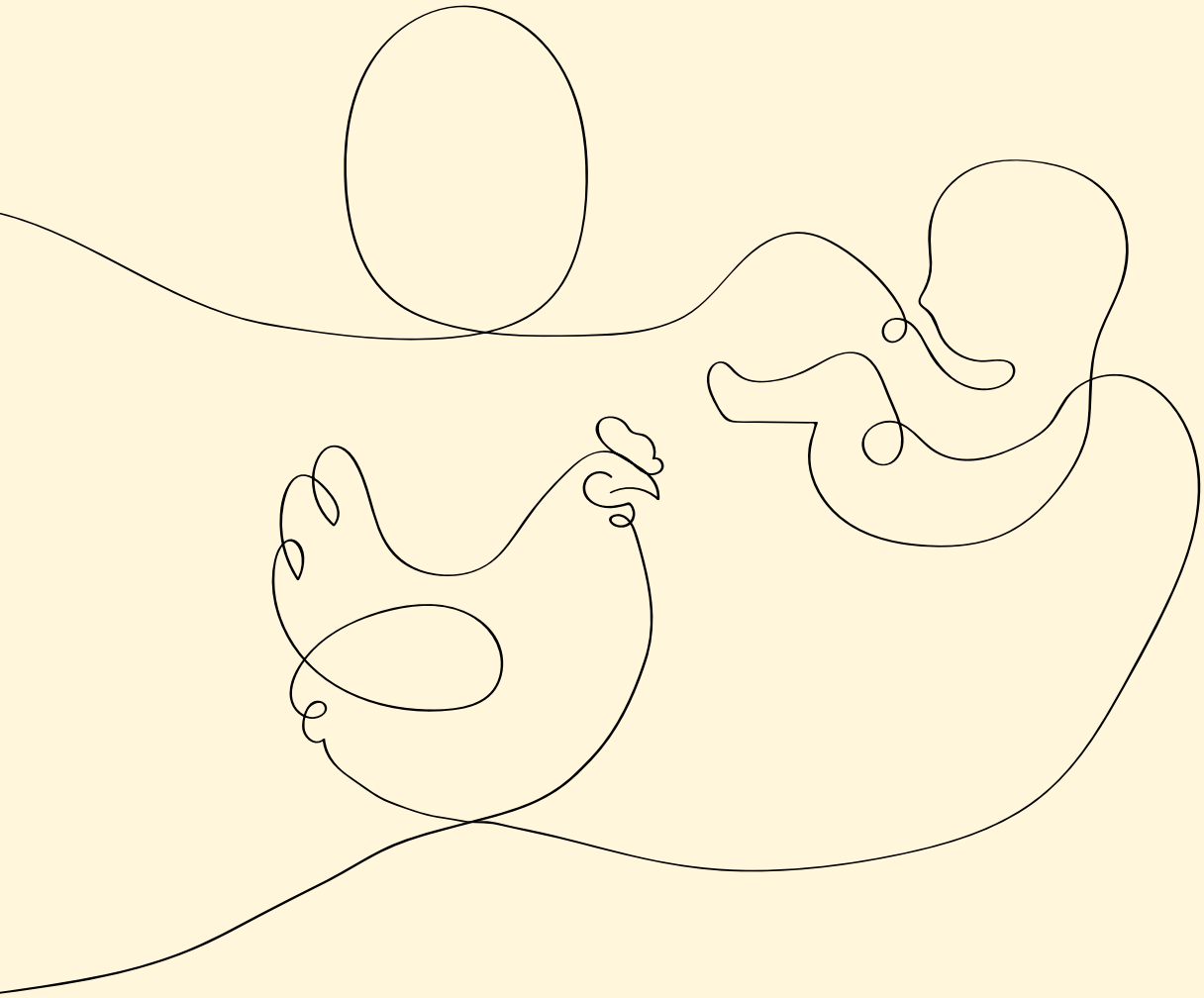
Addendum



Nederlandse samenvatting

De darmen spelen een belangrijke rol in de algemene gezondheid en het welzijn van mens en dier. In de darmen bevindt zich een enorme populatie microben, die samen de microbiota vormen. De microbiota hebben invloed op vele biologische processen, waaronder de vertering van voeding, de opname van voedingsstoffen, het onderhouden van de barrièrefunctie van de darmen, het afweersysteem en de stofwisseling. Kolonisatie van de darmen begint bij de geboorte van zoogdieren (in dit proefschrift: de mens) en bij het uitkomen van het ei voor gevogelte (de kip). De ontwikkeling van samenstelling van microbiota in de darmen wordt beïnvloed door vele factoren, zoals de genetische samenstelling van de gastheer en de leefomgeving. In de eerste levensfase van mens en dier ondergaan de darmmicrobiota dynamische veranderingen. Na 2-3 jaar bij de mens en 3-5 weken bij vleeskuikens is de microbiota divers en stabiel.

De samenstelling van de microbiota kan continu worden beïnvloed door bijvoorbeeld dieet, leefomgeving, de aanwezigheid van ziekteverwekkers, of antibioticagebruik. Over het algemeen herstelt de samenstelling van de microbiota weer, maar als het evenwicht ernstig wordt verstoord, dan leidt dit tot dysbiose. Dit kan gezondheidsproblemen, zoals infecties of ontstekingen in de darm en soms ook in andere delen van het lichaam, tot gevolg hebben. De verstoorde balans tussen goede en slechte bacteriën in vleeskuikens wordt geassocieerd met darmontstekingen en verkorting van vlokken (villi) van de dunne darm, resulterend in een afname van absorptie van voedingsstoffen en minder efficiënte groei van de dieren. Een veel voorkomende darmziekte in vleeskuikens is coccidiose. Als gevolg van coccidiose kan een ernstige darmontsteking, genaamd necrotische enteritis, ontstaan. Necrotische enteritis is geassocieerd met overgroei van de pathogene bacterie *Clostridium perfringens*. In veel gevallen hebben deze darmziekten een grote invloed op een groot deel van kippenpopulatie in een stal en worden antibiotica ingezet om ziekte en sterfte te beperken. Bij vroeggeboren baby's komt ook een darmziekte voor die geassocieerd is met *Clostridium perfringens*, genaamd necrotiserende enterocolitis. Deze darmaandoening is een ernstige darmontsteking die gepaard gaat met een verminderde doorbloeding van de darm, waardoor delen van de darmen kunnen afsterven en de pasgeboren baby kan overlijden.

Aangezien de darmmicrobiota zo'n veelzijdige en belangrijke rol spelen in de gezondheid van mens en dier, wijdt men veel onderzoek aan het onderhouden en verbeteren van de microbiële samenstelling en het onderhouden van de barrièrefunctie van de darm. Voor het verbeteren van de samenstelling van de microbiota in de darmen worden bijvoorbeeld niet-verteerbare oligosachariden, ook wel prebiotica, ingezet om gezondheidsvoordelen te leveren. Moedermelk bevat natuurlijke prebiotica en wordt geadviseerd ter voorkoming van necrotiserende enterocolitis. Maar er is meer onderzoek nodig om alternatieven voor moedermelk en antibiotica gebruik te vinden. In dit proefschrift wordt onderzoek gepresenteerd met twee bewezen prebiotica, namelijk 2'-fucosyllactose (2'-Fl), dat ook in moedermelk aanwezig is, en galacto-oligosachariden (GOS), één van de meest bekende oligosachariden die al wordt toegevoegd aan flesvoeding vanwege de prebiotische werking. Daarnaast is men op zoek naar andere prebiotica die de darmgezondheid kunnen onderhouden en verbeteren. Om nieuwe potentiële prebiotica te testen komen *in-vitro* technieken goed van pas. Daarom is in dit proefschrift aandacht besteedt aan een reeks van *in-vitro* testen, waarin fermentatiemodellen en darmmodellen worden gecombineerd voor zowel kip als mens, waaronder darmorganoïden (een soort 'mini-darmen') en co-cultuur cel-modellen. In deze modellen kunnen potentiële prebiotische effecten van oligosachariden worden gescreend. In dit proefschrift is dit gedaan voor isomalto/malto-polysachariden (IMMP) en drie verschillende citruspectines.

In **hoofdstuk 2** is het systeem CALIMERO-2, dat de blindedarm van de kip op microbieel niveau nabootst, geoptimaliseerd en vergeleken met blindedarmmonsters afkomstig uit vleeskuikens. Het systeem is gebaseerd op het gevalideerde TIM-2 model, dat is opgezet om de fermentatie in de dikke darm van de mens na te bootsen. Voor CALIMERO-2 zijn de temperatuur (41 °C) en pH (6,6) aangepast, zodat deze overeenkomen met de situatie in de blindedarm van kippen. Daarnaast zijn er verschillende mediumsamenstellingen getest, die worden toegevoegd aan het systeem om de voedingscomponenten na te bootsen die normaliter in de blindedarm van de kip aankomen. Hieruit bleek dat het medium genaamd SIEM, dat ook wordt gebruikt voor de experimenten in TIM-2, de meeste overeenkomsten gaf met de originele microbiotasamenstelling van monsters van de vleeskuikens.

In **hoofdstuk 3** is de optimalisatie van een ander *in-vitro* systeem te lezen, namelijk van de mini-darmen voor de kip. Deze kippendarmorganoïden bevatten diverse typen cellen die ook in de kippendarm voorkomen. Om deze organoïden goed te kunnen kweken, maar vooral ook voor langere tijd in kweek te kunnen houden, moesten er groeifactoren worden toegevoegd die hun oorsprong vinden in de kip, aangezien de veel gebruikte groeifactoren voor muizenorganoïden biologisch gezien te veel afwijken. Naast deze driedimensionale structuren, kunnen de kippendarmorganoïden ook tweedimensionaal worden gekweekt, waardoor ze gemakkelijker kunnen worden blootgesteld aan testproducten, die anders binnen in de driedimensionale organoïden geïnjecteerd zouden moeten worden.

In **hoofdstuk 4** is de samenstelling van vier oligosachariden, namelijk van drie citruspectinen en Isomalto/malto-polysacharide (IMMP) bepaald, en is onderzocht hoe deze oligosachariden de darmmicrobiota kunnen beïnvloeden in het *in-vitro* system CALIMERO-2, dat in hoofdstuk 2 is beschreven. Deze oligosachariden zijn vergeleken met het standaard medium (SIEM) en een positieve controle, waarvoor Mannan-oligosacharide (MOS) is gebruikt. De structurele analyse onthulde kleine verschillen in monosacharidesamenstelling en moleculair gewicht tussen de drie citruspectinen. Met name pectine 1 vertoonde een lage mate van methylverestering, terwijl pectine 2 en 3 een hoge methylverestering hadden. Deze overeenkomsten tussen pectine 2 en 3 waren ook te zien in de effecten op de diversiteit van de microbiota. Het fylum *Bacteroidetes* was het meest dominant, behalve in de twee controles SIEM en MOS, waarbij *Firmicutes* de overhand had. Gunstige bacteriën, met name *Lactobacillus*, bleven stabiel in alle monsters. Pectine 1 veroorzaakte een significant lagere totale productie van korte-keten vetzuren door de microbiota in vergelijking met SIEM, IMMP en pectine 3. Het effect van IMMP leek sterk op dat van de positieve controle MOS, vooral wat betreft het boterzuurgehalte. Deze studie onderstreept het potentieel van deze geteste prebiotica om via de microbiota de darmgezondheid bij vleeskuikens te bevorderen.

In **hoofdstuk 5** zijn de fermentatieproducten van de prebiotica 2'-FL en GOS, afkomstig uit TIM-2 met microbiota van 3 maanden oude baby's, getest in een co-cultuurmodel met darmepitheelcellen en witte bloedcellen om hun effecten te bestuderen op doorlaatbaarheid van de epitheelbarrière en de activiteit van de afweercellen. Deze fermentatieproducten bevatten onder andere verscheidene metabolieten die worden geproduceerd door de fermentatie van de prebiotica. De darmepitheelcellen zijn aan de bovenkant (apicaal) blootgesteld aan de fermentatieproducten van 2'-FL en GOS en van de controle genaamd i-SIEM (een variant van SIEM voor baby's). De fermentatieproducten van GOS verhoogden de doorlaatbaarheid van de epitheelcellen. Dit was geassocieerd met een toename van de afgifte van de ontstekingsmediator, interferon-gamma, door de onderliggende afweercellen. Zowel de controle fermentatieproducten, als de fermentatieproducten van 2'-FL en GOS, verhoogden de afgifte van ontstekingsmediator, interleukine-1 bèta, door geactiveerde afweercellen. De fermentatieproducten bevatten allemaal substantiële, maar vergelijkbare hoeveelheden lipopolysachariden (LPS). LPS is het belangrijkste bestanddeel van de buitenmembraan van gramnegatieve bacteriën. Wanneer LPS eruit

gefilterd werd of bij de toevoeging van een Toll-like receptor 4 (TLR4) antilichaam, dat de binding van LPS aan de TLR4-receptor blokkeert, resulteerde dit niet in een gunstiger effect op de doorlaatbaarheid van de epitheelcellen of afweerreacties van de witte bloedcellen. Hierdoor werd uitgesloten dat LPS de ontstekingsreacties veroorzaakte. Deze resultaten benadrukken de complexiteit van de interacties tussen de *in-vitro* gefermenteerde producten en de darmepitheelcellen en afweercellen.

Samenvattend presenteert het werk in dit proefschrift een *in-vitro* pijplijn die geavanceerde fermentatiemodellen voor kip en mens combineert met darmmodellen (darmorganoïden voor de kip of een co-cultuur celmodel voor de mens). Bij het testen van de effecten van potentiële prebiotica werd vooral duidelijk dat de interacties tussen fermentatieproducten, darmcellen en afweercellen complex is, maar werden wel potentieel gunstige effecten voor de darmgezondheid gezien. Deze *in-vitro* pijplijn voor darmgezondheid draagt bij aan het principe van de "drie V's" voor dierproeven: vervanging (dierproef wordt vervangen door een alternatief zonder proefdieren), vermindering van proefdieren en verfijning van dierproeven. In vergelijking met bijvoorbeeld *in-vivo* proefdiermodellen met muizen zullen dezen *in-vitro* pijplijnen voor de mens en kip een betere voorspellende waarde hebben, en kunnen ook voor andere zoogdieren worden ontwikkeld. Hiermee kunnen effecten van prebiotica en andere producten die de darmgezondheid kunnen bevorderen op efficiënte wijze worden getest, voordat deze in levende mensen of dieren worden toegepast

Summary

The intestine plays a crucial role in the overall health and well-being of humans and animals and contains a large number of microbial cells, known as the microbiota. The microbiota influences many biological processes such as digestion, nutrient absorption, metabolism, and immune function. Colonization of the gut begins at birth and at hatch, in humans and respectively in broiler chickens. The development of the gut microbiota composition is influenced by various factors, including genetics and the environment. During this early phase, the gut microbiota undergoes dynamic changes, resulting in a diverse and stable microbiota after 2-3 years in humans and 3-5 weeks in broiler chicks.

The composition of the microbiota can be influenced continuously during the development but also after a stable state is formed, by factors such as diet, the presence of pathogens, or antibiotic use. Generally, the microbiota can recover, but if the balance is severely disrupted, it can lead to dysbiosis, resulting in health issues such as intestinal infections or systemic inflammation. The disrupted balance between good and bad bacteria in broiler chickens is associated with intestinal inflammation and the shortening of the small intestinal villi, leading to reduced nutrient absorption and less efficient animal growth. A common intestinal disease in broilers is coccidiosis, which can lead to necrotic enteritis as a consequence. Necrotic enteritis is associated with overgrowth of the pathogenic bacterium *Clostridium perfringens* and has a significant impact on the entire flock. In premature babies, a gut disease is associated with the same bacterium, called necrotizing enterocolitis. This condition causes severe intestinal inflammation, leading to reduced blood flow to the intestine, which can result in parts of the intestine dying, and eventually death.

Considering the diverse and critical role of the gut microbiota in human and animal health, extensive research is dedicated to maintaining and improving the intestine's microbial composition and barrier function. Non-digestible oligosaccharides, also known as prebiotics, are used for this purpose, providing health benefits. For example, to prevent necrotizing enterocolitis, breastfeeding is recommended, since breastmilk contains natural prebiotics. But, additional research is needed to find alternatives next to breastmilk. This thesis focuses on two proven prebiotics, namely 2'-fucosyllactose (2'-FL), also present in breast milk, and Galacto-oligosaccharides (GOS), one of the most well-known oligosaccharides that are already added to formula milk due to their prebiotic effects. Furthermore, research is ongoing to find other prebiotics that can maintain and improve gut health. To test new potential prebiotics, *in-vitro* techniques are beneficial. Therefore, this thesis pays attention to an *in-vitro* pipeline that combines fermentation models and gut models, for both chickens and humans. This allows the screening of potential prebiotic effects of oligosaccharides. In this thesis this was done for Isomalto/malto-polysaccharide (IMMP) and 3 citrus pectins.

Chapter 2 focuses on the optimization and comparison with original cecal samples of the CALIMERO-2 system, which mimics the chicken cecum at the microbial level. The system is based on the validated TIM-2 system, designed to mimic colon fermentation in humans. For CALIMERO-2, temperature (41 °C) and pH (6.6) were adjusted to match the situation in the chicken cecum. Different medium compositions were tested and added to the system to mimic the nutrients passing to the chicken cecum. Samples obtained from fermentations with SIEM, the medium that is also used for TIM-2 experiments for the microbiota of humans, showed the most similarities with the microbial composition of the original chicken samples.

Chapter 3 describes the optimization of another *in-vitro* system, where chicken mini-intestines, also known as intestinal organoids, were created. These chicken intestinal organoids contain various types of cells present in the chicken intestine. To grow the cells and maintain this culture successfully for an

extended period, growth factors of chicken origin were added since commonly used mouse growth factors differ biologically. In addition to the three-dimensional structures, chicken intestinal organoids can also be cultures two-dimensionally, making them more accessible for exposure studies with test-products, which otherwise would have to be injected inside the three-dimensional organoids.

In **Chapter 4** we determined the composition of four oligosaccharides, namely three pectins and Isomalto/malto-polysaccharide (IMMP), and investigated how these oligosaccharides can influence the gut microbiota in the *in-vitro* system described in Chapter 2, CALIMERO-2. This was done in comparison with the standard medium (SIEM) and the control Mannan-oligosaccharides (MOS). Structural analysis revealed slight differences in monosaccharide composition and molecular weight between the pectins. Particularly, pectin 1 showed a low degree of methyl esterification, while pectins 2 and 3 had high methyl esterification. These similarities between pectins 2 and 3 were also observed in beta-diversity analysis of the microbiota-modulation. The phylum *Bacteroidetes* was the most dominant, except in the two controls SIEM and MOS, where *Firmicutes* prevailed. Beneficial bacteria, especially *Lactobacillus*, remained stable in all samples. Pectin 1 showed a significantly lower total production of short-chain fatty acids compared to SIEM, IMMP, and pectin 3, while IMMP closely resembled the positive control MOS, especially regarding butyrate content. This study highlights the potential of these oligosaccharides to promote gut health in poultry, via the gut microbiota.

In **Chapter 5** the fermentation products of the prebiotics 2′-FL and GOS, obtained from the *in-vitro* large intestine formation model TIM-2 inoculated with a microbiota of 3-month old babies, were studied on the epithelial barrier-integrity and immune activation in a Caco-2/Peripheral blood mononuclear cells co-culture cell-model. These fermentation products include several metabolites produced during fermentation of the prebiotics. The intestinal epithelial cells were exposed to the fermentation products of 2′-FL and GOS, and also to the control i-SIEM, a variant of SIEM for babies. GOS increased epithelial permeability, associated with increased release of interferon-gamma by underlying immune cells. Both the fermentation products of the control as well as those of 2′-FL and GOS further increased the inflammatory release of interleukin-1 beta from activated immune cells. The fermentation products all contained substantial but comparable amounts of lipopolysaccharides (LPS). However, filtering out LPS or adding a TLR4 antibody, which blocks the binding of LPS to its receptor, did not result in a more favorable effect on barrier integrity or immune responses. These results emphasize the complexity of interaction between *in-vitro* fermented products and intestinal epithelial cells and immune cells.

Overall, the work of this thesis presents an *in-vitro* pipeline combining sophisticated fermentation models for chicken and man with intestinal models (organoids [chicken] or co-culture cell model [man] to investigate the potential prebiotic effect of carbohydrates. This *in-vitro* test pipeline for intestinal health is compliant to the principle of the "Three Rs": replacement, reduction and refinement; is more predictive than e.g. *in-vivo* mouse models, and can also be implemented for other mammals.

Impact

Poultry, is one of the most widely consumed meats globally, providing a significant source of animal protein that is culturally accepted in many societies. However, broiler chickens are highly susceptible to developing an intestinal disease called coccidiosis caused by the protozoa *Eimeria*. Coccidiosis is one of the most prevalent and economically significant diseases in poultry. To reduce the impact of *Eimeria* infections, antimicrobial drugs are applied as feed additives throughout broilers' lives. However, objections against antimicrobial use in feed of food producing animals and resistance development to these drugs demand alternative control measures.

Coccidiosis has been linked to the overgrowth of *Clostridium perfringens* (*C. perfringens*), a bacterium that contributes to necrotic enteritis. Necrotic enteritis is an inflammatory condition of the intestine that disrupts the gut microbiota and can induce inflammation and damage to the gut lining, impairing its ability to function as a protective barrier and proper digestion. This can lead to the entry of harmful pathogens and undigested food particles into the system, causing severe health issues and even death. When necrotic enteritis is recognized, entire flocks are often needed to be treated with antibiotics. Both coccidiosis and necrotic enteritis impact broiler health and welfare and predispose for other diseases. They can significantly impact public health and food safety, by increasing the risk of foodborne disease by e.g, *Clostridium*, and of development of antimicrobial resistance. *C. perfringens* is also associated with necrotizing enterocolitis in humans, a life-threatening disease characterized by severe intestinal inflammation and bacterial overgrowth throughout the small and large intestines, primarily affecting preterm-born infants.

The intestinal microbiota has been shown to play a role in many physiological processes including digestion, metabolism, and immune function, in both humans and animals. The development of the intestinal microbiota starts at birth, and undergoes rapid and dynamic changes, with the establishment of a diverse and stable microbiota. A balanced microbiota composition is known to protect against pathogenic overgrowth, and in view of the above, could protect against *C. perfringens* overgrowth.

One way to modulate the microbiota, or increase its resilience, is by diet or specific dietary additives, thereby boosting intestinal health. Some carbohydrates are resistant to digestion by the host and absorption in the small intestine and can be fermented by the intestinal microbiota, conferring a health benefit, which is referred to as prebiotics. Prebiotics, besides modulation of the microbiota, can influence the immune system and intestinal barrier function.

The research presented in this thesis was carried out within the Carbohydrate Competence Center – Dutch Research Council "CarboBiotics: pre/probiotics mitigating the antibiotics burden" (CCC NWO CARBOBIOTICS: https://www.cccresearch.nl/ccc-nwo-carbobiotics/), where the overall aim was to study the health benefits of certain dietary non-digestible carbohydrates. Our research aimed to investigate the impact of specific non-digestible carbohydrates on the gut microbiota (composition and activity) and the effect of the modulation of the microbiota on the intestinal protective barrier and immune responses of the host. To achieve this, we developed and used a pipeline of various *in-vitro* tools. In general, *in-vitro* tools have long been instrumental in understanding biology and disease processes. The pursuit of novel *in-vitro* models is driven by the need for more accurate and cost-effective methods to study complex biological systems and test potential interventions. Moreover, it is important to minimize the use of animals in research as much as possible, therefore we performed experiments for which animal trials were not needed. In this thesis, fermentation *in-vitro* tools for humans, as well as chickens, optimized chicken intestinal organoids, and the use of a co-culture containing human intestinal epithelial cells in combination with immune cells, provide a valuable platform for future

investigations into the complex interactions between nutrients, the gut microbiome, and cellular/immune processes.

The knowledge gained from this research has significant implications for the scientific community and beyond. It has the potential to shape future studies in this field, enabling scientists to gain a deeper (mechanistic) understanding of the complexity of the digestive/fermentative system, including its microbiota, and develop targeted interventions to improve health outcomes. Specifically, our findings shed light on the effects of the non-digestible carbohydrates tested, such as Isomalto/malto-polysaccharide (IMMP) derived from potatoes, three different citrus pectins, for broilers and fermentation products of proven prebiotics, namely Galacto-oligosaccharides (GOS), and 2′-fucosyllactose (2′-FL) for infants.

IMMP and the pectins showed promising effects on the chicken microbiota composition and the production of metabolites during fermentation. The identified potential prebiotics can now be further tested in a co-culture of chicken intestinal organoids and immune cells, and in a later stage in field studies to validate their effects on gut health and explore their potential preventive and therapeutic applications in broiler flocks. This will contribute to a decrease in the occurrence of gut-related diseases in chickens, such as necrotic enteritis, leading to a drastic reduction in mortality, and thus costs and economic loss. Besides, the purported increase in health is of course beneficial for animal welfare.

The fermentation products of GOS and 2′-FL did not substantially improve the barrier function of the human intestinal cell cultures. These results highlight the complexity of the interactions between complete *in-vitro* large intestine-derived fermentation products in this *in-vitro* co-culture model combining intestinal epithelial cells and immune cells. We are one step closer to unraveling the effects of these non-digestible carbohydrate-derived fermentation products, but further studies are needed. It can be that a combination of microbiota-derived signals and direct (non-microbiota-derived) effects of the prebiotics in the host work in synergy to provide (intestinal) health benefits. In the current experimental *in-vitro* pipeline, these have not been combined, but both have helped to gain some insight into the separate processes related to the health benefits of dietary ingredients.

In summary, the implications of this research extend beyond the scientific community to e.g., poultry farmers, clinicians, and the general public. If the positive results observed in our models are confirmed through field trials, the implementation of the non-digestible carbohydrates studied in this thesis in the chicken diet can significantly contribute to improving the health of broiler chickens. This, in turn, will lead to healthier poultry products for consumers and reduce the economic burden associated with coccidiosis and necrotic enteritis in the poultry industry. Similarly, if we can increase the health of the infant microbiota, this can prevent necrotizing enterocolitis in preterm-born infants, but may also extend to benefits later in life, for instance, dysbiosis of the gut microbiota in infants has been linked to obesity at a later stage in life.

In conclusion, our research highlights the importance of understanding the impact of non-digestible carbohydrates on gut health and disease prevention in infants and broiler chickens. By employing a pipeline of innovative *in-vitro* models, we have contributed to the growing body of knowledge in this field and provided a platform for further investigations. The potential preventive and therapeutic applications of the non-digestible carbobiotics tested hold promise for the human health and poultry industry, with the aim of promoting a healthy gut.

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About the author

Miriam Jacomien Oost was born on 12 September 1990 in Harderwijk, the Netherlands. After finishing secondary school at Christelijk College Nassau Veluwe in Harderwijk, she started her first adventure abroad at Ghent University in 2010 where she started her BSc in Veterinary medicine. After two years, she decided to continue her studies in pharmaceutical science at Groningen University. She wrote her BSc thesis at the Department of Physiology and Pharmacology about the treatment of portal hypertension: current therapies, potential new compounds, and the use of mediators. She obtained her BSc degree in April 2016.

Miriam continued her studies at Utrecht University in the MSc program Drug Innovation. During this period, she did an internship in the pharmacology department at Utrecht University studying the safety of a potential bio-artificial kidney. After this, she did a 7-month internship at the physiology/pharmacology department at Western University in London, Ontario, Canada, where she focused on non-fatty liver diseases using spheroids of liver cells.

Miriam graduated from the MSc program in August 2018, after which she started as a Ph.D. candidate at the gut Microbiology group at Centre for Healthy Eating and Food Innovation at Maastricht University, Campus Venlo, in collaboration with Utrecht University and Hogeschool Utrecht. This research was performed in the public-private partnership 'Carbobiotics' coordinated by the Carbohydrate Competence Center. The research focused on improving intestinal health in chickens and infants using several *in-vitro* tools and is presented in this thesis.

List of publications

This thesis

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