Tuning Gut Health

Effects of Dietary Interventions on Porcine Intestinal Microbiota, Gut Epithelium and Immunity

Hugo de Vries

Propositions

1. When a probiotic strain is not present above molecular detection thresholds, it should be assumed that the effect on gut health is absent.
   (this thesis)

2. The use of dietary interventions to improve gut health is generally more impactful in early life.
   (this thesis)

3. The use of video capture is more adequate than traditional publishing media to explain the complex experimental procedures of *in vivo* studies.

4. Entrepreneurship should be stimulated more in the scientific community.

5. The global energy crisis cannot be tackled by politicians but requires the empowerment of the brightest engineering minds our societies have to offer.

6. Everybody can help reach sustainability goals by becoming more deeply invested in home growing and home fermenting.

Propositions belonging to the thesis, entitled

Tuning Gut Health – Effects of Dietary Interventions on Porcine Intestinal Microbiota, Gut Epithelium and Immunity

Hugo de Vries

Wageningen, 14 December 2022

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Hugo Johannes Antonius de Vries

Thesis

Submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University,

by the authority of the Rector Magnificus,

Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Wednesday 14 December 2022

at 4 p.m. in the Omnia Auditorium.

Hugo Johannes Antonius de Vries

Tuning Gut Health–Effects of Dietary Interventions on Porcine Intestinal Microbiota, Gut Epithelium and Immunity. 188 pages

PhD thesis, Wageningen University, Wageningen, The Netherlands (2022)

With references, with summary in English

ISBN: 978-94-6447-413-8

DOI: 10.18174/577493

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Chapter 1

General Introduction

Introduction

The world population is expected to rise to 9.7 billion by 2064 (Adam 2021), and with it the global food demand. Together with the increasing prosperity around the world, the consumption of meat proteins is projected to increase with 14% by 2030 (OECD/FAO 2021). This increasing demand for meat proteins will not only have an economic impact, but will also affect animal welfare as the number of animals needs to increase. The expanding human population and the intensification of livestock production in many countries will likely also impact animal and public health, due to increased pathogen flow at the wildlife-livestock-human interface and the emergence of infectious (zoonotic) diseases (Hassell, Begon et al. 2017). In the past, in-feed antibiotics were used to control harmful microorganisms and to increase feed efficiency in livestock. In-feed antibiotics were also extensively used in the pig industry, as they were associated with the reduction of weaning-associated problems, including post-weaning diarrhoea (Heo, Opapeju et al. 2013). However, the use of in-feed antibiotics caused great concern because of the emergence and spread of antimicrobial resistance in both human and animal pathogens. This eventually led to the European ban on the use of in-feed antimicrobial growth promotors (AGPs) that came into effect in 2006 (Commission 2005). Hence, sustainable alternatives to in-feed antibiotics such as pre- and probiotics gained increasing interest, as they have previously demonstrated to improve the structure and function of the gastro-intestinal tract (GIT) and the immune system (Heo, Opapeju et al. 2013). However, the modes of action of such dietary interventions are still under investigation as the interaction between diet, the gut microbiota and the immune system is extremely complex. There is increasing evidence that the gut microbiota, the gut epithelial barrier and the immune system are intrinsically linked to each other. In other words, dietary interventions that influence the GIT likely also impact the host's immune system. Their interaction determines beneficial or disadvantageous health outcomes to the host, and is largely dependent on other factors (e.g., genetics, sex, age, and environment). Therefore, interdisciplinary research has become increasingly central to animal health research. In pigs, many studies have investigated the effect of dietary interventions on the porcine GIT and the immune system. Most of these studies investigated the effects of dietary interventions on either the porcine gut microbiota or immune system and the majority included post-weaning animals. However, the potential of administering dietary interventions during the first period of life deserves further investigation, especially regarding the impact on the developing gut microbiota, the gut epithelial barrier and the immune system.

Gut microbiota in pigs

The GIT of pigs contains (partly digested) feed components, host derived components and gut microbiota. The host derived components consist of bile acids, digestive enzymes, and chemical and physical components of the mucosal defense such as antibacterial polypeptides, peptides, mucin and secretory immunoglobulin A (SIgA). The gut microbiota consists of bacteria, archaea, viruses, protozoa and fungi. Studies on the pig gut microbiota and associated functions have shown the importance of the microbiota for the pig host (Knecht, Cholewińska et al. 2020). The symbiotic members of the gut microbiota play an important role in colonization resistance against pathogens (Fons 2000). Furthermore, the gut microbiota plays a fundamental role on the induction, training and function of the host immune system (Zheng, Liwinski et al. 2020). Of the domains that comprise the pig's gut

microbiota, bacterial ecology has been the most widely studied. Important reasons for this focus are the advance of sequencing techniques based on the bacterial 16S ribosomal RNA gene, and the relative contribution of gut bacteria to the fermentative capacity of the pig's GIT. Although gut related perturbations can be caused by pathogens outside the bacterial domain (e.g. viruses or eukaryotes), pathogens within the bacterial domain are a substantial driver for economic losses and concerns for human and animal health (VanderWaal and Deen 2018). The domain of bacteria is divided into taxonomic groups called phyla, and the two most abundant phyla within the pig's gut microbiota are the *Firmicutes* and the *Bacteroidetes.* Similarly to humans (Risely 2020), pigs are considered to have prevalent core bacterial species in the intestinal microbiota. At least in terms of function, pigs share a core microbiota, with each pig harboring a diversity of bacterial species that are responsible for the degradation of a myriad of different compounds and macromolecules. These compounds and macromolecules can be carbohydrates, lipids or proteins, with specific bacterial species often being specialized in the breakdown of specific macromolecules and compounds. The pig's GIT harbors up to 1000 bacterial species (Patil, Gooneratne et al. 2020), with the highest density of bacteria in the pig's colon. In absolute numbers, an adult pig's small intestine harbors up to 10^5 bacteria per gram of luminal content (Tiwari, Singh et al. 2019), while the pig's colon harbors around 10^{10}-10^{11} bacteria per gram of luminal content (Guevarra, Lee et al. 2019). The high bacterial counts in the pig's proximal colon allow for the highest fermentative capacity, consistent with observations that most fiber compounds in feed are digested in the large bowel (Pu, Li et al. 2020). The pig's caecum also harbors significant amounts of gut bacteria (10^{10} bacteria per gram of luminal content) (Allison, Robinson et al. 1979), and their fermentative capacity comprises a significant contribution to the digestion of feed. However, the fermentative capacity of the caecum microbiota is lower than that of the microbiota residing in the colon or rectum (Bauer, Williams et al. 2004).

In this thesis, gut ecology was studied by determining the relative composition of bacterial species in luminal or faecal material of the intestinal tract. Throughout the thesis, the term microbiota exclusively applies to bacterial microorganisms within the GIT.

Role of gut microbiota in pigs

Due to its high bacterial counts, the gut microbiota has a large contribution to the presence of enzymes in the gut that help break down feed components. These enzymes can be proteases, lipases and carbohydrate active enzymes that are added to the pool of digestive enzymes produced by the host. The bacteria's metabolic activity also results in the production of bacteria-derived metabolites, notably short-chain fatty acids (SCFAs), amino acids, vitamins such as vitamin B and K (Hill 1997), and many other metabolites. SCFAs are an important energy source for intestinal epithelial cells (Parada Venegas, De la Fuente et al. 2019), and the utilization of amino acids that originate from both alimentary and endogenous proteins by gut bacteria results in amino acids becoming available to the host (Neis, Dejong et al. 2015). Disruptions in gut microbiota composition that impact the functional output of the gut microbiota are thought to be important drivers of gut related disorders (Neis, Dejong et al. 2015). This underlines the importance of the gut microbiota's role in the digestion of feed.

The community of gut microbiota mainly consists of non-pathogenic inhabitants of the host's GIT. With a well-established community of commensal bacterial species, most if not all ecological niches in the host's GIT are taken. This prevents the successful colonization of bacterial newcomers and as such also prevents the successful colonization of pathogenic microorganisms. This competitive exclusion of pathogens is most successful in adult hosts that no longer have a highly dynamic microbiota composition, but instead have a well-established, stable community of commensal bacterial species.

Characteristics of feed to alter gut microbiota composition in pigs

The most widely used method to alter the gut microbiota composition in pigs is by changing the composition of components in feed. An important factor is fibre content, with a higher fibre content promoting the growth of fibre-degrading microbiota. Since most pathogenic bacterial species are not specialized to degrade fibres, increasing the fibre content can be seen as increasing the selective pressure against most bacterial pathogens. Some bacterial pathogens perform well when the crude protein content in feed is relatively high, which is why reducing the crude protein content in feed can be beneficial to prevent the promotion of bacterial pathogens. There is a fine balance, however, because reducing the crude protein content in feed can also decrease the pig's growth performance due to nutrient limitations.

The acidity of feed is also an important contributor to altering the gut microbiota composition in pigs. As many pathogens, including the pathogenic species within the *Enterobacteriaceae* family, suffer when acidity is increased, this approach has been used to prevent expansion of these pathogens within the GIT. For species within the *Enterobacteriaceae* family, it is known that an acidified environment triggers intracellular acidification, which prevents replication. Additionally, the presence of SCFAs prevents the utilization of respiration for bacterial species within this family, which lowers their competitive advantage (Sorbara, Dubin et al. 2019). A second effect by which the acidity of feed contributes to alteration of the gut microbiota composition in pigs, is related to the highly acidic environment of the pig's stomach. With acidified feed, the dip in the stomach's acidity during feeding is not as pronounced, which means the stomach's acidity is better maintained. By reducing the window of opportunity that is related to dips in the stomach's acidity, fewer microorganisms will survive the highly acidic environment of the stomach, which additionally means fewer pathogens will be able to colonize the host's GIT.

Another important factor that impacts gut microbiota composition in pigs, is the structure of pig feed. With a coarse feed structure, meaning feed particles are relatively large, more feed components will reach the hindgut of the pig undigested. This promotes fermentative activity in the colon, increasing butyrate metabolism by colonic enterocytes and increasing epithelial transport of SCFAs. Coarse particle size increases stomach and hindgut acidification which may be beneficial to prevent expansion of bacterial species within the family of *Enterobacteriaceae (Kiarie and Mills 2019)*. On the other hand, finer feed particle sizes allow for better nutrient utilization, and the increased surface area allows for better contact with digestive enzymes in the GIT, meaning that choosing the right particle sizes for pig feed is a balancing act.

Dietary interventions to alter gut microbiota composition in pigs

Since the discovery and use of antibiotics such as penicillin for human health, antibiotics have been used as AGPs in livestock production. These AGPs are historically used at subtherapeutic levels to improve the rate of growth and efficiency of feed utilization, reduce mortality and morbidity, and improve reproductive performance (Cromwell 2002). To limit the emergence and spread of antimicrobial resistance (AMR) in human and animal microbiota and pathogenic species, the use of AGPs has been prohibited in many countries. Due to this ban, livestock farmers, feed producers and researchers are looking for alternative feed supplements that can mimic the effects of AGPs. The mechanisms by which AGPs are capable of increasing pig performance are not completely understood, however, such understanding would help to develop effective alternatives (Brown, Uwiera et al. 2017). It was expected that AGPs have a strong influence on microbiota composition in livestock animals, but several studies concluded that microbiota composition was not altered by AGP treatment, or was only modestly affected (Brown, Uwiera et al. 2017).

Zinc oxide (ZnO) is an inorganic compound that is known to increase pig's growth performance, feed intake and feed utilization (Hill, Mahan et al. 2001). At high inclusion rates, also called pharmacological levels, ZnO is expected to have an antimicrobial effect in the pig's gut, an effect that overlaps with the effects of AGPs. For ZnO it has been shown that its inclusion in pig feed can alter microbiota composition, as well as its metabolic activity (Starke, Pieper et al. 2014). An important reason for its use is that ZnO allows for efficient control of post-weaning diarrhoea (PWD) and has been shown to have a moderate antibacterial effect against *Escherichia coli* F4 (K88), which is an important causative agent of PWD (Bonetti, Tugnoli et al. 2021). Additionally, ZnO offers more beneficial effects to post-weaning pigs due to its multiple modes of action on feed digestion, the host's immune system, secretion of host enzymes, antioxidant activity, and changes to intestinal morphology (Bonetti, Tugnoli et al. 2021). Although ZnO has been successfully used on a large scale as an alternative for AGPs, the sustainability of this intervention is under debate. Zinc is a heavy metal and its increased release into the environment due to extensive use in pig production is an unwanted side-effect. Another detrimental side-effect of the extensive use of ZnO is its contribution to the spread of AMR (Bednorz, Oelgeschläger et al. 2013, Medardus, Molla et al. 2014, Slifierz, Friendship et al. 2015), which is yet another reason to reduce its inclusion in pig feed.

Prebiotics are compounds that have a relatively low digestibility. This means that a large proportion ends up in the hindgut, where they are fermented by the gut microbiota. Prebiotics are fibrous compounds that are not broken down by the host's enzymes or absorbed by the gut epithelium, but instead are broken down by specialized fibre-fermenting microbiota. Since prebiotics selectively stimulate the growth of fibre-fermenting microbiota, which nearly always are beneficial microorganisms, they are seen as an adequate instrument to steer the gut microbiota composition in the right direction. Prebiotics typically are non-digestible carbohydrates, and examples are fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, β-glucans, resistant starch, pectin, cello-oligosaccharides, lactulose and arabinoxylans (Shim 2005). When prebiotics are fermented, organic acids such as lactate, acetate, butyrate and propionate are produced (Shim, Verstegen et al. 2005), which can readily be taken up and metabolised by the gut epithelium as an energy source (Parada

Venegas, De la Fuente et al. 2019). The most practical implementation of prebiotics is by adding them as a supplement to feed.

Probiotics are live microorganisms that confer beneficial effects to the animal host by several modes of action. An important mode of action of many probiotic strains is the competitive exclusion of harmful microorganisms by either competing for a particular ecological niche, or by direct antagonistic effects. Another mode of action is the fermentation of feed components generating metabolic products that can be taken up by the host. Probiotics can also stimulate the development of gut physiology or the host's immune system (Verdu 2009, Sánchez, Delgado et al. 2017). Regularly used probiotics in livestock production include species within the bacterial genera of *Lactobacillus, Bifidobacterium, Enterococcus, Bacillus, Lactococcus, Leuconostoc, Pediococcus, Escherichia, Propionibacterium* and *Streptococcus*, as well as the yeast *Saccharomyces.* Probiotic formulations can be administered by direct feeding, by inclusion in feed, by inclusion in drinking water, or by using probiotic strains as inoculant for fermenting feed (e.g. inoculation of liquid feed). The latter option has the benefit of propagating the probiotic strain prior to feeding, and additionally liquid feed that is actively being fermented can be guaranteed to contain actively metabolizing bacteria, numerous bacterial enzymes, and metabolic products such as SCFAs.

Timing of dietary interventions

In pig production, dietary interventions are most frequently administered during the post-weaning phase. The reason for this is that mixing dietary interventions with the solid feed, and thus thereby supplementing solid feed, provides the most practical solution. Given that the amount of feed ingested by each individual animal is within the same order of magnitude, the dietary intervention will also be equally administered to each individual. During the pre-weaning phase, it is possible to provide dietary interventions to piglets by adding it to the creep feed, which is typically placed in nursery pens in the second week after parturition. However, piglets do not ingest creep feed in equal amounts (Choudhury, Middelkoop et al. 2021), which means the dietary intervention is not guaranteed to be administered to all individuals in equal and adequate amounts. Another option would be to manually feed the dietary intervention to piglets during the pre-weaning phase, but since that includes picking up animals, this option leads to investment of substantial labour, and stress to animals. To date, the majority of conducted *in vivo* studies have looked at effects of administering dietary interventions during the post-weaning phase. As piglets suffer most often from gut related disorders such as diarrhoea in the first six weeks of life, it may be useful to start promoting gut health during the pre-weaning phase, which typically lasts for three to four weeks.

Selected candidates as dietary interventions

Yeast-derived β-glucans

Yeast-derived β-glucans originate from the cell walls of baker's yeast, and due to the possibility of their large-scale production, have been extensively used in food and feed. As yeast-derived β-glucans are widely implemented in the food and feed chain, several yeast-derived β-glucan products have been tested in *in vitro* and *in vivo* studies. Yeast-derived β-glucans have been shown to stimulate immune cells (Hahn, Lohakare et al. 2006, Sonck 2011, Vetvicka and Oliveira 2014, Geervliet, Lute et al. 2020), and the general line of thought

is that most immune stimulating effects of β-glucans *in vivo* are established through their binding to cell receptors such as Dectin-1 and CR3 (Zhu, Du et al. 2015, Sahasrabudhe, Dokter-Fokkens et al. 2016, Kanjan, Sahasrabudhe et al. 2017), which in turn triggers immune pathways (Dennehy and Brown 2007, Jin, Zhang et al. 2019). The activation of immune pathways leads to expression of cytokines and chemokines by local immune cells.

In addition to acting as an immune stimulant, β-glucans are also thought to confer beneficial effects as prebiotic fibres. Due to their (1,3)-β-linked backbone with small numbers of (1,6)-β-linked side chains, yeast-derived β-glucans are not digested by the host's enzymes, but are instead fermented by fibre-fermenting bacteria in the hindgut. For this prebiotic effect to be noticeable, enough yeast-derived β-glucans need to be included in pig feed, i.e. at similar amounts as other prebiotic compounds are included. To summarize, when administered in adequate amounts, yeast-derived β-glucans provide a promising feed additive to both stimulate the immune system and to change the gut microbiota composition in a beneficial manner by its prebiotic effects.

EcN

Escherichia coli Nissle 1917 (EcN) is a probiotic strain that has been successfully used in human health for over 100 years (Mutaflor®), and that has been used in dairy health for over 15 years (Ponsocol®). EcN is one of the most extensively studied probiotic species, and has been found to confer its beneficial effects to the host due to a range of factors. These include the ability of EcN to compete against close relatives (i.e. pathogenic species in the family of *Enterobacteriaceae*), its ability to form biofilms, its ability to produce antimicrobial compounds like colibactin and microcins, its ability to scavenge iron in the lumen by the production of siderophores, and its ability to stimulate the host's local and systemic immune system (Sonnenborn and Schulze 2009, Behnsen, Deriu et al. 2013, Sonnenborn 2016, Wassenaar 2016). Given that EcN has been successfully used in humans of all ages (including neonates), and its successful use in the dairy industry (mainly by administration to calves), EcN is a promising probiotic candidate for use in pig production.

What shifts in gut microbiota composition are desired?

It is not easy to determine what the most beneficial composition of the pig's gut microbiota is at each stage of growth. However, a healthy pig is supposed to have a sufficiently diverse gut microbiota, so that all the digestive functions of the GIT that are required at the present growth stage are fulfilled. This means for example, that a suckling piglet's GIT contains sufficient lactobacilli, bifidobacteria and other strains to adequately assist with the digestion of sow milk contents such as lactose, oligosaccharides, fats and proteins. Another requirement for a resilient pig is colonization resistance, which is mostly exerted by antagonism to pathogens by the microbiota. This can be achieved by occupying most ecological niches within the pig's GIT, and for example by a sufficiently high production of SCFAs to adequately inhibit the growth of pathogenic strains within the family of *Enterobacteriaceae*.

A number of bacterial groups are generally regarded as having a beneficial impact on gut health. Members of this group are the lactic acid bacteria (e.g. *Lactobacillus, Bifidobacterium, Leuconostoc, Pediococcus, Lactococcus, Streptococcus, Enterococcus)*, the acetic acid bacteria (e.g. *Acetobacter*), the fibre-degraders that produce saccharolytic enzymes (e.g.

Ruminococcus, Prevotella), and a number of bacterial groups that each have their own unique but important function (e.g. *Propionibacter, Akkermansia)*, while bacterial species/ families that are generally regarded as detrimental to health are for example bacterial species in the family of *Enterobacteriaceae* such as *Escherichia coli* and *Salmonella* species. Not all *Escherichia coli* and *Salmonella* strains are detrimental to health though, as many of these strains belong to the group of gut commensals that have their own ecological niche within the pig's GIT. Yet, researchers and livestock producers typically test interventions with the aim of increasing the abundance of beneficial bacterial groups while reducing the abundance of bacterial groups that may be detrimental to health.

Role of gut epithelium in pig gut health

In order to prevent the entry of gut bacteria into the host's body, a physical barrier of gut epithelium is in place lining the entire GIT, and consisting mostly of enterocytes. Goblet cells within the gut epithelium continuously produce mucus, which provides a second barrier to prevent most members of the gut microbiota from approximating the gut epithelial cells. Behind the gut epithelium lies the lamina propria, which is a thin layer of connective tissue that contains numerous host immune cells, such as fibroblasts, lymphocytes, plasma cells, macrophages, leukocytes, mast cells and dendritic cells. Together with mesenteric lymph nodes (MLN), Peyer's patches, intra-epithelial lymphocytes and isolated lymphoid follicles, the lamina propria forms the gut-associated lymphoid tissue (GALT), that helps defend against microorganisms that have crossed the gut epithelium.

The pig's gut epithelium contains enterocytes, goblet cells and intra-epithelial lymphocytes, a number of other specialized host cells, namely enteroendocrine cells, stem cells, M-cells and Paneth cells. Especially Paneth cells are known to specialize in the production of antimicrobial proteins and peptides, and the gut epithelium is known to secrete a wide range of antimicrobial compounds such as alpha defensins, beta defensins, lysozyme, complement factors, cathelicidins and REG-III. Most of these compounds have a broad antimicrobial activity against both Gram-positive as well as Gram-negative bacteria, but also against other microorganisms such as fungi, yeasts, and viruses. With its action as a physical barrier, its generation of a mucus layer, its harbouring of an important part of the GALT, and its secretion of antimicrobial compounds, the gut epithelium forms an important defence against pathogenic microorganisms that reside within the GIT's lumen. For a visual representation of the content and components of the gut epithelium, its development over time, and the local immune system, see Figure 1 below.

Interaction between members of the gut microbiota and porcine mucosal barrier

As mentioned before, there exists a layer of mucus between the gut epithelium and the lumen of the GIT. This mucus layer physically separates members of the gut microbiota inhabiting the lumen from the host's cells. Even though in most situations there is no direct contact between members of the gut microbiota and host cells, there exists an interaction. The gut microbiota releases a broad range of metabolic products and microbe-associated molecular patterns (MAMPs) that permeate the mucus layer, of which the latter are recognized by gut epithelium cells and immune cells via receptors such as toll-like receptors (TLRs). Even though most members of the gut microbiota are not pathogenic, they can release MAMPs like lipopolysaccharides (LPS), flagellin and peptidoglycan. Exposure of host cells to MAMPs

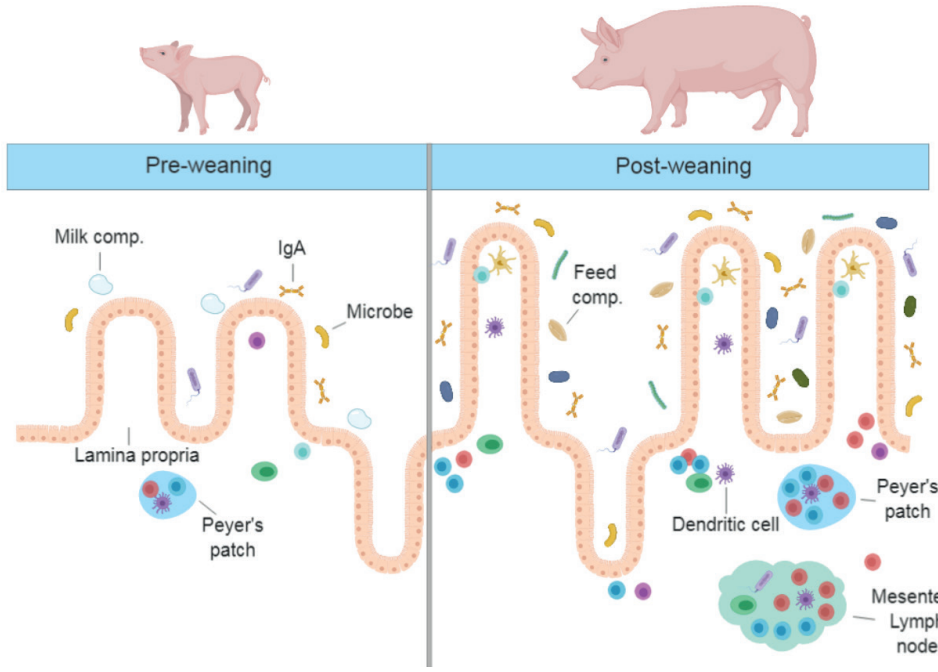

Figure 1. The early-life development of the gut microbiota, the gut epithelium and the local immune system in pigs. A schematic representation of the contents in the gut lumen, the gut epithelium, the lamina propria, and the local immune system which contains Peyer's patches and Mesenteric Lymph Nodes.

leads to activation of the innate immune system (Kumar, Kawai et al. 2011), and stimulates the maturation of immune cells like for example dendritic cells (Joffre, Nolte et al. 2009). Upon activation of the innate immune system, the gut epithelium is stimulated to produce and secrete antimicrobial compounds that enter the mucus layer and the GIT's lumen. Many of these antimicrobial compounds are membrane damaging peptides leading to cytoplasm leaking out of the cell that ultimately results in the bacteria's peril (Huttner and Bevins 1999). Apart from this, the gut epithelium is additionally known to secrete microRNAs (miRNA) that promote the growth of particular bacterial species, while they negatively impact the growth of other bacterial species (Bi, Zhang et al. 2020). A third route by which the host epithelium can interact with the gut microbiota, is via the stimulation of mucus production by goblet cells. By thickening the mucus layer, more distance is created between members of the gut microbiota in the lumen and the gut epithelium. Some bacteria, such as *Akkermansia muciniphila*, break down components of the host's mucus layer, which helps the mucus layer to continuously renew itself (Ouwerkerk, van der Ark et al. 2016). For this reason, the presence and activity of *Akkermansia muciniphila* is seen as contributing positively to gut health (Ouwerkerk, van der Ark et al. 2016). Since *Akkermansia muciniphila* resides within the mucus layer, it exists at a relatively close proximity to the gut epithelium. The same holds for biofilm forming gut commensals that form biofilms lining the intestinal wall. This has resulted in the gut epithelium's relatively high tolerance to nearby commensal bacteria and exposure to MAMPs (Pfefferle and Renz 2014). If it would react as dramatically to MAMPs as most systemic immune cells do, the gut epithelium would continuously become inflamed, and apoptosis of enterocytes would frequently be activated. Instead, when commensal

gut bacteria approximate the gut epithelium, it typically reacts by increasing its mucus production and increased release of antimicrobial compounds (Peng, Tang et al. 2021).

Aims and outline of this thesis

The work presented in this thesis was part of an integrated project entitled "Mucosal gut and airway immune competence, dietary modulation, and resilience against external induced and internal dysregulation". With its main focus on gut microbiota, the primary aim of the research described in this thesis was to investigate how early-life dietary interventions influence the gut microbiota and the gut epithelial barrier, with the ultimate goal to reduce weaning-associated health problems. It was hypothesized that, due to piglets not having a fully developed gut microbiota and host immune system in the first weeks of life, dietary interventions would have a greater chance to induce changes to the composition of the gut microbiota, to the gut epithelial barrier and to overall immunological responsiveness. Additionally, because piglets suffer from post-weaning diarrhoea just after weaning, the best time to induce the development of the gut microbiota and the host's immune system was thought to be during the pre-weaning phase. Yeast-derived β-glucans (MacroGard®) and the Gram-negative bacterial strain *E. coli* Nissle 1917 (Ponsocol®) were selected as suitable candidates to test our hypothesis, as they have previously been utilized to enhance gut health. Apart from this primary aim, this thesis furthermore aims to contribute to a more comprehensive understanding of gut microbiota and immune system development in early life. Overall, the research described in this thesis provides new insights in the host-microbe interactions in neonatal piglets and sets the stage for future microbiological and immunological research that involves dietary interventions.

In **Chapter 1**, I provide relevant background information and the aims and outline of this thesis are presented. In line with the main aim of this thesis, a more detailed review of the current state of the art with respect to effectors of microbiota development in the pig's GIT, and the non-microbial and microbial feed interventions that have been performed to date is then provided in **Chapter 2**. In this chapter, a division is made between the development of the gut microbiota during the pre-weaning phase versus the post-weaning phase. Dozens of porcine *in vivo* studies have been performed to assess the impact of a broad range of dietary interventions on gut microbiota composition. Additionally, current knowledge about the interaction of the gut microbiota with the host is summarised, as well as current understanding of the relation between microbiota composition and disease susceptibility.

In **Chapters 3 and 5** the results of the main porcine *in vivo* study conducted in the framework of this research project are presented to answer the question as to whether and to what extent different dietary interventions influence gut microbiota composition and the immune system in early life. To this end, we designed and carried out a 10-week study during which yeast-derived β-glucans, EcN or a tap water placebo treatment were perorally administered to pigs from day 2 to day 44. Blood, tissue, luminal content and faecal samples were collected at different pre- and post-weaning time points. **Chapter 3** describes the results with respect to the pre- and post-weaning effects of yeast-derived β-glucans on gut microbiota composition and the immune system. Additionally, we investigated whether yeast-derived β-glucans modulate the pig's immunocompetence (i.e. the ability to produce an effective immune response to an antigen, thereby providing protection in case of

exposure to a pathogen) by analysing the antibody responses induced after oral vaccination against *Salmonella enterica* serovar *Typhimurium*.

Designing and executing *in vivo* studies requires considerable preparation and planning. Therefore, a pilot study was conducted (**Chapter 4**) prior to the larger *in vivo* experiment (**Chapters 3 & 5**) to test and improve logistic and methodological procedures (e.g. dissection procedures). This also helped to select the most promising concentration of EcN to be administered during the main *in vivo* experiment. In addition, results from the pilot study provided valuable insights concerning the spatio-temporal presence of EcN in faeces, the GIT lumen and in the mucosal layer. It also allowed us to study potential cross-contamination of EcN between treatment and control animals, translocation of EcN to the MLN, and whether gut acidity is influenced by the presence of EcN. To summarize, the final outline, protocols and methods of the main *in vivo* study were refined by using the results that were obtained during this pilot study.

In **Chapter 5** the results of the main porcine *in vivo* study are presented to answer the question as to whether and how EcN influences the development of gut microbiota composition and the immune system in early life. Viable EcN cells were perorally administered to pigs from day 2 to day 44. Blood, tissue, mucosal scrapings, luminal content and faecal samples were collected at different pre- and post-weaning time points to study the pre- and post-weaning effects of this probiotic strain on gut microbiota composition, gene expression of the gut epithelium, and the immune system. Additionally, we investigated whether EcN modulates the pig's immunocompetence (i.e. the ability to mount an effective immune response to an antigen, thereby providing protection in case of exposure to a pathogen) by analysing the antibody responses induced after oral vaccination against *Salmonella enterica* serovar *Typhimurium*.

In the final chapter of this thesis, **Chapter 6**, I summarize and discuss the results in light of other scientific literature. In addition, I provide recommendations for future research and for the application of novel dietary interventions in livestock production.


2

Chapter 2

Microbiota Development in Piglets

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Published in "The suckling and weaned piglet" (ed. Chantal Farmer). Wageningen Academic Publishers 2020, Pages: 179–205; https://doi.org/10.3920/978-90-8686-894-0_7

Abstract

Starting at birth, the intestinal microbiota of piglets develops rapidly from a simple to a more stable, mature ecosystem, following a highly dynamic developmental trajectory that includes a strong disturbance particularly around the time of weaning. The microbiota in the gastro-intestinal tract of piglets plays numerous beneficial roles. Apart from its fermentation activity, the microbiota also stimulates a proper development of gut physiology, host immunity and general well-being of the piglet. Conventional methods to prevent gut-related issues include the use of antimicrobial growth promoters and the inclusion of zinc oxide in feed. As these conventional methods have several drawbacks, new methods are being developed to improve gut microbiota development and thereby prevent gut-related issues. Of these new methods, the inclusion of fibrous components in feed and the administration of probiotics are thought to be the most promising. To date, several in vivo studies have shown the effectiveness of including fibrous components or probiotics in feed. Although results are promising in a number of these studies, optimal inclusion rates of fibrous components are still to be found, and the use of probiotics also comes with some practical obstacles. This chapter reviews the available literature on the subject and offers a comprehensive understanding of the factors involved in the use of conventional and innovative methods to ameliorate impaired piglet gut health.

Keywords: gut health, early-life, livestock, prebiotics, probiotics

Introduction

The gut microbiota has numerous beneficial roles, such as fermentation of fibres, generation of vitamins, stimulation of immune development and prevention of pathogen colonization. To date, the piglet's microbial gut ecology has been extensively studied, and this has been made possible by the advent of molecular, cultivation-dependent approaches, and particularly the more recent rapid developments in next-generation sequencing methods. These methods have allowed several developmental mechanisms to be outlined. One of these mechanisms is that the composition of the microbiota becomes more diverse over time, especially during the first days of postnatal life as microbes from the environment colonize the gastrointestinal (GI) tract. Starting at birth, the microbiota in the GI tract develops in a highly dynamic environment, with the host gut physiology and the innate immunity developing in parallel (Kim, Hansen et al. 2012). As in other mammals, the GI tract of piglets is mostly oxic at birth, although the metabolizing activity of early colonizing microbiota results in oxygen depletion over time during the first postnatal week. Subsequently, new niches become available for anaerobic microbes, as large parts of the GI tract become anoxic (Jost, Lacroix et al. 2012). This changing environment results in a complex ecosystem with a dynamic composition that shifts over time and along the entire GI tract (Isaacson and Kim 2012).

Another important factor that strongly impacts the development of the gut microbiota in early life is the consumption of sow milk, which results in a milk-oriented microbiome (Frese, Parker et al. 2015). Host genotype and other factors, such as environmental factors, also impact microbiota composition, although the underlying mechanisms for selection of specific microorganisms are currently poorly understood. A new phase of microbiota development starts when the pigs are reared on solid feed, which occurs after weaning. The transition to solid feed leads to an enrichment of functional pathways that are involved in plant glycan metabolism (Frese, Parker et al. 2015), which relates to the plant-derived ingredients that are present in weaner diets. In addition to the changes in gut microbiota development, the sudden shift to solid feed can result in low or erratic feed intake, which is considered a risk factor for disease. Additional risk factors for disease include high dietary protein content, low levels of hygiene, low ambient air quality or low ambient air temperature (Lallès, Boudry et al. 2004). Just after weaning, the rapid changes in microbiota composition and activity result in a higher incidence of GI complications. These complications include a decrease in villus height (Hampson 1986), villus atrophy (Al Masri, Huenigen et al. 2015), reduced activity of intestinal digestive enzymes, disturbed intestinal permeability and the colonization of enteric pathogens such as *Escherichia coli* and rotaviruses (Lallès, Boudry et al. 2004).

Many pathogens found in pig herds are of viral origin, and vaccines are being developed to fight these viral diseases, such as Influenza (Romagosa, Allerson et al. 2011), African Swine Fever (ASF) (Argilaguet, Pérez-Martín et al. 2012), Porcine Reproductive Respiratory Syndrome (PRRS) (Meng 2000) and Porcine Circovirus type 2 (Rose, Andraud et al. 2016). Although comparatively less contagious than most viral diseases, bacterial-borne diseases, such as enterotoxigenic *E. coli* (ETEC), *Streptococcus suis*, *Salmonella enterica* and *Clostridioides difficile* are also of economic importance because they can lead to pig mortality. Notably, within the EU approximately 17% of liveborn piglets are lost and of these

losses, a substantial proportion can be associated with mucosal infections (Lallès, Bosi et al. 2007). The transition period of weaning is associated with GI tract infections, most often with colibacillosis diarrhoea (Gresse, Chaucheyras-Durand et al. 2017). As advised by Luppi in 2017, when dealing with colibacillosis, it is important to diagnose which ETEC type is responsible and to test for antimicrobial sensitivity, because antimicrobial sensitivity is highly variable among *E. coli* isolates (Luppi 2017). Enteric microbial infections can, although with clear drawbacks, typically be prevented and treated using antibiotics and/or antimicrobial supplements such as Zinc Oxide (ZnO) and copper sulphate. One of the drawbacks of the use of both antibiotics and antimicrobial supplements is the selection for antimicrobial resistant microbes (Moreno 2014).

Globally, the rising rate of antimicrobial resistance, caused by widespread use of antibiotics, is a significant concern. Antibiotics used in livestock production are closely related to those used for humans, and cross-species transmission of resistant bacteria or resistance-conferring genetic elements from animals to humans can occur (Tang, Caffrey et al. 2017). Furthermore, the use of zinc and copper in pig feed has been shown to result in the presence of multi-drug resistant *Salmonella* (Medardus, Molla et al. 2014), methicillin-resistant *Staphylococcus aureus* (Slifierz, Friendship et al. 2015) and multi-drug resistant *E. coli* (Bednorz, Oelgeschläger et al. 2013). Additionally, the use of zinc and copper at therapeutic dosage in pig feed is suspected to cause environmental pollution (Jondreville, Revy et al. 2003). Frequent use of antibiotics may result in the spread of multi-resistant bacteria (Landers, Cohen et al. 2012, Barton 2014), and concerns regarding public health have resulted in legislation reducing the use of antibiotics in livestock. To this end, the EU has banned the use of antibiotic growth promoters (AGPs) in 2006 and has more recently decided to limit the use of ZnO to a maximum of 150 mg/kg in feed (Starke, Pieper et al. 2014). However, according to several studies, AGPs are still used in a prophylactic manner. In addition, ZnO is also used at therapeutic levels, which often exceed 150 mg/kg (Barton 2014, Moreno 2014, Rhouma, Beaudry et al. 2016). Currently, the majority of antibiotics are still used in the livestock sector (Landers, Cohen et al. 2012), which emphasizes the need to develop non-antibiotic alternatives to prevent and control GI tract infections (Gresse, Chaucheyras-Durand et al. 2017). There exists an extensive body of literature on non-microbial and microbial dietary interventions that have been tested in *in vivo* trials to assess their impact on the porcine GI tract microbiota. Of the dietary interventions tested, a number were shown to have a beneficial effect and as such could possibly be further developed and used as nonantibiotic alternatives.

This chapter will provide an overview of both the development of the microbiota in the GI tract of piglets and the effectors involved. It will also describe findings regarding the interaction between the microbiota and the host, the relation of microbiota composition to disease (predisposition) and of microbial and non-microbial feed additives that are used to ameliorate gut-related issues in early life.

Effectors in early-life microbiota programming

The stomach functions as a pre-digestive organ that prepares the ingested feed for digestion at a low pH and that mixes solids with liquids. In the small intestine nutrients are hydrolysed by digestive enzymes and are transported across the epithelium. The large intestine acts as a fermentation chamber where mostly fibres and other indigestible feed components are broken down by anaerobic bacteria into components that can be absorbed and used by the host, such as short chain fatty acids (SCFAs). The SCFAs are important because they are used as an energy source by the host. In addition to allowing a proper fermenting capacity with the corresponding release of breakdown products in the GI tract, the gut microbiota is important for the development of gut physiology, immunity, and general well-being of the piglet (Guevarra, Lee et al. 2019). Most studies have demonstrated that *Firmicutes* and *Bacteroidetes* are the two main phyla with the highest relative abundance along the entire GI tract and in the faeces of piglets (Kim, Borewicz et al. 2011, Looft, Allen et al. 2014, Gresse, Chaucheyras-Durand et al. 2017). This is in contrast to some studies that showed exceptions to this finding, with one study that indicated *Firmicutes* and *Proteobacteria* to be the main phyla in both the jejunum and the ileum (Yang, Huang et al. 2016). Additionally, most studies have found an increasing trend of microbiota diversity as the piglets age (Chen, Xu et al. 2017, Wang, Tsai et al. 2019). At birth, the piglet's gut is exposed to microbes for the first time and these microbes originate from the sow's vagina during the piglet's passage through the birth canal. Subsequently, piglets are exposed to microbes in the housing environment, including microbes from the sow's faeces and microbes from the sow's skin during lactating. As few niches of the gut ecosystem are occupied in the first days after birth, many of these early microbial exposures lead to successful colonization. In general, exposure to microbes in the early stages of postnatal life is not considered detrimental, since the development of the gut microbiota requires continuous microbial exposure (Schmidt, Mulder et al. 2011).

As mentioned in the introduction, the GI tract is a largely oxic environment after birth. This results in the acquisition of oxygen tolerant bacteria during the first 24 hours, such as lactic acid bacteria, enterobacteria and streptococci (Lallès, Bosi et al. 2007). Other bacteria such as *Bacteroides, Lactobacillus, Bifidobacterium* and *Clostridium* colonize shortly after the first colonizers, namely, within one to five days after birth (Petri, Hill et al. 2010). In one study a stable microbiota composition was observed from the first day after birth and remained for the first three weeks. It mainly consisted of *Enterobacteriaceae, Lachnospiraceae, Bacteroidaceae, Clostridiaceae* and *Lactobacillaceae* (Frese, Parker et al. 2015). This is in contrast to what happens in human neonates, where early colonizers such as *Lactobacillus* and proteobacteria are supplanted during the first weeks of postnatal life by bacterial taxa that can feed on components of mother milk such as bifidobacteria and *Bacteroides* (Orrhage and Nord 1999, Dominguez-Bello, Costello et al. 2010). Several studies have shown the relevance of early-life gut microbiota development for gut health later on. A study by Dou et al. (Dou, Gadonna-Widehem et al. 2017) showed that a higher relative abundance of particular microbial groups during the nursing stage profoundly affects the emergence of post-weaning diarrhoea. Pigs that did not develop diarrhoea during the post-weaning period displayed a higher relative abundance of *Prevotellaceae, Lachnospiraceae, Ruminococcaceae* and *Lactobacillaceae* at day 7 of age compared to diarrhoeic pigs. In another study it was shown that administering multiple species of microbes to sows during

gestation and lactation influenced the composition of the microbiota and the concentration of SCFAs in the faeces of piglets during the post-weaning phase (Mori, Ito et al. 2011). These studies illustrate the potential for applying microbial interventions in the first weeks of postnatal life.

Host genotype was also shown to play a crucial role in the development of the microbiota along the GI tract of piglets. In a study by Xiao et al. (Xiao, Kong et al. 2018), the microbiota composition along the GI tract of piglets from two separate breeds (Jinhua vs Landrace) was investigated using 16S rRNA gene sequencing. The microbial diversity in the duodenum, jejunum and caecum was found to differ between the two breeds, whereas the ileal and colonic microbial diversity was similar. Furthermore, several bacterial groups were different in terms of relative abundance along the entire GI tract between the two breeds, with jejunal and ileal microbiota showing the greatest contrast. In addition to genotypic and microbiota differences between breeds, differences within pig breeds also exist, albeit smaller. Pig breeders typically select for general traits such as growth performance and overall health, but many breeders also take into account specific genotypic traits such as the absence of particular receptors along the gut lining that provide elevated colonization resistance against particular pathogens. One example is the breeding of F4 receptor-negative ($F4R^-$) pigs, which prevents the adhesion and subsequent infection by the diarrhoea causing pathogen ETEC harboring F4 $(K88)^+$ fimbriae (Zhou, Zhu et al. 2015). Although this Mucin 4 gene polymorphism has been shown to affect susceptibility to ETEC, it was also shown to have an effect on bacterial diversity in the jejunum, which might indicate that the interplay between the host and the microbiota is affected by the presence of this receptor (Messori, Trevisi et al. 2013). In addition to the Mucin 4 gene, the Mucin 13 gene is also of relevance because one of its two transcripts was reported to be associated with efficient binding by strains of *E. coli* that have F4 fimbriae (Ren, Yan et al. 2012).

Several effectors (i.e. diet, age) have a strong impact on composition of the microbiota along the GI tract. In order to find out which effectors are most important, the relative contribution of effectors can be compared. For example, in one study it was shown that age, introduction of solid feed and weaning were more important determinants of gut bacterial succession in piglets than breed and nursing mother (Bian, Ma et al. 2016). Nevertheless, piglet breed and nursing sow breed did lead to increasing differentiation of the piglet's faecal bacterial community, with specific bacterial taxa being associated with either piglet breed or sow breed, the latter most likely due to differences in milk composition (Bian, Ma et al. 2016). The introduction of creep feed is similarly expected to have a measurable impact on composition of the microbiota although, to the best of our knowledge, a trial specifically dedicated to exploring this has yet to be published. In addition to the more obvious factors affecting microbiota composition that were previously mentioned, factors like sanitary conditions (Montagne, Arturo-Schaan et al. 2010), dam parity (Carney-Hinkle, Tran et al. 2013) and housing (Kubasova, Davidova-Gerzova et al. 2017) also have a measurable impact on microbiota composition and as such should be taken into consideration.

Effectors of post-weaning development of microbiota

Weaning is a critical event in a pig's life. When a piglet switches from sow milk to solid feed as a source of nutrients, it is a sudden and stressful event which strongly impacts piglet health (Campbell, Crenshaw et al. 2013). Indeed, the piglet's health is affected by the change in diet and also by the accompanying social and environmental stresses. These stresses include the separation from the mother, handling, transport, changing physical environments and mixing of litters (Campbell, Crenshaw et al. 2013). In conventional pig husbandry practices, piglets are weaned at approximately 3-4 weeks of age, while natural weaning would occur much later at around 17 weeks of age (Jensen 1986). This difference between conventional weaning and natural weaning is mostly due to economic reasons but also exists to prevent damaging of sow teats, which occurs more frequently as piglets become older. Weaning can lead to anorexia, which contributes to local inflammation in the piglet's small intestine (McCracken, Spurlock et al. 1999). Furthermore, weaning is frequently associated with severe enteric infections and a high prevalence of enteric infections leads to overuse of antibiotics, which raises economic and public health concerns (Gresse, Chaucheyras-Durand et al. 2017). In addition to these concerns, antibiotics have been shown to have long-lasting effects on microbiota composition and host gene expression in the piglet, even when administered only once in the first week after birth (Schokker, Zhang et al. 2015). Similarly, administration of antibiotics to sows around parturition was shown to result in great differences in gut physiology of the offspring both during early neonatal life and at six months of age, and this especially in response to a dietary challenge with a high fat diet (Arnal, Zhang et al. 2014, Arnal, Zhang et al. 2015). Antibiotic use can be reduced if the frequency of enteric infections is reduced. To prevent enteric infections at weaning, it is important that the intestinal microbiota develops into a complex and stable community that generates a tight 'colonisation resistance' or 'competitive exclusion' against pathogens (Lallès, Bosi et al. 2007).

At weaning, the switch from highly digestible sow milk to less-digestible solid feed results in a shift from milk-derived glycan metabolism to plant-derived glycan metabolism along the GI tract (Frese, Parker et al. 2015). The microbiota composition changes dramatically (Konstantinov, Awati et al. 2006) with, for example, an increase in the relative abundance of obligate anaerobes (Inoue, Tsukahara et al. 2005). This shift of the gut microbiota is considered to be a main factor leading to post-weaning diarrhoea. As reviewed by Gresse *et al*. (2017), most studies conducted on the weaning transition have reported a decrease in bacteria of the *Lactobacillus* group accompanied by a loss of microbial diversity. In addition, an increase was reported for *Clostridium* spp., *Prevotella* spp. and *Proteobacteriaceae* (Gresse, Chaucheyras-Durand et al. 2017), while several studies reported a decrease of *E. coli* after weaning (Pajarillo, Chae et al. 2014, Mach, Berri et al. 2015, Wang, Tsai et al. 2019). The decrease in gut microbial diversity might be the leading cause for post-weaning diarrhoea, but the weaning transition is also associated with increased gut permeability and intestinal inflammation (Brown, Maxwell et al. 2006). Although the weaning transition entails a highly dynamic period in terms of microbiota succession that lasts approximately 1-2 weeks, changes in microbiota composition and further maturation still occur after this period. For example, it was found that the most predominant genus *Prevotella* represented up to 30% of all classifiable bacteria when pigs were 10 weeks of age, while this percentage

decreased to 3.5-4.0% when pigs were 22 weeks of age. With this decrease in *Prevotella*, an increase in *Anaerobacter* was observed. In the same time frame, an increase in the phylum *Firmicutes* was observed, whereas the proportion of bacteria in the phylum *Bacteroidetes* decreased (Kim, Borewicz et al. 2011). Most studies to date have focused on the period up to and during the weaning transition but only few studies have addressed later periods. As more longitudinal studies are carried out to investigate the dynamics of the GI tract microbiota of pigs well after weaning, hopefully a more complete picture can be obtained of the maturation of pig gut microbiota during the later stages of life.

Interaction of microbiota with the host

Commensal gut microorganisms are important for the fermentation of dietary components and for competitively excluding colonization and growth of pathogens. However, these microorganisms may also interact with the porcine host cells and the immune system. Of the porcine host cells, the first that the gut microorganisms come into contact with are the epithelial cells. Commensal gut microorganisms affect the intestinal epithelial barrier (i.e. activation of regulatory pathways) and, in turn, the intestinal epithelial barrier develops tolerance towards their presence (Sharma, Young et al. 2010). In addition, microbial colonization has a marked effect on the formation of lymphoid tissues and corresponding immune system development. Although this finding does not originate from a porcine study, the effect of microbial colonization was reported when using an alternative mammalian model, namely the comparison between germ-free and colonized mice (Hooper, Littman et al. 2012). With respect to porcine research, antibiotic treatment in the first week after birth was shown to have long-lasting effects on host intestinal gene expression (Schokker, Zhang et al. 2015). The presence of several taxonomical groups of bacteria, including *Turicibacter*, *Clostridiaceae, Streptococcaceae* and *Lactobacillaceae*, was positively correlated with body weight, which may partly be explained by interactions with the host immune system (Wang, Tsai et al. 2019). Another bacterium that was found to be positively correlated with pig body weight is *Lactobacillus mucosae,* which is known for its mucus-binding activity and for its ability to improve barrier function of the epithelium (Wang, Tsai et al. 2019). The possibility for specific microbiota to adhere to the gut lining of the host strongly dictates the interactions that take place. In this respect the genome of the host is an important player as it dictates the presence or absence of fimbriae-specific receptors on the small intestinal microvilli to which specific microorganisms can adhere. Of these fimbriae-specific receptors, the F4 and F18 fimbriae-specific receptors have been studied most intensively (Nagy and Fekete 2005) due to their relevance in post-weaning diarrhoea. The F4 receptors are expressed in intestinal tissue from birth, whereas F18 receptors are only fully expressed after three weeks of age (Fairbrother, Nadeau et al. 2005). Many pathogenic strains are adapted to bind to these receptors. For example, most ETEC strains that cause neonatal colibacillosis carry the F4 (K88), F5 (K99), F6 (987P) or F41 fimbriae, while most ETEC strains that cause post-weaning diarrhoea carry the F4 and F18 fimbriae, with which they bind to receptors that are present on the porcine host's enterocytes (Luppi 2017). Furthermore, F4 ETEC is able to colonize along the entire length of the jejunum and ileum, while F5, F6 and F41 ETEC mostly colonize the posterior jejunum and ileum (Luppi 2017). Research in human subjects has elucidated the chain of events that lead to diarrhoea caused by ETEC strains.

The ETEC strains produce enterotoxins that increase cellular electrolyte and fluid secretion, leading to hyper-secretory diarrhoea (Croxen and Finlay 2010). In response to exposure to F4 ETEC bacteria, the piglet's gut epithelium reacts by increasing the expression of specific genes, such as those encoding TLR4 and IL-8 in the jejunum and porcine β-defensin 2 in the ileum (Li, Piao et al. 2012). To summarize, there is an ongoing interaction between the intestinal gut microbiota and the porcine host, and by adjusting the expression of genes along the gut lining the porcine host is able to actively respond to the presence of pathogens.

Relation between microbiota status and disease susceptibility

The composition of the microbiota in animals is of importance as it determines to a certain extent whether these animals are susceptible to enteric infections. In one study it was shown that pigs that became low-*Salmonella*-shedding after a challenge with *Salmonella enterica* serovar Typhimurium, had a higher relative abundance of the *Ruminococcaceae* family before challenge than the high-shedding pigs. In addition, the high shedding pigs showed a decrease in relative abundance of *Prevotella* 2 days post-inoculation, and increases in relative abundance of various other genera, such as *Catenibacterium* and *Xylanibacter* (Bearson, Allen et al. 2013). Similarly, the inclusion of a *Saccharomyces cereviseae* fermentation product in the diet just after weaning altered the composition of the GI microbiota, which resulted in higher relative abundances of *Bacteroidetes* and *Lactobacillus* after infection with *Salmonella* (Price, Totty et al. 2010). In a more recent study, it was also found that colonization resistance to *Salmonella* was influenced by the intestinal microbiota. Members of *Clostridia* and cellulolytic microorganisms, such as *Ruminococcus* and *Prevotella,* were positively linked to colonization resistance to *Salmonella* while microorganisms associated with suckling, such as *Lactobacillus* and *Oscillospira,* were present at higher relative abundance in piglets that became infected (Argüello, Estellé et al. 2019). These last authors suggested that a lack of microbiome maturation increased susceptibility to infection. In summary, experiments that included a *Salmonella* challenge showed that the predisposition of the microbiota is of relevance for the subsequent succession of disease in piglets. Although these experiments do provide clues as to which taxonomic groups might be helpful to increase colonization resistance against particular pathogens, more research is required to better understand these interactions and to decipher through which means and when to best steer GI microbiota development towards desired endpoints.

Microbiota-inspired strategies to mitigate gut related issues

As previously discussed, the composition and functionality of the microbiota plays a crucial role for the health of the porcine host and is influenced by multiple effectors including colonization during and after delivery, diet and host genotype. Furthermore, feed efficiency and other growth traits are linked to microbiota composition or with specific members of the gut microbiota (Ramayo-Caldas, Mach et al. 2016, McCormack, Curião et al. 2017, Xiao, Yang et al. 2018). In addition to growth traits, gut related health issues such as pathogen infection, reduced gut barrier function and diarrhoea are linked to composition of the gut microbiota. Strategies that are aimed at reducing the incidence of microbiota-related complications

involve the timely presentation of creep feed, vaccination against enteric pathogens, limitation of protein content in feed, inclusion of feed ingredients that can reduce gastric pH, and exclusion of feed ingredients that can increase gastric pH. To date, several *in vivo* studies have been carried out to look at the effect of creep feed intake on post-weaning feed intake (Bruininx, Binnendijk et al. 2002) and on post-weaning occurrence of colibacillosis (Carstensen, Ersbøll et al. 2005). It was shown that consumption of an adequate (not too much and not too little) amount of creep feed during the nursing period stimulates early post-weaning feed intake, while occurrence of diarrhoea and faecal shedding of haemolytic *E. coli* were not conclusively associated with creep feeding.

Although vaccines are generally used to raise immunity against specific pathogens and are generally not designed to have an impact on the overall composition of the microbial community, the use of vaccines aimed at enteric pathogens can be identified as a microbiota-inspired strategy to mitigate gut related issues. Considering that several vaccines against enteric pathogens are widely used today and have proven their efficacy, the use of vaccinations is mentioned here amongst the possible strategies used by livestock producers to alter microbiota composition. Several vaccines were developed to protect piglets against pathogenic *E. coli* strains, such as strains that produce the Shiga-like toxin II variant (SLT-IIe) (Bosworth, Samuel et al. 1996). One such vaccine that was commercialized is Coliprotec. This vaccine provides sufficient protection to piglets against a challenge with ETEC F4 to significantly reduce the duration and severity of diarrhoea (Melkebeek, Goddeeris et al. 2013). Vaccination of piglets against *Salmonella* has resulted in mixed results, likely due to the complex infection strategy of *Salmonella*, with several studies showing reduced presence/shedding of *Salmonella* in the mesenteric lymph nodes (MLNs) or faeces (Denagamage, O'Connor et al. 2007). More recently, a number of vaccination trials showed promising results (Wales and Davies 2017), including the trial by Roesler and co-authors, where significant protection against clinical signs, shedding and tissue invasion was observed (Roesler, Stief et al. 2010). As has been reviewed, more recent approaches using mucosal presentation of antigens have been relatively successful, most likely due to the enhancement of cell-mediated immunity and mucosal immunity (Wales and Davies 2017).

A review by Rist and co-authors reported that avoiding excessive amounts of protein that reach the lower GI tract may reduce the incidence of post-weaning diarrhoea and inhibit proliferation of pathogenic bacteria (Rist, Weiss et al. 2013). In addition, a moderate reduction of dietary protein level may reduce the formation of detrimental fermentation products, such as ammonia, hydrogen sulfide and biogenic amines. Moreover, there are indications that the inclusion of fermentable carbohydrates could be promising to reduce fermentation of detrimental proteins and proliferation of potential pathogenic proteolytic bacteria (Rist, Weiss et al. 2013). In a pig trial focusing on the efficacy of a vaccine, the effect of dietary protein content was tested in parallel. Subclinical oedema disease developed in the pigs fed a low-protein diet, while pigs fed a high protein diet developed clinical oedema disease and death (Bosworth, Samuel et al. 1996). Reduction of post-weaning diarrhoea can also likely be achieved by limiting dietary protein content for a short period after weaning. However, caution should be taken to provide sufficient protein to match the piglet's requirements, as otherwise growth performance might be compromised (Halas, Heo et al. 2007). Furthermore, it has been shown that a greater dietary crude protein (CP) content (i.e.

230g CP/kg vs 130g CP/kg) increased coliforms in the faeces and decreased the lactobacilli to coliform ratio in the proximal colon (Wellock, Fortomaris et al. 2006). Although increasing crude protein content in feed led to an increase in faecal fluidity, it contrastingly also led to improvements in average daily gain. Consequently, with the inclusion of crude protein, there seems to be a trade-off between maximizing performance and minimizing risks of post-weaning diarrhoea. Interestingly, it was shown that feeding a low protein diet (173g CP/kg) supplemented with amino acids to conform to an ideal amino acid pattern can reduce post-weaning diarrhoea without compromising production (Heo, Kim et al. 2008). Taken together, these results indicate that limiting dietary protein content is a viable option to lower the abundance of coliforms and to reduce gut related issues in piglets, especially when a reduction in the use of antimicrobial growth promoters (AGPs) is desired.

The acidity of the stomach is the first line of defence against incoming pathogens and as such is important to take into account in the prevention of gut related issues. The incidence of pathogen survival and their transit to the small intestine are increased by the variability (between 2.2 and 4.2) of the pH in the piglet's stomach (Kim, Hansen et al. 2012). Overeating in the early weaning phase causes a greater risk for elevated gastric pH which may result in a higher incidence of pathogen survival (Halas, Heo et al. 2007). The use of feed ingredients that reduce gastric pH (e.g. organic acids), or the exclusion of feed ingredients that increase gastric pH (e.g. calcium carbonate) seem to be viable options to prevent gut-related problems. Of note, due to the buffering capacity of some amino acids, such as lysine, arginine and histidine, protein content is believed to have an influence on gastric pH as well (Partanen and Mroz 1999). In addition to the strategies mentioned, the incorporation of feed additives is a means of improving gut health and can be used to alter gut microbiota composition. Table 1 summarizes results from *in vivo* studies using currently fed additives and investigating their effects on the composition of the gut microbiota. One of the most widely used class of feed additives is the antimicrobial growth promoters (AGPs), which have been used since the 1950s. Although the modes of action of AGPs are still poorly understood, they have provided benefits such as increases in growth performance and reduction of disease incidence. Researchers have found that AGPs have an effect on bacterial load and composition and that they also interact with the immune system (Brown, Uwiera et al. 2017).

Promising alternatives to AGPs include feed additives such as prebiotics, probiotics, organic acids, minerals and essential oils (Pluske, Turpin et al. 2018). Prebiotics such as resistant starch, pectin, β-glucans and oligosaccharides are used to enhance bacterial fermentation of dietary fibres in the hindgut. Intestinal physiology and microbial composition, hence microbial fermentation, were shown to be influenced by these ingredients (Hopwood and Hampson 2003). Prebiotics have therefore provided livestock producers a "clean-label" feeding option that may be used to mitigate the negative effects of morbidity on growth and performance (Broadway, Carroll et al. 2015).

The effect of non-microbial feed interventions on microbiota composition

A number of fibrous feed components (e.g. β-glucans, cello-oligosaccharides and arabinoxylans) have been tested for their effects on the composition of the GI tract microbiota (Table 1). Generally, these fibrous components are not digested by the host's enzymes but are instead digested by the fermenting capacity of fibre-degrading bacteria in the hindgut. This microbial fermenting activity results in the production of metabolites such as SCFAs, of which butyrate is an important source of energy for enterocytes. In addition, some polysaccharides, such as yeast-derived polysaccharides, which are mostly comprised of α-D-glucans and β-D-glucans (Kogan and Kocher 2007), can also interact directly with host immune cells and are able to bind bacteria while blocking fimbriae of pathogenic bacteria and preventing their attachment to the mucous epithelium in the GI tract (Kogan and Kocher 2007, Ruiz-Herrera 2016). Short- and medium-chain organic acids such as lactic, citric, butyric, propionic and fumaric acids are used in piglet feed because of their bactericidal effect during feed storage, their effect at lowering gastric pH and because they help to improve growth performance (Suiryanrayna and Ramana 2015, Gresse, Chaucheyras-Durand et al. 2017). Proposed mechanisms by which growth performance is improved include the impact of these organic acids on digestive processes, their utilization as energy source and their effect on the composition of the intestinal microbiota. The use of organic acids in newly weaned piglets has been mostly related to a decrease in coliforms and an increase in *Lactobacillus* along the GI tract (Gong, Yu et al. 2008, Gresse, Chaucheyras-Durand et al. 2017), although some studies have shown a decrease in *Lactobacillus* (Gedek, Roth et al. 1992, Maribo, Jensen et al. 2000, Øverland, Kjos et al. 2008). Even though it presents some drawbacks, ZnO was proposed as one of the most effective feed additives to replace antibiotics and is already widely commercialized in several countries. High levels of ZnO were shown to have antimicrobial properties and are currently used to fight against post-weaning infections. The most severe effects of high dietary zinc were observed one week after weaning in the stomach and small intestine, with pronounced reductions of *Enterobacteriaceae* as well as lactobacilli (Starke, Pieper et al. 2014). Others have also found a reduction in *Lactobacillus* spp. after treatment with ZnO (Højberg, Canibe et al. 2005, Broom, Miller et al. 2006, Vahjen, Pieper et al. 2011), although the reduction of *Enterobacteriaceae* was not found in all studies (Højberg, Canibe et al. 2005, Vahjen, Pieper et al. 2011).

Another group of compounds that is used as a dietary supplement for pigs belongs to the phytogenic feed additives. This group comprises additives such as herbs, spices, essential oils and oleoresins. Although results are variable, their inclusion can result in improved feed intake by enhancing palatability, and can also increase storage preservation of the feed. Additional beneficial effects include improved digestive secretions, improved anti-oxidative status and antimicrobial effects (Jacela, DeRouchey et al. 2010). With respect to microbiota composition, *in vivo* studies that have included dietary essential oils generally resulted in a reduction in the amount of *E. coli* and/or other Gram-negative bacteria (Table 1). Another group of feed additives that has recently received attention involves the fungal kingdom. To date, there are two studies in which dehydrated and ground mushroom fruiting bodies (white button mushroom/oyster mushroom) have been added to feed and one study in which a particular polysaccharide (lentinan) from the Shiitake mushroom was used in feed for swine. Although results are variable between mushroom species, these

Table 1. Influence of non-microbial feed additives on porcine microbiota composition. Of note: This overview is not a result of a systematic approach.

Treatment	Effects	Location	Reference
Antibiotics			
Tylosin, post-weaning	> lactobacilli (only bacterial group that was tested using qPCR)	Ileum	Collier, Smiricky-Tjardes et al. (2003)
Chlortetracycline, sulfamethazine and penicillin, post-weaning	> proteobacteria, *Succinivobrio* and *Ruminococcus*, < *Bacteroidetes*	Ileum	Collier, Smiricky-Tjardes et al. (2003)
Chlortetracycline, post-weaning	< *Lactobacillus johnsonii* and *Turicibacter*, > *Lactobacillus amylovorus*	Ileum	Rettedal, Vilain et al. (2009)
Tulathromycin, pre-weaning	< lactobacilli (trend)	Jejunum	Schokker, Zhang et al. (2014)
Lincomycin, post-weaning	> *Lactobacillus* (only bacterial group that was tested using qPCR)	Ileum	Gong, Yu et al. (2008)
Heavy metals			
Zinc oxide, post-weaning	< *Enterobacteriaceae* and *Lactobacillaceae*	Ileum	Starke, Pieper et al. (2014)
Zinc oxide, post-weaning	< non-specific post-weaning diarrhoea	Faeces	Poulsen (1995)
Zinc oxide, post-weaning	> *Enterobacteriales*	Ileum, Colon	Yu, Zhu et al. (2017)
Zinc oxide, post-weaning	> *Weissella* spp., *Leuconostoc* spp., *Streptococcus* spp., < *Sarcina* spp., *Neisseria* spp., > gram-negative facultative anaerobic genera	Ileum	Vahjen, Pieper et al. (2010)
Zinc oxide, post-weaning	< *Bifidobacterium* spp.	Faeces	Mukhopadhya, O'Doherty et al. (2019)
Zinc oxide, post-weaning	< lactobacilli, > enterococci	Multiple segments GI tract	Højberg, Canibe et al. (2005)
Copper sulphate, post-weaning	< coliforms	Colon	Højberg, Canibe et al. (2005)
Organic acids			
Mix of organic acids: formic, benzoic, sorbic acid and butyrate, post-weaning	< coliforms, enterococci, lactic acid producing bacteria	Jejunum, Colon, Rectum	Øverland, Kjos et al. (2008)
Mix of organic acids, post-weaning	> *Lactobacillus* (only bacterial group that was tested using qPCR)	Ileum	Gong, Yu et al. (2008)
Prebiotics			
Resistant starch, post-weaning	> *Faecalibacterium prausnitzii* < *Escherichia coli, Pseudomonas*	Colon	Haenen, Zhang et al. (2013)
Inulin, pre-weaning	> *Lactobacillus* and *Bifidobacterium*	Caecum	Tako, Glahn et al. (2008)
Inulin, post-weaning	> Pigs harboring bifidobacteria	Colon	Loh, Eberhard et al. (2006)
Lactose, post-weaning	> *Bifidobacterium*	Faeces	Pierce, Sweeney et al. (2006)
Lactose, post-weaning	< *E. coli*	Faeces	O'Doherty, Dillon et al. (2010)
Lactulose, post-weaning	> lactobacilli	Colon	Guerra-Ordaz, González-Ortiz et al. (2014)
Lactulose, post-weaning	< proteobacteria, > *Clostridium*	Faeces	Chae, Pajarillo et al. (2016)
FOS, post-weaning	> *Bifidobacterium*, < *E. coli*	Colon	Gebbink, Sutton et al. (1999)

Treatment	Effects	Location	Reference
GOS, post-weaning	> *Bificobacterium*	Colon	Tzortzis, Goulas et al. (2005)
Oat-derived β-glucans, post-weaning	> lactobacilli and bifidobacteria	Colon	Metzler-Zebeli, Zijlstra et al. (2011)
Yeast-derived β-glucans, post-weaning	< *Enterobacteriaceae*	Colon	Sweeney, Collins et al. (2012)
Arabinoxylans, post-weaning	> *F. prausnitzii*, *Roseburia intestinalis*, *Blautia coccoides*, *Eubacterium rectale*, *Bifidobacterium* spp., *Lactobacillus* spp.	Faeces	Nielsen, Lærke et al. (2014)
Cello-oligosaccharides, post-weaning	> lactobacilli, < *Streptococcus suis*, < *E. coli*	Jejunum, Colon	Jiao, Ke et al. (2015)
Pectin, post-weaning	< *Lactobacillus*, > *Prevotella*	Colon	Tian, Bruggeman et al. (2017)
Mannanoligosacharides, post-weaning	< *Enterobacteriaceae*	Jejunum	Castillo, Martin-Orue et al. (2008)
Seaweed extract, post-weaning	< *E. coli*	Faeces	O'Doherty, Dillon et al. (2010)
Chicory forage and root, post-weaning	> lactic acid bacteria	Ileum	Liu, Ivarsson et al. (2012)
Chicory forage and root, post-weaning	> butyrate producing bacteria > *Megasphaera elsdenii*	Colon	Liu, Ivarsson et al. (2012)
Essential oils			
Carvacrol-thymol, post-weaning	> *Lactobacillus*, < *Enterococcus*	Jejunum	Wei, Xue et al. (2017)
Cinnamaldehyde-thymol, post-weaning	< *E. coli*, < total anaerobes	Rectum	Zeng, Xu et al. (2015)
Cinnamon-thyme-oregano extract, post-weaning	< coliforms	Faeces	Namkung, Li J. Gong et al. (2004)
Cinnamaldehyde-thymol, post-weaning	< *E. coli*, > ratio lactobacilli/*E. coli*	Caecum, colon and rectum	Li, Piao et al. (2012)
Cinnamaldehyde-thymol, post-weaning	< *E. coli* (based on counts of *Lactobacillus* and *E. coli* counts)	Faeces	Li, Ru et al. (2012)
Herbal extract, post-weaning	> *Lactobacillus* (only bacterial group that was tested using qPCR)	Ileum	Gong, Yu et al. (2008)
Fungal compounds			
Pulverized oyster mushroom (Pleurotus ostreatus), post-weaning	< Incidence of diarrhoea, < *Ruminococcaceae* (all doses), > *Veillonellaceae* (at 10 g/kg dose)	Faeces	Adams, Che et al. (2019)
Freeze-dried white button mushroom (Agaricus bisporus), post-weaning	> *Lachnospiraceae*, > *Ruminococcaceae*, < *Bifidobacteriaceae*	Faeces, proximal colon	Solano-Aguilar, Jang et al. (2018)
Lentinan (Mushroom polysaccharide from Lentinus edodes), post-weaning	< *Firmicutes*, > *Bacteroidetes*, > *Faecalibacterium*, > *Prevotella*,	Caecum	Wang, Wang et al. (2019)

fungal feed additives positively affected the composition of the piglet GI tract microbiota after weaning, with increases in fibre-degrading and SCFA-producing bacterial groups. In addition, a reduction in the incidence of diarrhoea with the administration of pulverized oyster mushroom powder was observed (see summary of findings in Table 1).

The effect of microbial feed interventions on microbiota composition

Apart from the use of antibiotics or other non-microbial feed components, the GI tract microbial composition can also be altered with microbial interventions. These can exist as natural or synthetic mixed culture inocula, fermented products, probiotics or synbiotics. Table 2 provides an overview of studies in which the effect of microbial interventions on gut microbiota composition was tested. Studies that were conducted before the year 2000 were not included in this table because a summary was made by Shim and colleagues in 2005 (Shim, Verstegen et al. 2005), showing that probiotics are beneficial to a certain degree and are able to influence some beneficial bacteria in the GI tract of young piglets. The mechanisms of action of microbial interventions include competition with pathogens for mucosal binding sites along the GI tract, competition for nutrients in the lumen, production of antimicrobial compounds such as microcins, and production of organic acids that inhibit pathogen growth (Vondruskova, Slamova et al. 2010). Additionally, some probiotics have strong adherence capacities to bind to intestinal epithelial cells or to the mucus layer (Bezkorovainy 2001), and several probiotics are thought to be capable of enhancing mucosal barrier integrity (Roselli, Pieper et al. 2017). Strongly adhering probiotics are expected to interfere with the adhesion of pathogenic bacteria and the adherence is also associated with stimulatory effects on the immune system (Isolauri, Sütas et al. 2001). Another effect that can occur through the use of microbial interventions is the modulation of the composition and activity of the resident microbiota (Gresse, Chaucheyras-Durand et al. 2017). Microorganisms that are commonly used as probiotics in livestock production systems are strains of *Lactobacillus, Bifidobacterium, Enterococcus, Bacillus, Pediococcus, Saccharomyces, Aspergillus* and *Escherichia coli*, and these can be administered via pelleted feed, as fermented feed, capsules, paste, powder and as granules (Ohashi and Ushida 2009). Additionally, probiotics can be administered in liquid form, given that they are administered in an amount of fluid that will be ingested entirely and rapidly to avoid loss of viability or contamination by pathogenic bacteria (Tournut 1989).

In recent years, on-farm fermentation of feed has become increasingly popular, as small-scale fermenting systems that can be used to perform controlled fermentations have become available. It is essential to perform the fermentation in a controlled manner because uncontrolled fermentation can lead to the production of acetic acid and deterioration of the feed conversion of weanling pigs (van der Peet-Schwering, Smolders et al. 2004). Typically an inoculum of a single (or multiple) Lactobacillus strain is used, thereby allowing the production of lactic acid and the lowering of pH in the feed. The resulting fermented feed contains a high abundance of the Lactobacillus strain that was used as inoculum, which is considered to elicit probiotic effects. This fermented feed is typically used as creep feed or as weaner feed and it would be interesting to study the effects of fermented feed on the composition of the GI tract microbiota and on development of the host immune system.

Table 2. Influence of probiotics on porcine microbiota composition. Of note: This overview is not a result of a systematic approach.

Treatment	Effects	Location	Reference
Probiotics			
Complex lactobacilli preparation, post-weaning	> *Escherichia coli*, < lactobacilli	Most sections of GI tract	Huang, Qiao et al. (2004)
Bifidobacterium longum, post-weaning	< anaerobes and clostridia, > bifidobacteria	Faeces	Estrada, Drew et al. (2001)
Lactobacillus sobrius, post-weaning	< ETEC	Ileum	Konstantinov, Smidt et al. (2008)
Enterococcus faecium, post-weaning	> lactobacilli	Ileum, caecum and faeces	Mallo, Rioperez et al. (2010)
Bacillus subtilis, post-weaning	< *Clostridium* spp. and coliforms	Caecum	Lee, Ingale et al. (2014)
Lactobacillus plantarum, post-weaning	> lactobacilli	Colon	Guerra-Ordaz, González-Ortiz et al. (2014)
Enterococcus faecalis, post-weaning	> lactobacilli	Faeces	Hu, Dun et al. (2015)
Lactobacillus johnsonii, post-weaning	> lactobacilli, < *E. coli*	Faeces	Chiang, Chen et al. (2015)
Lactobacillus mucosae, post-weaning	> lactobacilli, < *E. coli*	Faeces	Chiang, Chen et al. (2015)
Lactobacillus acidophilus, post-weaning	> lactic acid bacteria and bifidobacteria, < *E. coli* and clostridia	Faeces	Dowarah, Verma et al. (2017)
Pediococcus acidilactici, post-weaning	> lactic acid bacteria and bifidobacteria, < *E. coli* and clostridia	Faeces	Dowarah, Verma et al. (2017)
Multispecies probiotic mix, pre-weaning	> *Clostridium* spp.	Distal colon	Barszcz, Taciak et al. (2016)
Mix of *Bacillus licheniformis* and *Bacillus subtilis* followed by $F4^+$ ETEC challenge, post-weaning	> *Clostridium*, *Lactobacillus* and *Turicibacter*	Colon	Zhang, Zhu et al. (2017)
Lactobacillus rhamnosus GG, post-weaning	No effect on counts of lactic acid bacteria, enterobacteria and yeasts	Colon	Trevisi, Casini et al. (2011)
E. coli Nissle 1917, post-weaning	< Diversity of *E. coli* strains	Mucosal gut smears along GI tract	Šmajs, Bureš et al. (2012)
Synbiotics			
Oligofructose with probiotic mixture in creep feed, pre-weaning	< coliforms, > bifidobacteria	Ileum and colon	Shim, Verstegen et al. (2005)
Raw potato starch with *E. coli* probiotics, pre-weaning	Reduction of diarrhoea, increased microbial diversity	Ileum and colon	Krause, Bhandari et al. (2010)
Lactulose with *Enterococcus faecium*, post-weaning	> *Lactobacillus*, < *Enterobacteriaceae*	Faeces	Chae, Pajarillo et al. (2016)
FOS and *Bifidobacterium thermophilum*, post-weaning	> *Bifidobacterium* in synbiotic group compared to probiotic alone	Caecum and colon	Tanner, Lacroix et al. (2015)
Inulin with probiotic formulation (*E. faecium*, *L. salivarius*, *L. reuteri*, *B. thermophilum*), post-weaning	< *E. coli*, also changes in diversity and community profile	Multiple locations along GI tract	Sattler, Bayer et al. (2015)
Long-chain inulin combined with *Lactobacillus acidophilus* W37	> *Prevotellaceae*, < *Lactobacillaceae*	Faeces	Lépine, Konstanti et al. (2019)

Although the use of microbial feed interventions comes with challenges such as added cost, labour and logistical requirements, they can be considered to be one of the most direct methods to steer the development of the gut microbiota.

Conclusions

Given that the sow's microbiota dictates to a certain extent the microbiota development of her offspring, it should be possible to steer the microbiota development of the piglets through the sow. Additionally, many predisposing risk factors can be eliminated in the first days after birth by providing adequate housing, climate control and prevention of cross-contamination from infected pigs. Steering development of the microbiota can be done as early as in the first weeks of postnatal life through dietary interventions. As summarized in this chapter, many studies have investigated the effects of dietary interventions after weaning, with mixed outcomes on composition of the microbiome. However, relatively few studies were conducted to look at dietary interventions during the suckling period. Although less practical to implement in production environments, the administration of dietary components during the suckling period might be more promising in terms of early-life stimulation of mucosal and systemic immune development, steering microbial succession along the GI tract and subsequent disease prevention.

To this end, both non-microbial and microbial feed interventions can steer the GI tract microbiota composition into the 'right' direction. This generally means a reduction in the relative abundance of Gram negative bacteria, such as proteobacteria, enterobacteria, *Salmonella*, coliforms, *E. coli*, and more specifically pathogens, such as ETEC and *Streptococcus suis*, although a decrease in *E. coli* diversity was also seen (Šmajs, Bureš et al. 2012). During the suckling period, an increase in the relative abundances of milk-degraders such as lactobacilli and bifidobacteria is generally seen as a positive effect, while after weaning an increase in fibre-degraders such as *Prevotella* and clostridia is considered a positive sign of gut maturation. In several studies using a non-microbial feed intervention resulted in increased relative abundances of lactobacilli, bifidobacteria or strict anaerobes, and more specifically, some studies showed an increase in relative abundances of known probiotics (e.g. *Faecalibacterium prausnitzii*) after weaning. These are all considered as positive treatment effects. However, in a recent study it was found that when weaned piglets have a relatively high abundance of microbial members associated with suckling (*Lactobacillus* and *Oscillospira*), they are predisposed for infection by *Salmonella* (Argüello, Estellé et al. 2019).

Although some dietary fibres clearly confer beneficial health effects, recommendations for optimal inclusion rates are challenging (Pieper, Vahjen et al. 2015). Similarly, adequate administration of probiotics or fermented products might be even more challenging, as living microbes must be stored and administered in such a way that they at least partly survive until they reach the piglet's GI tract. In this respect, the use of postbiotic, i.e. microbial fermentation components, might be a viable option, especially since they were shown to elicit responses similar to those of probiotics (Tsilingiri and Rescigno 2012, Wegh, Geerlings et al. 2019). Despite these challenges, pre- and probiotics are expected to play increasingly

important roles in pig husbandry because of their potential to improve gut health and their potential to replace AGPs and other antimicrobial feed ingredients such as ZnO. The full potential of probiotics in pig husbandry has not yet been explored, as numerous porcine commensal bacteria remain uncultured and relatively few bacterial candidates have been tested for their positive effects on gut health as well as their safety as live microbial feed ingredients. One approach to finding promising candidates as probiotics in feed for piglets is by correlating growth/health traits with the abundance of particular microbial taxa. In one study, taxa such as *Christensenellaceae, Oscilibacter* and *Cellulosilyticum* were associated with a healthier host with increased feed efficiency (McCormack, Curião et al. 2017). The potential of discovering new candidates for probiotic, or prebiotic use and for alternative compounds, such as essential oils and fungal supplements, is high because they have not all yet been tested in controlled *in vivo* trials. With regard to microbial and non-microbial feed interventions that have been tested to date, findings from many published experiments are highly promising. As a result, implementation of these feed interventions in pig husbandry is likely to increase in the future.

Acknowledgements

We gratefully acknowledge funding by The Dutch Research Council (NWO) and Vereniging Diervoederonderzoek Nederland (VDN) through grant number: 868.15.032. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.


3

Chapter 3

Impact of Yeast-Derived β-Glucans on the Porcine Gut Microbiota and Immune System in Early Life

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Published in Microorganisms 2020, 8(10), 1573;
https://doi.org/10.3390/microorganisms8101573

Abstract

Piglets are susceptible to infections in early life and around weaning due to rapid environmental and dietary changes. A compelling target to improve pig health in early life is diet, as it constitutes a pivotal determinant of gut microbial colonization and maturation of the host's immune system. In the present study, we investigated how supplementation of yeast-derived β-glucans affects the gut microbiota and immune function pre- and post-weaning, and how these complex systems develop over time. From day two after birth until two weeks after weaning, piglets received yeast-derived β-glucans or a control treatment orally and were subsequently vaccinated against *Salmonella* Typhimurium. Faeces, digesta, blood, and tissue samples were collected to study gut microbiota composition and immune function. Overall, yeast-derived β-glucans did not affect the vaccination response, and only modest effects on faecal microbiota composition and immune parameters were observed, primarily before weaning. This study demonstrates that the pre-weaning period offers a 'window of opportunity' to alter the gut microbiota and immune system through diet. However, the observed changes were modest, and any long-lasting effects of yeast-derived β-glucans remain to be elucidated.

Keywords: β-glucans; porcine; gastro-intestinal tract; gut microbiota; immune system; early life

Introduction

Early in life and during weaning, pigs are at risk of developing diseases which compromise animal welfare and have major economic consequences for the pig industry. The early postnatal and weaning periods are critical, as the gastrointestinal (GI) system and the immune system are still undergoing major changes (Pluske, Turpin et al. 2018, Hornef and Torow 2020). Microbial colonization of the gut starts at birth with the transfer of maternal vaginal, gut, and skin microbiota to the piglet (Chen, Xu et al. 2018). Moreover, diet and environmental exposures (e.g., housing conditions) are major contributors to subsequent microbial colonization of the gut and development of the immune system (Schmidt, Mulder et al. 2011, Everaert, Van Cruchten et al. 2017). It is now recognized that pathogen and environmental exposures impact on the microbial community later in life and that early-life colonization of the mammalian host's mucosal surfaces plays an important role in the maturation of the host's immune system (Schokker, Zhang et al. 2014, Zheng, Liwinski et al. 2020). For example, many studies in different countries have confirmed that growing up on a farm has a protective effect on asthma and allergies in humans (Von Mutius and Vercelli 2010). Another study in mice has shown that low dose treatment with antibiotics can induce obesity later in life. Interestingly, the induction of obesity was not correlated with the antibiotic treatment itself, but with the changes in microbiota (Cox, Yamanishi et al. 2014). Similar results have been observed for early-life antibiotic treatment in pigs (Schokker, Zhang et al. 2015). This implies that changes and interventions in early life, affecting either the microbiota or the immune system, may have long-lasting effects on health, welfare and performance.

Diet constitutes a pivotal determinant of gut microbial colonization and development, which makes it a compelling target for modulation of the gut microbiota and immune function (Zmora, Suez et al. 2019). Thus, dietary interventions (e.g., prebiotics and probiotics) attracted much interest after the EU wide ban on antimicrobial growth promotors (AMGPs) for production animals in 2006. The notion was that dietary interventions in early life could improve gut health and immune competence, thereby supporting health throughout the vulnerable period post-weaning.

A relatively well studied dietary intervention in both animals and humans are β-glucans. β-glucans are a group of glucose polymers and are structural components of fungi, algae, bacteria, and plants. Being the subject of numerous studies on their application in animal feed formulations, a lot is known about the molecular mechanisms of β-glucans and their binding to specific cell receptors (e.g., Dectin-1) along the gut lining and specific immune cells, such as antigen-presenting cells (APCs) (Goodridge, Wolf et al. 2009, Iliev 2015). As reviewed by Thompson et al., several in vitro and in vivo studies showed that β-glucans possess immunomodulatory properties, dependent on their structure, solubility and molecular weight (Thompson, Oyston et al. 2010). Especially particulate β-glucans derived from fungi (including yeast), which consist of a (1,3)-β-linked backbone with small numbers of (1,6)-β-linked side chains, are known for their immune-stimulating effects (Volman, Ramakers et al. 2008, Stier, Ebbeskotte et al. 2014).

Besides the observed immunological effects, there is increasing evidence that β-glucans may have prebiotic traits, and as such might modulate the microbiota (Lam and Cheung 2013). As recently reviewed by Atanasov et al., several fungal polysaccharides, including yeast-derived β-glucans, are already being sold as commercial products due to their high bio-activity (Atanasov, Schloermann et al. 2020). In the last few decades, a small number of studies investigated the effects of yeast-derived products on the intestinal health of pigs (Elghandour, Tan et al. 2020). In a recent study that used an autolyzed dried yeast cell product, no effects were shown on caecal microbiota composition (Berto, TSE et al. 2020). However, it should be noted that that study only investigated the effects of a yeast-derived product in the post-weaning phase.

As outlined above, several studies have investigated the development of the gut microbiota, and the effects of dietary fibres on porcine gut health and the immune function in the post-weaning phase. However, only a few studies have focused their research on the immediate and early period (Arfken, Frey et al. 2020). To the best of our knowledge, no in vivo studies have investigated the effects of yeast-derived β(1,3/1,6)-glucans (MacroGard®) on both the porcine gut microbiota and immune system in early life, despite the promising results from several in vitro studies (Sonck, Devriendt et al. 2011, Geervliet, Lute et al. 2020). In this study, we investigated whether early-life supplementation of yeast-derived β-glucans affects the gut microbiota as well as the immune system. In addition, we examined the temporal dynamics of these complex systems. We hypothesized that early life consumption of yeast-derived β-glucans alters gut microbiota development (composition) and the immune system. Further developed and mature gut microbiota and immune system in early life could make the animals more resilient against invasive pathogens during the weaning phase and later in life.

Materials and Methods

Ethical Statement

The animal experiment was conducted in accordance with the Dutch law on animal experimentation and ethical requirements, which complies with the European directive 2010/63/EU on the protection of animals used for scientific purposes. The project license application was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) (Permit Number: AVD1040020173948) and the animal experiment and associated protocols were approved by the Animal Care and Use Committee of Wageningen University and Research (Wageningen, The Netherlands). Pigs were euthanized by intravenous injection of 20% sodium pentobarbital (Euthasol®) followed by immediate exsanguination according to Good Veterinary Practice (GVP). All efforts were made to minimize suffering.

Study Design

To determine the effect of β-glucans on gastro-intestinal development and the immune function in early life, an in vivo study was performed at the Swine Research Centre (Trouw Nutrition, Sint Anthonis, The Netherlands). The experiment was conducted with 33 sows (Hypor Libra, Boxmeer, The Netherlands) and their litters (Maxter × Hypor Libra sow). Sperm from a single boar was used to inseminate all sows to reduce genetic variation in

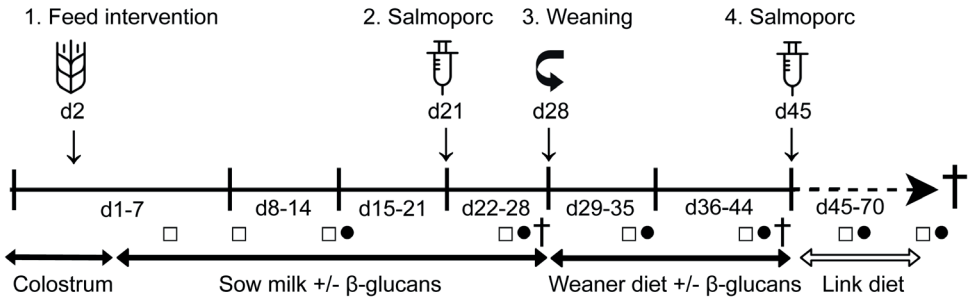
the litters. Shortly after parturition, the piglets received an ear tag, an intramuscular iron injection, their tails were docked, and their birth weight and sex were determined. Calcium carbonate-based powder (Power-Cal®, Power-Cal, Sint-Oedenrode, The Netherlands) was added to all pen floors to reduce the infection pressure in the stable. Approximately 24 h after parturition, 96 female piglets were selected and cross-fostered to minimize possible confounding effects such as genetic background, sow parity, litter size, birth weight and time of birth. During cross-fostering, all piglets were randomly allocated to either the control- or treatment (β-glucan) group, while at the same time making efforts to have groups as equal as possible in terms of body weight, sow parity, and time of birth. This resulted in six animals per pen (experimental unit) with 16 pens in total (eight per group) which were divided over four farrowing rooms (balanced for treatment). The complete study timeline and a schematic representation of the experimental design are presented in Figure 1. Non-experimental (male) piglets were equally divided over the pens, with on average six non-experimental animals per pen. All suckling piglets were kept together with their fostering mother until weaning (approximately day 28), each sow being housed in farrowing pens in a room with a computer-controlled climate system. Weaner diet (Table S1) was administered three days prior to weaning (day 25–27) to allow piglets to become familiar with the consumption of solid feed prior to weaning. At weaning, three pigs per pen (48 pigs in total) were randomly selected and reallocated to a clean nursery facility. During the entire post-weaning period all pens were provided with solid feed and water *ad libitum*. From day 28 until day 44, all pigs received a weaner diet in combination with the feed intervention, and from day 45 until the end of the study the piglets solely consumed a nursery diet (Table S1). Pigs with a history of medication, leg/claw problems, or growth retardation were excluded from the selection. At day 27, 44, and 70 (dissection), sixteen piglets were sacrificed (*n* = 8 per treatment group, at each time point).

Experimental Procedures

Dietary Intervention and Oral Vaccination

From day 2 until day 44 of the study, piglets received either β-glucans (MacroGard®) or a control treatment (tap water) every other day. Piglets received the dietary interventions orally using disposable syringes (Discardit II, BD). MacroGard® (Orffa Additives B.V., Werkendam, The Netherlands), which consisted of 100% yeast (*Saccharomyces cerevisiae*) cell walls, contains a minimum of 60% particulate β(1,3/1,6)-glucans with an approximate molecular weight of 200 kDa (Sonck, Stuyven et al. 2010). These β-glucans were not present as soluble molecules but were instead in particulate form as part of yeast cell wall fragments. Especially these (yeast-derived) particulate β-glucans were known for their immune-stimulating effects (Volman, Ramakers et al. 2008, Stier, Ebbeskotte et al. 2014). Other components of MacroGard® were lipids (max. 18%), proteins (max. 8%) and raw ash (max 10%). In addition, the batch did not contain lipopolysaccharides as was assessed in a previous study (Geervliet, Lute et al. 2020). The dosage of β-glucans was gradually increased every week with 50 mg, starting from 50 mg per administration in week one to 300 mg per administration in week six. These dosages of β-glucans were chosen by taking previous studies into consideration (Sonck 2011). To investigate the effect of the yeast-derived β-glucans on the immune function, an oral live attenuated vaccine (Salmoporc® STM (lot number 0270617), IDT Biologika GmbH, Dessau-Rosslau, Germany) was administered on day 21 (primary vaccination) and day 45 (booster vaccination). This vaccine was registered for

A



B

Department	# of pens	d1-d14	d15-d28	d29-d44	d45-d70
1	2	6/pen	4/pen	3/pen	2/pen
1	2	6/pen	4/pen	3/pen	2/pen
2	2	6/pen	4/pen	3/pen	2/pen
2	2	6/pen	4/pen	3/pen	2/pen
3	2	6/pen	4/pen	3/pen	2/pen
3	2	6/pen	4/pen	3/pen	2/pen
4	2	6/pen	4/pen	3/pen	2/pen
4	2	6/pen	4/pen	3/pen	2/pen
Animals per treatment:		48	32	24	16
Total number of animals:		96	64	48	32

Figure 1. Study timeline (**A**). From day 2 until day 44, all piglets orally received either β-glucans or a control treatment (tap water) every other day (1). A sub-selection of piglets was weaned on day 28 (3). Pigs were vaccinated with Salmoporc® on day 21 (primary vaccination; 2) and on day 45 (booster vaccination; 4). Faecal samples were collected on days 4, 8, 14, 26, 35, 43, 59 and 69 (squares; □). Blood samples were taken on days 14, 26, 35, 43, 59, and 69 (circles; ●). A subset of animals were sacrificed on days 27, 44, and 70 (cross; †). Schematic representation of the experimental design showing the remaining number of animals per treatment over time as determined by deselection or dissection of animals (**B**). The control and the β-glucan groups are presented as yellow and green, respectively. The dashed line separates the pre-weaning and post-weaning time points.

oral application in pigs to diminish bacterial colonization, excretion, and clinical symptoms of infection with *Salmonella* Typhimurium. The vaccine suspension, containing 5×10^8–5×10^9 CFU/mL of the live attenuated *Salmonella* enterica serovar Typhimurium, was freshly prepared according to manufacturer's instructions prior to oral administration.

Blood and Faecal Sampling

Blood and faecal samples were collected at different time points during the study for the evaluation of immune function and microbiota composition, respectively. Faecal samples were collected on days 4 (n = 24 per treatment group), 8 (n = 24), 14 (n = 24), 26 (n = 24), 35 (n = 16), 43 (n = 16), 59 (n = 8), and 69 (n = 8). Rectal stimulation was performed by inserting the tip of a wetted (with sterilized H_2O) cotton swab (PurFlock Ultra, Puritan) into

the rectum, by making small, gentle movements (circular and back- and forward). These fresh faecal samples were collected in cryotubes, immediately placed on dry ice and stored at −80 °C until further processing. DNA isolation from faeces was done on all faecal samples from animals that were dissected. Blood was collected on days 14, 26, 43, and 69 from the jugular vein of the pig using Natrium Heparin tubes (S-monovette®, Sarstedt, Germany). Blood samples were either kept at room temperature (RT) until further processing for cell analysis or centrifuged at 2000× *g* for 10 min to collect serum. Serum was stored at −20 °C until further use. Piglets were sampled in a random order and weighed directly before sample collection.

Dissection

After euthanasia and exsanguination, the ileocecal mesenteric lymph node (MLN) was removed and stored on ice-cold (4 °C) RPMI 1640 Medium (with GlutaMAX™ supplement, Gibco®), containing 10% fetal calf serum (FCS, Gibco®) and 1% L-Glutamine (Gibco®). Subsequently, the GI tract was removed from the abdominal cavity of the piglet, and the jejunum, ileum, and caecum were identified and segmented accordingly. Digesta samples from these GI segments were taken on day 27 (pre-weaning), and day 44 and 70 (post-weaning) by gently squeezing the segment content into a plastic container. Next, the collected digesta was completely homogenized using a clean spatula. Approximately 1 g of homogenized digesta was stored in a sterile cryogenic vial, snap-frozen on dry ice and stored at −80 °C until further processing. The remainder of the digesta from each GI segment was mixed with H_2O for pH measurement (Figure S1) using a pH meter (ProLine B210).

Measurements

Microbiota Analysis

DNA was extracted from faecal and digesta samples using a customized Maxwell 16 Total RNA protocol (Promega Corp., Madison, WI, USA) with Stool and Transport and Recovery Buffer (STAR; Roche Diagnostics Corp., Rotkreuz, Switzerland). Briefly, 50 mg of previously frozen (−80 °C) faeces or 100 mg of digesta was homogenized with 0.25 g of sterilized 0.1 mm zirconia beads and three 2.5 mm glass beads in 300 µL STAR buffer for 3 × 1 min at 5.5 m s^{-1} using a bead beater (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France), with a waiting step of 15 s in between. Samples were incubated with shaking at 300 rpm for 15 min at 95 °C and centrifuged for 5 min at 4 °C and 16,100 × *g*. The supernatant was removed and the pellets were processed again using 200 µL fresh STAR buffer. Samples were incubated at 95 °C and centrifuged as before. The supernatant was removed, pooled with the first supernatant and 250 µL was used for purification with Maxwell 16 Tissue LEV Total RNA Purification Kit, catalogue no. AS1220 (Promega Corp.) customized for DNA extraction in combination with the STAR buffer. DNA was eluted with 50 µL of DNAse- and RNAse-free water (Qiagen). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and adjusted to 20 ng μL^{-1} with DNAse- and RNAse-free water. PCR amplification was carried out with barcoded primers directed to the V4 region of the bacterial and archaeal 16 S rRNA gene, namely EMP_515 F (5'-GTGYCAGCMGCCGCGGTAA), with linker 'GT', and EMP_806 R (5'-GGACTACNVGGGTWTCTAAT), with linker 'CC'. PCR reactions were done in duplicate, each in a total volume of 50 µL and containing 20 ng of template DNA. In order to distinguish samples between sequencing reads, each sample was amplified with a unique barcoded

primer pair (200 nM each per reaction), 1 × HF buffer (Thermo Fisher Scientific, Waltham, MA, USA), 1 µL dNTP Mix (10 mM each; Roche Diagnostics GmbH, Baden-Württemberg, Germany), 1 U Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Fisher Scientific), and 36.5 µL of DNAse- and RNAse-free water. The amplification program included 30 s initial denaturation at 98 °C, followed by 25 cycles (with the exception of jejunum digesta samples which were processed with 30 cycles to yield sufficient amplicon fragments) of denaturation at 98 °C for 10 s, annealing at 56 °C for 10 s, elongation at 72 °C for 10 s, and a final extension at 72 °C for 7 min. The PCR product presence and size (≈290 bp) was confirmed with gel electrophoresis using a 1% agarose gel. In each library, 69 unique barcode tags were used and two artificial (mock) communities were included in addition to a water (blank) control (Ramiro-Garcia, Hermes et al. 2016). PCR products were purified using the HighPrep PCR kit (MagBio Genomics Inc., Gaithersburg, MD, USA), and DNA concentrations were measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). Of each barcoded sample, 200 ng DNA was added to an amplicon pool that was subsequently concentrated using the HighPrep PCR kit to a volume of 20 µL. The DNA concentration of the amplicon pool was measured using the Qubit dsDNA BR Assay Kit and the libraries were sent for Illumina HiSeq sequencing (Eurofins Genomics, Ebersberg, Germany). Amplicon sequence data were processed and analysed using NG-Tax 2.0 (Poncheewin, Hermes et al. 2019) and annotated using the SILVA 132 database (Quast, Pruesse et al. 2012).

Serology

To analyse the antibody response against *Salmonella* Typhimurium, an in-house ELISA was optimised and performed as described previously (Van Altena, Peen et al. 2016). In short, Salmoporc®, containing a live attenuated strain of *Salmonella enterica* serovar Typhimurium, was streaked on a MacConkey agar (Sigma Aldrich) plate and grown overnight at 37 °C. Then, a single colony was used to inoculate 2 mL of lysogeny broth (LB) medium, and grown overnight under aeration on a shaker (200 rpm) at 37 °C. The following day, 1 mL of the overnight culture was transferred to a 50 mL tube containing 15 mL of LB medium. The 50 mL tube was placed under aeration on a shaker (200 rpm) at 37 °C until reaching the exponential growth phase ($OD_{600\ nm}$ = 0.6–0.8). Thereafter, the bacteria were pelleted by centrifugation at 10,000 rpm for 5 min and washed two times with cold PBS. Medium-binding 96 well plates (clear, flat bottom, Greiner Bio-One, Vilvoorde, Belgium) were coated with 100 µL of the bacterial suspension (2 × 10^8 bacteria/mL) and incubated overnight at 4 °C. The following day, non-attached bacteria were removed and attached bacteria were fixed with 4% paraformaldehyde for 2 h at RT. Plates were blocked overnight at RT with a blocking solution consisting of 5% milk powder (ELK, FrieslandCampina, Amersfoort, The Netherlands) in demi water, and stored at 4 °C until usage. After blocking, plates were washed with PBS/Tween20 (0.05%) and 100µL of diluted serum samples were added. Serum samples were diluted 250 × (IgG) and 50 × (IgA, IgM) in blocking solution, and incubated for 1 h at RT. Plates were washed two times, and 100 µL of 50,000 times diluted (in blocking solution) horseradish peroxidase (HRP) conjugated goat anti-Porcine IgG, IgM or IgA (Novus Biologicals, Centennial, CO, USA) was added. After 30 min, plates were washed five times and incubated with 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Enhanced K-Blue®, Neogen, Lansing, MI, USA). After 15 min the reaction was stopped with 100 µL of a 2% HCl stop solution, and the optical density (OD) of the plates was measured at a wavelength of 450 nm (Multi-Mode Microplate Reader FilterMax F5).

Cell Isolation

Collected blood was diluted 1:1 with PBS (containing 0.5 mM EDTA) within 4 h of collection and transferred to Leucosep® tubes using a 60% FICOLL-PAQUE™ Plus density-gradient to isolate the peripheral blood mononuclear cells (PBMCs). Remaining red blood cells were lysed with ACK lysis buffer (Gibco®). MLN cells were collected by cutting the nodules in smaller pieces, and gently squeezing and flushing the cells through a sterile Falcon® cell strainer (100 µm, Corning®, New York, NY, USA), using a syringe plunger and sterile Mg^{2+} and Ca^{2+} free PBS (Lonza, Basel, Switzerland). Both isolated PBMCs and MLN cells rested overnight at 4 °C until further processing.

Stimulation Assay

After resting overnight, 1 × 10^6/well of PBMCs and MLN cells were separately transferred to a 96 well clear round bottom plate (Greiner Bio-One). Plates containing wells with LPS (serotype O55:B5/L2880, Sigma-Aldrich, St. Louis, MO, USA), with Concanavalin A (Con-A, C2010, Sigma-Aldrich), or without a stimulus (cell culture medium only) were prepared the previous day and stored at 4 °C overnight. The next day, the plates were placed at 37 °C for 30 min before addition of the cells. Cells were incubated with 0.1, 1 or 10 µg/mL of LPS, with 5, 2.5, or 1.25 µg/mL or without stimuli for 24 h at 37 °C (5% CO_2). After 24 h of incubation, the plates were centrifuged at 300× *g* for 3 min to spin the cells down. Subsequently, the cell culture supernatant was collected and immediately stored in 96 well polypropylene plates (Nunc®, MicroWell™, Sigma-Aldrich) at −80 °C until further processing.

Flow Cytometry

Isolated PBMCs and MLN cells were stained with a DC or T lymphocyte/NK cell antibody panel (Table 1). To analyse these cells, 5 × 10^6 (DC panel) or 1 × 10^6 (T lymphocyte/NK) freshly isolated PBMCs or MLN cells (per animal) were placed in a 96 well polypropylene plate (Nunc®, MicroWell™, Sigma-Aldrich). Per well, 200 µl FACS buffer (Mg^{2+} and Ca^{2+} free PBS; Lonza), 2 mM EDTA (Merck, Kenilworth, NJ, USA), 0.5% BSA fraction V (Roche, Basel, Switzerland) was added and the plates were centrifuged at 400× *g* for 3 min at 4 °C to wash the cells. After washing, extracellular cell surface markers (Table 1) were stained with antibodies for 30 min on ice (in the dark), followed by three washing steps with cold PBS. Subsequently, cells were stained with (in PBS diluted) Fixable viability dye eFluor™ 506 (eBioscience™) and Streptavidin-BV421 (DC panel) for 30 min, followed by a washing step with FACS buffer. Then, 100 µL Fix/Perm buffer (eBioscience™) was added to each well and incubated for 45 min at RT, to allow for intracellular staining. After incubation, cells were washed three times in Perm buffer (eBioscience™), followed by the incubation with the intracellular antibody mix (Table 1) in 35 µL Perm buffer for 30 min at 4 °C. After two other washing steps with Perm buffer, cells were resuspended in 200 µL FACS buffer and measured for 150 s (NK cells/T cells) or 300 s (DCs) on the FACS CANTO II at a medium flow rate. Beads were used for single stained controls using compensation beads (UltraComp eBeads™, ThermoFisher), and cells were used for Fluorescence Minus One (FMO) controls to control for spectral overlap. Flow cytometry data analysis was performed by using FlowJo™ software (Version 10). Gating of DC subsets, NK cells and T cell subsets was performed in line with previous studies (Gerner, Käser et al. 2009, Auray, Keller et al. 2016, Vreman, Auray et al. 2018). Examples of the gating strategies can be found in Figure S2 (DCs) and Figure S3 (T lymphocytes and NK cells).

Table 1. Antibodies used to identify DC subsets, NK cells and T cell subsets in PBMCs and mesenteric lymph node (MLN) cells.

Antibody	Host/Isotype	Clone	Fluorochrome	Company	Dilution
CD14	Mouse, IgG2b	MIL-2	FITC	Bio-Rad	1:50
CD172a	Mouse, IgG2b	74-22-15A	PE	BD Biosciences	1:40
CD4a	Mouse, IgG2b	74-12-4	PerCP-Cy5.5	BD Pharmingen™	1:320
CADM1	Chicken, IgY	3E1	Biotin	MBL	1:200
Streptav.	n/a	n/a	BV421	BD Horizon™	1:50
CD152 *	Mouse, IgG2a	n/a	APC	Ancell	1:320
CD3ε	Mouse, IgG2a	BB23-8E6-8C8	PE-Cy™7	BD Pharmingen™	1:160
CD4a	Mouse, IgG2b	74-12-4	PerCP-Cy5.5	BD Pharmingen™	1:320
CD8a	Mouse, IgG2a	76-2-11	FITC	BD Pharmingen™	1:10
FoxP3	Rat, IgG2a	FJK-16s	Alexa Fluor® 700 **	eBioscience™	1:20
Ki67	Mouse, IgG1	B56	BV421 **	BD Horizon™	1:80
CD25	Mouse, IgG1	K231.3B2	Purified ***	Bio-Rad	1:200
γδ T cells	Rat, IgG2a	MAC320	PE	BD Pharmingen™	1:20

*; Human CD152 (CTLA-4) murine Ig fusion protein was used to detect CD80 and CD86 on DC subsets. **; Antibodies against intracellular antigens. ***; The antibody CD25 (purified) was labelled with the ReadiLink™ Rapid iFluor™ 647 Antibody Labeling Kit (Aat Bioquest, Sunnyvale, CA, USA).

Statistical Analysis

All R-scripts, data files and pdf files with extensive information on the performed analyses can be accessed through the following DOI: 10.4121/12999620. Alternatively, these files can be found under the following Github page: https://github.com/mibwurrepo/de-Vries-et-al-2020-porcine-study-beta-glucans

Microbiota Analysis

In order to estimate the impact of yeast-derived β-glucans on alpha diversity of the microbiota in piglets' faeces and digesta, Shannon and InvSimpson diversities were calculated for each sample. Shannon and InvSimpson diversities were both chosen as Shannon's diversity index gives more weight to rare species and Simpson's index gives more weight to common species. It should be noted that really rare sequences are missing from the dataset as NG-Tax 2.0 uses an abundance threshold of 0.1% for amplicon sequence variants (ASVs) in a given sample. Shannon and InvSimpson diversities were used in a Linear Mixed-Effects Model (i.e., Shannon~Day_of_study * Treatment) to test for significant differences between time points and treatment groups (nlme package v3.1-140) (Pinheiro, Bates et al. 2015). To estimate the impact of β-glucans on overall microbiota composition, PERMANOVA tests were performed using Bray-Curtis dissimilarities at ASV level (9999 permutations). The homogeneity assumption was tested by calculating Bray-Curtis dispersion for each group followed by ANOVA tests. To visualize the beta diversity in faecal samples over time, a Principal Coordinates Analysis (PCoA) plot was generated using weighted Unifrac dissimilarities. In order to estimate the impact of β-glucans as a dietary intervention on the relative abundance of specific genera in piglet faeces, reads of the ASVs were first aggregated to genus level using the tax glom function of the phyloseq R package (McMurdie and Holmes 2013), after which read frequencies were transformed to compositional data using the transform function of the microbiome R package (Lahti and Shetty 2017). Genera

were filtered to exclusively include genera that had a relative abundance of 0.1% in at least 50% of the samples. Resulting genera were tested for differential relative abundance using the Generalized Additive Models for Location, Scale and Shape (GAMLSS) with a zero-inflated beta family (function taxa.compare) with GAMLSS-BEZI as a statistical method in the metamicrobiomeR package (Ho, Li et al. 2019). Dietary intervention was used as the main variable for comparison and day of study and department (farrowing rooms) were used as adjusting variables. Ear tag was indicated as the identifier to enable a longitudinal approach and "fdr" (False Discovery Rate) was chosen as the method for multiple testing adjustment (p.adjust < 0.05 as the threshold). This analysis was performed on three different datasets: a dataset containing only pre-weaning faecal samples, one with only post-weaning faecal samples and one dataset containing faecal samples from all time points (both pre- and post-weaning). To get a better overview of the genera that mostly contributed to the differences in microbiota composition between the treatment groups over time, weighted Unifrac distance-based Principal Response Curve (dbPRC using package Vegan (Van den Brink and Braak 1999, Shankar, Agans et al. 2017)) analysis was performed. For these dbPRC figures, pre- and post-weaning datasets were used to investigate these distinct periods in the piglet's microbiota development.

Immunological Analysis

To assess if yeast-derived β-glucans alters the immune system, a Student's t-test (unpaired) was performed using R statistical software (version 3.6.2). Additionally, a Two-way ANOVA followed by a Tukey post-hoc test was performed to assess the effects over time and the interaction between time and treatment. Normality of data (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were checked prior to statistical testing. Skewness values between −2 to +2 were considered acceptable (George 2011). Extreme outliers (indicated by R) were removed from the analysis. When the statistical assumptions were not met, data were log transformed. Data are presented as untransformed means ± SEM. Results with an adjusted p-value below 0.05 ($p < 0.05$) were considered statistically significant and results between 0.05 and 0.1 ($0.05 < p < 0.10$) were considered a trend. Significant results were tested for confounding factors, including body weight at birth, using an ANCOVA.

Correlation Analysis

To assess possible correlations between microbial composition, study factor- and immunological data, first microbiota ASVs were aggregated at the genus level and transformed to compositional abundances. Then, a prevalence filter was applied with a 0.1% abundance threshold in at least 50% of the samples. Resulting genera were combined with study factor data and immunological data and a test for association was performed for each parameter pair, using a Pearson moment correlation (95% CI). p-values were corrected for multiple testing using the 'Benjamini-Hochberg' method.

Results

Microbial Colonization

Alpha Diversity

To assess potential differences in microbiota composition between β-glucan treated animals and control animals, both faecal and luminal samples were collected at multiple time points (Figure 1). No significant differences for alpha diversity were observed between the treatment groups when including all faecal time points (Figure 2; $p = 0.11$ for Shannon, $p = 0.60$ for InvSimpson). However, when including exclusively pre-weaning faecal time points, a significant difference in alpha diversity between the treatment groups was observed for the Shannon diversity ($p < 0.04$ for Shannon, $p < 0.10$ for InvSimpson). The significant difference for Shannon's diversity indicates that low abundance ASVs are mostly contributing to the differences in alpha diversity. As expected, piglet faecal and luminal microbiota composition showed different temporal trends in alpha diversity, with alpha diversity in faeces increasing up to day 35 before reaching a plateau that lasted until the end of the study. Furthermore, digesta from different gut segments all showed a similar pattern with a higher α-diversity on day 27 in comparison to day 44 ($p < 0.001$).

Microbiota Composition Over Time

The dynamic shifts in the microbiota composition in the faeces and digesta over time were examined at the phylum and family classification level. At the phylum level (Figure 3A), the microbiota composition in faeces transitioned from a *Firmicutes*, *Bacteroidetes*, *Proteobacteria,* and *Fusobacteria* predominated community on day 4 to a *Firmicutes* and *Bacteroidetes* predominated community at later time points. Both the jejunum and ileum digesta were characterized by a *Firmicutes* predominated community at all time points (day 27, 44, and 70), whereas the caecal microbiota was found to be predominated by *Firmicutes* and *Bacteroidetes* at all time points. Furthermore, the average relative abundance of the *Euryarchaeota* in faeces (Figure S5) increased over time during the pre-weaning period from below 0.03% on day 4 to reach an average relative abundance of over 6% on day 26. Post-weaning faecal samples show a more stable pattern of the *Euryarchaeota* with a relative abundance of 1–2% on each time point until day 69. Interestingly the *Euryarchaeota* were present in caecal digesta with a similar abundance (1.5%) on day 27, while at later time points the mean relative abundance in caecal digesta was drastically lower (<0.1% at both day 44 and day 70). In jejunum and ileum digesta, the relative abundance of the *Euryarchaeota* was very low at all time points, except for jejunum digesta on day 70 (Figure S5). At the family level (Figure 3B), a dramatic shift of taxa could be observed in faeces over time, with large shifts occurring from day 4 to day 8 and during the weaning transition, from day 26 to day 35. Jejunum digesta was predominated by *Lactobacillaceae* at all time points, while ileum digesta was predominated by *Lactobacillaceae* at day 27 and day 44 followed by *Clostridiaceae*_1 on day 70. Caecum digesta was predominated by *Lactobacillaceae, Ruminococcaceae,* and *Prevotellaceae* on day 27, by *Lactobacillaceae* and *Prevotellaceae* on day 44 and by *Prevotellaceae* on day 70. For a more detailed overview of the relative abundance of several families in faecal samples over time, see Figure S6.

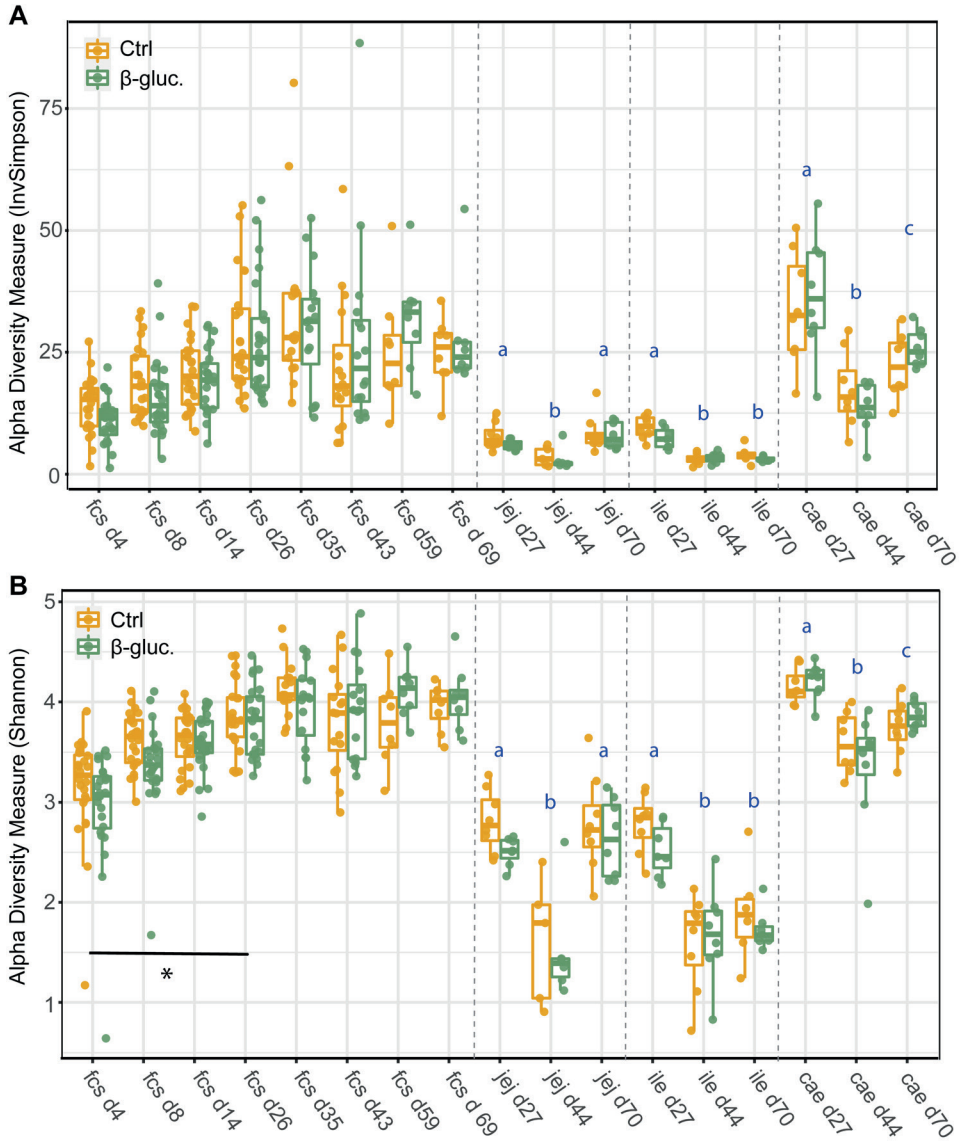


Figure 2. Alpha-diversity of faecal and luminal microbiota between treatments and over time. Every dot represents a single animal with the control animals in red and the β-glucan treated animals in green. InvSimpson diversity values (**A**) and Shannon diversity values (**B**) are given by sampling time point (d4-70) and by faeces (fcs) or gut segment; jejunum (jej), ileum (ile), caecum (cae). Letters (a, b, c) represent significant differences ($p < 0.05$) between time points. The horizontal line with the corresponding asterisk represents the significant difference between treatment groups in the pre-weaning period, calculated using a Linear Mixed-Effects Model (*; $p < 0.05$).

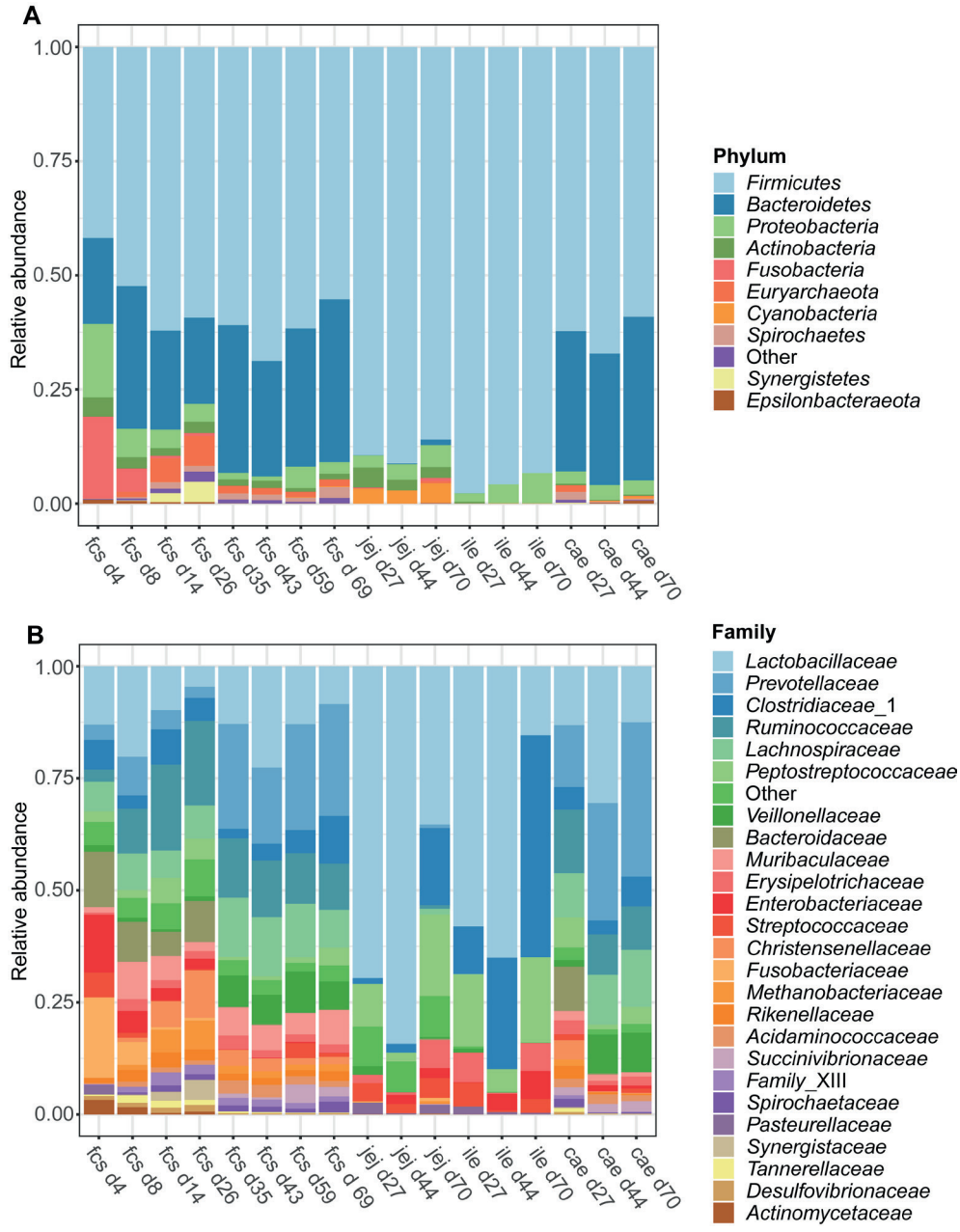


Figure 3. Taxonomic composition of the piglet faeces and digesta over time at phylum (**A**) and family (**B**) level. Data are given as the mean relative abundance by sampling time point (d4-70) and by faeces (fcs) or gut segment; jejunum (jej), ileum (ile), caecum (cae). Data includes samples from both treatment groups and shows the 25 most abundant families and 10 most abundant phyla.

Beta Diversity

Beta diversity was calculated at the ASV level using Bray-Curtis dissimilarities and a PERMANOVA test was performed on all faecal samples with treatment and time point as model parameters. The effect of treatment was not significant ($p > 0.14$), but a strong time effect was observed ($p < 0.001$). When including only pre-weaning faecal samples, however, the effect of treatment was significant ($p < 0.042$), but the contribution of treatment to the variance was low ($R^2 < 0.0031$). This was not observed when only post-weaning samples were included. Bray-Curtis dispersions of pre- and post-weaning faecal samples were compared and showed to be significantly different (ANOVA, $p < 0.001$), with dispersion being lower in post-weaning faecal samples. Principal Coordinates Analysis (PCoA) based on weighted Unifrac distance of the piglet faecal microbiota revealed significant temporal shifts and a distinct separation between pre- and post-weaning faecal samples (Figure 4). The wide distribution of the samples collected on days 4, 8, and 14 reinforced the notion that the overall microbiota composition of piglet faeces changes dramatically in the first two weeks after birth.

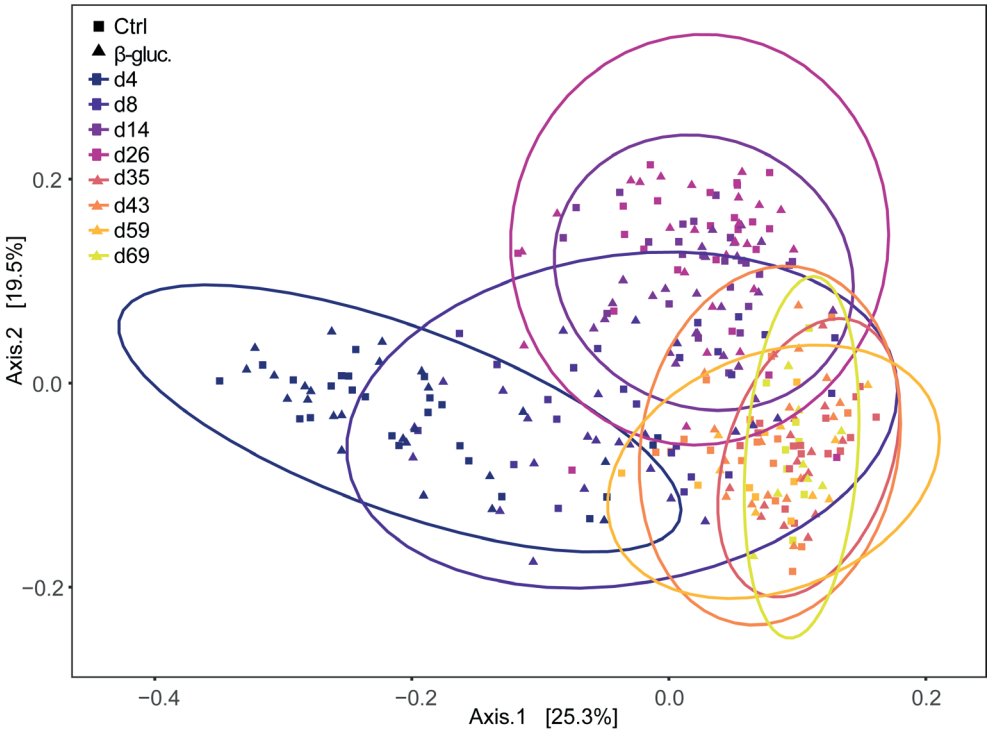
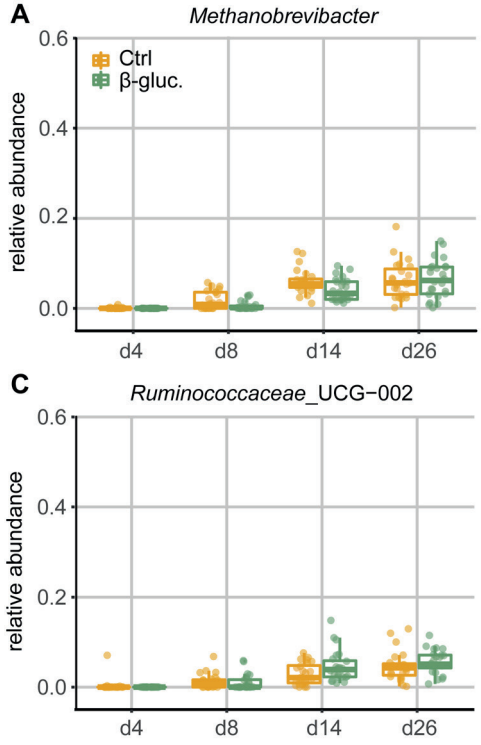


Figure 4. Principal Coordinates Analysis (PCoA) plot of beta-diversity based on weighted Unifrac dissimilarities in faecal samples over time (d4-69). Every dot represents a faecal sample from a single animal and samples are enveloped per time point. Control and β-glucan treated animals are represented by squares (■) and triangles (▲), respectively.

Differentially Abundant Genera between Treatment Groups

When comparing the relative abundances of individual taxa in pre-weaning faecal samples at the genus level, three out of a total of 31 genera that passed the prevalence filter were found to be differentially abundant between the β-glucan and control group (Figure 5). Especially on days 8 and 14, the genus *Methanobrevibacter* was less abundant in animals that received β-glucans, while the genera *Fusobacterium* and *Ruminococcaceae*_UCG-002 were more abundant at a number of time points in animals that received β-glucans. No differentially abundant genera were found in faecal samples of the post-weaning period, nor were differentially abundant genera found when all faecal samples were used in the GAMLSS model. The dbPRC analysis (Figure S4) revealed several additional genera that contributed to the changes between treatment groups over time.



Genus	Estimate	SE	t-value	p.adjust
A. *Methanobrevibacter*	-0.26	0.09	-2.86	0.05
B. *Fusobacterium*	0.33	0.10	3.18	0.05
C. *Ruminococcaceae*-UCG−002	0.29	0.10	2.89	0.05

Figure 5. Relative abundances (by fraction) of differentially abundant genera in faeces during the pre-weaning period (d4-26). Shown genera resulted from comparing β-glucan animals to control animals using a GAMLSS model. Dietary intervention was used as the main variable for comparison and day of study and department were used as adjusting variables. Ear tag was used as an identifier and "fdr" was chosen as the method for multiple testing adjustment (*p*.adjust < 0.05).

Immunological Analysis

Immunological Response to Oral Vaccination

To analyse if supplementation of β-glucans in early life induces changes to the host's innate and adaptive immune system, different types of analysis were performed to assess this on a local and systemic level. In this study, an oral vaccination against *Salmonella*

Typhimurium was administered to measure whether yeast-derived β-glucans have an effect on the responsiveness of the immune system. As expected, a clear and significant increase of *Salmonella*-specific IgM and IgA antibodies could be observed after both the primary and the secondary (booster) vaccination (Figure 6A,B). An increase in antigen-specific IgG antibodies was only observed after the second vaccination (Figure 6C). Despite the clear increase of antibody titres, no altered antibody response to the *Salmonella* vaccine strain was observed between the β-glucan and control group during the pre- and post-weaning phase of the study (Figure 6A–D).

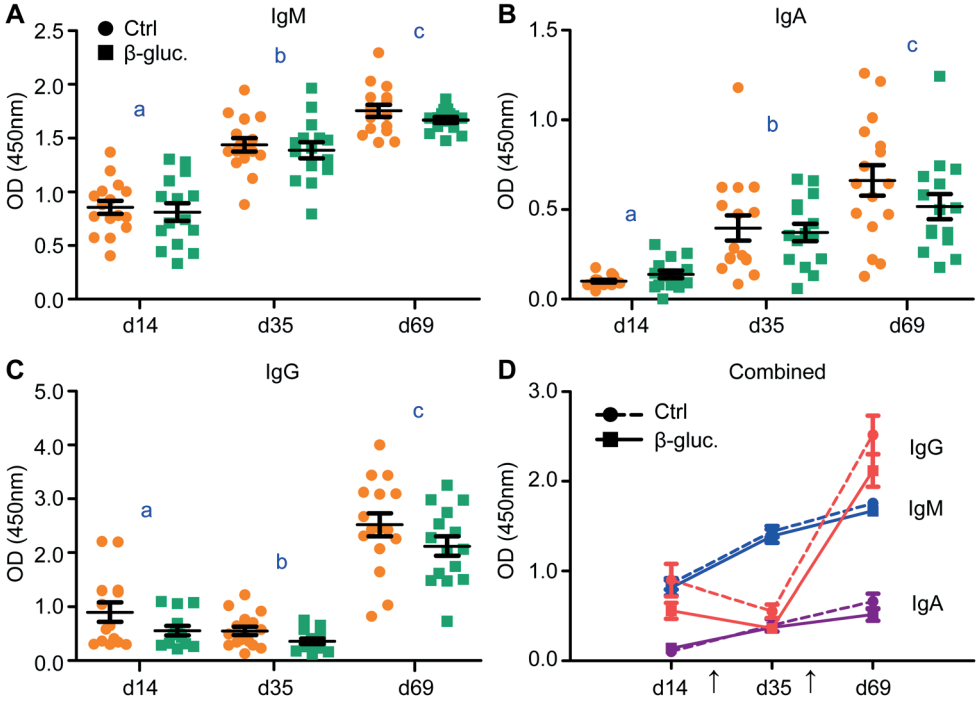


Figure 6. Levels of vaccine-specific IgM (**A**), IgA (**B**) and IgG (**C**) were measured in blood serum prior to vaccination (day 14), three weeks after vaccination (day 35) and 3 weeks after the booster vaccination (day 69). Figure 6**D** incorporates the results of Figure 6**A–C**. The arrows in plot 6**D** indicate the time of vaccine (Salmoporc®) administration (d21 and d45). From every pen, two control animals (circles; ●, dashed lines) or two β-glucan treated animals (squares; ■, solid lines) were randomly selected and followed over time (n = 16 per treatment group). Every symbol represents a single observation. No significant differences were observed between the treatment groups. Letters (a, b, c) represent significant differences ($p < 0.05$) between time points.

Ex Vivo Stimulation of MLN Cells and PBMCs

Figure 7 shows the production of the anti-inflammatory cytokine IL-10 by MLN cells and the production of the pro-inflammatory cytokine TNFα by PBMCs, upon stimulation with LPS or Con-A. The cytokines were measured at three different time points (one pre- and two post-weaning) during the study. Significant differences were only observed pre-weaning. On day 27, MLN cells from β-glucan treated animals produced higher levels of IL-10 upon stimulation with 10 µg/mL LPS (p = 0.008; T-test) in comparison to LPS stimulated MLN cells from non-treated animals (Figure 7A). Using a lower concentration of LPS (1 µg/mL)

showed the same pattern but resulted in a lower level of IL-10 production (Figure S7). In accordance with these results, Con-A stimulated MLN cells from β-glucan treated animals showed an increased IL-10 production on day 27 (Figure 7C), albeit not significant (p = 0.09; T-test). No differences in IL-10 production were observed for stimulated PBMCs (Figure S8). Interestingly, MLN cells from β-glucan treated animals also produced more IL-10 when left unstimulated (medium only) compared to unstimulated MLN cells from the control animals (p = 0.057; T-test). No such differences were observed for the post-weaning time points (day 44 and 70, respectively) and the variation between individual animals increased in the post-weaning phase. No differences in TNFα production by MLN cells were observed between the treatment groups (Figure S8), but stimulation of PBMCs obtained at day 27 from β-glucan treated animals showed a decreased TNFα production upon stimulation with Con-A (p = 0.024; T-test) and LPS (not significant) (Figure 7B,D). Furthermore, IL-10 production changed over time as observed for Con-A stimulated MLN cells (Figure 7C), independent of the different treatment groups. As for LPS stimulated PBMCs (Figure 7C), a significant increase of TNFα was only observed for β-glucan treated animals (time-treatment interaction).

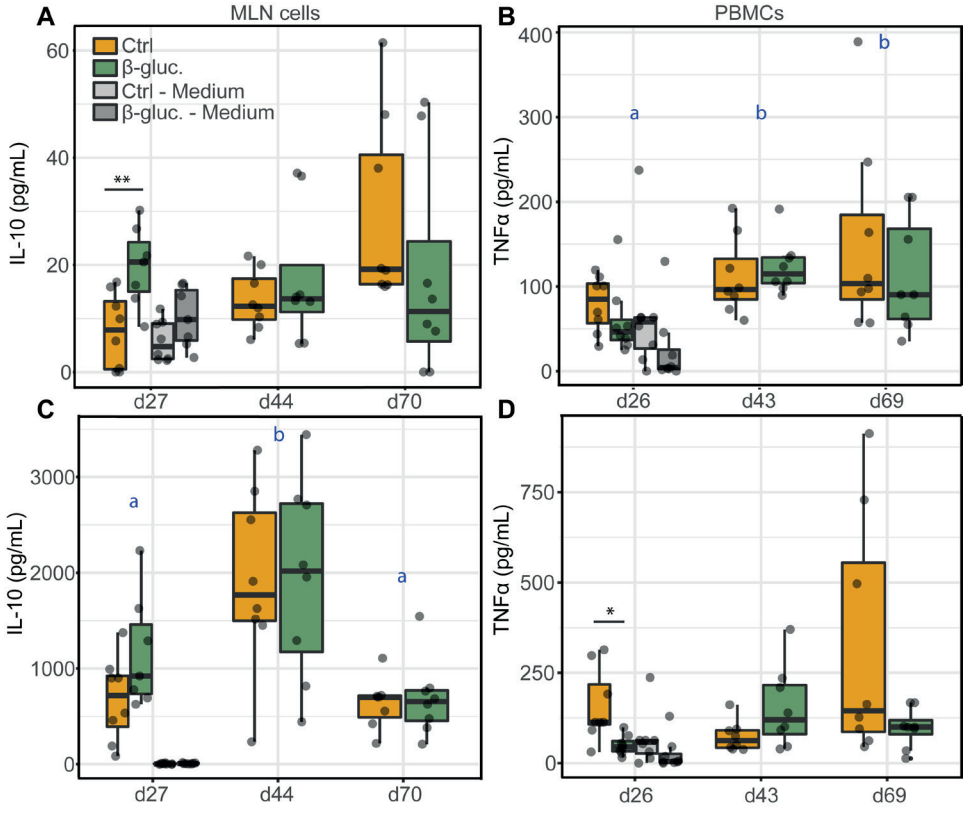


Figure 7. Levels of cytokines IL-10 (**A**,**C**) and TNFα (**B**,**D**) from stimulated MLN cells and PBMCs. MLN cells and PBMCs were stimulated with 10 µg/mL LPS (**A**,**B**) or 5 µg/mL Con-A (**C**,**D**), or left unstimulated (cell culture medium only) for 24 h. Significant differences between the treatment groups are indicated by asterisks (**; p < 0.01 and *; p < 0.05). Letters (a, b) represent significant differences (p < 0.05) between time points. Every dot represents a single animal from a different pen (n = 7 or 8 per treatment group) and error bars represent standard deviations. Statistical analysis was performed for every time point (T-test) and over time (Two-way ANOVA). Data were checked for normal distribution and equal variances and log-transformed when required.

Analysis of Immune Cells in Blood and the MLN

Cell analyses of PBMCs and MLN cells were used to investigate the potential effects of β-glucans on the abundance (percentage of cells) or function (maturation or proliferation) of different immune cell populations, including DCs, NK cells and T cells (Table 2: PBMC, Table 3: MLN). No significant differences were observed for the DC subsets in PBMCs at any of the time points. However, a decreased percentage of proliferating NK cells (p = 0.009; T-test, p = 0.010; Two-way ANOVA) and γδ T cells (p = 0.049; T-test, p = 0.034; Two-way ANOVA) was observed for β-glucan treated animals on day 26 (Figure 8A,D). As for MLN cells, the same immune cell populations were analysed, with the exception of NK cells. On day 44, a higher percentage of cDC1 (p = 0.011; T-test) and cytotoxic (CTL) cells (p = 0.035; T-test) was observed in β-glucan treated animals. In contrast, a lower expression of the cell maturation marker CD80/86 was observed for pDCs on day 27 (p = 0.024; T-test) and 70 (p = 0.034; T-test). Interestingly, correlation analysis showed that the *Romboutsia* genus positively correlates with the expression of the cell maturation marker CD80/86 on cDC1 cells (Figure S9). Furthermore, a significant time effect was observed for almost all analysed cell populations (Tables 2 and 3), generally with clear differences between day 14 and day 26 (pre-weaning) and between the pre- and post-weaning time points.

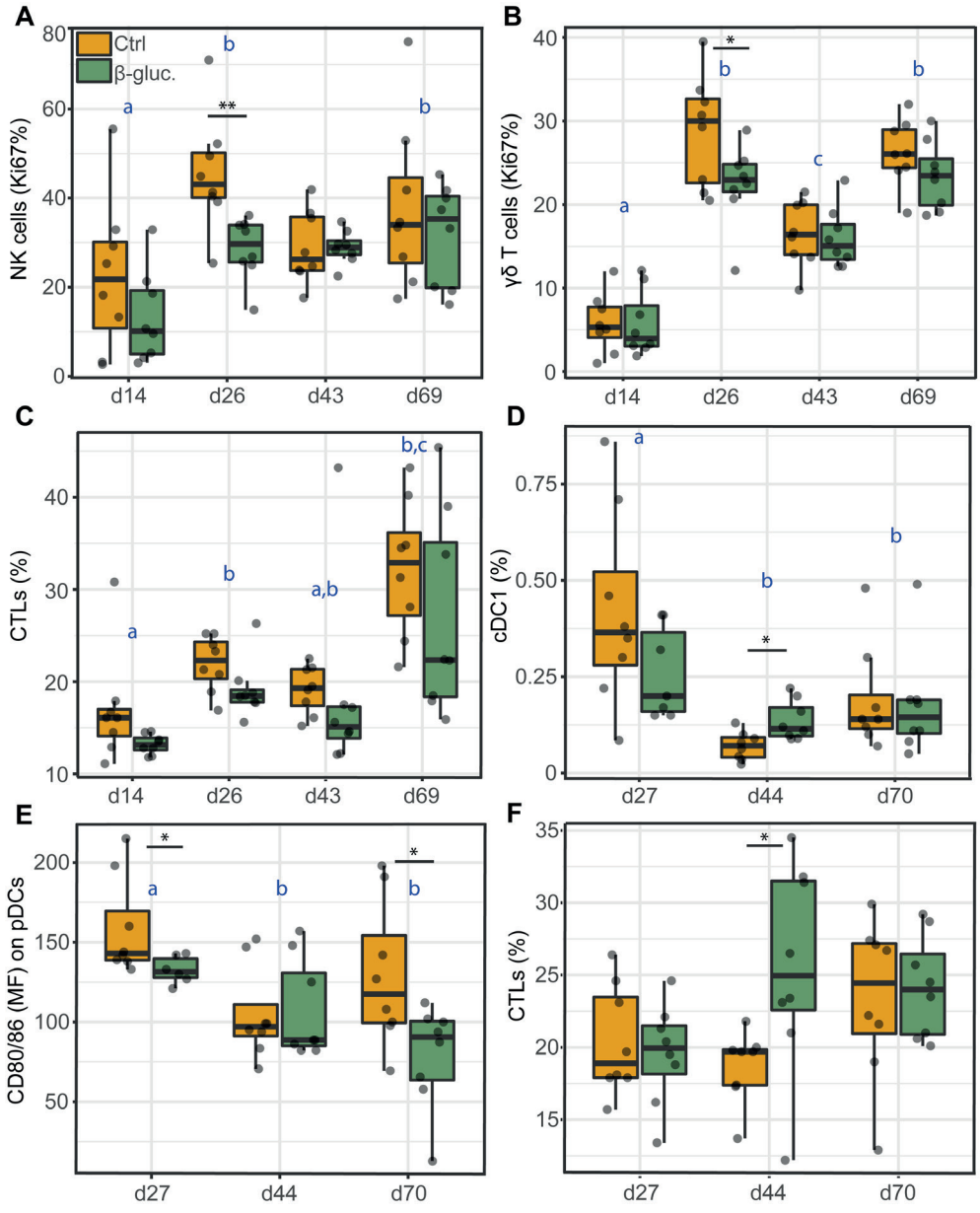


Figure 8. Cell analysis of different immune cell populations from PBMCs (**A**–**C**) and MLN cells (**D**–**F**). Significant differences between treatments are indicated by asterisks (**; $p < 0.01$ and *; $p < 0.05$). Letters (a, b, c) represent significant differences ($p < 0.05$) between time points. Data were presented as the percentage of proliferating cells (Ki67%; **A**,**B**), percentage of cells (%; **C**,**D**,**F**), or mean fluorescent intensity (MFI; **E**). Every dot represents a single animal from a different pen ($n = 7$ or 8 per treatment group) and error bars represent standard deviations. Statistical analysis was performed for every time point (T-test) and over time (Two-way ANOVA). All data were checked for normal distribution and equal variances and log-transformed when required.

Table 2. Cell analysis of DCs, NK cells and T cells in PBMCs between treatment groups over time.

Subset	Phenotype	Unpaired T-Test				Two-Way ANOVA
		D14	D26	D43	D69	
		p-value [CI]	*p*-value [CI]	*p*-value [CI]	*p*-value [CI]	
pDCs (%)	$CD14^-CD4^+CD172\ a^+CADM1^-$	ns	ns	ns	$0.060^{\downarrow}$ [−0.66 to 34.14]	T (<0.001), I (0.020)
pDCs (mat.)	$CD14^-CD4^+CD172\ a^+CADM1^-CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
cDC1 (%)	$CD14^-CD4^-CD172\ a^{low}CADM1^+$	ns	ns	ns	ns	T (<0.001)
cDC1 (mat.)	$CD14^-CD4^-CD172\ a^{low}CADM1^+\ CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
cDC2 (%)	$CD14^-CD4^-CD172\ a^+CADM1^+$	ns	ns	ns	ns	T (0.092)
cDC2 (mat.)	$CD14^-CD4^-CD172\ a^+CADM1^+\ CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
NK cells (%)	$CD3^-CD8^{\alpha}+$	ns	$0.060^{\downarrow}$ [−0.41 to 15.68]	ns	ns	G (0.080), T (0.063)
NK cells (act.)	$CD3^-CD8\alpha^+CD25^+$	ns	ns	ns	ns	T (<0.001)
NK cells (Ki67%)	$CD3^-CD8\alpha^+Ki67^+$	ns	$0.009^{\downarrow}$ [5.15 to 28.53]	ns	ns	G (0.010), T (<0.001)
γδ T cells (%)	$CD3^+TCR\text{-}\gamma\delta^+$	ns	ns	ns	ns	T (0.005)
γδ T cells (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^+Ki67^+$	ns	$0.049^{\downarrow}$ [0.02 to 12.75]	ns	ns	G (0.034), T (<0.001)
CTLs (%)	$CD3^+TCR\text{-}\gamma\delta^-CD8\alpha^+$	ns	$0.090^{\downarrow}$ [−0.53 to 6.13]	ns	ns	G (0.008), T (<0.001)
CTLs (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^-CD8\alpha^+Ki67^+$	ns	ns	ns	ns	T (<0.001)
T helper (%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+$	ns	ns	ns	ns	T (<0.001)
T helper (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+Ki67^+$	ns	ns	ns	ns	T (<0.001)
Mem./Act. (%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+CD8\alpha^+$	ns	ns	ns	ns	T (<0.001)
Mem./Act. (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+CD8\alpha^+ki67^+$	ns	ns	ns	ns	T (<0.001)
Tregs (%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+CD25^{high}Foxp^3+$	ns	ns	ns	ns	T (0.003)
Tregs (Ki67%)	$CD3^+\ TCR\text{-}\gamma\delta^-CD4^+CD25^{high}Foxp3^+Ki67^+$	ns	ns	ns	ns	T (<0.001)

Ns; not significant, $0.05 < p < 0.1$; trend, $p < 0.05$; significant. An unpaired Student's T-test and a Two-way ANOVA were conducted to analyse the group effect per time point, and the effects over time, respectively. G; group effect, T; time effect, I; group-time interaction, act.; activation, mat.; maturation, Mem./Act.; Memory/Activated T cells. Arrows indicate if the values (e.g., percentage of cells) in the treatment group (β-glucan, $n = 7$ or 8) are significantly higher ($^{\uparrow}$) or lower ($^{\downarrow}$) than the control group ($n = 7$ or 8). The 95% confidence intervals (CI) on the difference between the means are shown for all significant results or results which show a trend.

Table 2. Cell analysis of DCs, NK cells and T cells in PBMCs between treatment groups over time.

Subset	Phenotype	Unpaired T-Test				Two-Way ANOVA
		D14	D26	D43	D69	
		p-value [CI]	*p*-value [CI]	*p*-value [CI]	*p*-value [CI]	
pDCs (%)	$CD14^-CD4^+CD172\ a^+CADM1^-$	ns	ns	ns	$0.060^{\downarrow}$ [−0.66 to 34.14]	T (<0.001), I (0.020)
pDCs (mat.)	$CD14^-CD4^+CD172\ a^+CADM1^-CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
cDC1 (%)	$CD14^-CD4^-CD172\ a^{low}CADM1^+$	ns	ns	ns	ns	T (<0.001)
cDC1 (mat.)	$CD14^-CD4^-CD172\ a^{low}CADM1^+\ CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
cDC2 (%)	$CD14^-CD4^-CD172\ a^+CADM1^+$	ns	ns	ns	ns	T (0.092)
cDC2 (mat.)	$CD14^-CD4^-CD172\ a^+CADM1^+\ CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
NK cells (%)	$CD3^-CD8^{\alpha}+$	ns	$0.060^{\downarrow}$ [−0.41 to 15.68]	ns	ns	G (0.080), T (0.063)
NK cells (act.)	$CD3^-CD8\alpha^+CD25^+$	ns	ns	ns	ns	T (<0.001)
NK cells (Ki67%)	$CD3^-CD8\alpha^+Ki67^+$	ns	$0.009^{\downarrow}$ [5.15 to 28.53]	ns	ns	G (0.010), T (<0.001)
γδ T cells (%)	$CD3^+TCR\text{-}\gamma\delta^+$	ns	ns	ns	ns	T (0.005)
γδ T cells (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^+Ki67^+$	ns	$0.049^{\downarrow}$ [0.02 to 12.75]	ns	ns	G (0.034), T (<0.001)
CTLs (%)	$CD3^+TCR\text{-}\gamma\delta^-CD8\alpha^+$	ns	$0.090^{\downarrow}$ [−0.53 to 6.13]	ns	ns	G (0.008), T (<0.001)
CTLs (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^-CD8\alpha^+Ki67^+$	ns	ns	ns	ns	T (<0.001)
T helper (%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+$	ns	ns	ns	ns	T (<0.001)
T helper (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+Ki67^+$	ns	ns	ns	ns	T (<0.001)
Mem./Act. (%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+CD8\alpha^+$	ns	ns	ns	ns	T (<0.001)
Mem./Act. (Ki67%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+CD8\alpha^+ki67^+$	ns	ns	ns	ns	T (<0.001)
Tregs (%)	$CD3^+TCR\text{-}\gamma\delta^-CD4^+CD25^{high}Foxp^3+$	ns	ns	ns	ns	T (0.003)
Tregs (Ki67%)	$CD3^+\ TCR\text{-}\gamma\delta^-CD4^+CD25^{high}Foxp3^+Ki67^+$	ns	ns	ns	ns	T (<0.001)

Ns; not significant, $0.05 < p < 0.1$; trend, $p < 0.05$; significant. An unpaired Student's T-test and a Two-way ANOVA were conducted to analyse the group effect per time point, and the effects over time, respectively. G; group effect, T; time effect, I; group-time interaction, act.; activation, mat.; maturation, Mem./Act.; Memory/Activated T cells. Arrows indicate if the values (e.g., percentage of cells) in the treatment group (β-glucan, $n = 7$ or 8) are significantly higher ($^{\uparrow}$) or lower ($^{\downarrow}$) than the control group ($n = 7$ or 8). The 95% confidence intervals (CI) on the difference between the means are shown for all significant results or results which show a trend.

Table 3. Cell analysis of DCs, NK cells and T cells in MLN cells between treatment groups over time.

Subset	Phenotype	Unpaired T-Test			Two-Way ANOVA
		D27	D44	D70	
		p-value [CI]	*p*-value [CI]	*p*-value [CI]	
pDCs (%)	$CD14^{-}CD4^{+}CD172\ a^{+}CADM1^{-}$	ns	ns	ns	T (<0.001)
pDCs (mat.)	$CD14^{-}CD4^{+}CD172\ a^{+}CADM1^{-}CTLA4\text{-}Ig^{+}$	0.034 [3.21 to 67.89]↓	ns	0.024 [7.75 to 92.70]↓	G (0.007), T (0.001), I (0.08)
cDC1 (%)	$CD14^{-}CD4^{-}CD172\ a^{low}CADM1^{+}$	ns	0.011 [−0.11 to −0.02]↑	ns	T (<0.001), I (0.028)
cDC1 (mat.)	$CD14^{-}CD4^{-}CD172\ a^{low}CADM1^{+}CTLA4\text{-}Ig^{+}$	ns	ns	ns	T (<0.001)
cDC2 (%)	$CD14^{-}CD4^{-}CD172\ a^{+}CADM1^{+}$	ns	ns	ns	T (0.001)
cDC2 (mat.)	$CD14^{-}CD4^{-}CD172\ a^{+}CADM1^{+}CTLA4\text{-}Ig^{+}$	0.084 [−62.71 to 4.50]↑	ns	ns	ns
NK cells (%)	$CD3^{-}CD8\ a^{+}$	n/a	n/a	n/a	n/a
NK cells (act.)	$CD3^{-}CD8\alpha^{+}CD25^{+}$	n/a	n/a	n/a	n/a
NK cells (Ki67%)	$CD3^{-}CD8\alpha^{+}Ki67^{+}$	n/a	n/a	n/a	n/a
γδ T cells (%)	$CD3^{+}TCR\text{-}\gamma^{\delta}+$	ns	ns	ns	T (<0.001)
γδ T cells (Ki67%)	$CD3^{+}TCR\text{-}\gamma\delta^{+}Ki67^{+}$	0.089 [−15.45 to 1.26]↑	ns	ns	T (<0.001), I (0.086)
CTLs (%)	$CD3^{+}TCR\text{-}\gamma\delta^{-}CD8\alpha^{+}$	ns	0.035 [−13.03 to −0.60]↑	ns	T (0.076)
CTLs (Ki67%)	$CD3^{+}TCR\text{-}\gamma\delta^{-}CD8\alpha^{+}Ki67^{+}$	ns	ns	ns	T (<0.001)
T helper (%)	$CD3^{+}TCR\text{-}\gamma\delta^{-}CD4^{+}$	ns	ns	ns	T (<0.001)
T helper (Ki67%)	$CD3^{+}TCR\text{-}\gamma\delta^{-}CD4^{+}Ki67^{+}$	0.079 [−12.53 to 0.77]↑	ns	ns	T (<0.001), I (0.019)
Mem./Act. (%)	$CD3^{+}CD4^{+}CD8\alpha^{+}$	ns	ns	ns	T (<0.001)
	$CD3^{+}CD4^{high}CD8\alpha^{low}$	ns	ns	ns	T (0.002)
	$CD3^{+}CD4^{high}\ CD8\alpha^{low}ki67^{+}$	ns	ns	ns	T (<0.001)
	$CD3^{+}CD4^{low}CD8\alpha^{high}$	ns	ns	ns	ns
	$CD3^{+}CD4^{low}\ CD8\alpha^{high}ki67^{+}$	ns	ns	ns	T (<0.001)
Tregs (%)	$CD3^{+}TCR\text{-}\gamma\delta^{-}CD4^{+}CD25^{high}Foxp3^{+}$	ns	ns	ns	T (<0.001)
Tregs (Ki67%)	$CD3^{+}\ TCR\text{-}\gamma\delta^{-}CD4^{+}CD25^{high}Foxp3^{+}Ki67^{+}$	n/a	n/a	n/a	n/a

Ns; not significant, $0.05 < p < 0.1$; trend, $p < 0.05$; significant. An unpaired Student's T-test and a Two-way ANOVA were conducted to analyse the group effect per time point, and the effects over time, respectively. G; group effect, T; time effect, I; group-time interaction, act.; activation, mat.; maturation, Mem./Act.; Memory/Activated T cells. Arrows indicate if the values (e.g., percentage of cells) in the treatment group (β-glucan, $n = 7$ or 8) are significantly higher (↑) or lower (↓) than the control group ($n = 7$ or 8). The 95% confidence intervals (CI) on the difference between the means are shown for all significant results or results which show a trend.

Discussion

The microbiota plays an important role in in the development of the innate and adaptive immune system (Fung, Olson et al. 2017). However, the interplay between diet, the gut microbiota, and the immune system is still poorly understood, especially in early life. In this study, we investigated the impact of yeast-derived β-glucans on the porcine gut microbiota and the immune system from birth to six weeks post-weaning. We hypothesized that early-life supplementation of yeast-derived β-glucans (MacroGard®) alters gut microbial development and potentiates immunological responses in pigs. Overall, supplementation of yeast-derived β-glucans had no profound effect on the development of the gut microbiota and the immune system, and any observed differences were modest. However, clear time effects were observed in the gut microbiota and the immune system between the pre- and post-weaning time points, which gives valuable information regarding the development of these complex systems. Importantly, no adverse effects of orally administered β-glucans were observed, and there were no indications that β-glucans had a negative effect on physiological parameters (e.g., body weight and pH), gut microbiota, or the immune system in early life.

Yeast-derived β-glucans are being used as functional ingredients in pig nutrition to boost gut health. Although β-glucans have been shown to impact the immune system by binding to specific host receptors, β-glucans can also be considered as dietary fibres. Dietary fibres can potentially be used to impact gut microbiota composition by stimulating the growth of saccharolytic or fibrolytic microorganisms (Zhang, Mu et al. 2016). Similarly, β-glucans may, depending on solubility, escape digestibility in the upper GI tract and may arrive in the lower GI tract where they are broken down by saccharolytic bacteria into short-chain fatty acids (SCFAs) (Kedia, Vázquez et al. 2008). A meta-analysis on the impact of the inclusion of cereal-derived β-glucans in feed has indeed shown a positive correlation with the presence of the SCFA butyrate in the colon (Metzler-Zebeli, Zijlstra et al. 2011). In the present study, three genera were found to be differentially abundant over time in faeces during the pre-weaning phase when given yeast-derived β-glucans as dietary intervention, including *Methanobrevibacter, Fusobacterium,* and a genus within the family of *Ruminococcaceae.* Interestingly, in a porcine study in which dietary fibres were administered during the pre-weaning period (day 14 until weaning), effects were seen on the relative abundance of several genera in the mid-colon, amongst which two genera in the *Ruminococcaceae* family (Van Hees, Davids et al. 2019), highlighting the potential of microbiota modulation by dietary fibres. In a study where the effects of three purified β-glucans were tested on members of the GI tract, all three were shown to reduce the *Enterobacteriaceae* population (Sweeney, Collins et al. 2012). In the current study, this shift in *Enterobacteriaceae* was, however, not observed.

Besides the differentially abundant genera, minor but significant differences in alpha and beta diversity were found in faeces between treatment groups in the pre-weaning period. These differences were not found in digesta from jejunum, ileum or caecum, which might be explained by the smaller sample size. As expected, a clear temporal pattern was observed for both treatment groups. This includes a clear separation between pre- and post-weaning time points as demonstrated before in similar studies (Frese, Parker et al. 2015, Chen, Xu

et al. 2017, Wang, Tsai et al. 2019). The alpha diversity increased in the early pre-weaning phase (day 4) up to the first post-weaning time point (day 35), after which a plateau was reached. This is in line with previous studies (Chen, Xu et al. 2017, Wang, Tsai et al. 2019). In another study, alpha diversity reached a plateau from day 24 onwards (Arfken, Frey et al. 2020). This outcome likely has to do with the earlier time of weaning (day 21), demonstrating the dominant role that the weaning transition has on gut microbiota maturation. The GI tract's microbiota composition found in the present study is comparable to that observed in other studies in terms of main phyla in faeces and caecum, both pre- and post-weaning (Kim, Borewicz et al. 2011, Looft, Allen et al. 2014, Gresse, Chaucheyras Durand et al. 2019). In addition, main phyla found in the jejunum and ileum post-weaning were comparable to the results from another study (Yang, Huang et al. 2016). In addition, a study by Kim et al. found that the *Prevotellaceae* family is predominant at ten weeks of age (Kim, Borewicz et al. 2011), which was also observed in our study. Moreover, the temporal pattern of the composition and relative abundance of archaeal taxa is comparable with data from multiple studies (Gresse, Chaucheyras Durand et al. 2019, Mi, Peng et al. 2019).

Both the composition and development of the microbiota as well as the innate and adaptive immune maturation are influenced by exposure to commensal microorganisms and dietary antigens (Everaert, Van Cruchten et al. 2017). Dietary β-glucans are recognized by pathogen recognition receptors, the most important one being Dectin-1 (Taylor, Tsoni et al. 2007). Sonck et al. demonstrated that Dectin-1 is expressed in the spleen, MLN, lungs, and along the digestive tract of pigs (Sonck, Stuyven et al. 2009). Immune cells, including DCs, are also known to express Dectin-1 in human and mice, but this has not yet been shown for porcine immune cells (Ariizumi, Shen et al. 2000, Hernanz-Falcon, Arce et al. 2001). Recognition of β-glucans by immune cells may induce altered immune responses upon a secondary stimulation, thereby influencing the immune competence of the pig. To test this, we assessed if pigs treated with β-glucan showed an enhanced immune response to a live attenuated *Salmonella* vaccine (Salmoporc®). A clear vaccine-induced increase in *Salmonella* specific antibodies was observed, but no differences were found between the control and β-glucan group. This could imply that the vaccination induced an immunological response that was too strong to determine the subtle effects of this dietary intervention on the systemic immune competence, or that yeast-derived β-glucans do not enhance the systemic immune system. In line with these results, we did not see major differences in the percentage and proliferation of T-cell subsets in both blood and the MLN. These results are in accordance with a previous study that investigated the effect of (purified) yeast-derived (1,3/1,6)-β-d-Glucan (Wellmune WGP®) on the vaccination response and T-cell subsets in neonatal piglets. The authors did not find any effect of this dietary supplement on intestinal or immune development and it did not improve the antibody response to vaccination in neonatal piglets (Hester, Comstock et al. 2012).

Interestingly, when we stimulated PBMCs from β-glucan treated animals ex vivo with LPS or Con-A, we observed a reduction in the production of the pro-inflammatory cytokine TNFα on day 26. In line with these findings, ex vivo stimulated MLN cells from β-glucan treated animals produced higher levels of the anti-inflammatory cytokine IL-10 on day 27. These results suggest that yeast-derived β-glucans induce a more anti-inflammatory or tolerant state of the systemic immune system. This is also suggested by the reduced percentages

of CTLs and proliferating NK cells and γδ T cells in PBMCs from β-glucan treated animals on day 26. The observed reduction of TNFα in β-glucan treated animals has been observed in other porcine in vivo studies. Vetvicka et al. showed a significant reduction of TNFα in blood serum after an endotoxin challenge with LPS (Vetvicka and Oliveira 2014). In addition, Li et al. observed a decrease of TNFα production by blood lymphocytes from β-glucan treated pigs which were stimulated ex vivo with LPS (Li, Xing et al. 2005). Taken together, these findings suggest that β-glucans may alter immune cell responses upon a secondary stimulus as was observed in a study by dos Santos et al. (Dos Santos, de Figueiredo et al. 2019), in which they showed a significant increase in IL-10 production after 2 and 4 h of infection with *L. braziliensis* in β-glucan trained macrophages. The modest effects of yeast-derived β-glucans on the immune system were primarily observed in the pre-weaning phase, which is in line with the findings from the microbiota analysis (Figures 2 and 3). A more complex diet after weaning may impair the ability to identify any subtle changes induced by yeast-derived β-glucans in the post-weaning period.

Most studies suggest that yeast-derived β-glucans improve the overall health of pigs. However, strong and consistent evidence is lacking, and no studies have investigated its impact on both the gut microbiota and the immune system. The present study shows that yeast-derived β-glucans primarily induce effects in the gut microbiota and immune system early in life. During the pre-weaning period, the diversity of the faecal microbiota was lower in animals that received β-glucans. This is unexpected, as fibrous components typically result in increased microbial diversity (Makki, Deehan et al. 2018). As previously discussed, three genera were found to be differentially abundant in the same period, including *Methanobrevibacter*, *Fusobacterium,* and a genus within the *Ruminococcaceae* family. At the genus level, it is hard to state whether these are positive effects, as a species within these genera can either act in a commensal or beneficial fashion, or can have adverse effects for the host. Interestingly, the genus *Romboutsia* showed a positive correlation with the expression of the cell maturation marker CD80/86 on cDC1 cells (Figure S9). This may imply that *Romboutsia* contributes to the maturation of the (innate) immune system. Several *Romboutsia* spp. are commensals of the mammalian GI tract and have been shown to be flexible anaerobes that are adapted to the small intestine (Gerritsen 2015, Gerritsen, Hornung et al. 2017). As for the immune system, the increased production of the anti-inflammatory cytokine IL-10 by MLN cells, and the decreased production of TNFα by PBMCs, suggest an anti-inflammatory effect. However, it should be noted that only a small number of animals were sampled and that the level of LPS induced IL-10 produced by MLN cells was relatively low (0–61 pg/mL). Nevertheless, these results are in line with other in vivo studies that showed limited or no effects of yeast-derived β-glucans on the (mucosal) immune system or vaccination response (Hahn, Lohakare et al. 2006, Stuyven, Van Den Broeck et al. 2010, Hester, Comstock et al. 2012, Pence, Hester et al. 2012).

Taken together, this study reinforces that the pre-weaning period constitutes a window of opportunity to alter the immune system through diet, which may set the stage for immune homeostasis and subsequent host–microbial interaction. However, the observed changes induced by dietary β-glucans were modest, and any long-lasting health effects of yeast-derived β-glucans remain to be elucidated.

Acknowledgements

The authors would like to thank the Swine Research Centre staff for their technical assistance during the in vivo experiment. A Special thanks goes to Carlijn de Bruijn for her support regarding the logistics, organization and execution of the study. The authors also would like to thank Giacomo Antonello and Caifang Wen for their help with sample collection, processing and data analyses, Ben Meijer and Gael Auray for their help with flow cytometry analyses, and Gerben Hermes for his constructive recommendations on the statistical analyses.

Supplementary Materials

	Weaner Diet Day 25-44	**Nursery Diet** Day 45-70
Ingredient composition (%)		
Barley	25.00	30.00
Wheat	25.00	22.00
Corn	15.28	12.00
Soybean meal (48% crude protein)	10.00	9.90
Sweet whey powder	9.29	4.29
Soy protein concentrate [1]	4.30	4.00
Soya oil	3.20	3.31
Cane molasses (> 47.5% sugar)	1.00	1.00
Sucrose	1.50	1.50
Wheat bran	1.50	5.13
Sunflower seed meal (27% crude protein)	0	3.00
Sodium chloride	0.58	0.46
Sodium bicarbonate	0	0.29
Mono-calcium phosphate	0.29	0.03
Limestone (calcium carbonate)	0.47	0.56
Organic acids [2]	0.15	0.15
Phytase [3]	0.05	0.05
Vitamins and trace minerals [4]	1.05	1.12
Synthetic amino acids	1.34	1.21
Total	100.00	100.00
Calculated nutrients, g/kg		
Moisture	110	113
Crude protein	170	175
Crude fat	50	52
Crude fibre	28	40
Crude ash	47	47
Starch (Ewers method)	384	378
Total dietary fibre	141	165
Soluble dietary fibre	64	79
Insoluble dietary fibre	80	94
Lactose	65	30
Calcium	5.50	5.50
Phosphorus	4.50	4.36
Digestible Phosphorus	4.10	3.50
Na	3.00	3.00
Cu (total, mg)	165	167
Zn (total, mg)	124	129
Metabolic energy (MJ)	14.22	13.84
Net energy (MJ)	10.46	10.11
Standardized ileal digestible lysine	12.35	12.03

	Weaner Diet Day 25-44	**Nursery Diet** Day 45-70
Analysed nutrients, g/kg		
Moisture	101	97
Crude protein	170	176
Crude fibre	28	40
Crude fat	53	53
Crude ash	46	47
Zinc (mg/kg)	118	116

Table S1. Experimental diets fed during the experimental period. 1HP 300 (Hamlet protein, Horsens, Denmark); 2 Fylax Forte HC-SP (Trouw Nutrition Selko, Tilburg, The Netherlands) 3 Phyzyme XP 5000 TPT (Danisco Animal Nutrition, Marlbourough, UK) providing 600 FTU 6-phytase per kg feed; 4 Farmix (Trouw Nutrition, Putten, The Netherlands), provided per kg feed: 8000 IU vit A, 2000 IU vit D3, 100 (weaner) or 150 (nursery) IU vit E-acetate, 1.5 mg menadione, 1 mg thiamine mononitrate, 4 mg riboflavin, 1 mg pyridoxine, 30 µg cyanocobalamin, 20 mg niacin, 12 mg pantothenic acid, 300 µg folic acid, 150 mg choline chloride, 50 mg betain.

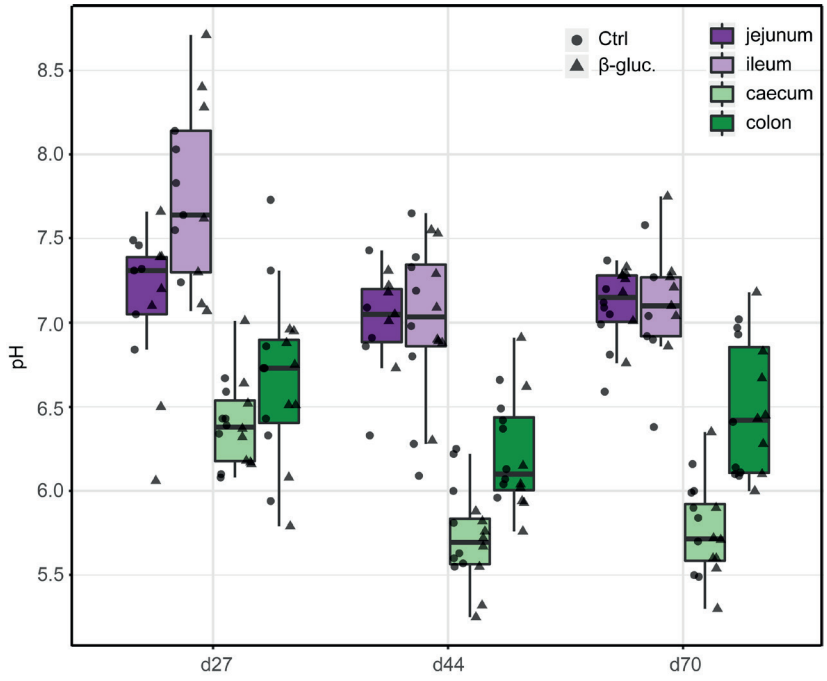


Figure S1. The pH of digesta from four different gut segments (jejunum, ileum, caecum and colon). The pH of digesta was measured on day 27, 44 and 70 of the study. Samples from control animals (circles; ●) and β-glucan treated animals (triangles; ▲) are presented in this figure (n = 8 per group).

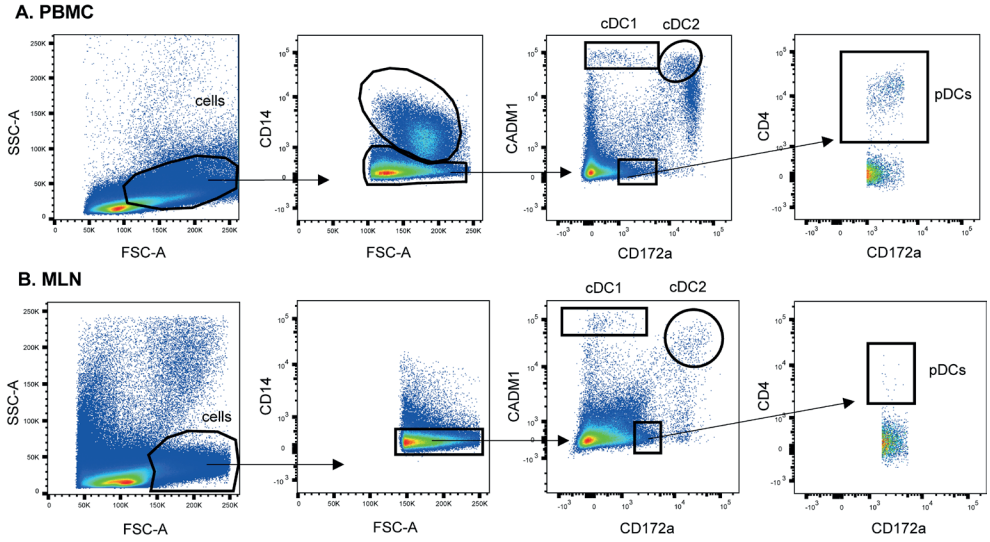


Figure S2. Gating strategy for the identification of DC subsets following five-color flow cytometry. Antibodies against CD14, CD172a, CADM1 and CD4 in were used to identify DC subsets in PBMCs (A) and MLN cells (B). After doublet discrimination and selection for viable cells, gates were set on cells with high forward and side scatter (large cells). DC subsets (CD14−), were defined as pDC (CD172a+CADM1-CD4+), cDC1 (CD172alowCADM1+CD4− cells) and cDC2 (CD172a+CADM1+CD4−).

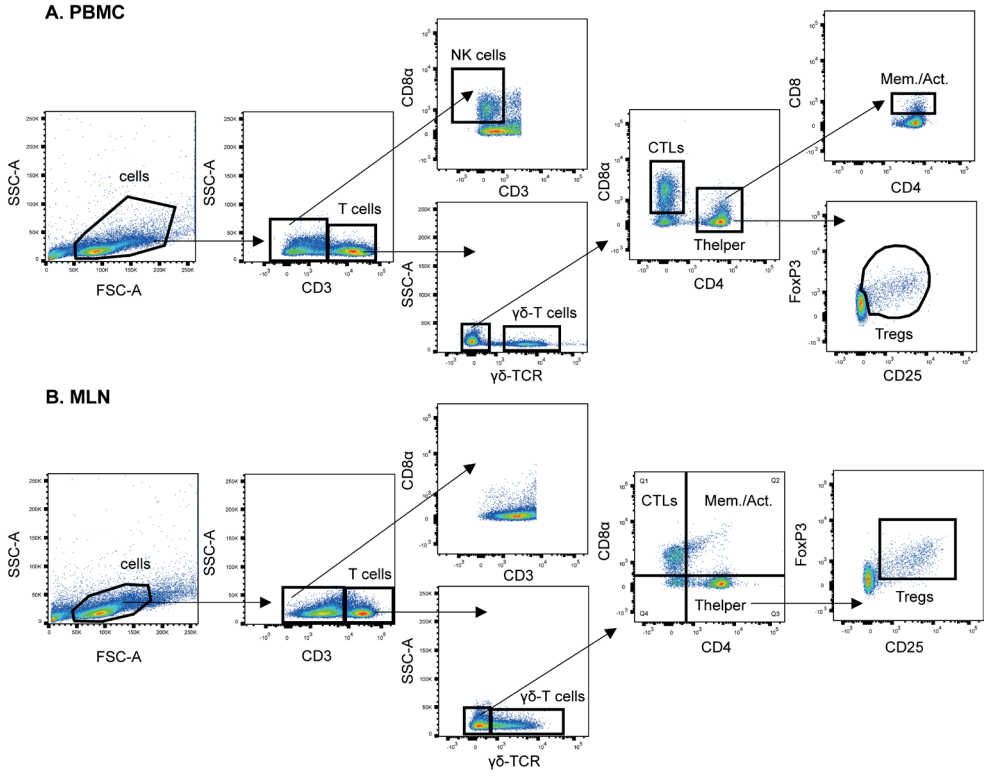


Figure S3. Gating strategy for the identification of T lymphocytes and NK cells following six-color flow cytometry. Antibodies against CD3, CD8α, TCR-γδ, CD4, FoxP3 and CD25 were used to identify different cell populations in PBMCs (A) and MLN cells (B). After doublet discrimination and selection for viable cells, gates were set on cells with medium/high forward and side scatter to select for lymphocytes and exclude debris. NK cells were defined as CD3-CD8α+, γδ T cells as CD3+TCR-γδ+, CTLs as CD3+TCR-γδ-CD8a+, T helper cells as CD3+TCR-γδ-CD4+, Memory/Activated (Mem./Act.) T cells as CD3+TCR-γδ-CD4+CD8a+ and T regulatory cells (Tregs) as CD3+TCR-γδ-CD4+CD25highFoxp3+.

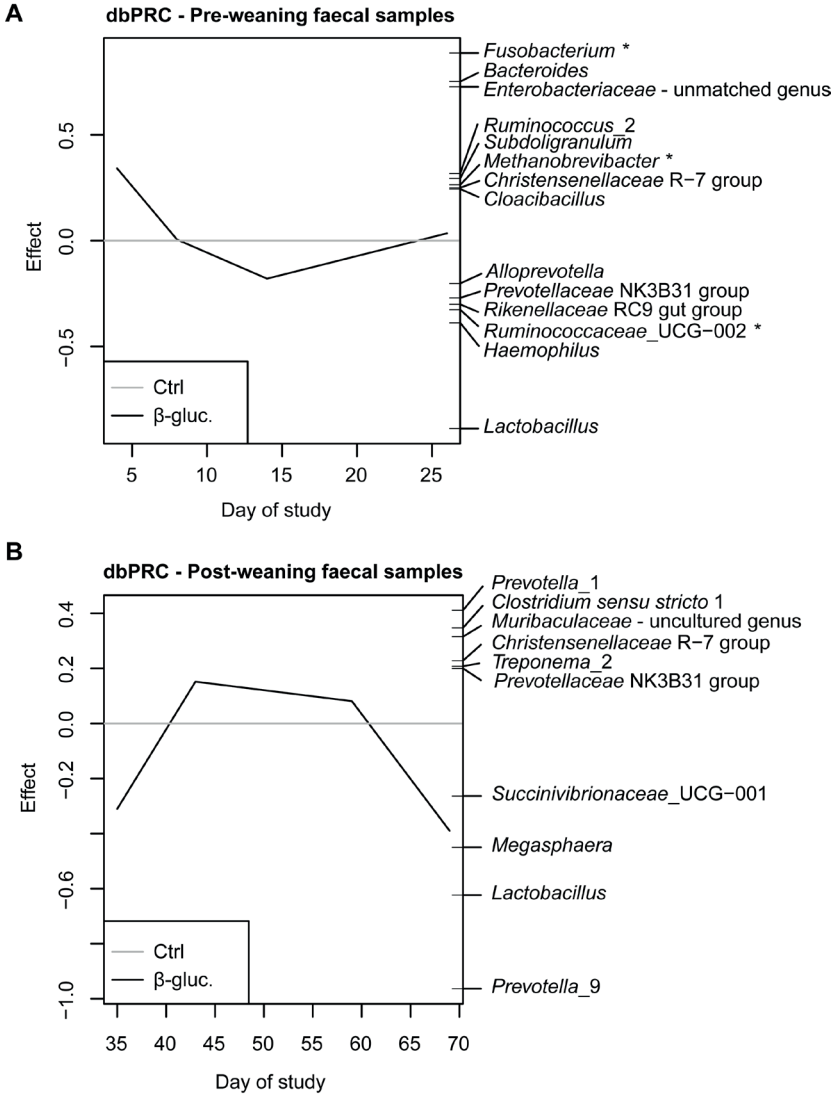


Figure S4. Weighted Unifrac distance-based Principal Response curves (dbPRC) of differences in pre-weaning (A) and post-weaning (B) faecal microbiota composition between β-glucan (black line) and control (reference baseline with zero PRC values) animals. The horizontal axis represents time, and the vertical axis represent PRC score values. Calculations were performed at the genus level, genera with a score lower than -0.2 or higher than 0.2 are shown on the right y-axis. Asterisks represent genera that were significantly differentially abundant.

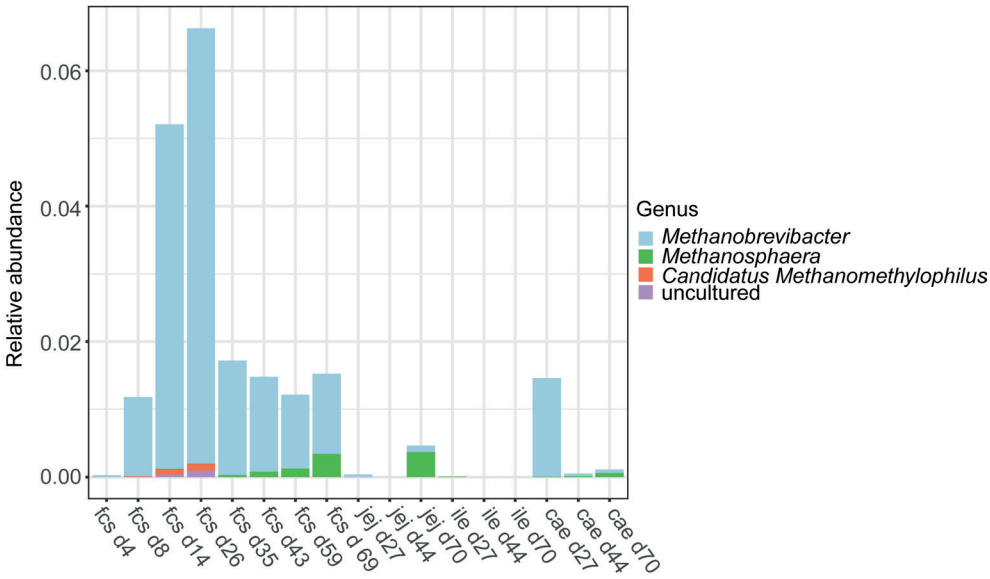


Figure S5. Taxonomic composition of Archaea within the piglet faeces and digesta over time. Data are given as the mean relative abundance at the genus level by sampling time point (d4-70) and by faeces (fcs) or gut segment; jejunum (jej), ileum (ile), caecum (cae). Data includes samples from both treatment groups.

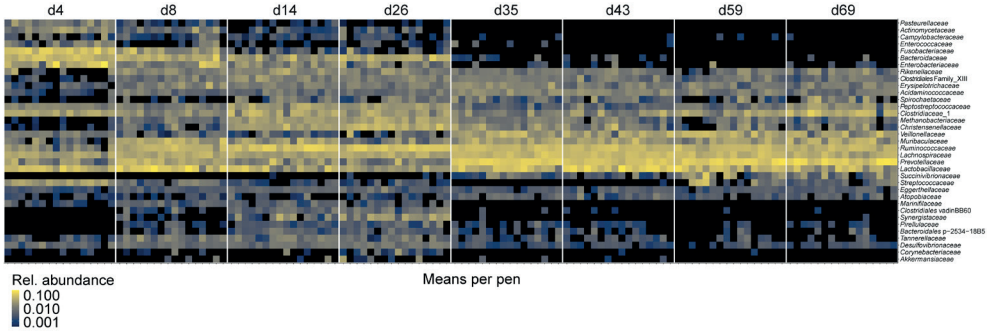


Figure S6. Heatmap of the relative abundance of the 35 most prevalent families in faecal samples over time. Each time point includes the means of 16 pens (8 control pens and 8 β-glucan pens).

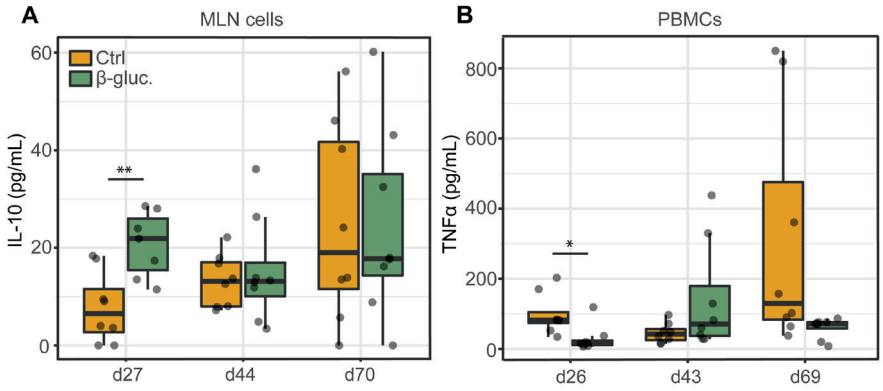


Figure S7. Levels of cytokines IL-10 (A) and TNFα (B) from LPS stimulated MLN cells and Con-A stimulated PBMCs, respectively. MLN cells and PBMCs were stimulated with 1 µg/mL of LPS or 2.5 µg/mL of Con-A for 24 h. Significant differences between treatments are indicated by asterisks (**; P<0.01 and *; P<0.05). Every dot represents a single animal (n = 7 or 8 per group) and error bars represent standard deviations. Statistical analysis was performed for every time point (T-test) and over time (Two-way ANOVA). Data were checked for normal distribution and equal variances and log-transformed when required.

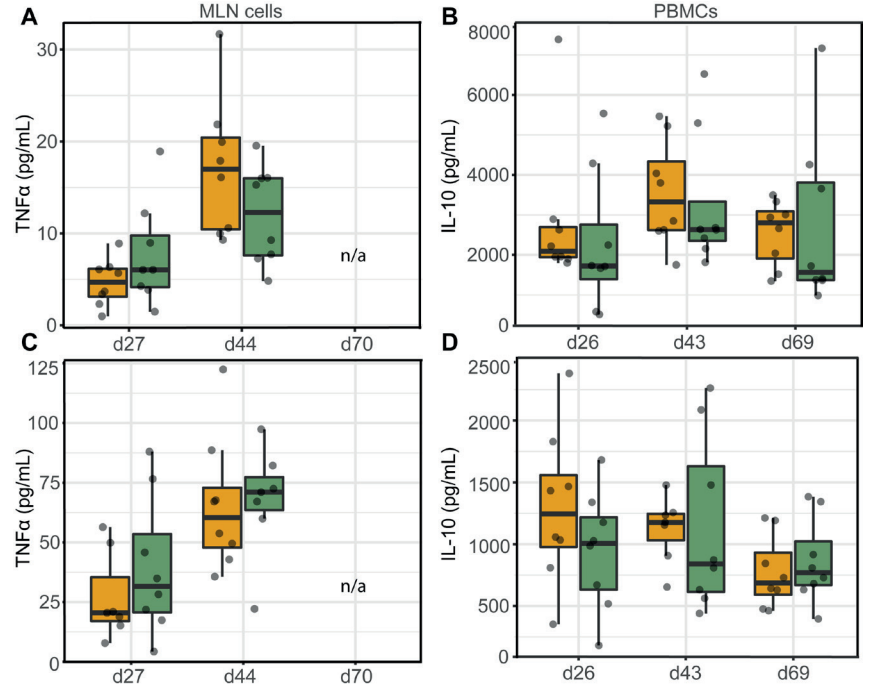


Figure S8. Levels of cytokines TNFα (A, C) and IL-10 (B, D) from stimulated MLN cells and PBMCs. MLN cells and PBMCs were stimulated with 10 µg/mL LPS (A, B) or 5 µg/mL Con-A (C, D) for 24 h. Every dot represents a single animal (n = 7 or 8 per group) and error bars represent standard deviations. Statistical analysis was performed for every time point (T-test) and over time (Two-way ANOVA). Data were checked for normal distribution and equal variances and log-transformed when required. No cytokine levels were detected on day 70 (MLN cells) as indicated by n/a.

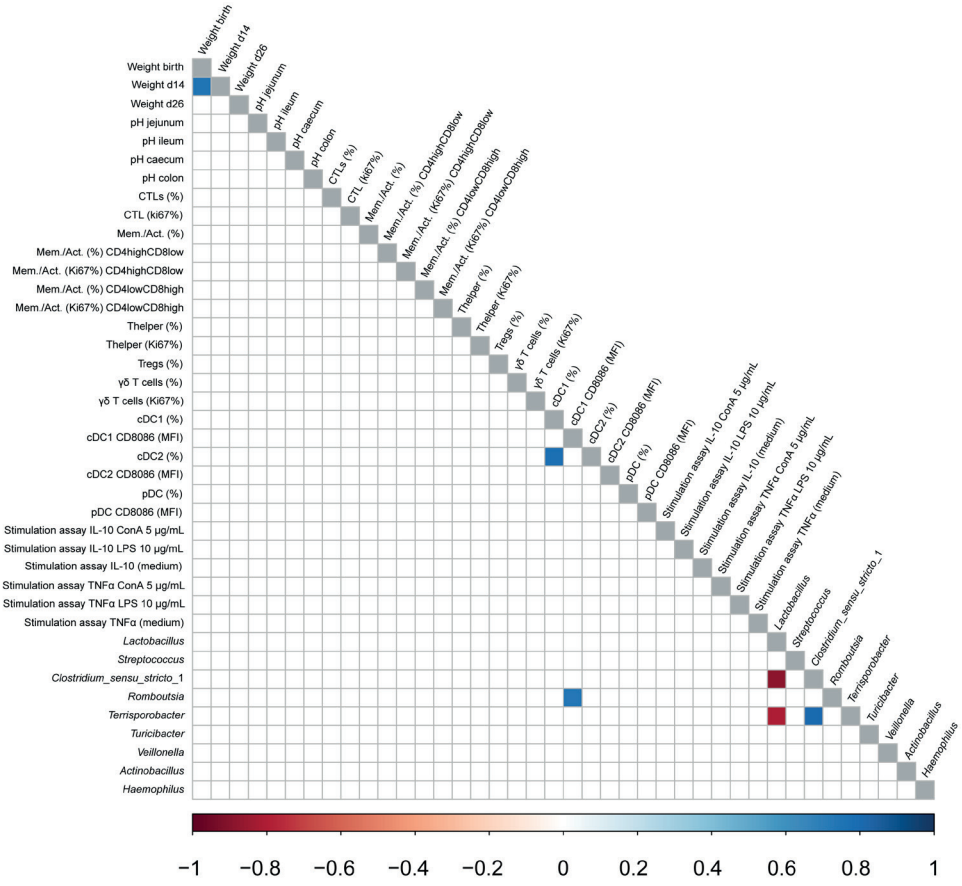


Figure S9. Correlation plot of several study parameters, including physiological parameters (weight and pH), immunological parameters (MLN cell analysis and stimulation assay) and ileal microbiota composition abundances (genus level). This figure only includes data from day 27 of the study. All correlations with an adjusted p-value below 0.05 are shown in the correlation plot. Color intensity is proportional to the correlation coefficient.


4

Chapter 4

Oral Administration of *E. coli* Nissle 1917 in Neonatal Pigs – Implications for Future Research

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Abstract

E. coli strain Nissle 1917 (EcN) has been used for over 100 years as a probiotic against a variety of intestinal disorders, but no studies have investigated its effects in neonatal piglets. In this pilot study, we have investigated the presence, abundance, and location of EcN in the porcine gastrointestinal tract, mesenteric lymph nodes, and faeces after oral administration of different concentrations of EcN. In addition, the pH of different gut segments was measured to determine the effect of EcN on the gut environment. Overall, no adverse health effects related to EcN were observed during the study. EcN was detected in faeces of all piglets that received EcN, and no major differences were observed between the piglets that received a high, medium, or low concentration of EcN (10^9, 10^8, or 10^7 CFU/mL, respectively). Albeit at a low abundance, EcN was also detected in one of the control animals. Therefore, measures to prevent cross-contamination are highly recommended when designing *in vivo* experiments that involve microbial products such as probiotics. Moreover, EcN was also detected in the mesenteric lymph node of a single animal that received the highest concentration of EcN, suggesting that EcN is able to (spontaneously) translocate to (secondary) lymphoid organs. In conclusion, EcN can be found in faeces, in all segments of the gastrointestinal tract, and in the mesenteric lymph node after oral administration of EcN. These results provide valuable insights into the behaviour of EcN and may contribute to a better comprehension of its effects on the gastrointestinal tract.

Keywords: *E. coli* Nissle 1917, porcine, microbiota, gastrointestinal tract, immune system

Introduction

Early life is a critical phase during which microbes colonize the neonatal piglets' gastrointestinal tract (GIT), settle down as different communities, evolve as a stable ecosystem, and ultimately compete for the most suitable niche (Baker, Davis et al. 2013). In addition, neonatal piglets are immunologically 'immature' and are highly dependent on passive immunity through uptake of immunoglobulins from colostrum (Bailey, Haverson et al. 2005, Stokes 2017). During the post-natal period, the piglet's microbiome and immune system have to change from a simple, unstable composition to a complex, stable and functional system, the development of which is strongly influenced by environmental factors (Buddington, Sangild et al. 2012, Schokker, Zhang et al. 2014). It has been hypothesized that these environmental factors modulate the early development of the gut microbiota and can thereby induce long-lasting effects on the host's immune development (Thompson, Wang et al. 2008, Mulder, Schmidt et al. 2009). For example, a study in pigs demonstrated that early life perturbations (antibiotics and stress) have long-lasting effects on the gastrointestinal system, both on gut microbiota composition and intestinal gene expression, including genes involved in immune-related processes (Schokker, Zhang et al. 2015). As it is now widely recognized that the gut microbiota is closely linked to metabolic processes and the development of the immune system, it is not surprising that probiotics receive major interest (Lallès, Bosi et al. 2007, Rooks and Garrett 2016). In this context, early life administration of probiotics could be a valuable strategy to enhance the maturation of the piglet's microbiome and immune system, which may reduce the incidence and magnitude of gastrointestinal diseases during weaning and potentially later in life.

Probiotics are defined as 'live microorganisms' that, when administered in adequate amounts, confer a health benefit for the host (FAO 2006, Bajagai, Klieve et al. 2016). One of the most well studied Gram-negative probiotics is *E. coli* Nissle 1917 (EcN), which was isolated in 1917 from the intestinal microbiota of a young soldier by the German Professor Alfred Nissle (Wassenaar 2016). Interestingly, a more recent study demonstrated the presence of a natural isolate of EcN in a swine herd in Germany (Kleta, Steinrück et al. 2006). In experimental studies, EcN was shown to reduce diarrhoea in calves (Von Buenau, Jaekel et al. 2005). In addition, EcN inhibited colonisation by enteric bacterial pathogens by adhering strongly to the porcine epithelial surface (Schroeder, Duncker et al. 2006, Barth, Duncker et al. 2009). However, it is currently unknown if EcN can pass the GIT of conventional pigs when orally administered in the neonatal period. There is also little information available regarding EcN's presence in different porcine GIT segments and whether it is present in mucosal scrapings from those segments. In addition, as EcN might influence the pH in the GIT, the pH should be measured in digesta of different GIT segments. Moreover, EcN is also known to stimulate specific immune cells (e.g., dendritic cells) that underlie the epithelial barrier. These immune cells may interact directly or indirectly (through the microbiota) with probiotic bacteria, as has been previously hypothesized for EcN (Scaldaferri, Gerardi et al. 2016). To examine if orally administered EcN might directly interact with immune cells in gut-associated lymphoid tissues (GALT), bacterial translocation of EcN to mesenteric lymph nodes (MLNs) should be investigated. MLNs are known as key sites for oral tolerance induction and T-cell activation and act as a barrier to prevent intestinal bacteria from penetrating the systemic immune system. (Macpherson and Smith 2006). Bacterial translocation of EcN to

MLNs has previously been described in murine studies but has yet to be investigated in pigs (Bereswill, Fischer et al. 2013). This pilot study aimed to fill the previously mentioned knowledge gaps and to develop methodologies that improve the design of larger future *in vivo* studies.

Materials and Methods

Ethical statement

This animal experiment was conducted in accordance with the Dutch animal experimental and ethical requirements and the project license application was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) (Permit Number: AVD1040020173948). The protocol of the experiment was approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, The Netherlands) (Protocol Number: 2017.W-0076). Pigs were euthanized with Euthasol® according to Good Veterinary Practice (GVP), and all efforts were made to minimize suffering.

Study design

This pilot study was performed at the Swine Research Centre (Trouw Nutrition, Sint Anthonis, The Netherlands). The study was conducted with eight Hypor*Maxter newborn female piglets that were selected from two multiparous sows. Both sows were housed in farrowing pens with steady temperature, humidity, and light, and *ad libitum* access to water. Shortly after parturition, all newborn piglets received an ear tag, an intramuscular iron injection, their tails were docked, and their birth weight and sex were determined. Twenty-four hours after parturition (day 1), four female newborn piglets were selected from each sow and were then housed together in a farrowing pen with of the sows. Subsequently, all eight piglets were randomly allocated to the treatment groups, with two animals receiving 1 mL of Ponsocol® solution containing 10^7 Colony Forming Units (CFU) of EcN, two animals receiving 10^8 CFU/mL of EcN, two animals receiving 10^9 CFU/mL, and two animals receiving tap water (control treatment). All EcN and control treatments were orally administered with a syringe every other day until the end of the study (day 25). From day 23 onwards, suckling piglets received creep feed to get used to solid feed intake. All piglets were housed with a (foster) sow during the entire study period of 25 days. A graphical presentation of the study design, including the time points of faecal sample collection, is shown in Figure 1.

Preparation of *E. coli* Nissle 1917 inocula

E. coli Nissle 1917 (Ponsocol®) was kindly provided by Ardeypharm GmbH, Herdecke, Germany. Inocula were freshly prepared and administered every other day starting from day two onwards. Three EcN concentrations were tested: 10^7, 10^8, and 10^9 CFU per mL. To prepare 10^8 and 10^9 CFU per mL of EcN, the Ponsocol® solution was centrifuged at 5200g for 5 min. Next, excess supernatant was removed and used for the resuspension of bacterial pellets up to the desired concentrations. To verify the number of CFU per mL, serial dilutions of the Ponsocol® preparations were plated on Lysogeny Broth (LB) agar plates. A volume of 0.05 mL of each diluted solution was plated (using sterile disposable spreaders) in a laminar flow hood. Each dilution chosen was plated in triplicate. Plates were incubated upside down at 37 °C overnight. On the following day, plates with apparent CFU counts between 30 and 300

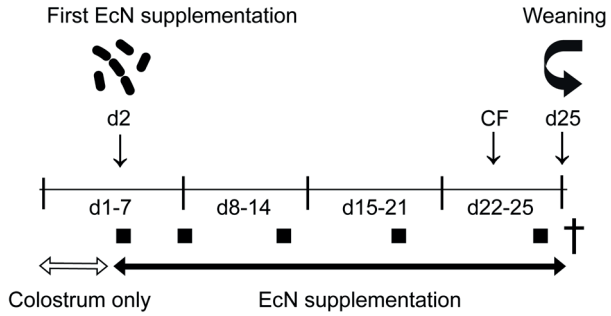

Figure 1. Study design. Eight female piglets were included in the study and housed with the sow for 25 days. Oral administration of 10^7, 10^8, and 10^9 CFU/mL of EcN or water (control treatment) started on day 2 of the study and was repeated every other day until sacrifice and dissection (d25 after birth). Creep feed (CF) was provided from day 23 onwards. Faecal samples were collected on d4, d8, d13, d19, and d25 (indicated by black squares).

per plate were counted. Results were corrected by the volume plated and dilution applied. CFU counts (log10 CFU per mL) were averaged, and standard deviations were calculated.

Experimental procedures

Faecal sampling

Individual faecal samples were collected following rectal stimulation on days 4, 8, 13, 19, and 25 of the study. All faecal samples were immediately snap-frozen using dry ice and stored in a sterile cryo-tube at -80°C until further processing. During dissection, digesta and mucosal scrapings of the epithelium were collected from the jejunum, ileum, duodenum, and colon to determine the presence of EcN along the GIT. In addition, the pH was measured in the digesta samples of all GIT segments, since it is an important determinant of bacterial survival. To determine bacterial translocation of EcN to the mesenteric lymph node (MLN), the ileocecal MLN (adjacent to the ileum) was aseptically collected and stored overnight at 4°C in sterile PBS until further processing. Zootechnical parameters, including general health status of the animals were evaluated daily by animal caretakers. Faecal scoring was performed during the collection of faecal samples (days 4, 8, 13, 19, and 25). The body weight was measured at birth, 24 hours after birth, and on days 13, 19, and 25. All experimental procedures were performed by trained staff and according to standardized operation procedures.

Quantification EcN in faeces, digesta and mucosal scrapings using qPCR

For relative quantification of EcN in faeces, digesta and mucosal scapings, SYBR green qPCR assays were performed with extracted sample DNA using an iCycler iQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). All qPCR analyses were carried out with a reaction volume of 25 µL, using optical-grade PCR plates and sealing film. The reaction mixture contained 2x iQ SYBR green Supermix (Bio-Rad Laboratories B.V.), 400 nM (final concentration) of each primer (Table S1), and 0.5 µL of either the DNA template (20ng/µL) or PCR grade water. The bacterial amplification program consisted of an initial denaturation at 95°C for 3 min followed by 39 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 45 s. The fluorescent products were detected at the last step of each cycle. Following amplification, melting curve analysis of PCR products was performed to

determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.5°C/s increments from 65 to 95°C, with continuous fluorescence collection. EcN specific and total bacteria standard curves (10pg–10ng genomic DNA/reaction) for the assays were prepared by diluting genomic DNA isolated from an EcN culture.

Cultivation of bacteria from the MLN
The ileocecal MLN was homogenized using cold PBS and a cell strainer (BD Falconä Nylon 100µm Cell Strainer), and 1mL of the cell suspension was stored overnight at 4 °C in a sterile Eppendorf tube. The next day, 100 µl of the tissue homogenates were cultured overnight on LB agar plates at 37°C. After 24 h, the bacteria were visually inspected, and a subset of colonies was selected for colony PCR.

Identification of EcN colonies by colony PCR.
A subset of bacterial colonies was selected, and a colony PCR (cPCR) was performed to identify EcN colonies using EcN-specific primers (see table S1). For each reaction, 12.5 µl Onetaq buffer, 0.5 µl dNTPs (10 mM), 0.5 µl of each primer (10 µM), 0.125 µl Onetaq polymerase were combined with H_2O to reach a final volume of 25 µl and were incubated at 95 °C for 5 min followed by 35 cycles of 95 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s. The resulting amplicons were visualized using a 1% agarose gel.

Results

Relative abundance of EcN in faeces
The amount of EcN present in faecal material at different time points was determined by qPCR. Moreover, we investigated if control animals were able to pick up the probiotic strain from the pen environment when co-housed with EcN-treated piglets. A measurable amount of EcN was present in the faeces of each animal after oral administration of 10^8 or 10^9 EcN CFU/inoculation (Figure 2). Importantly, no adverse health effects were observed at any of the doses of EcN during the study. In addition, oral administration of 10^7 EcN (CFU/inoculation) resulted in a low relative abundance of EcN in one of two animals (pig 8) during the first two weeks of the study. The relative abundance of EcN showed to be more constant in the second half of the study (days 19 and 25) in comparison to the first half of the study (days 4, 8, and 13). Interestingly, EcN could also be detected in the faeces of the two control animals (pigs 1 and 2) that did not receive EcN. On day 19 of the study, EcN was detected in the faeces of piglet 1, whereas faeces of piglet 2 showed to contain a relatively high abundance of EcN at the start of the study (day 4) and a low but measurable amount of EcN at the end of the study (day 25).

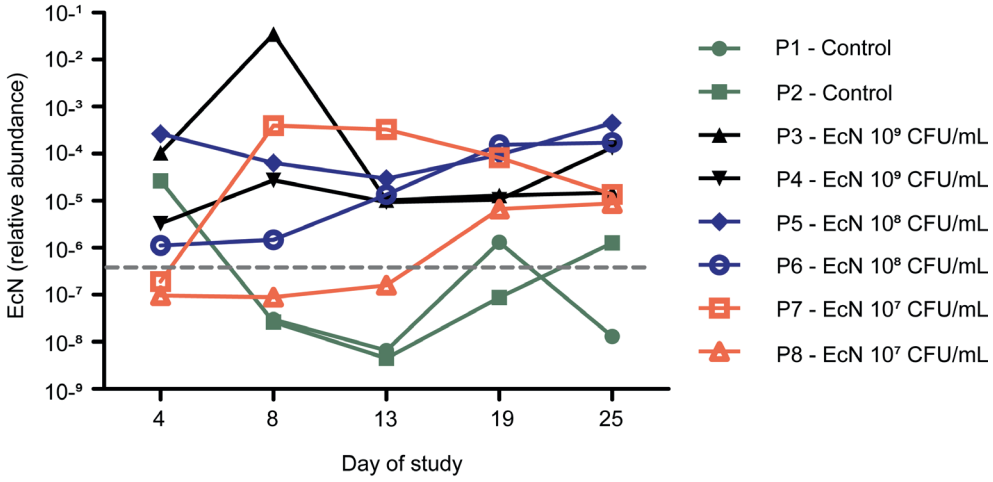

Figure 2. Oral administration of high amounts of EcN shows a consistently high relative abundance of EcN in the faeces of piglets. Neonatal piglets received either EcN at different doses (10^7, 10^8, or 10^9 CFU/inoculation) or water (control treatment) every other day for the entire duration of the study. Faecal samples were taken on days 4, 8, 13, 19, and day 25 and analysed for the relative abundance of EcN by qPCR. No faecal material could be retrieved from pig 1 (P1) on day 4 of the study. Data are represented as the relative abundance of EcN compared to the total amount of bacteria (log-scale). Below the indicated threshold line, measurements had a cycle value (Cq) above 31 at which the development of amplicon artefacts could have occurred, which can potentially lead to false positives.

Presence of EcN in digesta and mucosal scrapings of different GIT segments

To investigate the presence of EcN in different segments of the GIT, digesta and mucosal scrapings were collected from the jejunum, ileum, caecum, and colon (Figure 3A). EcN was detected in all EcN-treated piglets but was especially abundant in the digesta of the caecum and colon. This is in contrast with the jejunum and ileum, which contained no or lower amounts of EcN. Independent of the gut segmentation, a maximum of around 10^{-3} (1:1000) EcN (relative to the total amount of bacteria) was detected in digesta. In addition, EcN was detected in the jejunum of both piglets that received 10^9 CFU per dose of EcN. In general, piglets that received 10^7 CFU per dose of EcN showed a lower relative abundance of EcN in comparison to the piglets that received 10^8 or 10^9 CFU per dose of EcN. EcN was not detected in most of the digesta of the control animals, with the exception of the caecum and colon of one of the control animals, albeit at relatively low abundance. In addition to the analysis of the digesta, a small selection of mucosal scrapings from different gut segments were collected to get an impression of the presence of EcN in the mucus layer (Figure 3B). In general, the relative abundance of EcN in the mucus layer was comparable to its presence in the digesta. In two cases (ileum, pig 5 and colon, pig 6), EcN was detected in the digesta but not in the mucus layer of the respective gut segments.

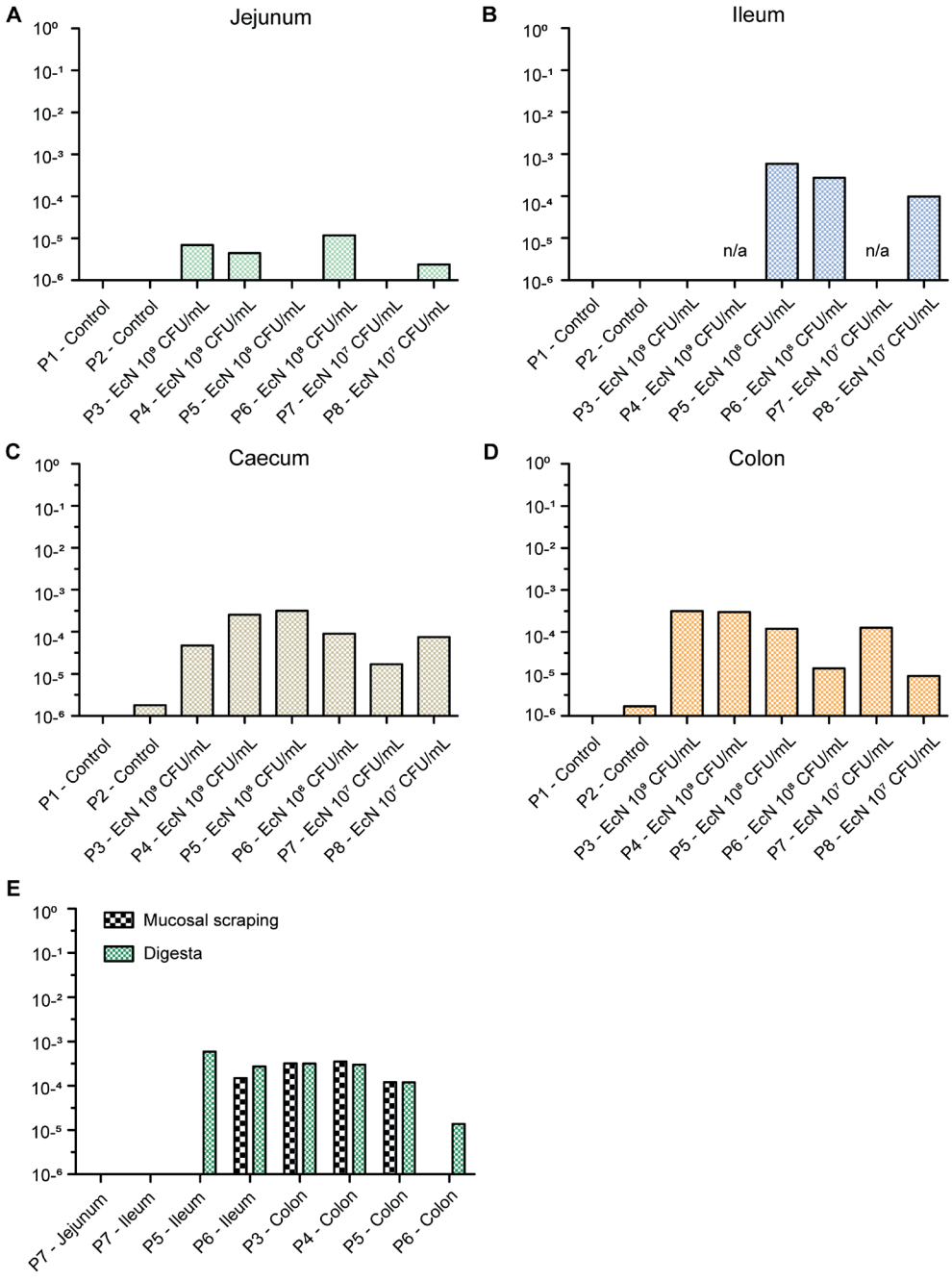

Figure 3. Presence of EcN in digesta and mucosal scrapings of different gut segments. Neonatal piglets received different doses of EcN; 10^9 CFU (pigs 3 and 4), 10^8 CFU (pigs 5 and 6), 10^7 CFU (pigs 7 and 8), or a control treatment (water) every other day for the entire duration of the study. Digesta and a small selection of mucosal scrapings from different gut segments (jejunum, ileum, caecum, and colon) were collected to determine the presence of EcN in the GI tract at the end of the study (day 25). Data are represented as relative abundance of EcN compared to total amount of bacteria (log10-scale). Luminal content from the ileum could not be retrieved from two piglets (P4 and P7) as indicated by not available (n/a).

pH in different GIT segments

To determine if peroral administration of different concentrations of EcN affect the acidity levels in the gut, the pH was measured in the luminal content of the jejunum, ileum, caecum, and colon (Figure 4). The luminal content of the jejunum showed to have a pH ranging from 6.4 to 9.3 with an average pH of 7.1. In contrast to the jejunum, the ileum showed to have a slightly basic pH, ranging from 7.46 to 7.9 with an average pH of 7.62. The pH of the caecum and colon was more acidic. The pH of the luminal content from the caecum ranged from 6.79 to 6.41 with an average pH of 6.11, and for the colon the pH ranged from 5.69 to 6.95 with an average pH of 6.14. No apparent effects of different EcN concentrations on the pH were observed.

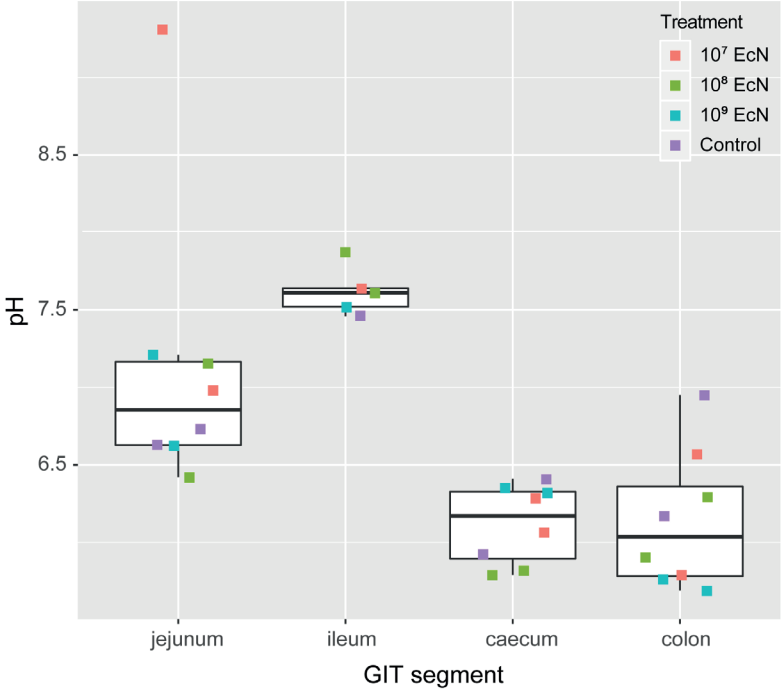

Figure 4. pH levels of gut segments from different treatment groups. Neonatal piglets received different amounts of EcN; 10^9, $10^{8,}$ and 10^7 CFU (per dose) or a control treatment every other day until dissection at the end of the study (day 25). Luminal content from different gut segments were collected to determine the pH. The y-axis indicates the pH (5.5 – 9.5) and the x-axis shows the different gut segments (jejunum, ileum, caecum, and colon). Every point represents an individual piglet and the colours correspond to the treatment group (administered dosage of EcN).

Bacterial translocation to the MLN

Translocation of EcN to the MLN was investigated to better understand its behaviour and its potential interaction with the immune system in early life (Figure 5). From all thirty bacterial colonies tested, one was confirmed by colony PCR to be EcN (lane 25). This bacterium was obtained from the MLN of a single animal that received the highest concentration of EcN (10^9 CFU/mL).

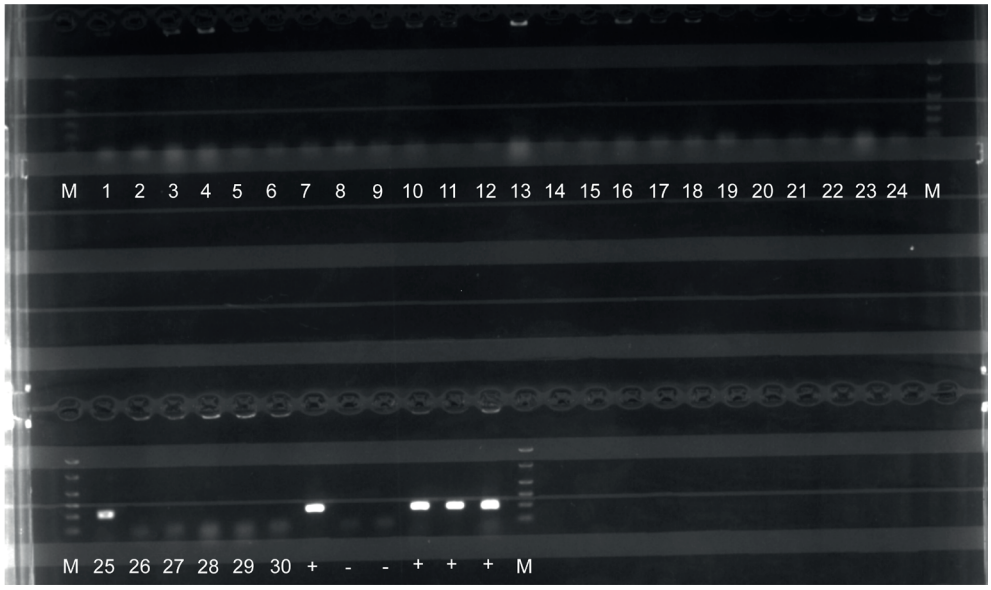

Figure 5. Detection of EcN in the MLN by colony PCR. The numbers indicate the bacterial isolates. M: 100 bp DNA ladder. Positive controls (samples containing EcN) are indicated by plus signs (+), and negative controls (samples without EcN) are indicated by minus signs (-).

Discussion

Early in life and around weaning, piglets are vulnerable to gastrointestinal disorders such as diarrhoea. The incidence of these gastrointestinal disorders increases around weaning due to weaning-associated stressors such as maternal separation, transportation, and change of diet (Moeser, Pohl et al. 2017). In the past decades, there has been increasing interest in the effects of probiotics to confer a health benefit to the host. Probiotics are generally known to support a healthy gut microbiota, digestive tract, and immune system (Isolauri, Sütas et al. 2001, Behnsen, Deriu et al. 2013, Roselli, Pieper et al. 2017). However, to date, there is only limited data available on the presence, localization, and persistence of probiotic bacteria in the GIT when administered orally in early life. In this pilot study we used *E. coli* Nissle 1917 (EcN), a Gram-negative bacterial strain that is known to prevent gastrointestinal disorders in different host species. A previous study showed that when EcN was inoculated prior to experimental infection with enterotoxigenic *E. coli,* faecal shedding of the challenge strain decreased, and the piglets were protected from developing diarrhoea (Schroeder, Duncker et al. 2006). In the current pilot study, no changes in the consistency of the faeces between the treatment groups were detected, and no adverse health effects were observed upon administration of EcN. Moreover, no major differences were observed between the different concentrations of EcN administered, with regard to the presence, abundance and localization of EcN along the GIT.

To investigate if the presence of EcN in piglets is time and dose-dependent, faecal material was collected at different time points of the study. EcN was detected at all time points of the

study when animals received EcN at a concentration of 10^8 or 10^9 CFU/mL. In contrast, EcN could not be detected at all time points when animals received EcN at a concentration of 10^7 CFU/mL. In line with these results, the relative abundance of EcN in faeces on day 4 was lowest in piglets that received EcN at a concentration of 10^7 CFU/mL. Furthermore, from day 4 onwards the variation between individuals increased, but in the second half of the study the individual variation decreased. This could be explained by the temporal development of the gut microbiome as it will attain diversity and complexity of composition over time (Wang, Tsai et al. 2019). Interestingly, on days 4, 19, and 25, EcN was detected in one of the control animals. Cross-contamination between animals could have occurred due to direct contact between piglets after oral administration (e.g., during suckling) or piglets consuming faeces from piglets who received EcN. This observation opts for the proper division of the treatment and the control groups in similar experiments and to test all control animals for the presence of EcN or whichever probiotic strain is used.

In this pilot study we also investigated the presence and abundance of EcN along the GIT. Digesta and mucosal scrapings were collected from the jejunum, ileum, caecum and colon. EcN was detected in all GIT segments at the time of dissection , with the highest relative abundances found in the ileum, caecum and colon. This partially overlaps with previous research that demonstrated that commensal *E. coli* strains are predominantly located in the hindgut, especially in the caecum and the colon (Poulsen, Lan et al. 1994). Another factor that could influence the presence, abundance and localization of EcN in the GIT is the pH (Bezkorovainy 2001). The colon and caecum were found to have a slightly more acidic environment (approximately pH 6) in comparison to the jejunum and ileum (approximately pH 7 and 7.5, respectively). This is in line with other studies (Merchant, McConnell et al. 2011, Sciascia, Daş et al. 2016). However, it is hard to determine if pH is a determinant of EcN abundance in the gut as many factors may contribute (e.g., genetics, nutrition, and age). Furthermore, no apparent effects of EcN on the pH were observed for any of the EcN concentrations administered. In addition to the collection of digesta, a small number of mucosal scrapings from different GIT segments were collected to compare the relative abundance of EcN between digesta and mucosa of the same segment. For most of the piglets, the relative abundances of EcN in digesta and mucosal scrapings were similar. The presence of EcN in mucosal scrapings can be explained by the expression of different types of fimbriae by EcN (e.g., F1C fimbriae), which are known to facilitate adhesion of EcN to the mucus layer or gut epithelial cells (Blum, Hacker et al. 1995, Lasaro, Salinger et al. 2009).

It is well established that bacteria are able to pass the epithelial barrier and translocate to secondary lymphoid organs, including MLNs (Schultz, Watzl et al. 2005, Nagpal and Yadav 2017). However, it is not known if EcN is able to translocate to the MLN when administered in early life, a period where the integrity of the epithelial barrier is suboptimal. Interestingly, in this pilot study, viable cells of EcN were detected in the MLN of one of the animals that received the highest amount of EcN (10^9 CFU/inoculation). Bacterial translocation of EcN to the MLN has been described in murine ileitis and colitis models (Schultz, Strauch et al. 2004, Bereswill, Fischer et al. 2013). In an inflamed gut, bacterial translocation is more likely to occur as the gastrointestinal barrier is disrupted and bacteria are able to penetrate the epithelial barrier more easily. However, 'spontaneous' translocation of bacteria also takes place in healthy subjects on a continuous basis, a process that is influenced and controlled

by the host's microbiota and immune system (Jeppsson, Mangell et al. 2004, Gronbach, Eberle et al. 2010, Ley, Desseyn et al. 2019). These findings could explain why EcN was detected in the MLN of a single animal. This observation provides new insights into the behaviour of EcN and its link with the mucosal immune system in a conventional pig model.

In conclusion, we could show in this pilot study that EcN can be detected in both the digesta and mucosa of different gut segments and in the faeces of EcN-treated piglets. No adverse health effects were observed in any of the animals. Future *in vivo* studies that involve probiotics should pay special attention to cross-contamination to other experimental groups, as it may affect the outcome and interpretation of the study. This study contributes to the quality of future *in vivo* studies that will help to better understand the effects of EcN on the microbiota, gastrointestinal tract, and the mucosal immune system.

Supplementary material

Table S1. Primers and annealing temperatures (T_m) that were used in this study.

Application	Primer	Primer sequence (5'-3')	T_m (°C)	Target	Length (nucleotides)	Reference
Total Bacteria	Bact1369f	CGGTGAATACGTTCYCGG	60	16S rRNA region	123	(van Lingen, Edwards et al. 2017)
	Bact1492r	GGWTACCTTGTTACGACTT	60			
EcN specific	Muta 9	GCG AGG TAA CCT CGA ACA TG	60	EcN Plasmid pMUT2	313	(Blum-Oehler, Oswald et al. 2003)
	Muta 10	CGG CGT ATC GAT AAT TCA CG	60			

Acknowledgements

The authors would like to thank the Swine Research Centre staff for their technical assistance during the pilot study. A Special thanks goes to Carlijn de Bruijn for her support regarding the logistics, organization and execution of the study.


5

Chapter 5

Effects of *E. coli* Nissle 1917 on the Porcine Gut Microbiota, Intestinal Epithelium and Immune System in Early Life

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Published in Frontiers Microbiol. 2022 Feb 25; 13:842437.
https://www.frontiersin.org/articles/10.3389/fmicb.2022.842437

Abstract

Early in life and particularly around weaning, piglets are susceptible to infections because of abrupt social, environmental, and dietary changes. Dietary interventions with probiotic bacteria have gained popularity because of the increased awareness of the direct link between diet and health. In this study, piglets received the probiotic strain *Escherichia coli* Nissle 1917 (EcN) or a control treatment perorally from day 2 after birth until 2 weeks post-weaning. To investigate spatio-temporal effects of EcN on the gut microbiota composition, intestinal epithelial gene expression and immune system, faeces, digesta, blood, scraping material and mesenteric lymph node tissue were collected at different time points. In addition, oral vaccinations against *Salmonella enterica* serovar Typhimurium were administered on days 21 and 45 of the study to assess the immunocompetence. EcN-treated pigs showed a reduced diversity of taxa within the phylum Proteobacteria and a lower relative abundance of taxa within the genus *Treponema* during the pre-weaning period. Moreover, EcN induced T cell proliferation and Natural Killer cell activation in blood and enhanced IL-10 production in *ex vivo* stimulated mesenteric lymph node cells, the latter pointing toward a more regulatory or anti-inflammatory state of the local gut-associated immune system. These outcomes were primarily observed pre-weaning. No significant differences were observed between the treatment groups with regards to body weight, epithelial gene expression, and immune response upon vaccination. Differences observed during the post-weaning period between the treatment groups were modest. Overall, this study demonstrates that the pre-weaning period offers a 'window of opportunity' to modulate the porcine gut microbiota and immune system through dietary interventions such as EcN supplementation.

Keywords: *E. coli* Nissle 1917, porcine, gastrointestinal tract, gut microbiota, immune system, early life, immunomodulation, probiotic

Introduction

Abrupt social, environmental, and dietary shifts associated with weaning lead to transient lower feed intake, activation of the hypothalamic-pituitary-adrenal (HPA) axis, increased intestinal permeability, diarrhoea, and increased abundance of *Enterobacteriaceae* in the gut (Trevisi, Luise et al. 2021). These physiological responses increase susceptibility to viral and bacterial pathogens (e.g., enterotoxigenic *Escherichia coli* or ETEC) and inflict potential welfare issues over the following weeks. From the 1950's onwards, the addition of antibiotics to pig feed has been used to combat these problems and to improve the growth of young pigs and the reproductive performance of sows (Li 2017). However, the use of antibiotics increases the selective pressure on intestinal bacteria, resulting in antimicrobial resistance (AMR) and hence increased risk of transmission of antibiotic-resistant zoonotic pathogens to humans. In 2006, the EU prohibited the use of antibiotics as growth promoters in livestock to reduce the spread of antimicrobial resistance. Since then, other countries have prohibited the use of medically important antibiotic growth promotors (AGPs), but this is not the case in all major livestock producing countries (Kirchhelle 2018). Effective alternative strategies in a One Health setting that support a healthy development and growth of piglets would help drive the changes needed to reduce AMR worldwide.

Probiotics are becoming increasingly popular as alternatives to AGPs (Bajagai, Klieve et al. 2016). However, research outcomes in this field have been largely inconsistent, and there is still a need to optimize such probiotic strategies (Barba-Vidal, Martín-Orúe et al. 2019). One of the oldest well-studied probiotics is the Gram-negative bacterial *E. coli* strain Nissle 1917 (EcN), initially isolated by Alfred Nissle during the First World War from a healthy soldier during an outbreak of diarrhoea (Blum, Hacker et al. 1995). Since its discovery, EcN has been used to treat intestinal diseases such as acute diarrhoea, ulcerative colitis, Crohn's disease and constipation in humans (Sonnenborn and Schulze 2009). Clinical studies showed that oral administration of EcN attenuates acute diarrhoea in infants and toddlers, and prevents diarrhoea in neonatal calves (Von Buenau, Jaekel et al. 2005, Henker, Laass et al. 2008). The beneficial effects of EcN are considered to be mediated through production of anti-microbial colicins and microcins, competition with pathobionts and pathogens for nutrients and adhesion sites, and immunomodulatory activities (Patzer, Baquero et al. 2003, Barth, Duncker et al. 2009, Jacobi and Malfertheiner 2011, Deriu, Liu et al. 2013, Sassone-Corsi, Nuccio et al. 2016, Wassenaar 2016). In humans, intestinal inflammation leads to a bloom of *Enterobacteriaceae* and increased abundance of B2 phylotype *E. coli* that adhere to the intestine and elicit damage through hemolysin production, which further promotes mucosal inflammation and intestinal barrier disruption. EcN is of the same phylotype as these adherent and invasive *E. coli* (AIEC) and can compete for adherence without affecting gut integrity (Mirsepasi-Lauridsen, Du et al. 2016).

Studies with EcN and intestinal cell lines report increased expression of tight junction protein zonula occludens 2 (ZO-2) (Zyrek, Cichon et al. 2007), induction of β-defensin-2 expression through NF-kB- and AP-1-mediated signaling (Wehkamp, Harder et al. 2004), and production of interleukin (IL)-8 (Lammers, Helwig et al. 2002). Moreover, EcN has shown to modulate the activity of cells of the porcine innate and adaptive immune systems (Vlasova, Shao et al. 2016, Michael, Paim et al. 2021). However, the exact molecular mechanisms are

still under investigation. *In vitro*, EcN enhances dendritic cell (DC) maturation and cytokine production, and induces activation, cell cycling and expansion of human γδ T cells (Guzy, Paclik et al. 2008, Geervliet, Lute et al. 2020). Moreover, several studies in different animal species demonstrated that EcN alters host cytokine responses upon stimulation, particularly by inducing high levels of the anti-inflammatory cytokine IL-10 (Cross, Ganner et al. 2004, Güttsches, Löseke et al. 2012, Kandasamy, Vlasova et al. 2016). These immunomodulatory effects are considered to be primarily induced through a Toll-like receptor 4 (TLR-4) dependent signaling pathway (Grabig, Paclik et al. 2006, Adam, Delbrassinne et al. 2010). *In vivo* studies using gnotobiotic pigs showed that colonization with EcN mediated greater protection against human rotavirus challenge than *Lactobacillus rhamnosus* GG (LGG), possibly due to stimulation of the innate immune system and activation of the DC-IL-12-NK immune axis (Kandasamy, Vlasova et al. 2016, Vlasova, Shao et al. 2016).

Although EcN has been studied for over a century (Wassenaar 2016), little is published about the *in vivo* effects of EcN on the gut microbiota, gut epithelial barrier and immune system of pigs. Moreover, there is limited data available on the presence, abundance and persistence of EcN in the gastrointestinal tract (GIT) of conventional pigs. The aim of this research was therefore to determine whether peroral administration of EcN to postnatal piglets would impact on the development of the porcine gut microbiota, intestinal barrier function and the innate and adaptive arms of the immune system. A more mature or established gastrointestinal system could ultimately facilitate the establishment of a vigilant immune system that counteracts pathogens during the weaning and post-weaning period.

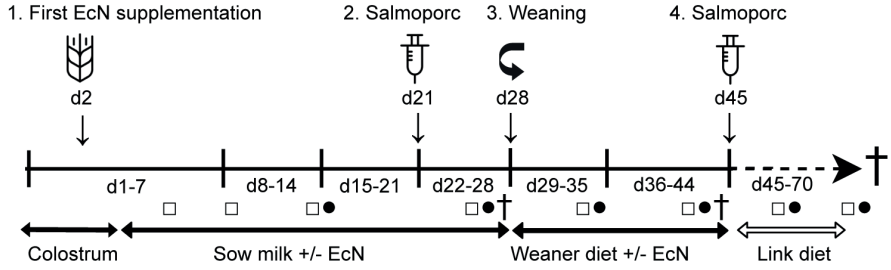
Materials and Methods

Ethics Statement and Study Design

This study complied with the European directive 2010/63/EU on the protection of animals used for scientific purposes, and was conducted in accordance with the Dutch law on animal experimentation and ethical requirements. The project was approved and licensed (Permit Number: AVD1040020173948) by the Dutch Central Authority for Scientific Procedures on Animals (CCD), and all associated protocols were approved by the Animal Experimentation Committee of Wageningen University & Research (Wageningen, Netherlands). Pigs were euthanized by intravenous injection with 20% sodium pentobarbital (Euthasol®), followed by immediate exsanguination. Pigs were euthanized according to Good Veterinary Practice (GVP), and all efforts were made to minimize suffering or discomfort.

The study was performed at the Swine Research Center (Trouw Nutrition, Sint Anthonis, Netherlands) and included 33 sows (Hypor Libra) and their litters (Maxter × Hypor Libra sow). To reduce genetic variation in the litters, sperm from a single boar was used to inseminate all sows. Shortly after birth, all piglets received an intramuscular injection of iron, an ear tag for identification, and their tails were docked. In addition, their sex and birth weight were recorded. Approximately 24 h after birth, 192 female piglets were selected and cross-fostered to minimize possible confounding effects, including the day of birth, weight at birth, litter size, sow parity and genetic background. At cross-fostering, from the original 192 piglets, 96 piglets were randomly allocated to either the control- or treatment (EcN)

group, resulting in 16 pens (pen is the experimental unit) with one sow and six piglets each (eight pens per treatment group). Pens were distributed over four farrowing rooms and balanced for treatment. Male and non-experimental female piglets were housed together with the focal piglets by equally dividing them over the pens. A schematic representation of the experimental design and the study timeline is presented in Figure 1. All piglets were housed together with their fostering mother until weaning (approximately day 28), in a room with a computer-controlled climate system. To keep the floors sufficiently dry in the farrowing rooms, calcium carbonate-based powder (Power-Cal®, Power-Cal, Netherlands) was added to all pen floors in the first few days post-farrowing. In addition, various measures were taken to prevent cross-contamination of EcN between the farrowing pens, including pen separators, separate equipment (e.g., boots and overalls), and hygiene protocols. A weaner diet was fed 3 days before weaning (from days 25 to 27) to allow piglets to become acquainted with the consumption of solid feed (de Vries, Geervliet et al. 2020). At weaning, a subset of pigs (48 pigs, 3 per pen) was randomly selected and reallocated to



Department	# of pens	d1-d14	d15-d28	d29-d44	d45-d70
1	2	6/pen	4/pen	3/pen	2/pen
1	2	6/pen	4/pen	3/pen	2/pen
2	2	6/pen	4/pen	3/pen	2/pen
2	2	6/pen	4/pen	3/pen	2/pen
3	2	6/pen	4/pen	3/pen	2/pen
3	2	6/pen	4/pen	3/pen	2/pen
4	2	6/pen	4/pen	3/pen	2/pen
4	2	6/pen	4/pen	3/pen	2/pen
Animals per treatment:		48	32	24	16
Total number of animals:		96	64	48	32

Figure 1. Timeline of the study **(A)**. From day 2 until day 44, piglets received an oral dose of EcN or were sham-dosed with tap water (Control) every other day (1). A subset of piglets was weaned on day 28 (3). All pigs received an oral vaccination (Salmoporc®) against *Salmonella enterica* serovar Typhimurium on days 21 [primary vaccination; (2)] and 45 [booster vaccination; (4)]. Faecal sample collection was performed on days 4, 8, 14, 26, 35, 43, 59, and 69 (squares; □) and blood samples were taken on days 14, 26, 35, 43, 59, and 69 (circles; •). Subsets of animals were sacrificed on days 27, 44, and 70 (cross; †). Schematic representation of the experimental design showing the remaining number of animals per treatment over time as determined by deselection or dissection of animals **(B)**. The control and the EcN groups are presented as gray and blue, respectively.

a nursery facility. Pigs that received medication or suffered from leg/claw injuries or growth retardation were excluded from this selection. All pigs received a weaner diet from day 28 until day 44, followed by a nursery diet until the end of the study (de Vries, Geervliet et al. 2020). During the entire post-weaning period all piglets had access to solid feed and water *ad libitum*. On days 21 and 45 all piglets perorally received a *Salmonella enterica* serovar Typhimurium (Salmoporc®) vaccination and on days 27, 44, and 70, sixteen piglets were sacrificed (eight per treatment, at each time point).

Experimental Procedures

EcN Administration and Oral Vaccination With Salmoporc®

Escherichia coli Nissle 1917 (Ponsocol®) was kindly provided by Ardeypharm GmbH (Herdecke, Germany) and stored until use at 4°C. From day 2 onwards, pigs perorally received either a control treatment (tap water) or EcN (10^9 CFU/administration) every other day until day 44 of the study. EcN was administered at increasing volume over time ranging from 1 to 5 mL, using disposable syringes with a volume of 2 and 5 mL (Discardit II, BD). The sham-dosed control piglets similarly received water. To prepare EcN at the required concentration, Ponsocol® vials (containing 10^8 CFU/mL) were gently agitated and pooled, followed by centrifugation at 5,200 × g for 5 min. The bacterial pellet was resuspended to the required concentration using excess supernatant. CFU counts of Ponsocol® and the final solution were confirmed by plating serial dilutions on lysogeny broth (LB), followed by counting.

To investigate the effect of EcN on the immune response to vaccination, all pigs were vaccinated against *Salmonella enterica* serovar Typhimurium (Salmoporc® STM, lot number 0270617, IDT Biologika GmbH) on days 21 (primary vaccination) and 45 (booster vaccination). This oral live-attenuated vaccine is registered for pigs to inhibit bacterial colonization, excretion, and clinical symptoms of *Salmonella enterica* serovar Typhimurium. The vaccine was reconstituted in water before oral administration according to the manufacturer's instructions.

Blood and Faecal Sampling

For both faecal- and blood samples, piglets were sampled in random order and weighed shortly before sample collection. Faecal samples were collected in cryotubes on days 4, 8, 14, 26, 35, 43, 59, and 69 by gentle rectal stimulation with cotton swabs (PurFlock Ultra, Puritan) pre-wetted with sterile water. The cryotubes were immediately placed on dry ice and stored at −80°C until further processing.

Blood samples were collected from the jugular vein of sixteen animals on days 14, 26, 43, and 69 using Serum Gel tubes (S-Monovette®, Sarstedt) that were centrifuged at 2,500 × g for 10 min to separate serum. Serum was stored at −20°C until further use. Blood samples for flow cytometry were collected in Sodium heparin tubes (S-Monovette®, Sarstedt) and stored at room temperature (RT) until further processing.

Dissection

Immediately after euthanasia and exsanguination of a pig, the ileocecal mesenteric lymph node (MLN) was removed and stored on ice in cold (4°C) RPMI 1640 Medium with

GlutaMAX™ supplement (Gibco®), 2 mM L-Glutamine (Gibco®), and 10% fetal calf serum (FCS, Gibco®). Next, the GIT was removed from the abdominal cavity, then the jejunum, ileum, caecum and colon were identified and segmented accordingly (Supplementary Figure 1). Digesta samples from each gastrointestinal segment were taken by gently squeezing the content of a 40 cm segment into a plastic container (see Supplementary Figure 1 for a visual overview). Next, the digesta were homogenized using a sterile spatula, and approximately 1 g was aliquoted and stored in sterile cryogenic vials. The vials were immediately snap-frozen on dry ice and stored at −80°C until further processing. Leftover digesta from each gastrointestinal segment were mixed with water for pH measurement using a pH meter. Mucosal scrapings of the ileal and colonic gut epithelium were collected by taking 5 cm long gut segments adjacent and proximal to the gut segments used for digesta sampling (Supplementary Figure 1). First, the 5 cm segment was cut open longitudinally and cleaned using sterile Phosphate Buffered Saline (PBS). Then, mucosae were collected by scraping using a scalpel. Care was taken not to include muscular tissue. Mucosal scrapings were directly stored in Snaplock tubes containing RNA-later (Sigma-Aldrich, St. Louis, MO, United States), placed on dry ice, and stored at −80°C.

Measurements

Quantification of EcN by qPCR

A qPCR with primers specific to EcN (Table 1) was used to determine the relative abundance of EcN in faecal samples. All qPCR analyses were performed in triplicate in a reaction volume of 10 µL, using Hard-Shell® 384-Well PCR plates (Bio-Rad). The reaction mixture contained 2x iQ SybrGreen Supermix (Bio-Rad Laboratories B.V., Lunteren, Netherlands), 200 nM of each primer (Table 1), and 2 µL of the DNA template (1 ng/µL). The amplification program consisted of an initial denaturation at 94°C for 10 min followed by 39 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s using a CFX384TM thermocycler (Bio-Rad Laboratories B.V., Lunteren, Netherlands). The fluorescent products were detected at the last step of each cycle. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating with 0.5°C/min increments from 60 to 95°C with continuous fluorescence reading. For both EcN-specific and total bacterial primers, standard curves for the qPCR assays were prepared with tenfold serially diluted EcN genomic DNA as a template. The EcN qPCR was performed on a subset of the faecal and digesta samples to validate the EcN-specific ASV in the NGS dataset.

Table 1. Primers used for the quantification of EcN

Name	Target	Sequence (5'-3')	Annealing Temp (°C)	Length of PCR product (bp)	Reference
BACT1369f	16S rRNA region	CGG TGA ATA CGT TCY CGG	60	123	(van Lingen, Edwards et al. 2017)
BACT1492r	16S rRNA region	GGW TAC CTT GTT ACG ACT T	60		
EcN_for	EcN chromosome	GAA GAA ATT GAC GCA CCC C	60	110	(Reister, Hoffmeier et al. 2014)
EcN_rev	EcN chromosome	CGT GCA AGA CAT GGA GAG AC	60		

Determination of Microbiota Composition
A personalized Maxwell 16 Total RNA protocol (Promega Corp., Madison, WI, United States) was used to extract DNA from faecal and digesta samples. DNA extraction and further processing were performed exclusively on samples from animals that were dissected during the study. In addition to samples from this study, synthetic mock communities, blank water negative controls and EcN spiked samples were included in the sequencing libraries. Spiked samples (spk1 – spk5) were generated by mixing 0.05 mL 10-fold serial dilutions of Ponsocol® to 0.05 g of faecal material that was tested negative for the presence of EcN (Supplementary Figure 2). This resulted in five spiked faecal samples, ranging from 10^6 CFU/mL of EcN in the most diluted sample (spk1) to 10^{10} CFU/mL of EcN in the most concentrated sample (spk5).

For PCR amplification, barcoded primers directed to the V4 region of the bacterial and archaeal 16S rRNA gene were used, namely EMP_515F (5'-GTGYCAGCMGCCGCGGTAA), with linker 'GT,' and EMP_806R (5'-GGACTACNVGGGTWTCTAAT), with linker 'CC.' PCR reactions were done in duplicate for digesta samples and in triplicate for faecal samples. The amplification program included 30 s initial denaturation at 98°C for 10 s, annealing at 50°C for 10 s, elongation at 72°C for 10 s, and a final extension at 72°C for 7 min. For a detailed description of the DNA extraction protocol and the PCR protocol, see de Vries et al., 2020 (de Vries, Geervliet et al. 2020). DNA extraction from jejunum digesta samples occasionally resulted in low DNA yields (i.e., <1 ng/µL). For this reason, the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States) was used to measure the DNA concentration of all jejunum samples instead of a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). The latter was used for all ileum, cecal and faecal samples. An overview of DNA concentrations and quality readings can be found in Supplementary File 1. For the purification of PCR products, the HighPrep PCR kit (MagBio Genomics Inc., Gaithersburg, MD, United States) was used. DNA concentrations were measured with the Qubit dsDNA BR Assay Kit. The amplicon pool was generated by combining 200 ng of PCR amplified DNA from each sample and then concentrated to a final volume of approximately 20 µL using the HighPrep PCR kit. The Qubit dsDNA BR Assay Kit was used to measure the DNA concentration of the amplicon pool, and libraries were sent for Illumina HiSeq sequencing (Sequence mode used: NovaSeq 6000 S2 PE150 XP, Eurofins Genomics, Ebersberg, Germany). Amplicon sequence data were processed and analyzed using NG-Tax 2.0 (Poncheewin, Hermes et al. 2019), and annotated using the SILVA (release 132) database (Quast, Pruesse et al. 2012). The resulting dataset, containing microbiota composition data, will be referred to as the NGS dataset and contains taxonomic assignments up to the level of amplicon sequence variants (ASVs) that each has a unique ASV number. Each ASV is aimed to represent one bacterial species, and, where possible, were annotated at the genus level.

Gastrointestinal Mucosal Gene Expression Analysis
Mucosal scrapings of the epithelium were thawed on ice and excess RNA-later was removed. Then, a small portion (approximately 3 by 3 mm) was taken using a scalpel and tweezers, and added to 500 µL RLT (RNeasy lysis) buffer (Qiagen, Hilden, Germany). The sample was homogenized for 90 s using an Ultra-Turrax IKA T-10 Basic (IKA-Werke GmbH, Staufen, Germany). After homogenization of each sample, the shaft of the Ultra-Turrax IKA T-10 Basic was cleaned twice by running the device in a tube containing an excessive amount of fresh

PBS. This was done to prevent cross-contamination of samples. Next, 100 µL of homogenate was thoroughly mixed with 600 µL ice-cold RLT buffer. Then, 700 µL of 70% ethanol was added, followed by thorough mixing. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions including DNAse treatment to remove host DNA. The concentration of purified RNA was measured using the Qubit BR RNA Assay Kit (Thermo Fisher Scientific), and the integrity value of each RNA sample was assessed using the Qsep 100 protocol (GC Biotech, Waddinxveen, Netherlands). The RNA Quality Number was higher than 8.0 for all samples. After isolation, RNA was directly stored at −80°C. The QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) was used for cDNA synthesis (in duplicate) with 500 ng of RNA, and included the additional removal of genomic DNA (gDNA Wipeout Buffer). Genes of interest were chosen based on functionality within the mucosal barrier and functionality related to local and systemic immune responses. Distribution and lengths of introns and exons within genes of interest were found using Ensembl (https://www.ensembl.org/index.html). Primers were designed to span intron/exon boundaries using Primer3 version 0.4.0 (https://bioinfo.ut.ee/primer3-0.4.0/) if possible, and checked for interspecies-variation using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were synthesized by Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, United States). A total of 96 primer pairs were used for gene expression analysis. Primer sequences, corresponding genes, primer efficiency, and amplicon lengths can be found in Supplementary File 2.

For pre-amplification, three µl TaqMan PreAmp Master Mix (Applied Biosystems, Waltham, MA, United States), 2.5 µl 200 nM mix of each of the 96 primer pairs, 2 µl low-EDTA TE-buffer (Panreac Applichem, Darmstadt, Germany) and 2.5 µl diluted cDNA (1:10 in low-EDTA TE-buffer) was mixed and incubated at 95 °C for 10 min followed by 19 cycles of 95 °C for 15 s and 60 °C for four min. The pre-amplified cDNA was treated with 16 U Exonuclease I (New England Biolabs, Ipswich, MA, United States) for 30 min at 37 °C, followed by 80 °C in 15 min. Prior to qPCR analysis, the pre-amplified cDNA was diluted 1:10 in low-EDTA TE-buffer. Microfluidic qPCR was performed using a Biomark HD Reader (Fluidigm, CA, United States) with the following cycle parameters: 50 °C for 2 min, 95 °C for 10 min followed by 35 cycles at 95 °C for 15 s and 60 °C for 1 min. Melting curves were generated as described previously. after the 35 cycles of amplification. For each time point and tissue type, data were pre-processed and normalized separately using GeneEx5 Real-Time PCR Analysis software (MultiD, Göteborg, Sweden), as described earlier by Brogaard et al. (Brogaard, Klitgaard et al. 2015). For each tissue origin (ileum or colon), reference genes were selected using the algorithms geNorm and NormFinder (Vandesompele, De Preter et al. 2002, Andersen, Jensen et al. 2004). Primer pairs that resulted in inconsistent replicates were excluded from the dataset. Paired t-tests were performed using Excel 2012 (Microsoft, Redmond, WA, United States), and a dataframe with resulting p-values was imported into R statistical software (version 3.6.1). P-values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (fdr) correction, and resulting adjusted p-values (p.adjust) were used in combination with mean fold-change values (between treatments) to create volcano plots. Exclusively genes with a fold change > 2 were provided with a label and genes were considered to be statistically differentially expressed if p-adj. < 0.05.

Serology

The antibody response against *Salmonella enterica* serovar Typhimurium vaccination was measured using an ELISA. The live attenuated strain of *Salmonella enterica* serovar Typhimurium (Salmoporc®) was grown overnight at 37°C on MacConkey agar (Sigma-Aldrich). Next, a single colony was inoculated into 2 mL of lysogeny broth medium and incubated overnight at 37°C with shaking (200 rpm). The following day, 1 mL of this overnight culture was transferred to a 50 mL tube that contained 15 mL of fresh LB medium. The 50 mL tube was then placed on a shaker (200 rpm) at 37°C under aeration until exponential growth phase (OD_{600nm} = 0.6 to 0.8) was reached. Bacteria were then pelleted by centrifugation at 10,000 rpm for 5 min, followed by two washes with cold PBS. Next, this bacterial suspension (2×10^8 bacteria/mL, 100 µL/well) was used to coat 96 well plates (medium-binding flat bottom clear wells, Greiner Bio-One) that were incubated overnight at 4°C. The following day, the bacterial suspension was removed and bacteria still attached to the plate were fixed with 4% paraformaldehyde in PBS for 2 h at RT. Plates were then blocked overnight at RT with a blocking solution consisting of 5% milk powder (ELK, FrieslandCampina) in demi water. After overnight blocking, plates were stored at 4°C until use. Just before use, plates were washed with PBS/Tween20 (0.05%) and 100 µL of sera diluted in blocking solution (250× to determine IgG levels and 50× for IgA and IgM) were added. After 1 h of incubation at RT, plates were washed two times. Next, 100 µL horseradish peroxidase (HRP) conjugated goat anti-Porcine IgM, IgA, or IgG (Novus Biologicals) diluted 1:50,000 in blocking solution was added to the plates. After approximately 30 min, plates were washed five times and incubated with 100 µL of 3,3',5,5'- tetramethylbenzidine (TMB) substrate solution (Enhanced K-Blue®, Neogen). After 15 min the reaction was stopped by adding 100 µL of stop solution (2% HCl). The optical density (OD) of the plate content was measured at 450nm (Multi-Mode Microplate Reader FilterMax F5).

Isolation of Immune Cells

Peripheral blood mononuclear cells (PBMCs) were isolated within 4 h using 50 mL Leucosep™ tubes (Greiner Bio-One, Alphen a/d Rijn, Netherlands) filled with 60% FICOLL-PAQUE™ Plus density-gradient according to manufacturer's protocol. All blood samples were diluted 1:1 with PBS (containing 0.5 mM EDTA) prior to cell isolation. Any remaining red blood cells were lysed with ACK lysis buffer (Gibco®). To collect MLN cells, lymph nodules were cut into small pieces, gently squeezed with a syringe plunger, and passed through a sterile Falcon® cell strainer (100 µm, Corning®) using sterile Mg^{2+} and Ca^{2+} free PBS (Lonza, Basel, Switzerland). Isolated PBMCs and MLN cells were stored overnight in PBS at 4°C until further processing.

Cell Stimulation Assay

Dilutions of LPS (serotype O55:B5/L2880, Sigma-Aldrich), Concanavalin A (ConA, C2010, Sigma-Aldrich), or cell culture medium only (no stimulus) were prepared the previous day and stored at 4°C overnight. The following day, plates were placed at 37°C for 30 min for temperature adjustment before adding cells. Next, PBMCs and MLN cells were seeded into 96 well clear round-bottom plates (Greiner Bio-One) at a final concentration of 1×10^6 cells/200 µL. Cells were incubated with 5, 2.5, or 1.25 µg/mL of ConA, with 10, 1, or 0.1 µg/mL of LPS, or without stimuli for 24 h at 37°C (5% CO2). After 24 h of incubation, plates were centrifuged at 300 × g for 3 min, and cell culture supernatant was collected and stored in 96 well polypropylene plates (Nunc®, MicroWell™, Sigma-Aldrich) at −80°C until further

processing. On the day of analysis, cell culture supernatant was thawed for measurement of Tumor Necrosis Factor alpha (TNFα) and Interleukin 10 (IL-10) using DuoSet ELISA kits (R&D systems, Minneapolis, MN, United States) according to the manufacturer's instructions.

Flow Cytometry

The PBMCs and MLN cells were detected with DC or T lymphocyte/NK cell antibody panels (Table 2). For cell analysis, 5×10^6 (DC panel) or 1×10^6 (T lymphocyte/NK) PBMCs or MLN cells were transferred to 96 well polypropylene plates (Nunc®, MicroWell™, Sigma-Aldrich). Next, 200 µL/well FACS buffer (Mg^{2+} and Ca^{2+} free PBS; Lonza), 2 mM EDTA (Merck), 0.5% BSA fraction V (Roche) was added, and cell were washed by centrifugation at 400 × g for 3 min at 4°C. Next, the expression of markers specific for the various subsets of immune cells was determined by extracellular staining of cells with an antibody mix for 30 min on ice (in the dark), followed by one washing step with FACS buffer and two washing steps with cold PBS. Then, cells were stained with (in PBS diluted) Streptavidin-BV421 (DC panel) to detect CADM1 and Fixable viability dye eFluor™ 506 (eBioscience™) to discriminate live and dead cells. After incubation of 30 min, cells were washed with FACS buffer. To stain intracellular proteins, 100 µL Fix/Perm buffer (eBioscience™) was administered to each well, followed by a 45 min incubation at RT. Next, cells were washed three times in Perm buffer (eBioscience™), followed by incubation with an intracellular marker-specific antibody mix (Table 2) in 35 µL Perm buffer for 30 min at 4°C. Next, cells were washed two times with Perm buffer, and resuspended in 200 µL FACS buffer. Cells were measured for 300 s (DCs) or 150 s (NK cells/T cells) on the FACS CANTO II (medium flow rate). Beads (UltraComp eBeads™, Thermo Fisher Scientific) were used for single-color compensation controls. All panels were validated using Fluorescence Minus One (FMO) controls. Flow cytometry data analysis was performed using FlowJo™ software (Version 10). Identification of DCs, NK cells, and T cells was done according to previous studies (Gerner, Käser et al. 2009, Auray, Keller

Table 2. List of antibodies used for identifying DCs, NK cells, and T cells in PBMCs and MLN cells.

Antibody	Host/Isotype	Clone	Fluorochrome	Company	Dilution
Antibody panel for the identification of DC subsets and DC					
CD14	Mouse, IgG2b	MIL-2	FITC	Bio-Rad	1:50
CD172a	Mouse, IgG2b	74-22-15A	PE	BD Biosciences	1:40
CD4a	Mouse, IgG2b	74-12-4	PerCP-Cy5.5	BD Pharmingen™	1:320
CADM1	Chicken, IgY	3E1	Biotin	MBL	1:200
Streptav.	n/a	n/a	BV421	BD Horizon™	1:50
CD152 [a]	Mouse, IgG2a	n/a	APC	Ancell	1:320
Antibody panel for the identification of NK cells and T cell subsets					
CD3ε	Mouse, IgG2a	BB23-8E6-8C8	PE-Cy™7	BD Pharmingen™	1:160
CD4a	Mouse, IgG2b	74-12-4	PerCP-Cy5.5	BD Pharmingen™	1:320
CD8a	Mouse, IgG2a	76-2-11	FITC	BD Pharmingen™	1:10
FoxP3 [b]	Rat, IgG2a	FJK-16s	Alexa Fluor® 700	eBioscience™	1:20
Ki67 [b]	Mouse, IgG1	B56	BV421	BD Horizon™	1:80
CD25 [c]	Mouse, IgG1	K231.3B2	Purified	Bio-Rad	1:200
γδ T cells	Rat, IgG2a	MAC320	PE	BD Pharmingen™	1:20

a. Human CD152 (CTLA-4) murine Ig fusion protein for the identification of CD80 and CD86 on DCs.
b. Antibodies against intracellular antigens.
c. The ReadiLink™ Rapid iFluor™ 647 Antibody Labeling Kit (Aat Bioquest) was used to identify CD25.

et al. 2016, Vreman, Auray et al. 2018), and details of the gating strategies are shown in Supplementary Figure 3 (DCs) and Supplementary Figure 4 (NK cells/T cells).

Colony PCR
During dissection, the ileocecal MLN was removed, rinsed with Milli-Q water, and stored in ice-cold PBS. Within 12 h after dissection, separate nodules of the MLN were passed through a cell strainer (BD Falcon™ Nylon 100 µm Cell Strainer) in a Biosafety Cabinet, and 1 mL of the MLN cell suspension was stored in an Eppendorf tube at 4°C. The next day, 100 µL of the MLN cell suspension was plated on MacConkey (MAC), incubated overnight at 37°C, and stored at 4°C for later use. A subset of red/pink colored colonies was picked, and a colony PCR (cPCR) was performed to identify EcN colonies using EcN-specific primers (see Table 1). For each reaction, 12.5 µL Onetaq buffer, 0.5 µL dNTPs (10 mM), 0.5 µL of each primer (10 µM), 0.125 µL Onetaq polymerase were combined with H2O to reach a final volume of 25 µL and were incubated at 95°C for 5 min followed by 35 cycles of 95°C for 45 s, 60°C for 45 s and 72°C for 45 s. Resulting amplicons were visualized using a 1% agarose gel.

Statistical Analyses
To estimate the impact of the probiotic intervention on alpha diversity of the microbiota in pig faeces, Shannon diversity and Observed Species richness were calculated for each sample at ASV resolution. In addition, Shannon diversity and Observed Species richness were similarly calculated for the phylum Proteobacteria, by first filtering out ASVs from the phylum Proteobacteria using the 'prune_taxa' function of the phyloseq R package (McMurdie and Holmes 2013). The Observed Species index sums the number of bacterial taxa identified in each sample, while the Shannon diversity index additionally takes into account the relative abundance of each taxon. It should be noted that rare sequences are missing from the dataset as NG-Tax 2.0 uses an abundance threshold of 0.1% for ASVs in a given sample. Shannon diversity and Observed Species richness were used in a Linear Mixed-Effects Model (i.e., Shannon ~ Day_of_study * Treatment) to test for significant differences between treatment groups (lme function of nlme package) (Pinheiro, Bates et al. 2015). For this analysis, complete faecal sample datasets were tested, but also subsets thereof (pre- or post-weaning faecal samples exclusively). To provide additional insights into the relative abundances of ASVs within the Proteobacteria phylum, a heatmap was generated that contains all faecal samples of the pre-weaning period, with a clear separation between treatment groups. To estimate the impact of EcN on overall microbiota composition, PERMANOVA tests were performed using Bray-Curtis dissimilarities at ASV level (9,999 permutations). The homogeneity assumption was tested by calculating Bray–Curtis dispersion for each group followed by ANOVA tests. To visualize the beta diversity in faecal samples over time, a Principal Coordinates Analysis (PCoA) plot was generated using Bray-Curtis dissimilarities (Supplementary Figure 5). Beta diversity was calculated at the ASV level using Bray-Curtis dissimilarities and a PERMANOVA test was performed on all faecal samples with treatment and time point as model parameters (adonis function of the vegan R package (Shankar, Agans et al. 2017). In order to estimate the impact of EcN as a probiotic intervention on the relative abundance of specific genera in pig faeces, ASV counts were first aggregated at genus level using the 'tax_glom' function of the phyloseq R package (McMurdie and Holmes 2013), after which read frequencies were transformed to compositional data using the 'transform' function of the microbiome R package (Lahti

and Shetty 2017). Genera were filtered to exclusively include genera that had a relative abundance of 0.1% in at least 30% of the samples (prevalence filter). Resulting genera were tested for differential relative abundance using the Generalized Additive Models for Location, Scale and Shape (GAMLSS) with a zero-inflated beta family (function taxa.compare) with GAMLSS-BEZI as a statistical method in the metamicrobiomeR package (Ho, Li et al. 2019). Probiotic intervention was used as the main variable for comparison and day of study and department (farrowing rooms) were used as adjusting variables. Ear tag was indicated as the identifier to enable a longitudinal approach, and "fdr" (False Discovery Rate) was chosen as the method for multiple testing adjustment (p.adjust < 0.05 as the threshold). For this analysis, complete faecal sample datasets as well as subsets (pre- or post-weaning faecal samples exclusively) were tested.

A Linear Mixed Model (LMM) was used to assess the immunological effects over time and the interaction between time and treatment, using R statistical software (version 3.6.2). Additionally, unpaired Student's t-tests were performed to analyze the differences between the treatment groups per time point. Normality of data (Shapiro–Wilk test) and homogeneity of variances (Levene's test) were checked prior to statistical testing. Skewness values between −2 to +2 were considered acceptable (George 2011). Extreme outliers (indicated by R) were removed from the analysis. When normality of data or homogeneity of variances were not met, data were log-transformed but presented as untransformed means. Results with an adjusted p-value below 0.05 were considered statistically significant and results between 0.05 and 0.1 were considered a trend.

For a complete and detailed overview of the statistical analyses, all R-scripts, data files, and pdf files with extensive information on the performed analyses can be accessed through the following doi: 10.4121/15060177. Alternatively, these files can be found under the following Github page: https://github.com/mibwurrepo/Geervliet-et-al-2022-porcine-study-EcN.

Results

Spatio-Temporal Dynamics of EcN Occurrence

To investigate the spatio-temporal dynamics of EcN following peroral administration, faeces and digesta were collected at different time points during the study. After analyzing faecal samples that were spiked with EcN, we identified only a single EcN-specific ASV that corresponded to the amount of EcN in spiked samples (Supplementary Figure 2A). The amount of EcN identified with this EcN-specific ASV correlated well (R^2 = 0.84) with the amount of EcN identified by qPCR (Supplementary Figure 2B). Therefore, the EcN-specific ASV in the NGS data was used to assess the relative abundance of EcN in all faecal samples. The relative abundance of EcN was variable among pigs in the EcN treatment group, and in only a small number of treated animals EcN was detected at all pre-weaning time points (Figure 2). EcN was not detected in digesta or faeces of control animals, with the exception of one animal with a very low relative abundance of EcN (6.2×10^{-5}) in faeces on day 4 of the study. EcN could not be detected in faeces of two EcN-treated pigs throughout the pre-weaning period. Peak abundances of EcN were observed on days 8 and 14 of the study, followed by a substantial decline toward day 26. Interestingly, EcN was also detected by

colony PCR in the MLN of one pig on day 27, as indicated by the arrow in Supplementary Figure 6. EcN was not detected in post-weaning faecal samples, despite the fact that the treatment group still received EcN up to day 44 (data not shown). EcN was detected in jejunum, ileum, and caecum digesta in a few individuals on day 27, while on day 44 no EcN was found in any of the gut segments. Interestingly, EcN was detected in jejunal or ileal digesta of three pigs at the end of the study (day 70), which illustrates that EcN persisted in some individuals for at least 26 days after the final administration (Supplementary Figure 7). Finally, no adverse health effects were observed after peroral administration of EcN, and there were no indications that EcN had a negative effect on body weight (Supplementary Figure 8).

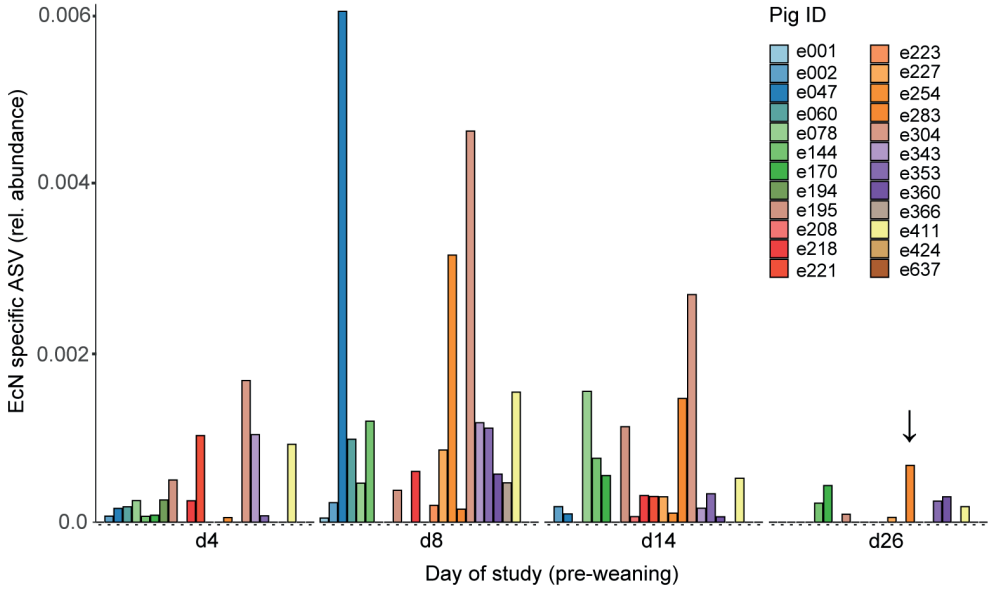


Figure 2. Presence of EcN in faeces from EcN-treated pigs during the pre-weaning period. Data are presented as the relative abundance of the EcN-specific ASV, as calculated from the NGS dataset. Colors correspond to an individual pig (Pig ID, n = 24). The arrow indicates the pig (e254) from which we also found EcN in the MLN by using colony PCR. EcN was not detected in the post-weaning period and therefore only the pre-weaning data is presented.

Effect of EcN Treatment on the Development of Intestinal Microbiota

Alpha and Beta Diversity

To assess potential effects of EcN on microbiota composition and diversity, faecal samples were collected at multiple time points during the study (Figure 1). No significant differences in alpha diversity were observed between the EcN treatment and control groups when including all time points and all microbial taxa (Supplementary Figure 9). Similarly, no significant differences in alpha diversity were observed when only including pre-weaning time points. As ASVs within the phylum of Proteobacteria were previously reported to be affected by EcN (Šmajs, Bureš et al. 2012), the alpha diversity of this phylum was investigated

in more detail after removing ASVs belonging to other phyla. Interestingly, a significantly lower number of Proteobacteria ASVs were detected in EcN-treated animals during the pre-weaning period ($p < 0.04$, Figure 3). A more detailed heatmap with all Proteobacteria ASVs that includes all pre-weaning time points is presented in Supplementary Figure 10. Especially ASVs within the family of *Enterobacteriaceae* contributed to a higher number of Proteobacteria ASVs in the control group (Figure 3).

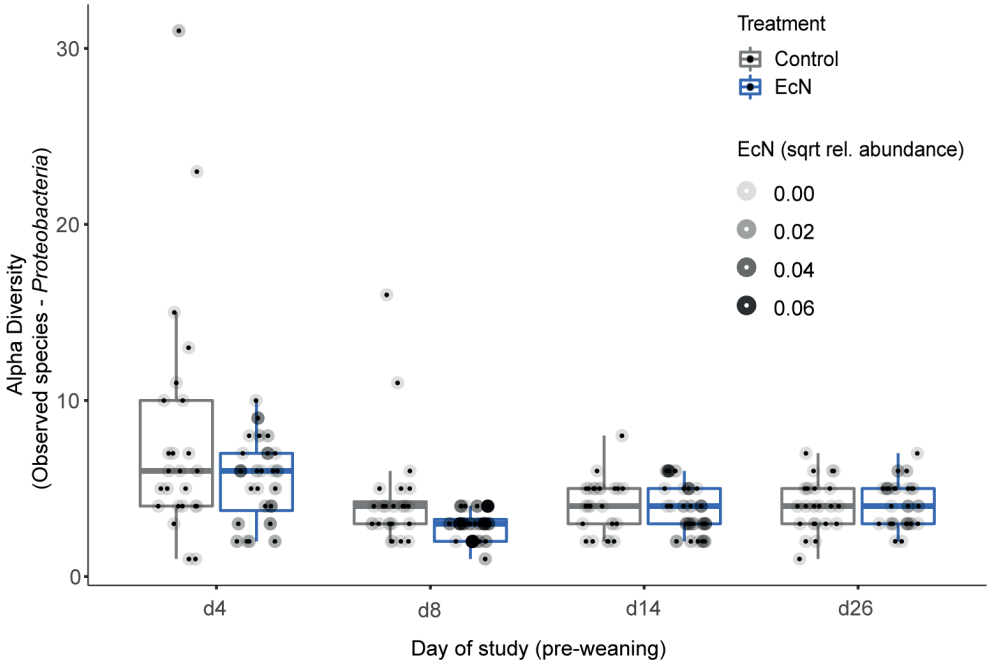


Figure 3. Comparison of the number of observed ASVs in the Proteobacteria phylum in faecal samples between treatments and over time (pre-weaning). Every point represents a single animal within the control group (grey box) and the treatment group (blue box). Observed Species richness values are given by sampling time point (d4–26). A significant difference in the amount of Proteobacteria ASVs was found when including only pre-weaning time points. For each sample, the relative abundance of the EcN-specific ASV is visualized by tinting the outer circle (a darker tint means a higher abundance of EcN).

The effect of the probiotic treatment on microbiota beta diversity was significant ($p < 0.015$). In addition, a clear time effect was observed ($p < 0.0001$), which is also apparent from Supplementary Figure 5. When only including pre-weaning faecal samples, the effect of treatment was more significant ($p < 0.005$), but with a low coefficient of determination ($R^2 < 0.011$). A statistically significant treatment effect was not observed when only post-weaning samples were included ($p > 0.075$, $R^2 < 0.014$), which indicates that EcN had a more pronounced effect on microbial composition during the pre-weaning period.

Differentially Abundant Genera Between the Treatment Groups

To investigate the effects of EcN on specific genera, the relative abundance of individual genera in faeces were compared between the treatment groups. A GAMSLS model was used to identify significantly differently abundant genera. This resulted in the detection of six (out of 71) genera, including *Treponema_2, Holdemanella, Olsenella, Coprococcus, Catenisphaera*, and an unclassified genus in the family of *Prevotellaceae*, that were differentially abundant between the treatment groups (Figure 4). When only including pre- or post-weaning timepoints, six genera (out of 60) and two genera (out of 87) were found to be differentially abundant in faecal samples, respectively (Supplementary Figures 11, 12). Furthermore, a correlation was observed between the amount of EcN present in faecal samples, and the relative abundance of *Treponema_2*; samples that had a relative abundance of EcN higher than 0.05% did not contain the *Treponema_2* genus (Figure 4B). This relation was only observed during the pre-weaning period, since EcN was not detected in any of the post-weaning faecal samples. A complete overview of the presence of ASVs within the *Treponema_2* genus in faeces from both treatment groups can be found in Supplementary Figure 13A. As it was previously demonstrated that ASVs within the genus of *Treponema* can be characterized up to species level using 16S rRNA amplicon sequencing (Hallmaier-Wacker, Lüert et al. 2019), each ASV's forward sequence was run through BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This resulted in the detection of two ASVs with a 100% match with an earlier described invasive *Treponema* species (Mølbak, Klitgaard et al. 2006), namely *Candidatus Treponema suis* (Supplementary Figure 13B). Taken together, these results show that EcN affected the relative abundance of specific genera in the gut, and that most of these changes were observed during the pre-weaning period.

Intestinal Epithelial Gene Expression

Gene expression analysis of mucosal scrapings revealed several differentially expressed (>2-fold) genes between the treatment groups (Figure 5, orange points). In the ileum, the genes IDO1 and IL1RN had a lower mean expression in EcN-treated animals on day 27, whereas IFNG showed a higher mean expression in the EcN group on day 44 (Figures 5A,B). As for the colon, a larger number of genes were differentially expressed between the treatment groups (Figures 5C,D). However, after correction for multiple testing, none of these genes were significantly differentially expressed. Comparison of data from day 27 (pre-weaning) with data from day 44 (post-weaning) demonstrated that twenty genes in the colon (Figure 5E) and 11 genes in the ileum (Figure 5F) were significantly differentially expressed (green points) between the time points.

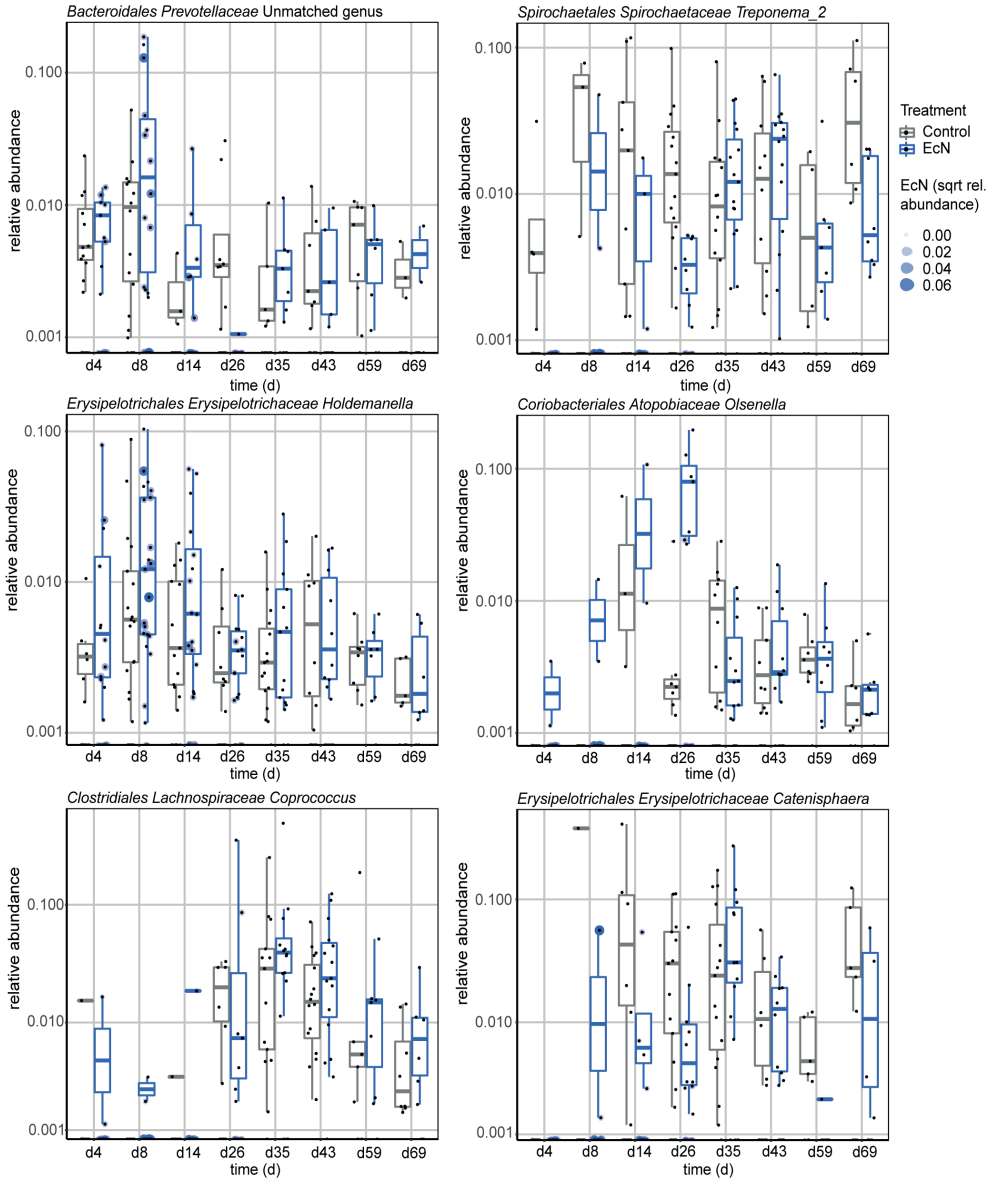


Figure 4. Relative abundances of differentially abundant genera in faeces during the complete study period, including an unclassified genus in the family of *Prevotellaceae* **(A)**, *Treponema_2* **(B)**, *Holdemanella* **(C)**, *Olsenella* **(D)**, *Coprococcus* **(E)**, and *Catenisphaera* **(F)**. Shown genera resulted from comparing EcN-treated animals to control animals using a GAMLSS model. Probiotic intervention was used as the main variable for comparison and day of study and department were used as adjusting variables. Ear tag was used as identifier and "fdr" was chosen as the method for multiple testing adjustment (p.adjust < 0.05).

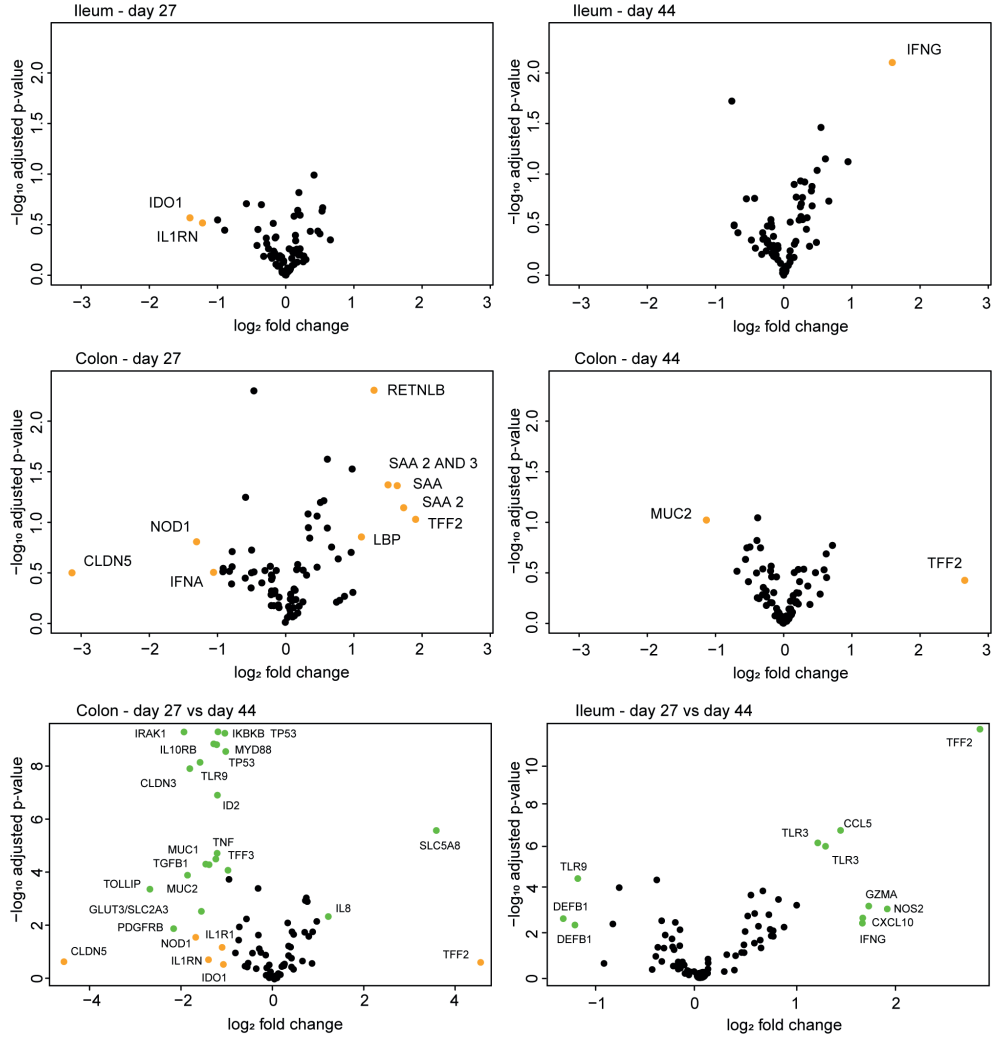


Figure 5. Volcano plots of differentially expressed genes between the control group and the EcN group (n = 8 per group) in mucosal scrapings of the ileum **(A,B)** and colon **(C,D)** on day 27 (pre-weaning) and day 44 (post-weaning) of the study. No significantly differentially expressed genes were observed between the treatment groups in any gut segment or time point. Fold change on the x-axis depicts the fold-change difference in the EcN treatment group compared to the control group. When comparing all samples from the colon **(E)** or ileum **(F)** from day 27 with those of day 44 (both treatment groups), several differentially expressed genes were identified. Fold change on the x-axis depicts the fold-change difference between time points. Orange points indicate the differentially expressed genes with a fold change > 2. Green points indicate differentially expressed genes with a fold change > 2 and a p-adj. < 0.05.

Effect of EcN Treatment on the Immune System

Vaccine Specific Antibody Responses

To assess the effect of EcN on the responsiveness of the immune system, a live-attenuated vaccine against *Salmonella enterica* serovar Typhimurium (Salmoporc®) was administered perorally on days 21 (primary vaccination) and 45 (booster vaccination) of the study. On days 14, 35, and 69 blood serum was collected and analyzed for vaccine-specific IgM, IgA, and IgG (Figures 6A–D). As expected, *Salmonella*-specific immunoglobulins increased significantly upon vaccination. *Salmonella*-specific IgM and IgA increased after both the primary and booster vaccination, whereas *Salmonella*-specific IgG increased only after the booster vaccination. Temporal analysis revealed that *Salmonella*-specific IgM in serum of EcN-treated was significantly lower than the control group ($p = 0.001$). A lower vaccine-specific IgM level could already be observed on day 14 of the study ($p = 0.031$), which was 1 week before the first vaccination with Salmoporc®. No significant differences between the EcN and control groups were found for *Salmonella*-specific IgA and IgG.

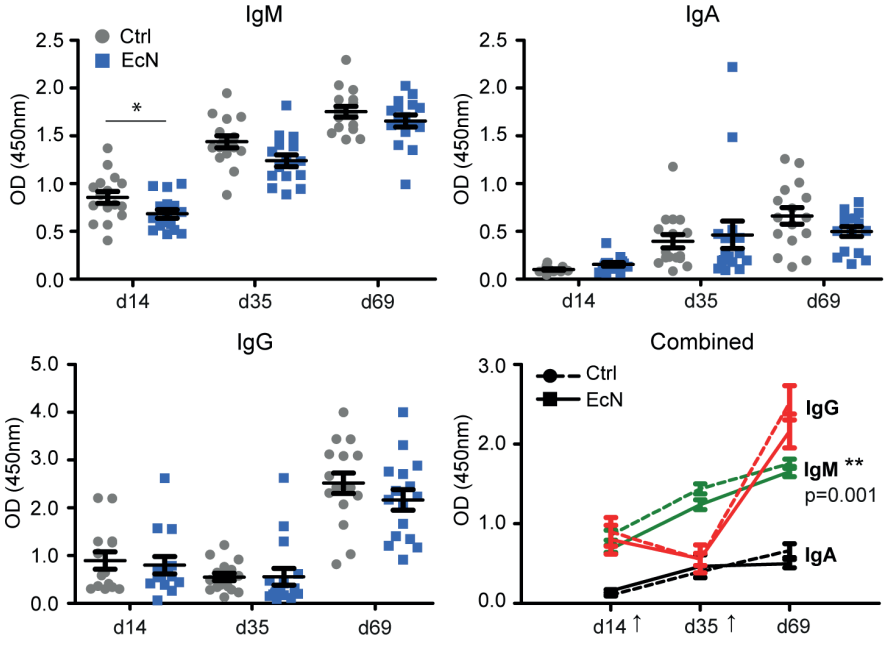


Figure 6. Serum levels of *Salmonella*-specific IgM, IgA, and IgG **(A–C)** prior to vaccination (day 14), 2 weeks after the initial vaccination (day 35), and 3 weeks after the booster vaccination (day 69). Plot **(D)** incorporates all results **(A–C)**, with the arrows indicating the time of vaccination (days 21 and 45). Every symbol represents a single animal. Two EcN-treated animals (squares; ■, solid lines) and two control animals (circles; •, dashed lines) were randomly selected from every pen, and followed over time (n = 16 per treatment group). Data are presented as the mean ± standard error of the mean (SEM). Asterisks are used to demonstrate significant differences between treatment groups after taking into account single time points (*$p < 0.05$ and **$p < 0.01$), and p-values indicate significant differences between treatment groups over time.

***Ex vivo** Stimulation of Peripheral Blood Mononuclear Cells and Mesenteric Lymph Node Cells*

To assess whether EcN modulated TNFα and IL-10 secretion, different concentrations of LPS and ConA were used for *ex vivo* stimulation of isolated immune cells. Temporal analysis (using a LMM) illustrated that EcN did not affect TNFα production by stimulated PBMCs and MLN cells (data not shown). In contrast, PBMCs from EcN-treated animals produced a lower amount of IL-10 upon stimulation with LPS in comparison to the control group (Figures 7A–C). This result was observed after stimulation with 10, 1, as well as 0.1 µg/mL LPS ($p = 0.057$, $p = <0.001$ and $p = 0.001$, respectively). PBMCs stimulated with ConA also produced a lower amount of IL-10 (Supplementary Figure 14). There were no significant differences in IL-10 production by stimulated MLN cells. However, LPS stimulated MLN cells from EcN-treated animals produced significantly higher amounts of IL-10 when only taking into account the pre-weaning time point (day 27) (Figures 7D–F). The same trend was observed after stimulation with 5 µg/mL of ConA ($p = 0.07$, Supplementary Figure 14). Interestingly, unstimulated MLN cells from EcN-treated animals also produced higher levels of IL-10 in comparison to unstimulated MLN cells from control animals ($p = 0.016$), and again this was only observed pre-weaning.

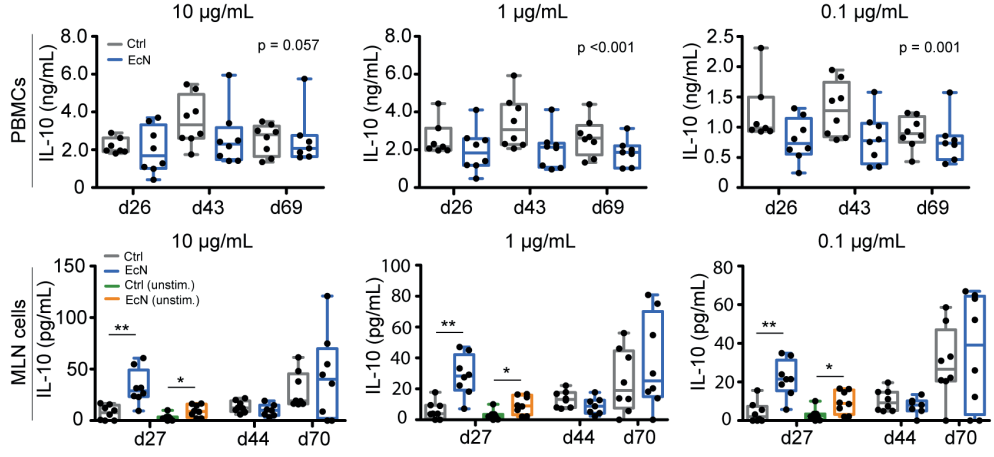


Figure 7. IL-10 production upon *ex vivo* stimulation of PBMCs and MLN cells. PBMCs **(A–C)** and MLN cells **(D–F)** were stimulated for 24 h with 10, 1, and 0.1 µg/mL of LPS, or left unstimulated (cell culture medium only). P-values indicate significant differences between treatment groups over time **(A–C)**, and asterisks are used to demonstrate significant differences between treatment groups after taking into account single time points (*$p < 0.05$ and **$p < 0.01$, **D–F**). Every point represents an individual animal from a separate pen (n = 7 or 8 per treatment group), and error bars represent standard deviations. Normal distribution and equal variances of data were checked and log-transformed when necessary. No differences in IL-10 production by unstimulated MLN cells were observed post-weaning and are therefore not presented.

Innate and Adaptive Immune Cell Analysis

To determine whether there was an effect of EcN on specific innate and adaptive immune cell populations, cell analysis was performed on isolated PBMCs (Supplementary Table 1) and MLN cells (Supplementary Table 2) from a subset of animals. The number of DCs (pDC, cDC1, and cDC2) or DC maturation status (determined by CD80/86 upregulation) was not significantly different between the EcN and control groups. In addition, no differences between the control and treatment group were observed for other immune cells from the MLN (Supplementary Table 2). Temporal analysis of immune cells from PBMCs revealed that EcN significantly ($p = 0.010$) enhanced expression of the activation marker CD25 on NK cells ($CD3^{-}CD8\alpha^{+}$), which was most evident on day 26 pre-weaning ($p = 0.015$, Figure 8A). However, EcN treatment did not significantly increase NK cell numbers in PBMCs (Supplementary Table 1). Furthermore, temporal analysis of specific T cell populations showed that the percentage of memory/activated T cells ($CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD8\alpha^{+}$) was significantly lower ($p = <0.001$) in the EcN treatment group (Figure 8B). Moreover, analysis of single time points showed a significant reduction of memory/activated T cells on day 44 ($p = 0.042$). Similarly, a significant reduction of regulatory T cells ($CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD25^{high}Foxp3^{+}$) was observed on day 44 of the study ($p = 0.016$, Figure 8C). Next to cell number, maturation and activation, proliferative responses of different T cell populations were investigated by using the proliferation marker Ki67. EcN-treated animals had a significantly higher percentage of Ki67-positive T helper cells ($CD3^{+}TCR\gamma\delta^{-}CD4^{+}$) and memory/activated T cells ($CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD8\alpha^{+}$) on day 14 of the study ($p = 0.011$ and $p = 0.030$, respectively) (Figures 8D,E). Albeit not significant, a similar pattern could be observed for $CD3^{+}TCR\gamma\delta^{+}$ $CD8\alpha^{+}Ki67^{+}$ T cells (cytotoxic T cells, Figure 8F).

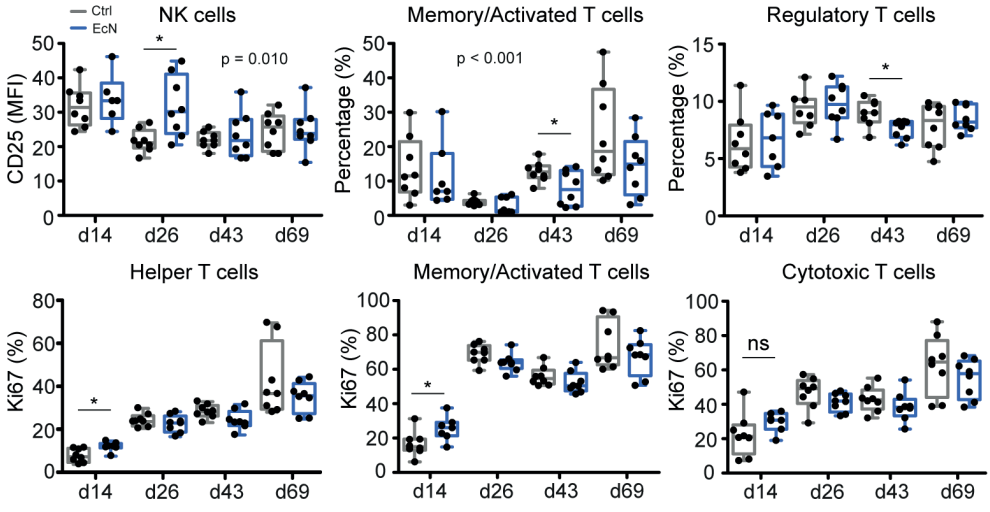


Figure 8. Analysis of innate and adaptive immune cells in PBMCs. P-values indicate significant differences between treatment groups over time **(A,B)**, and asterisks are used to demonstrate significant differences between treatment groups after taking into account single time points (*$p < 0.05$, **C–E**). Data were presented as the mean fluorescent intensity (MFI; **A**), percentage of cells (%; **B,C**), or percentage of proliferating cells (Ki67%; **D–F**). Every point represents an individual animal from a separate pen (n = 7 or 8 per treatment group), and error bars represent standard deviations. Normal distribution and equal variances of data were checked and log-transformed when necessary.

Discussion

For over 100 years EcN has been extensively studied *in vitro* or in animal models to understand its probiotic properties, but to date, no studies have investigated its effects on the porcine gut microbiota and the immune system when administered in early life. It is well known that the conditions (e.g., diet and housing) in the neonatal phase and during early life shape the development of the GIT and the immune system (Zmora, Suez et al. 2019). Therefore, we hypothesized that by administration of EcN in the immediate postnatal period leads to colonization of the intestine and competition with overt pathogens and pathobionts that often increase in abundance due to stressors in the post-weaning period. In addition, we hypothesized that gut-adherent EcN would interact with the gut epithelial barrier and the innate and adaptive arms of the immune system.

The EcN was detected in faecal samples for at least 4 weeks (primarily pre-weaning) after administration of EcN every other day from day 2 until day 44. EcN reached highest abundance in faeces on days 8 and 14 but was not detected after weaning. This may be due to the increasing complexity of the gut microbiota over time, and the dramatic shift of the gut microbiota as a result of weaning (due to changes in diet and housing). These factors may hinder growth, subsequent colonization and detection of EcN in the GIT. However, in three piglets EcN was detected in jejunal or ileal digesta at the end of the study (day 70), demonstrating that in some pigs EcN can persist in the GIT. This is consistent with other studies that showed that EcN could be detected in faeces of post-weaning pigs after oral administration (Duncker, Lorentz et al. 2006), and in pigs that were not deliberately treated with EcN (Kleta, Steinrück et al. 2006). In another study where EcN was administered to 3-month-old pigs for seven consecutive days, EcN was detected in faeces approximately 4 weeks after the last administration (Barth, Duncker et al. 2009). This may be due to the larger number of EcN administered (i.e., 3.75–37.5 × 10^8 CFU EcN per kg of body weight per day) or the use of a different detection method. Another study administering EcN (3.5 × 10^{10} CFU) for 14 consecutive days to 4–5 months old pigs, reported that EcN was not recovered from intestinal contents using cultivation-dependent methods (Šmajs, Bureš et al. 2012). Collectively these studies indicate that EcN can persist in the intestine during the pre-weaning period, but is not efficiently colonizing the porcine GIT. This is consistent with studies on EcN colonization in humans (Wassenaar 2016), and can also be explained by the fact that facultative anaerobic bacterial species typically represent only 0.1% of the bacteria that are present in the anaerobic environment of the colon (Eckburg, Bik et al. 2005). Colonization of EcN in an established intestinal microbiome may also be more challenging, which could explain why EcN administration does not lead to colonization or persistence post-weaning.

Since EcN was primarily present pre-weaning, it was considered likely that EcN exerted effects on the gut microbiota composition during this period. We found that EcN reduced the diversity within the phylum of Proteobacteria in the porcine GIT during the pre-weaning period, as shown in another study (Šmajs, Bureš et al. 2012). These results are in line with the antagonistic effects of EcN on growth of closely related taxa in the family of *Enterobacteriaceae* (Lodinová-Žádníková and Sonnenborn 1997, Jacobi and Malfertheiner 2011, Sassone-Corsi, Nuccio et al. 2016, Sonnenborn 2016). Moreover, our results show

that presence of EcN altered the relative abundance of specific genera, with a particularly interesting correlation for the genus *Treponema_2*; relative presence of EcN higher than 0.05% in faecal samples corresponded with absence of the *Treponema_2* genus. This is an interesting finding, given *Treponema* is often associated with pathogenic species (Mølbak, Klitgaard et al. 2006, Hallmaier-Wacker, Lüert et al. 2019, Giffin, Lankapalli et al. 2020). The antagonistic effect against the *Treponema* genus is in line with observed effects from other known probiotics such as *Bifidobacterium* species (Belkacemi, ALOU et al. 2020), *Lactobacillus fermentum* and *Pediococcus acidilactici* (Wang, Yao et al. 2019). The genus *Treponema* contains a number of pathogenic and symbiotic bacteria that can be found in vastly different anatomical and environmental habitats (Hallmaier-Wacker, Lüert et al. 2019). In 2006, the 16S rRNA gene of a *Treponema* strain was PCR amplified after isolation in pig host tissue. This strain, also known as *Candidatus Treponema suis,* was found to be associated with colitis considering its invasion of the surface epithelium as well as superficial parts of the mucosa (Mølbak, Klitgaard et al. 2006). Two of the *Treponema_2* ASVs within our dataset were identical in sequence to the 16S rRNA gene of this strain. When plotting these two ASVs over time, a very interesting temporal pattern could be discerned in both treatment groups (Supplementary Figure 13B), with higher abundances observed in the immediate post-weaning period up to 2 weeks post-weaning (days 35 and 43, respectively). As piglets are known to suffer from post-weaning diarrhoea especially in the first week post-weaning up to the second-week post-weaning, the potential role of *Candidatus Treponema suis* in post-weaning diarrhoea warrants further investigation. Although *Treponema* and Proteobacteria include harmless commensal bacterial species, they are primarily known as potential pathogens. Thus, the association between EcN and reduced Proteobacteria diversity (pre-weaning) as well as the potential antagonistic effect against *Treponema* suggest a beneficial effect on the porcine gut microbiota composition.

In contrast to the little information available regarding the effects of EcN on the gut microbiota, more is known about the bacterial–epithelial crosstalk between EcN and the intestinal epithelial cells (Sonnenborn and Schulze 2009, Wassenaar 2016). In mice with inflammatory colitis, EcN strengthened the epithelial barrier by enhancing expression of tight junction proteins ZO-1 (Ukena, Singh et al. 2007). In addition, EcN altered the expression and distribution of ZO-2 protein in *in vitro* cultured human epithelial cells (Zyrek, Cichon et al. 2007). Differential expression of these zonulin genes was not observed in our study, possibly due to the use of conventionally reared healthy animals. Significant differences in gene expression were observed between days 27 and 44, particularly in the colon. Albeit not significant, our data also showed that on day 27 nine genes were differentially expressed (at least twofold) in the colon of EcN-treated piglets, while on day 44 this was the case for only two genes. This could imply that EcN is more likely to induce changes in the gut epithelial barrier in the pre-weaning period.

Supplementation of EcN in early life aims at providing the gut with advantageous commensal bacteria that directly or indirectly (by affecting the gut microbiota composition or gut barrier function) influence the immune responsiveness. Considering the decrease of Proteobacteria diversity, EcN may have hindered pathogenic enterobacteria, thereby influencing the immune response. As for direct effects, several studies indicated that EcN activates Toll-like Receptor 4 (TLR-4) that recognizes the lipid A component of semi-rough type LPS present in the outer

cell membrane of EcN (Grozdanov, Zähringer et al. 2002, Grabig, Paclik et al. 2006, Adam, Delbrassinne et al. 2010), and has been identified along the porcine GIT and on porcine blood and lymphoid DCs (Gourbeyre, Berri et al. 2015, Auray, Keller et al. 2016, Shao, Fischer et al. 2016). Conversely, TLR-4 expression in the ileum, colon or MLN of 1-week-old gnotobiotic piglets was not altered by EcN (Splichal, Donovan et al. 2019). We measured no significant effects of EcN on genes involved in gut barrier function, or on DC maturation (upregulation of CD80/86). In our previous *in vitro* study, we showed an EcN concentration-dependent increase of the DC maturation marker CD80/86 upon stimulation with EcN (Geervliet, Lute et al. 2020), but in that study, DCs were in direct contact with the bacteria. Interestingly, we did detect a significant increase of the activation marker CD25 on NK cells in blood, which was most evident on day 26 of the study (pre-weaning). Activated NK cells produce a variety of inflammatory cytokines (e.g., IFN-γ), followed by cytokine-induced downstream signaling cascades that contribute to their immune response against infection (Abel, Yang et al. 2018). Previous studies have demonstrated that EcN is able to enhance the frequency and function of NK cells in systemic tissues and blood from gnotobiotic pigs after a virulent human rotavirus challenge (Vlasova, Shao et al. 2016, Michael, Paim et al. 2021). Moreover, EcN biofilm treatment also showed to enhance NK cell activity in blood mononuclear cells from malnourished piglets transplanted with human infant faecal microbiota (Michael, Paim et al. 2021). Our results demonstrate that EcN altered the immune responsiveness in the pre-weaning period, but further (challenge) studies are required to determine if enhanced NK cell responses induced by EcN indeed provide protection against infectious agents.

EcN did not only modulate innate immune responses, but also affected adaptive immune responses, the latter being the basis for effective immunization against infections. For example, a modest but significantly enhanced proliferation of T helper cells and Memory/Activated T cells in PBMCs was observed on day 14 of the study. On the other hand, EcN reduced the number of Memory/Activated T cells and regulatory T cells in blood, and enhanced IL-10 production by LPS stimulated MLN cells. These results suggest that EcN induced a more regulatory or anti-inflammatory state of the local gut-associated immune system. In contrast, temporal analysis revealed that LPS stimulated PBMCs from EcN-treated animals produced lower levels of IL-10, indicating different effects of EcN on the local (MLN) and systemic (PBMC) immune system. These differences could be explained by the fact that the MLN is the main site for tolerance induction, and a large number of cells (e.g., DCs) that reside in the MLN are still immature or naive. Moreover, the reduction of *Salmonella*-specific IgM in EcN-treated animals may also imply immune tolerance. However, a significant reduction of *Salmonella*-specific IgM was already observed prior to vaccination (day 14), which could mean that *Salmonella*-specific antibodies were already present in the pigs' blood or that antibodies to other *Enterobacteriaceae* cross-react with components of the ELISA. Our observation that EcN primarily induced changes pre-weaning supports our hypothesis that repeated administration of EcN in early life affects the porcine gut microbiota and the developing innate and adaptive arms of the porcine immune system. Our results also illustrated that EcN did not consistently persist during the post-weaning period, and that EcN did not affect the gut barrier function or the vaccination response. Compared to the pre-weaning period, effects observed post-weaning were modest. EcN's ability to reduce the diversity of taxa within the phylum of Proteobacteria, its seemingly antagonistic effect against *Treponema*, and its effect on cells (e.g., NK cell activation) and cell

responses (e.g., IL-10 production by MLN cells and PBMCs) imply beneficial effects, which should be further investigated in future infection studies. Taken together, the results of this study support the concept that the pre-weaning period provides a 'window of opportunity' to modulate the gut microbiota and the immune system through dietary interventions such as EcN.

Data Availability Statement

The data presented in the study are deposited in the 4TU.ResearchData repository, under the following doi: https://doi.org/10.4121/15060177.v1.

Ethics Statement

The animal study was reviewed and approved by Dutch Central Authority for Scientific Procedures on Animals (CCD).

Author Contributions

MG, HFJS, HS, ET, HV, and JW: conceptualization. MG, HV, GA, CW, and KS: data curation. MG, HV, CJ, KS, HFJS, HS, ET, and JW: methodology. MG and HV: software, validation, formal analysis, writing—original draft preparation, writing and editing, and visualization. MG, GA, ET, and HV: investigation. HH, MG, HFJS, HS, and HV: resources. CJ, VR, HFJS, HS, ET, and JW: supervision. HFJS, HS, and JW: project administration and funding acquisition. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This research was funded by The Dutch Research Council (NWO) and Vereniging Diervoederonderzoek Nederland (VDN), through grant number 868.15.030.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Acknowledgements

We would like to acknowledge and thank the staff of the Swine Research Centre for their technical assistance during the study. We would especially like to thank Carlijn de Bruijn for her contribution on the logistics, organization and execution of this study. In addition, we want to thank Rudolf von Bünau and Birgit Klinkert for providing Ponsocol®, the EcN specific primers, and all the fruitful discussions. We want to acknowledge Natalie Groothuis for her help with sample collection, processing, and data analyses, Ben Meijer and Gael Auray for their assistance with flow cytometry analyses, and Gerben Hermes for his recommendations for statistical analyses.

Supplementary Materials

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.842437/full#supplementary-material

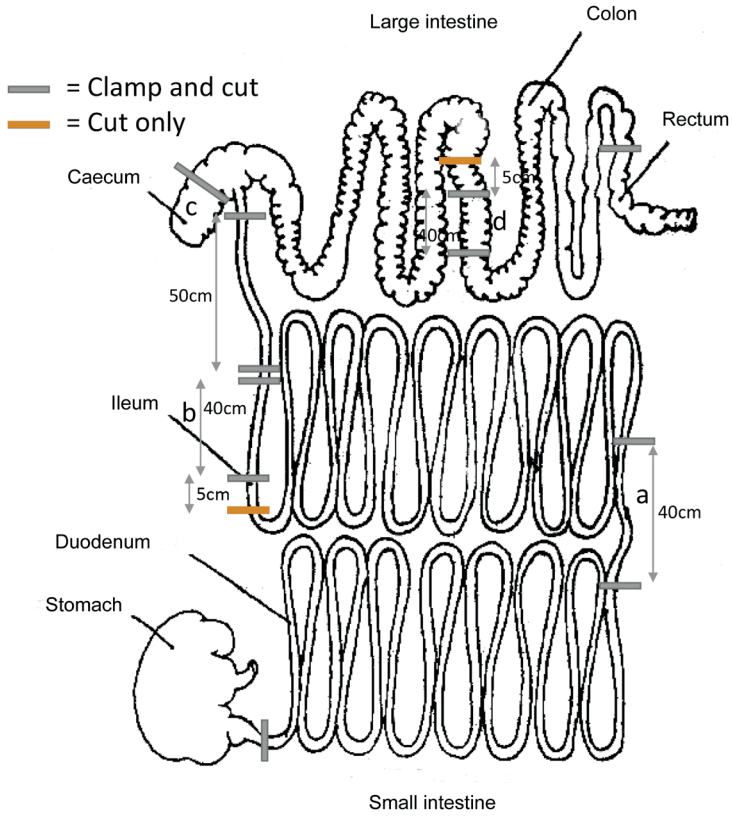


Figure S1. Visual representation of the standardized division of GIT segments at dissection. The jejunum segment (a) was taken by clamping off a 40 cm piece halfway the small intestine after which a cut was made on both sides in order to place the 40 cm piece in a separate container. The ileum segment (b) was taken by placing clamps at 50 cm and 90 cm proximal to the ileocaecal valve. Caecum luminal content (c) was taken by first isolating the caecum content using a clamp after which a cut was made in order to place the caecum in a separate container for further processing. Halfway the colon, a 40 cm segment (clamped off) was taken distally from mid-point. For both ileum and colon, a 5 cm segment was taken proximally to the 40 cm segment that was to be used for transcriptomics.

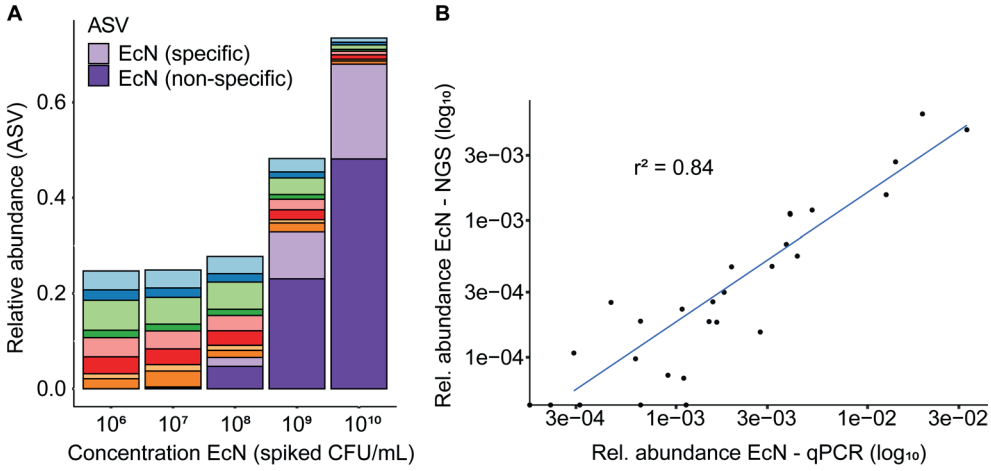


Figure S2. (A) Bar plot of top 10 abundant ASVs in the spiked samples. The abundance of two 16S rRNA gene sequences (ASVs), both of which are present in the EcN genome, increased in proportion to the amounts of EcN added to the spiked samples. One of these two ASVs was found to be specific to EcN in the NGS dataset and was therefore used to identify EcN in faeces. It should be noted, however, that by including solely the EcN-specific ASV, the relative abundance of EcN is underestimated by a two-to-three fold. (B) Validation of EcN in NGS dataset using qPCR. Relative abundance of EcN calculated using the specific EcN qPCR compared to the relative abundance of EcN calculated using the EcN-specific ASV in the NGS dataset. From the regression line (blue), the correlation determinant $r^2 = 0.84$, indicates a high correlation of the two methods for EcN quantification in faecal samples. Additionally, with the use of a CT value of 33 as threshold with qPCR, each positive outcome of EcN in the NGS dataset was validated.

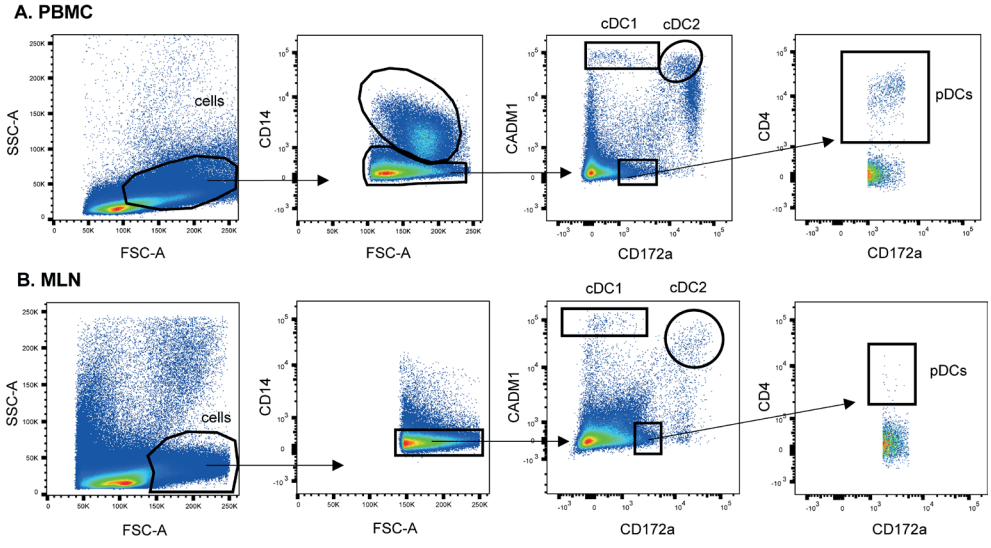


Figure S3. Gating strategy for the identification of DC subsets in porcine PBMCs and MLN cells. After doublet exclusion and discrimination of viable (live) and non-viable (death) cells, live cells were gated based on scatter light (forward scatter; FSC, and side scatter; SSC). Antibodies that specifically recognize CD14, CD172a, CADM1 and CD4 in were used to identify DC subsets in PBMCs (A) and MLN cells (B).

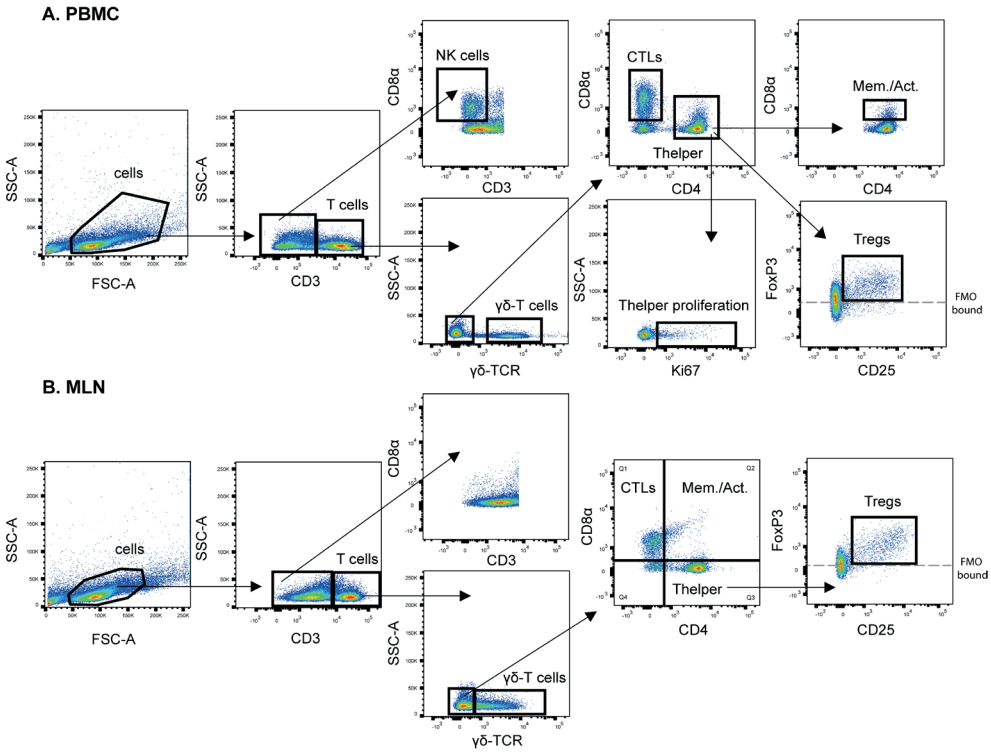


Figure S4. Gating strategy for the identification of NK cells and T cell subsets in porcine PBMCs and MLN cells. After doublet exclusion and discrimination of viable (live) and non-viable (death) cells, live cells were gated based on scatter light (forward scatter; FSC, and side scatter; SSC). Antibodies that specifically recognize CD3, CD8α, TCRγδ, CD4, FoxP3 and CD25 were used to identify NK cells and T cell subsets in PBMCs (A) and MLN cells (B). Ki67 was used to determine cell proliferation. The gating strategy for determining T helper cell proliferation is presented as an example (A).

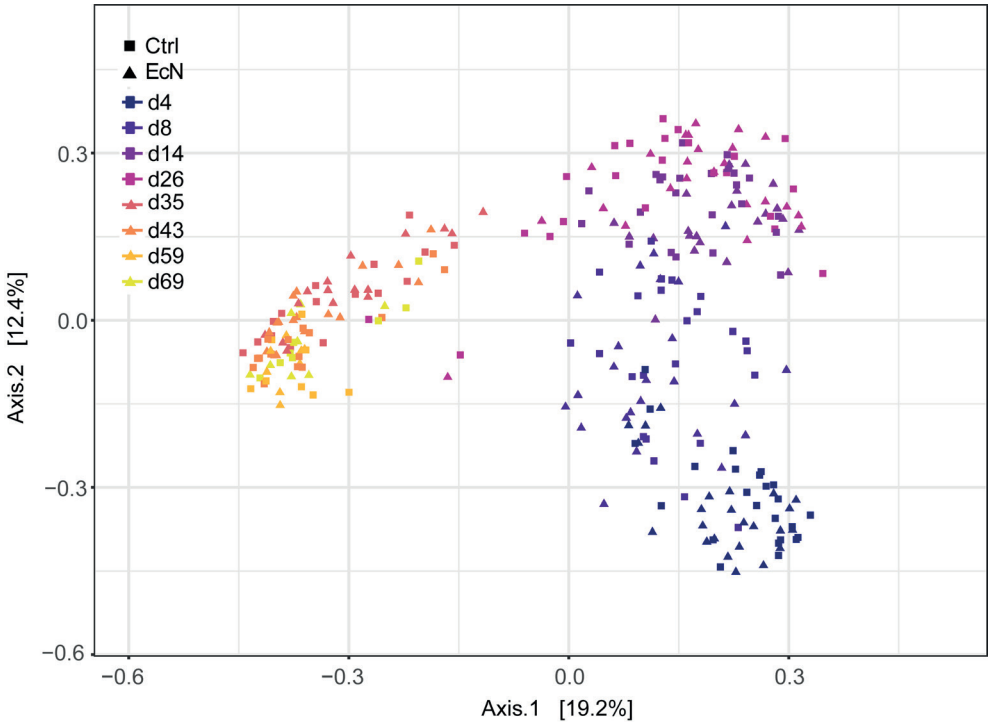
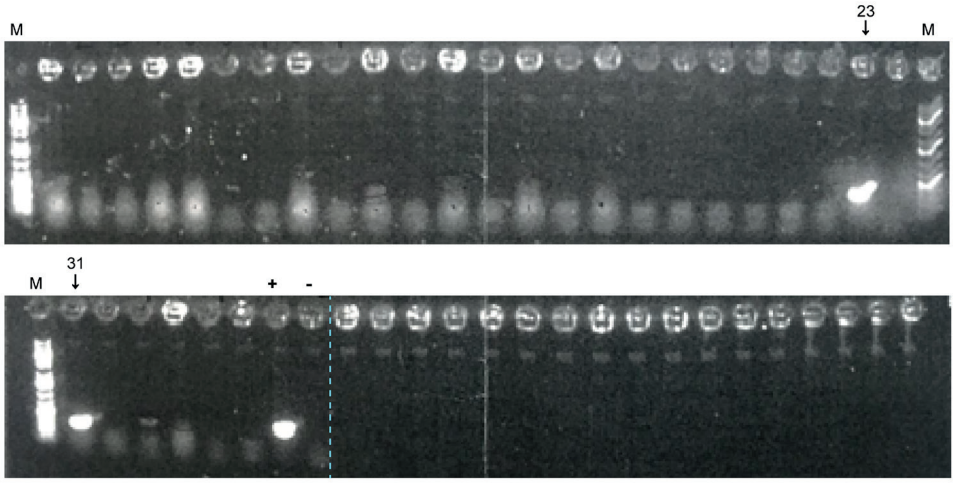


Figure S5. Principal Coordinates Analysis (PCoA) of beta-diversity values (Bray-Curtis dissimilarities) in faecal samples over time (d4-69). Every point represents a faecal sample from an individual animal, and colors correspond to time points. Control and EcN-treated animals are represented by squares (■) and triangles (▲), respectively.

A



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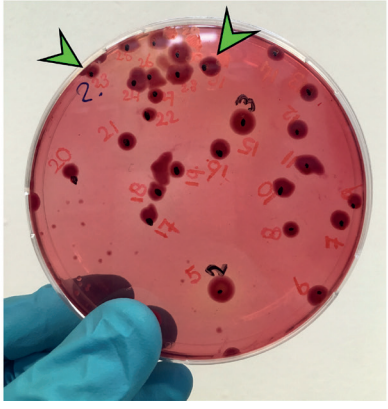

C

No. of animals with white colonies / total number of animals			
	d27	d44	d70
Control	6/8	0/8	1/8
EcN	7/8	0/8	0/8
No. of animals in which EcN was detected / total number of animals			
	d27	d44	d70
EcN	1/8	0/8	0/8

Figure S6. Translocation of EcN to the MLN. Detection of EcN in the MLN of EcN-treated animal using colony PCR (A). Arrows indicate the detection of EcN specific colonies, 'M'; marker, + and -; positive control and negative control, respectively. Bacteria from the MLN grown on MacConkey agar (B). Arrows (in green) point at EcN specific colonies that were identified by colony PCR. Bacterial translocation to the MLN between the treatment groups over time (C). Pre-weaning (day 27) off-white opaque colonies were detected in the majority of both control and EcN-treated animals, which likely represent *Salmonella* colonies. EcN specific colonies were only detected on day 27 and in a single animal.

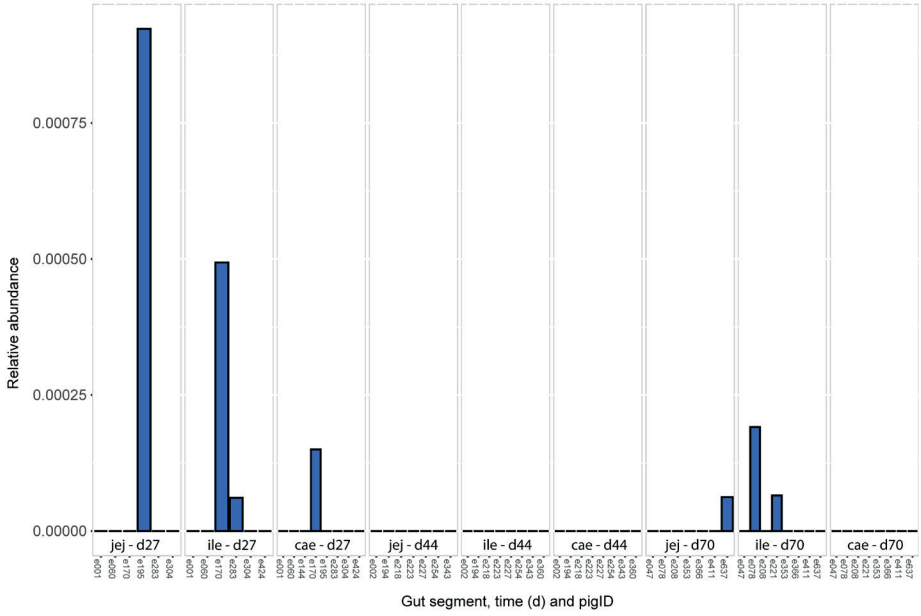

Figure S7. Presence of EcN in digesta of different gut segments (jejunum; jej, ileum; ile and caecum; cae) from EcN-treated pigs over time, sacrificed at the indicated days (d; day). Data is presented as the relative abundance of the EcN-specific ASV, as calculated from the NGS dataset.

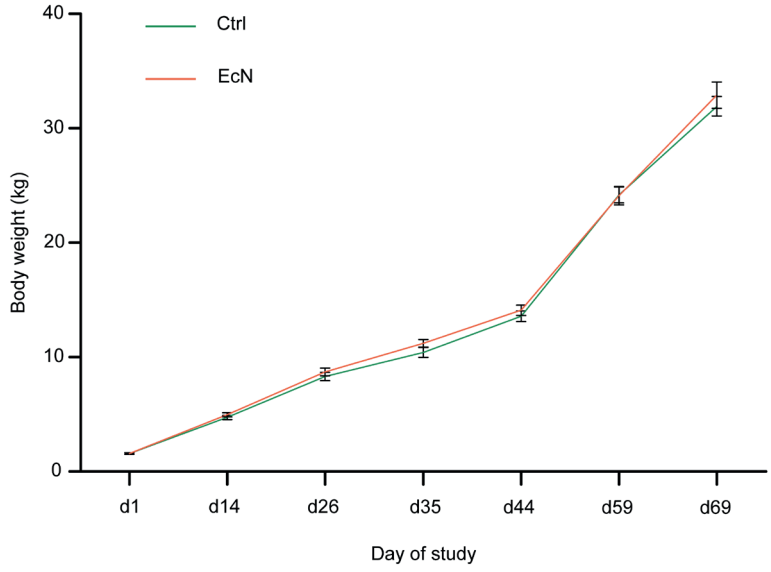

Figure S8. Body weight (kg) over time between the treatment groups. Animals included in this graph were followed during the entire study (n=16 per treatment group). At the start of the study, groups were made as equal as possible in terms of body weight, time of birth and sow parity. Data are shown as the means ± the standard error of the mean (SEM). No significant differences were observed between the treatment groups.

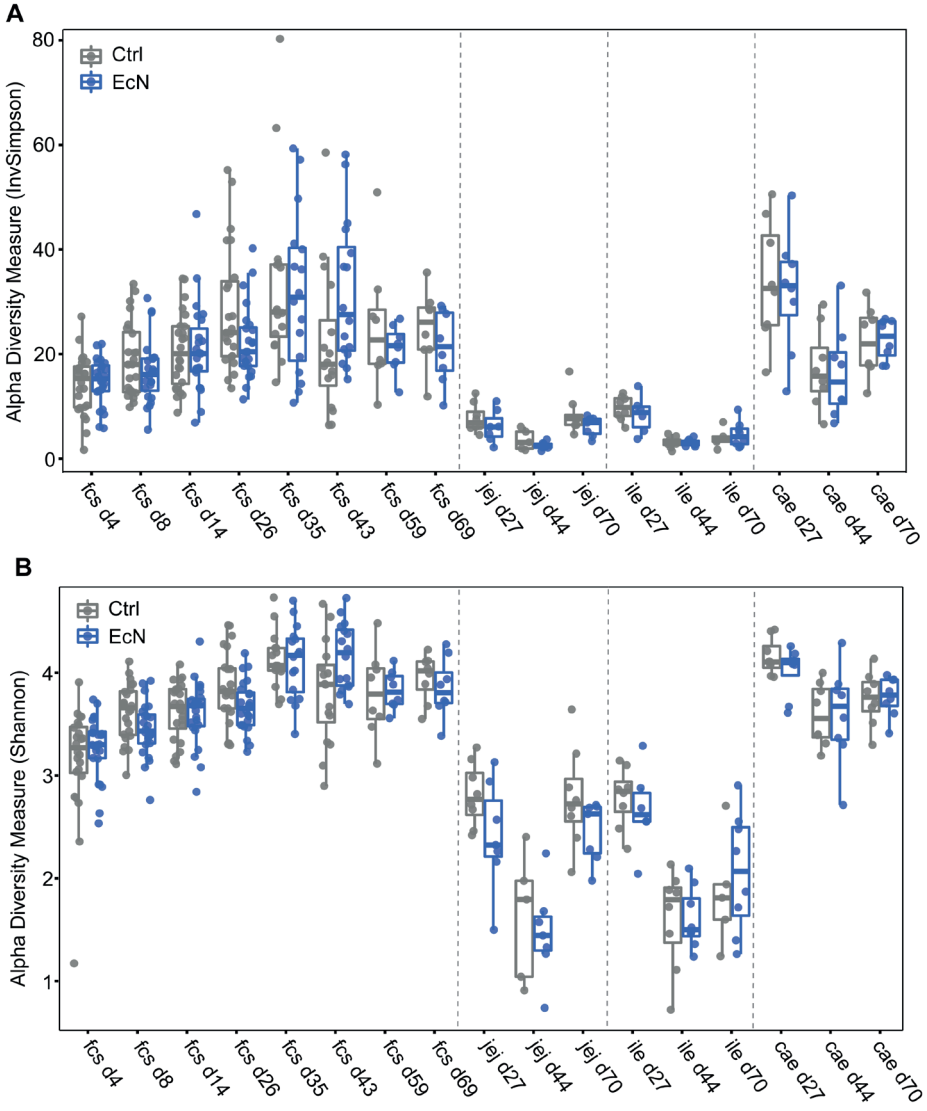

Figure S9. Alpha-diversity of porcine gut microbiota in faeces and digesta between treatments over time. Every point represents an individual animal with the control animals in grey and the EcN-treated animals in blue. InvSimpson (A) and Shannon (B) diversity values are presented by sampling time point (day 4 – day 70), and by faeces (fcs) or gut segment (jej; jejunum, ile; ileum, cae; caecum).

Figure S10. Heatmap of the relative abundance of all ASVs belonging to the phylum Proteobacteria in pre-weaning faecal samples over time. Each time point includes 24 faecal samples per treatment group (8 control pens and 8 EcN pens, 3 animals per pen). This figure shows that especially ASVs in the family of *Enterobacteriaceae* contribute to a higher number of Proteobacteria ASVs in the control group.

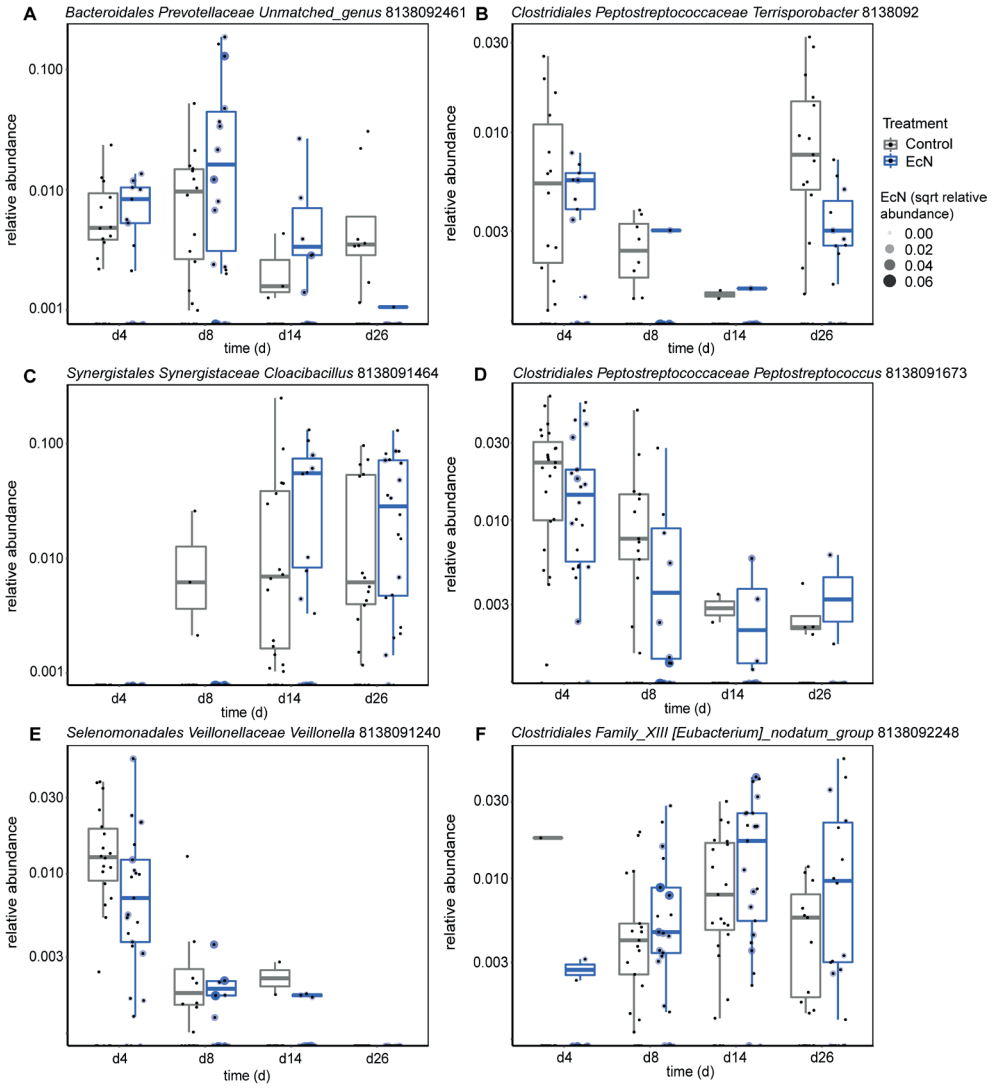

Figure S11. Identification of differentially abundant genera in faeces during the pre-weaning period. Data are presented as relative abundances (by fraction) over time. Shown genera resulted from comparing control animals (grey) to EcN-treated animals (blue) using a GAMLSS model and after adjustment for multiple testing (p.adjust < 0.05). Every point represents an single animal, and the size of the points gives the relative abundance (sqrt) of EcN detected in this individual.

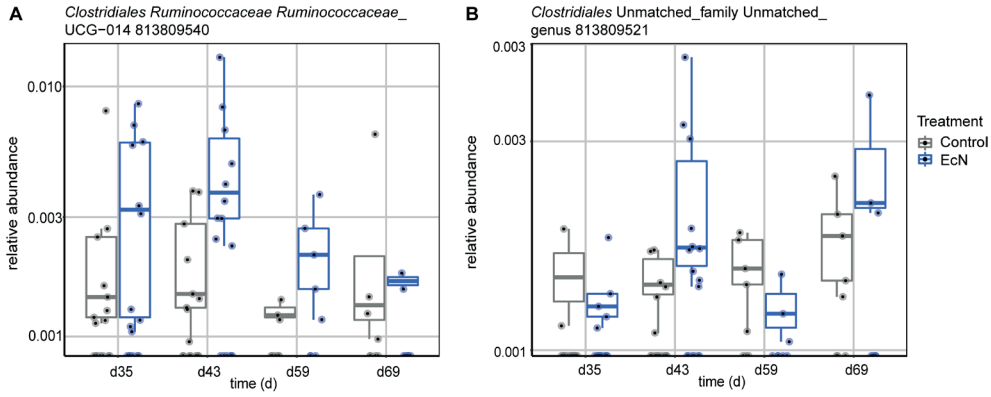

Figure S12. Identification of differentially abundant genera in faeces during the post-weaning period. Data are presented as relative abundances (by fraction) over time. Shown genera resulted from comparing control animals (grey) to EcN-treated animals (blue) using a GAMLSS model and after adjustment for multiple testing (p.adjust < 0.05). Every point represents a single animal, and the size of the points gives the relative abundance (sqrt) of EcN detected in this individual.

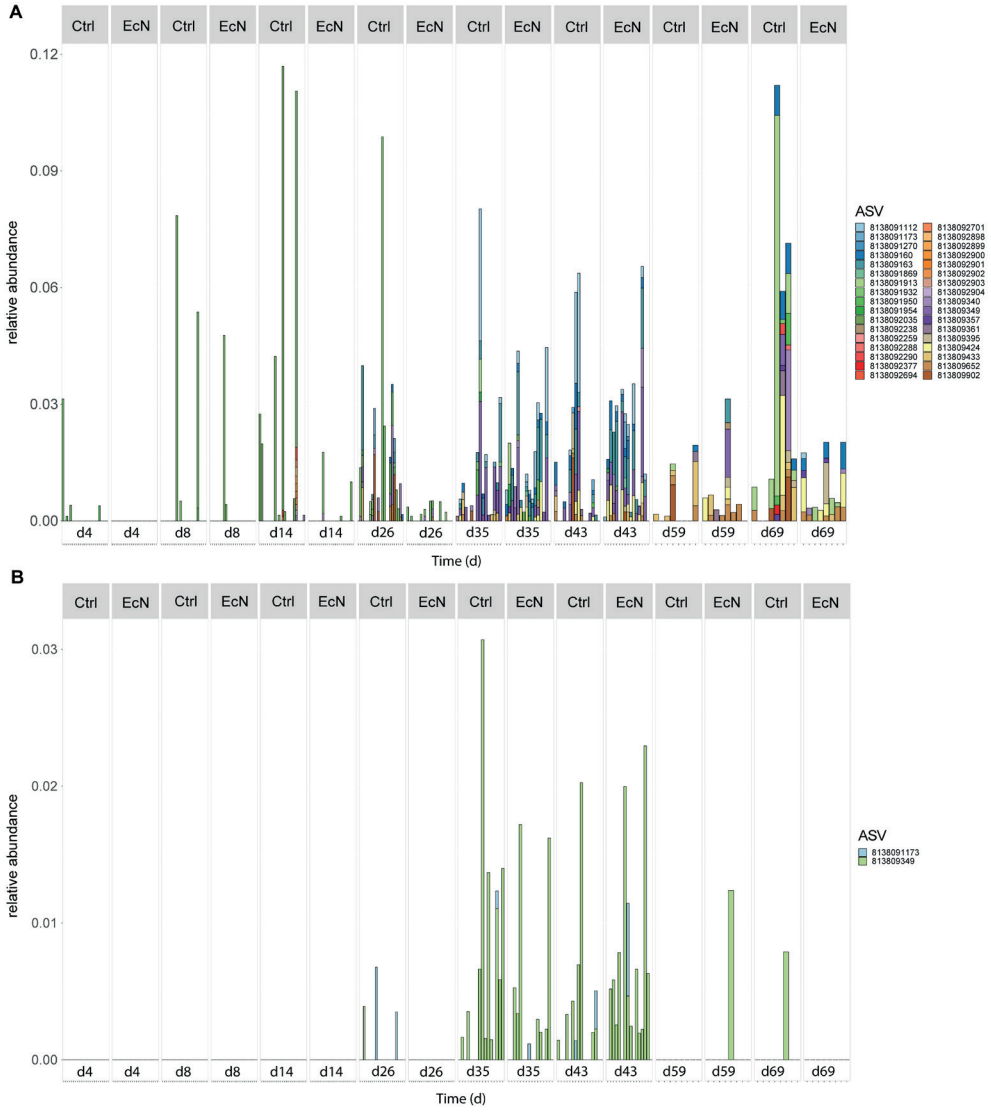

Figure S13. Relative abundance of all ASVs within the *Treponema_2* genus over time in faecal samples (A), and the relative abundance of the two ASVs that both correspond to the sequence of the earlier reported invasive *Treponema* species, namely *Candidatus Treponema suis* (B). This *Treponema* ASV is especially abundant in the immediate post-weaning period up to two weeks post-weaning (d35 and d43). Samples are ordered chronologically and are separated by treatment group (Ctrl versus EcN).

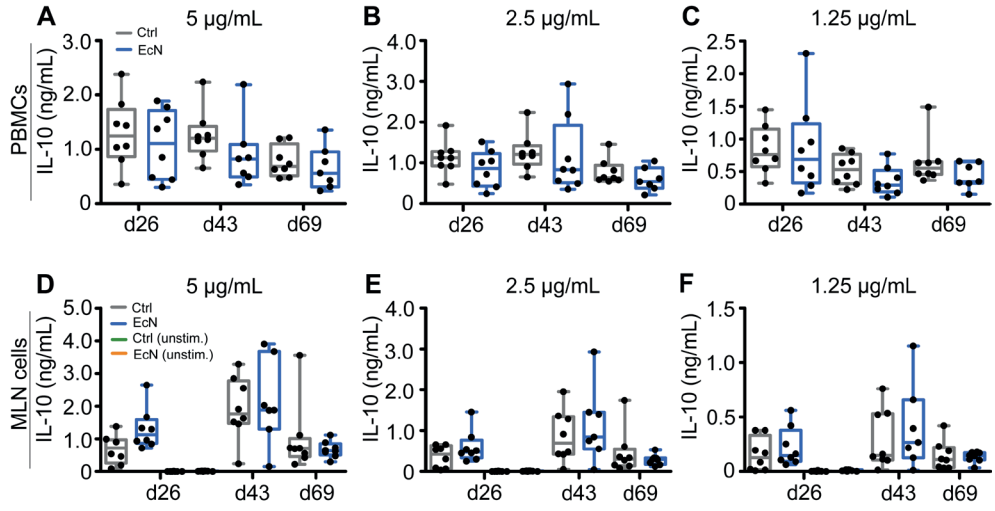

Figure S14. IL-10 production by *ex vivo* stimulated PBMCs and MLN cells. PBMCs (A-C) and MLN cells (D-F) were stimulated with 5 µg/mL, 2.5 µg/mL, 1.125 µg/mL of ConA or left unstimulated (cell culture medium only) for 24 h. Every point represents a single animal from a different pen (n = 7 or 8 per treatment group). No significant differences were found between the treatment groups. Normal distribution and equal variances of data were checked and log-transformed when necessary.

Table S1. Analysis of innate and adaptive immune cells in PBMCs between treatment groups over time.

Subset	Phenotype	Unpaired T-test				Linear Mixed Model
		D14	D26	D43	D69	
		p-value [CI]	p-value [CI]	p-value [CI]		p-value [CI]
pDCs (%)	$CD14^-CD4^+CD172a^+CADM1^-$	ns	ns	ns	ns	T (<0.001)
pDCs (mat.)	$CD14^-CD4^+CD172a^+CADM1^-CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
cDC1 (%)	$CD14^-CD4^-CD172a^{low}CADM1^+$	ns	ns	ns	ns	T (<0.001)
cDC1 (mat.)	$CD14^-CD4^-CD172a^{low}CADM1^+$ $CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (0.018)
cDC2 (%)	$CD14^-CD4^-CD172a^+CADM1^+$	ns	ns	ns	ns	T (0.035)
cDC2 (mat.)	$CD14^-CD4^-CD172a^+CADM1^+$ $CTLA4\text{-}Ig^+$	ns	ns	ns	ns	T (<0.001)
NK cells (%)	$CD3^-CD8\alpha^+$	ns	ns	ns	ns	T (0.051)
NK cells (act.)	$CD3^-CD8\alpha^+CD25^+$	ns	**0.015↑[-17.7 to -2.45]**	ns	ns	**G (0.010)**, T (<0.001)
NK cells (Ki67%)	$CD3^-CD8\alpha^+Ki67^+$	ns	ns	ns	ns	T (<0.001)
γδ T cells (%)	$CD3^+TCR\gamma\delta^+$	ns	ns	ns	ns	T (<0.001)
γδ T cells (Ki67%)	$CD3^+TCR\gamma\delta^+Ki67^+$	ns	ns	ns	ns	T (<0.001)
CTLs (%)	$CD3^+TCR\gamma\delta^-CD8\alpha^+$	ns	ns	ns	ns	T (<0.001)
CTLs (Ki67%)	$CD3^+TCR\gamma\delta^-CD8\alpha^+Ki67^+$	ns	ns	ns	ns	T (<0.001)
T helper (%)	$CD3^+TCR\gamma\delta^-CD4^+$	ns	ns	ns	ns	T (<0.001)
T helper (Ki67%)	$CD3^+TCR\gamma\delta^-CD4^+Ki67^+$	**0.011↑ [-7.11 to -1.11]**	ns	ns	ns	T (<0.001)
Mem./Act. (%)	$CD3^+TCR\gamma\delta^-CD4^+CD8\alpha^+$	ns	ns	**0.042↓[0.014 to 0.587]**	ns	**G (<0.001)**, T (<0.001)
Mem./Act. (Ki67%)	$CD3^+TCR\gamma\delta^-CD4^+CD8\alpha^+ki67^+$	**0.030↑ [-17.1 to -1.01]**	ns	ns	ns	T (<0.001), I()
Tregs (%)	$CD3^+TCR\gamma\delta^-CD4^+CD25^{high}Foxp3^+$	ns	ns	**0.016↓ [0.31 to 2.47]**	ns	T (<0.001)
Tregs (Ki67%)	$CD3^+TCR\gamma\delta^-CD4^+CD25^{high}Foxp3^+Ki67^+$	ns	ns	ns	ns	T (<0.001)

An Unpaired Student's T-tests and a Linear Mixed Model (LMM) were performed to analyse differences between treatment groups per time point and over time, respectively (n = 7 or 8 per treatment group). Abbreviations: G; group effect, T; time effect, I; group-time interaction. Ns; not significant, $p < 0.05$; significant, $0.05 > p < 0.1$; trend. Arrows indicate if effects (e.g., percentage of cells) in the treatment group are higher (↑) or lower (↓) compared to the control group. For all results that are significant or show a trend, the 95% confidence intervals (CI) on the difference between the means are given.

Table S2. Analysis of innate and adaptive immune cells in MLN cells between treatment groups over time.

		Unpaired T-test			Linear Mixed Model
		D27	D44	D70	
Subset	**Phenotype**	p-value [CI]	p-value [CI]	p-value [CI]	
pDCs (%)	$CD14^{-}CD4^{+}CD172a^{+}CADM1^{-}$	ns	ns	0.064↓ [-0.021 to 0.659]	T (<0.001)
pDCs (mat.)	$CD14^{-}CD4^{+}CD172a^{+}CADM1^{-}CTLA4\text{-}Ig^{+}$	ns	ns	0.092↓ [-6.092 to 72.39]	T (<0.001)
cDC1 (%)	$CD14^{-}CD4^{-}CD172a^{low}CADM1^{+}$	ns	ns	ns	T (<0.001)
cDC1 (mat.)	$CD14^{-}CD4^{-}CD172a^{low}CADM1^{+}CTLA4\text{-}Ig^{+}$	ns	ns	ns	T (<0.001)
cDC2 (%)	$CD14^{-}CD4^{-}CD172a^{+}CADM1^{+}$	ns	ns	ns	T (<0.001)
cDC2 (mat.)	$CD14^{-}CD4^{-}CD172a^{+}CADM1^{+}CTLA4\text{-}Ig^{+}$	ns	ns	ns	ns
NK cells (%)	$CD3^{-}CD8a^{+}$	n/a	n/a	n/a	n/a
NK cells (act.)	$CD3^{-}CD8\alpha^{+}CD25^{+}$	n/a	n/a	n/a	n/a
NK cells (Ki67%)	$CD3^{-}CD8\alpha^{+}Ki67^{+}$	n/a	n/a	n/a	n/a
γδ T cells (%)	$CD3^{+}TCR\gamma\delta^{+}$	ns	ns	ns	T (<0.001)
γδ T cells (Ki67%)	$CD3^{+}TCR\gamma\delta^{+}Ki67^{+}$	ns	0.093↓ [-0.643 to 7.300]	ns	T (0.022)
CTLs (%)	$CD3^{+}TCR\gamma\delta^{-}CD8\alpha^{+}$	ns	0.091↑ [-6.011 to 0.5034]	ns	T (0.004)
CTLs (Ki67%)	$CD3^{+}TCR\gamma\delta^{-}CD8\alpha^{+}Ki67^{+}$	ns	ns	ns	T (<0.001)
T helper (%)	$CD3^{+}TCR\gamma\delta^{-}CD4^{+}$	ns	ns	ns	T (<0.001)
T helper (Ki67%)	$CD3^{+}TCR\gamma\delta^{-}CD4^{+}Ki67^{+}$	ns	ns	ns	T (0.006)
Mem./Act. (%)	$CD3^{+}CD4^{+}CD8\alpha^{+}$	ns	ns	ns	T (<0.001)
	$CD3^{+}CD4^{high}CD8\alpha^{low}$	ns	ns	ns	T (<0.001)
	$CD3^{+}CD4^{high}\ CD8\alpha^{low}ki67^{+}$	ns	ns	ns	T (0.002)
	$CD3^{+}CD4^{low}CD8\alpha^{high}$	ns	ns	ns	T (0.038)
	$CD3^{+}CD4^{low}\ CD8\alpha^{high}ki67^{+}$	ns	ns	ns	T (<0.001)
Tregs (%)	$CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD25^{high}Foxp3^{+}$	ns	ns	ns	T (<0.001)
Tregs (Ki67%)	$CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD25^{high}Foxp3^{+}Ki67^{+}$	n/a	n/a	n/a	n/a

An Unpaired Student's T-tests and a Linear Mixed Model (LMM) were performed to analyse differences between treatment groups per time point and over time, respectively (n = 7 or 8 per treatment group). Abbreviations: G; group effect, T; time effect, I; group-time interaction. Ns; not significant, $p < 0.05$; significant, $0.05 > p < 0.1$; trend. Arrows indicate if effects (e.g., percentage of cells) in the treatment group are higher (↑) or lower (↓) compared to the control group. For all results that are significant or show a trend, the 95% confidence intervals (CI) on the difference between the means are given

Chapter 6

General Discussion

In recent years, porcine gut microbial ecology has received increasing interest, mostly due to its perceived relevance for porcine gut health. An important aspect of the gut microbiota is its fermentative capacity, which greatly aids in feed digestion. Therefore, it is vital that the fermentative capacity of the porcine gut microbiota is not at suboptimal levels. Technological advances in the research field of microbial ecology provide the necessary basis for achieving increased understanding of the relevant players within the porcine gut microbiota, which will ultimately lead to improvements in porcine gut health. Nowadays, antibiotics and zinc oxide are still widely used in livestock production, which are practices that can hopefully be minimized in the not-too-distant future. Alternatives to these compounds will likely be substances or live microbial cultures that modulate gut microbiota composition, function and activity and/or substances that have a direct effect on the host's mucosal tissues and the immune system. By increasing our understanding of the impact of candidate substances and live cultures on the porcine host's gut microbiota, mucosal tissue and immune system, we can provide vital tools to livestock producers to counteract gut diseases.

The aims of this thesis were:

1. To provide an overview of used microbial and non-microbial dietary interventions to alter gut microbiota composition in pigs (**Chapter 2**).

2. To investigate the effects of early-life administered yeast-derived β-glucans and EcN on the porcine gut microbiota, the gut epithelial barrier, and the immune system *in vivo* (**Chapters 3, 4, & 5**).

3. To describe the implications of these findings for porcine gut health and gut microbiota composition (**Chapter 6**).

As piglets do not have a fully developed gut microbiota and host immune system in the first weeks of life, dietary interventions in this period are expected to have a greater chance to induce significant changes to gut microbiota composition, the gut epithelial barrier and to the overall immunological competence. The results of the β-glucans and EcN *in vivo* studies confirm the first part of the hypothesis, as both dietary interventions mostly affected gut microbiota composition during the first weeks of life (Chapters 3 and 5). Effects on the gene expression levels in the gut epithelial barrier were exclusively tested for animals that received EcN (Chapter 5). To this end, the effects of EcN on the gut epithelial barrier did not result in statistically significant differences, suggesting that EcN did not impact the gut epithelial barrier in our study. As for the immunological responsiveness, a number of local and systemic immunological factors were tested (Chapters 3 & 5). For both dietary interventions, most immunological parameters that were significantly affected by the dietary intervention were observed in the pre-weaning period. Together with the results on the gut microbiota, this strongly suggests that dietary interventions such as β-glucans and EcN can be best administered early in life in order to optimize animal health and performance.

In this chapter, I will summarize the findings of our *in vivo* studies, I will discuss the implications of our findings considering other literature, and I will describe what these results might mean for porcine (gut) health. Additionally, I will cover practical implications

of our *in vivo* findings and will provide recommendations for future research. Finally, other promising feed additives are discussed, including alternative compounds with antibiotic activity, fermented feed, microbial consortia, and live mushroom spawn.

Discussion on findings of the β-glucan *in vivo* study

Differences in alpha diversity between treatment groups

One of the important research questions was whether the administration of yeast-derived β-glucans in early-life would affect the pig's gut microbiota composition. An important indicator to answer this question is alpha diversity. To this end, we observed a difference in alpha diversity during the pre-weaning phase between treatment groups when using the Shannon diversity index, but not when using the InvSimpson diversity index. Shannon's diversity index gives more weight to rare species, which means that especially low abundance ASVs were impacted by the administration of β-glucans. The fact that the Shannon alpha diversity was on average lower in β-glucan treated animals during the pre-weaning phase, while this difference was not observed for the InvSimpson diversity index, indicates that fewer low abundance ASVs were present in the β-glucans treated animals. The relative importance of these low abundant ASVs to microbiota functions such as fermentative capacity is debatable, and any possible implications would be purely speculative. To put things into perspective, in several studies of microbiota composition, the low abundant ASVs are filtered out prior to further analyses (Surendran Nair, Eucker et al. 2019, Sibai, Altuntaş et al. 2020, Wynne, Thakur et al. 2020), which shows that several researchers choose to ignore the low abundant ASVs. In fact, the data processing pipeline used in this study (NG-Tax) has a default setting to remove ASVs that have a relative abundance no greater than 0.1%, meaning low abundant ASVs were also not taken into account in this study.

Differences in relative abundance of bacterial families pre- and post-weaning

It is commonly accepted that weaning induces a rapid and large change in the gut microbiota composition of piglets (Gresse, Chaucheyras Durand et al. 2019). Even though we did not observe differences in microbiota composition between treatment groups post-weaning, it is interesting to investigate in what manner the porcine microbiota composition changes around the weaning period. Supplementary Figure S6 of Chapter 3, which contains the 35 most predominant bacterial families up to week 10, provides a good overview of the dynamics of the relative abundance of these bacterial families in the first weeks of life, but also provides a clear view on what happens to these bacterial families during the weaning phase. As weaning took place on day 28, time points d26 and d35 were considered the most interesting to analyse regarding the impact of weaning on microbiota composition. Several bacterial families do not show a dramatic change in relative abundance between d26 and d35 (such as *Rikenellaceae, Clostridialis* Family_XIII, *Erysipelotrichaceae*, etc.), but several bacterial families show a dramatic reduction in relative abundance after weaning, such as the *Actinomycetaceae, Campylobacteraceae, Fusobacteriaceae, Bacteroidaceae, Enterobacteriaceae, Streptococcaceae, Marinifilaceae, Clostridiales* vadinBB60, *Synergistaceae* and *Corynebacteriaceae*. Interestingly, of these bacterial families, the *Streptococcaceae* become abundant again from d59 onward. A few bacterial families show a dramatic increase in relative abundance after weaning, such as the *Veillonellaceae, Prevotellaceae, Lactobacillaceae* and *Succinivibrionaceae*. In a study that specifically focused on the microbiota composition changes around the weaning transition in healthy

piglets and piglets that developed post-weaning diarrhoea, it was found that an increase in the relative abundance of *Prevotellaceae* was characteristic of healthy weaned piglets (Karasova, Crhanova et al. 2021). Furthermore, it is interesting to compare the changes at the family level during the weaning transition in healthy animals of that study with animals of the current study. When comparing Supplementary Figure S6 of Chapter 3 with healthy animals present in Figure 1 of the other study (Karasova, Crhanova et al. 2021), it is observed that there is clearly overlap in the bacterial families that are increased, reduced or that are stable before and after weaning (see Table 1 below). Of the families mentioned in Figure 1 of the article by Karasova et al., a reduction in relative abundance of the *Synergistaceae, Enterobacteriaceae, Fusobacteriaceae, Clostridiaceae*_1*,* and *Bacteroidaceae* was observed in both studies. The clear reduction in *Spirochaetaceae* observed by Karasova et al., was not observed in the current study. In both studies, a slight increase in *Acidaminococcaceae* and *Lachnospiraceae* is observed, and a clear increase in *Lactobacillaceae* was observed. In both studies, a high prevalence of the *Ruminococcaceae* was found pre- and post-weaning, with no obvious change around the weaning period. Furthermore, the *Porphyromonadaceae* were quite abundant in the other study pre- and post-weaning, while they were not at all abundant in the current study. In both studies, the *Bacteroidales* and *Coriobacteriaceae* were not abundant during the pre- or post-weaning period. To summarize, even though both *in vivo* studies were conducted in different European countries (The Netherlands and Czechia), the shifts in relative abundance of bacterial families during the weaning transition observed by Karasova et al. shares many similarities with the shifts observed in this study.

Table 1. Comparison of temporal changes in relative abundance of top abundant families around the weaning period between the study by Karasova et al., and this study.

Bacterial family	Study by Karasova et al.	This study
Acidaminococcaceae	↑	↑
Bacteroidaceae	↓↓	↓↓
Bacteroidales	Low abundance	Low abundance
*Clostridiaceae*_1	↓↓	↓↓
Coriobacteriaceae	Low abundance	Low abundance
Enterobacteriaceae	↓↓	↓↓
Fusobacteriaceae	↓↓	↓↓
Lachnospiraceae	↑	↑
Lactobacillaceae	↑↑	↑↑
Porphyromonadaceae	↑	Low abundance
Ruminococcaceae	Stable	Stable
Spirochaetaceae	↓↓	Stable
Synergistaceae	↓↓	↓↓

Differences in microbiota composition between gut segments and faeces

Even though no significant differences between treatment groups were found for the microbiota composition in individual gut segments, the analysis of the microbiota composition in several gut segments and faeces in the same study provides interesting insight into which bacterial groups play the most important roles along the GIT of pigs. Figure 3 of Chapter 3 provides such an overview at the phylum level (Figure 3A) and at

the family level (Figure 3B). When looking at the phylum level, a clear distinction becomes apparent between the small intestine and the hindgut (caecum, faeces), with *Firmicutes* clearly dominating in the small intestinal gut segments, while this dominance is shared in the hindgut with the phylum *Bacteroidetes*. When looking at the global overview at the family level, what stands out is that a number of families that are present in the hindgut, are practically absent in the small intestinal gut segments. These are the *Prevotellaceae, Ruminococcaceae,* and *Muribaculaceae*, which are bacterial families that are especially known for their fiber-degrading capabilities (Guo, Li et al. 2019, Lagkouvardos, Lesker et al. 2019, Precup and Vodnar 2019, Jiang, Lu et al. 2020). What furthermore stands out, is that the *Lactobacillaceae* comprise the vast majority of bacteria in the small intestine on d27 and d44, which underlines the importance of this bacterial family for early-life gut health and fermentative capacity in the small intestine.

***Methanobrevibacter** less abundant in β-glucan group*
An interesting finding in the β-glucans study is that the archaeal genus *Methanobrevibacter* had a reduced relative abundance in β-glucan treated animals during the pre-weaning period (Figure 5, Chapter 3). This difference was especially apparent on d8 and d14. Researchers worldwide are trying to reduce methane emissions in livestock production, because it is an important greenhouse gas, meaning its presence in the atmosphere affects the earth's temperature and climate system. Since ruminants are the principal source of livestock methane emissions (Tapio, Snelling et al. 2017), it may be interesting to administer β-glucans to ruminants to see whether a reduction in the genus of *Methanobrevibacter* can be repeated. Alternatively, instead of lowering the abundance of all archaea within the genus of *Methanobrevibacter,* β-glucans may have an impact on the composition of the archaeal community of ruminants, which is also a strong factor in relation to methane emissions. For instance, animals harbouring the *Methanobrevibacter gottschalkii* clade tend to be associated with greater methane emissions (Zhou, Chung et al. 2011, Tapio, Snelling et al. 2017).

Discussion on findings of the EcN *in vivo* study
Being able to follow the temporal presence of EcN after administration of EcN to pigs was instrumental in understanding when and where EcN most likely contributes its effects. To this end, EcN was especially present in the first four weeks of life, and was found in all sampled GIT segments (Chapters 4 & 5). In the main study, which lasted until day 70 of life, EcN was additionally found in the jejunum and ileum of piglets that were dissected on day 70. This is an interesting finding, as EcN was not found in any caecal or faecal sample that was taken during the post-weaning period. An important reason could be that the microbial density is much lower in the foregut (jejunum, ileum) compared to the hindgut (caecum, colon & rectum), which allows for easier detection of the probiotic strain in the foregut. It is, however, somewhat unexpected, as *E. coli* strains, and therefore also EcN, are known to be adapted to mammals with hindgut microbial fermentation chambers (Gordon 2004), and so the expectation was that EcN would primarily be present in the hindgut.

When looking at EcN's effect on overall gut microbiota composition, the apparent effect on close relatives such as other *E. coli* strains was the most prominent. This was evidenced by a reduced Proteobacteria diversity in faeces of EcN-supplemented animals during the pre-

weaning period. In an earlier study it was found that EcN can lower the diversity of strains in the family of *Enterobacteriaceae* (Šmajs, Bureš et al. 2012), which is a finding that is consistent with what was found in the main study of this thesis.

When comparing the effect of β-glucans and EcN on overall gut microbiota composition, the effect of EcN was more pronounced. This was evidenced by the effect of the probiotic treatment on microbiota beta diversity being significant ($p < 0.015$), while this was not found for the β-glucan treatment. However, this effect was significant for both treatments when including exclusively pre-weaning samples. Additionally, using a GAMLSS model, six genera were found to be differentially abundant in faecal samples of the EcN group when compared to the control group. When only including pre- or post-weaning timepoints, six genera and two genera were found to be differentially abundant in faecal samples of the EcN group. In contrast, in the β-glucan group such differences (in six genera) could only be observed when including exclusively pre-weaning faecal sampling timepoints. The administration of EcN also showed a potential antagonistic effect against *Treponema*, which in combination with a reduced Proteobacteria diversity suggests a beneficial effect on the porcine gut microbiota composition.

EcN's effect on gene expression in gut epithelium
Although no statistically significant differences in gene expression of the gut epithelium were found for EcN-treated animals in any of the 96 genes tested, there was one gene that deserves mentioning. The average expression of Trefoil factor 2 (TFF2) encoding gene was higher in the colon of EcN-treated animals, on both day 27 and day 44 of the study. In addition, for both tissue types tested (ileum and colon), TFF2 had the most dramatic fold-increase in expression when comparing the expression of day 27 with that of day 44. What should be noted is that even though TFF2 has a more than 22-fold increase in gene expression in the colon when comparing day 27 to day 44, the result was not statistically significant due to high variability between animals (Figure 5E, of Chapter 5). This high variability in gene expression was found for both tissue types and both time points, which is likely the reason why TFF2 is only significantly differentially expressed in ileum samples when comparing day 27 to day 44. In humans, TFF peptides are known to be produced by mucus-producing goblet cells and are known to have a broad protective effect on the mucosa, which is why they are considered candidates for therapeutic restoration of diseased or damaged mucosa (Behnsen, Deriu et al. 2013). As EcN's presence may increase the expression of TFF2 in mucosa of the colon, elevated presence of TFF peptides could be achieved by the administration of EcN. As such, EcN could be an interesting alternative to administering TFF peptides as therapeutic agents.

Of all genes tested, the expression of SAA (Serum amyloid A) variants showed greatest variability, with gene expression between individual pigs consistently varying as much as multiple hundred-fold at both timepoints tested and with both tissue types tested (ileum and colon mucosal scrapings). This variability in gene expression was observed for each of the amplicons tested of the SAA variants (SAA, SAA 2, SAA 2 AND 3). This is in line with other studies in mammals, where researchers found SAA genes to be highly variably expressed in different tissue types, especially when exposed to inflammatory agents such as LPS (Goldberger, Bing et al. 1987, Zhang, Ahsan et al. 2005, Vels, Røntved et al. 2009). The

variability in gene expression of these proteins can be explained by their role as acute-phase proteins, which means that they play an important role in acute inflammatory responses (Zhang, Zhang et al. 2019). According to De Buck et al. in 2016, gene expression of SAA variants can be used as a diagnostic marker for many diseases, and increases in production are triggered by infection and inflammatory reactions (De Buck, Gouwy et al. 2016). The order of magnitude of gene expression increase is dependent on the level of inflammation: From a 10- to 100-fold increase during limited inflammatory events to a 1000-fold increase during severe bacterial infections. Even though all three SAA variants tested showed an average higher gene expression in the colon of EcN-treated animals on day 27 (Figure 5C, of Chapter 5), this result was not statistically significant.

Practical implications of our *in vivo* findings

Administer dietary interventions in early life

One of the main findings of the *in vivo* studies in this thesis is that the dietary interventions had the highest impact on gut microbiota composition in the first weeks of life. These changes in microbiota composition were almost exclusively found in the pre-weaning period for both dietary interventions. A likely explanation for this observation is that the gut microbiota is not yet fully developed in early life (Patil, Gooneratne et al. 2020). The diversity of the gut microbiota in early life is still low when compared to that in an adult pig, as can be seen from the alpha diversity figures in both Chapter 3 and Chapter 5. This means that due to having a relatively low microbiota diversity in early life, there is room for newcomers to colonize. Additionally, in the first weeks of life, the gut microbiota is subject to continuous compositional changes, which makes it a dynamic environment with potential for ecological shifts. Although it would be hard to study, another effect may be that the gut of an adult pig contains mostly well-established microbiota species which have had ample time to adapt to the porcine host's GIT environment. A probiotic "newcomer" like EcN is propagated in a biotechnological process, and thus has not yet had time to adapt its metabolic processes to compete effectively against the already established members of the gut microbiota. What should be noted is that this effect, which is largely based on speculation, would not exclusively affect the effectiveness of EcN, but would affect the effectiveness of any probiotic strain used, since all probiotic products are produced in a biotechnological process and not in a mammalian host.

Additionally, what should not be underestimated, is that in absolute microbiota cell numbers, an adult pig contains a larger number of microbial cells in its gut than a piglet in the first weeks of life. The total luminal content in an adult's pig gut is many times greater, as their GIT is much larger in terms of volume. This may explain why the administration of 10^9 EcN cells to a pre-weaning pig resulted in presence of EcN on numerous occasions, in both the amplicon sequence dataset and in the qPCR results, while the same administration in adult pigs did not result in presence of EcN in any of the faecal samples. Given that both EcN-detection assays used are quite sensitive, with EcN detection limits around ten thousand cells per gram luminal content, it can be argued that EcN was present at low concentrations during the post-weaning period in faeces or was not present at all. This implies that researchers and pig farmers may need to administer higher amounts of EcN during the post-weaning period in order to allow for temporal tracking of EcN in faeces, or when the aim is to confer gut ecological effects, such as competitively excluding pathogenic microbial species.

Another difference between pre-weaning and post-weaning pigs is that the pH of the stomach is typically lower in a post-weaning pig (Ravindran and Kornegay 1993). Especially with the administration of probiotics, this may be an important factor, as some probiotic species are not capable of surviving highly acidic environments. For EcN, in a number of porcine *in vivo* studies, live EcN was detected in faeces after administration to post-weaning pigs (Duncker, Lorentz et al. 2006, Barth, Duncker et al. 2009), which means that at least a portion of administered EcN is able to withstand the acidity in the stomach of post-weaning pigs. This implies that the higher stomach acidity is unlikely to explain the differential presence of EcN in pre- and post-weaning pigs. Some prebiotics may also not be able to withstand highly acidic environments, and so may have higher effects in the pre-weaning period opposed to administration during the post-weaning period.

Especially with dietary interventions that share resemblance with ingredients present in post-weaning feed, administration during the pre-weaning period may prove to result in the highest impact. In the β-glucan *in vivo* study, yeast-derived β-glucans were used, which share resemblance with oat and wheat grain derived β-glucans that are present in the post-weaning diet during the β-glucan *in vivo* study (see Chapter 3, Table S1 for diet composition). Yeast- and wheat-derived β-glucans share structural features, and thus may exert similar effects on host cells. The activation of immune responses, due to the Dectin-1 signalling pathway are known to be caused by both yeast- and wheat-derived β-glucans (Sonck, Stuyven et al. 2010). Post-weaning pigs are exposed to wheat-derived β-glucans from the feed, which may be an important reason why effects of yeast-derived β-glucans were seen mostly during the pre-weaning period in this thesis' *in vivo* study. The explanation is that pigs in the control group are already exposed to wheat-derived β-glucans during the post-weaning period, which removes the relative impact of introducing yeast-derived β-glucans to the diet.

To conclude, even though it may introduce more labour for pig farmers or may require further innovations, the implementation of dietary interventions during the pre-weaning period as opposed to the post-weaning period, is promising. For the probiotic strain EcN, it was found that its temporal presence gravitates strongly towards a higher presence in the pre-weaning period, so its efficacy will likely be highest when administered in early life. Whether this also stands true for other dietary interventions and other probiotic strains, such as *Lactobacillus* strains, still needs to be investigated. With the successful introduction of a dietary intervention to a young pig herd, farmers may find that the added labour and cost of administering a dietary intervention in early life is easily compensated by an increase in pig health, with reduced economic losses, and reduced labour and costs for treating animals.

Relevance of pilot studies

Pilot studies are usually small-scale experiments that aim to test the logistics of carrying out a main experiment and to generate preliminary data that is used to better plan that main experiment. Pilot studies can potentially save a lot of time, money and animals (Festing 2011). Before the main porcine study was conducted, a pilot study was carried out to get better insights into many aspects related to performing an *in vivo* study, including logistics and the methods to be used. In addition, there were a few specific questions that

needed answering and there were several assumptions that needed verifying. An important question was whether EcN mostly resides in the intestinal lumen or rather resides in the mucosal layer of the gut epithelium. If EcN would mostly reside in the mucosal layer of the gut epithelium, mucosal scrapings would need to be used instead of luminal samples for the analysis of gut microbiota composition. Because earlier porcine studies showed that EcN could be detected in pig's faeces (Duncker, Lorentz et al. 2006, Barth, Duncker et al. 2009), it was assumed that EcN would also end up in piglets' faeces during the current study. As the plan was to administer a high dose of 10^9 EcN CFU per piglet from the first days of life, we had to eliminate the possibility that the probiotic administration would harm the young piglets. The assumption was that it would not harm young piglets, as EcN has a long track record of use in human neonates (Sonnenborn and Schulze 2009), but still needed verification for neonatal piglets.

Furthermore, probiotic strains are known to have the capacity to spread to control individuals during *in vivo* studies (Costeloe, Bowler et al. 2016), and so it was assumed that cross-contamination of EcN to control animals might take place. The observation during the pilot study that cross-contamination took place when EcN-treated and control piglets are in close proximity to one another (Chapter 4, Figure 2), strengthened the awareness that robust physical barriers between pens were needed and that workers' clothing and tools should be switched or disinfected after performing any operations in pens that contain EcN-treated piglets. By adhering to these procedures, we almost completely prevented EcN cross-contamination during the main *in vivo* study.

Risk factors for probiotic cross-contamination
When running a porcine *in vivo* study that is as close to the practical situation as possible, the experiment's layout will likely include multiple neighbouring pens within each department. In order to maintain a random assignment of treatments to pens, the result will nearly always be that control pens will be neighbouring probiotic treatment pens. Separating treated animals and control animals by department is typically not advisable, since it is hard to guarantee that all factors related to gut health are equal in each department. A solution to prevent cross-contamination of the probiotic strain is to use robust physical barriers between pens. All care should be taken that no faecal material or saliva can be introduced to neighbouring pens and additionally care should be taken that the vast majority of dust particles does not spread to neighbouring pens. Preventing the spread of all dust particles including very fine dust particles between pens in the same department is practically impossible, because very fine dust particles can spread all over the department. So with a randomized study design, where control and probiotic pens are situated in the same department, there is always a potential of probiotic strain cross-over.

Another important factor that determines the potential for cross-contamination of the probiotic strain, is the movement and actions of staff that enter the *in vivo* study's departments. What was practically feasible in the current study, was to use two sets of clothing (boots and overalls). One set was used with all tasks for which the entering of pens was not required, and when entering pens of control animals, while the other set was used when entering pens of probiotic-treated animals. In addition to using two sets of clothing, staff was also required to wear latex gloves and protective glasses when entering

pens of probiotic-treated animals. Gloves were discarded after handling of probiotic-treated animals was finished and boots were disinfected whenever leaving or entering a department. Any tools that were used in pens of probiotic-treated animals or on the probiotic-treated animals themselves, were thoroughly cleaned and disinfected afterwards. Any surfaces that probiotic-treated animals touch against outside of their pens (e.g. during sampling), was disinfected afterwards. For small tools and metal surfaces, the disinfectant of choice was a 70% ethanol solution. Whenever moving animals outside of their pens, they typically become stressed and start to defecate. All care should be taken that no droppings from probiotic-treated animals are left on the flooring, such as in the hallway. Flooring should be disinfected after all work with probiotic-treated animals is finished.

Tracking temporal presence of EcN

Being able to identify and enumerate the administered probiotic strain in faecal and luminal samples arguably improves the interpretation of the outcomes of an *in vivo* study. Being able to additionally follow the temporal pattern of the probiotic strain and localizing it in specific gut segments, further improves the interpretation of the outcomes of an *in vivo* study. With EcN, highly specific qPCR methods are available, with some primers being more specific than others (Blum-Oehler, Oswald et al. 2003). Additionally, after tweaking and re-iterating the 16S ribosomal RNA (rRNA) gene amplicon sequence data processing through MIB's in-house data pipeline (NG-Tax) (Ramiro-Garcia, Hermes et al. 2016, Poncheewin, Hermes et al. 2019), and with the use of the EcN-spiked samples, an ASV was found that in the current study showed to be specific to EcN. To be more precise, the sequence was specific to a number of EcN's rRNA gene copies. To verify that the EcN-specific ASV coincided with the presence of EcN, a theoretically 100% EcN-specific primer pair (verified using BLAST with the guidance of the probiotic's manufacturer) was used in several qPCR runs. After verifying that the EcN-specific taxon in the amplicon sequence dataset really did correspond to EcN's presence, it became possible to visualize its temporal presence in faecal samples over time, and it became possible to state that the probiotic strain did practically not cross-over to control pens. The resulting observation that EcN is exclusively present in piglet's feaces during the pre-weaning period, strengthened the theory that EcN's impact on gut microbiota composition and host health is highest during the pre-weaning period.

Relative importance of dietary interventions

Pre- and probiotics are interesting ingredients that can be used as dietary intervention. In numerous studies, they have been shown to positively impact general health and gut health in particular (Shim 2005). With the right administration, pre- and probiotics may be a more sustainable alternative to AGPs. Aspects that should be considered, are time of administration, dose, and method of administration. In order to place the use of dietary interventions in perspective, one should be aware that many other factors influence porcine health, such as: Housing, indoor climate, air quality, sanitary conditions, density of animals, moment of weaning and quality of feed. When the aim is to improve pig health, these factors may be more important to focus on than the use of dietary interventions. However, if all care is taken that for none of these aspects issues may develop, dietary interventions may be an important approach. This can translate itself in better growth performance, lower incidence of disease, and reduced antibiotics use. Additionally, dietary interventions have shown in several studies the potential to influence pigs in such a way that they become

more resilient against adverse outside factors (Adams, Che et al. 2019, Lépine, Konstanti et al. 2019, Berto, TSE et al. 2020, Bonetti, Tugnoli et al. 2021). This shows that health related factors not only differentially impact health, but that they are also inter-connected.

Future recommendations

Looking at specific biomarkers

With the advance of culture-independent methods to characterize gut microbiota composition, as well as the complete functional repertoire of the gut microbiome using metagenomics, datasets related to *in vivo* studies are becoming increasingly complex. For exploratory purposes and to identify novel biomarkers, such studies with related datasets can be invaluable. However, if the intention is to frequently screen novel feed ingredients or novel probiotic formulations, amplicon- and metagenome sequence datasets may prove to be too complex in many use-cases, as they require specialized and intensive data processing, analysis and interpretation. Instead, after having identified a number of important biomarkers, i.e. biomarkers for disease (or health), it may be more useful to focus on these and perform PCR-based testing to enumerate the presence and abundance of disease biomarkers in faecal and luminal samples. For instance, the *Treponema2* genus, with the taxon *Candidatus Treponema suis* in particular, may prove to be an interesting health biomarker. Designing a specific qPCR for such biomarkers may result in a more efficient tool for screening of candidate dietary interventions and probiotic formulations.

Organoids as model to study interaction of gut epithelium with dietary interventions

The scope of the research described in this thesis included *in vitro* work which entailed the study of the interaction between dietary interventions and porcine organoids. Since EcN was one of the dietary interventions to be used during the *in vivo* studies, the interaction of this probiotic strain with porcine organoids was first studied. As described by van der Hee et al. in 2018 (Van der Hee, Loonen et al. 2018), first ileum-derived porcine organoids were generated from a donor animal, and then propagated by growing them in 3D. When 3D organoids were sufficiently propagated, they were dissociated and seeded onto a 2D surface, which over time yields a confluent 2D organoid monolayer (see Figure 1 below). These 2D organoid monolayers contain cell lineages that are present in the tissue of origin (e.g. enterocytes, goblet cells, Paneth-like cells), which creates a near physiological model to study innate immunity (Van der Hee, Loonen et al. 2018). The goblet cells that are present in the monolayer produce mucus, creating a thin layer of mucus on top of the monolayer. This thin layer of mucus on top of the monolayer allows for the exposure of compounds or microbes to the monolayer, without the compounds or microbes directly contacting the epithelial cells that make up the monolayer.

When exposing the 2D organoid monolayer to EcN cells, it was observed that EcN cells would rapidly die in the organoid monolayer's growth medium (within hours), without any cells surviving. This effect was not observed when exposing EcN to an IPEC-J2 monolayer that was grown in the same growth medium. The EcN cells were not dying due to antibiotics added to the tissue culture medium, as the culture medium was prepared as described by van der Hee et al. in 2018 (Van der Hee, Loonen et al. 2018), without the addition of antibiotics. After growing the organoid 2D monolayer for 24 hours, the growth medium supernatant was removed, which hereinafter is referred to as 'conditioned supernatant'.

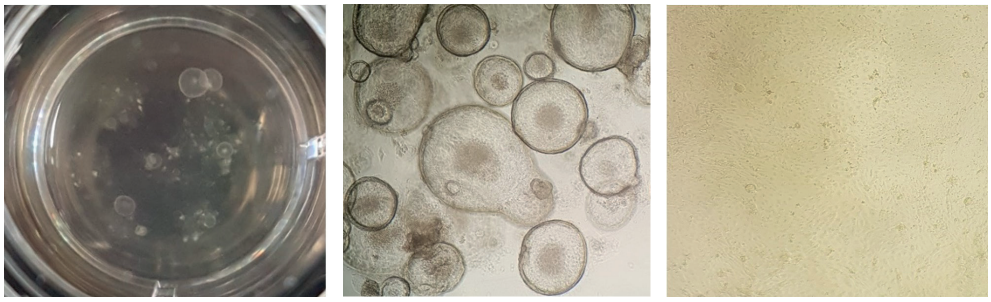

Figure 1. Pictures of propagating 3D organoids in a 24-well plate, as can be seen with the naked eye (left) and as can be seen using a light microscope (middle). On the right, a confluent 2D organoid monolayer in a 96-well plate is shown.

Upon investigation of the antibacterial effect of the conditioned supernatant, it was found that the growth of EcN, *E. coli* LF82, and *Salmonella enterica* serovar *Typhimurium* was drastically reduced, while the growth of *Lactobacillus plantarum, Streptococcus suis* P17, *Streptococcus suis* LS89, and *Pseudomonas aeruginosa* was slightly reduced, and the growth of a *Cellulosimicrobium* strain was not significantly reduced (see Figure 2 below). An interesting finding was that autoclaving the conditioned supernatant for 30 min at 121 °C did not remove its antibacterial effect, suggesting that the antimicrobial factor produced by the organoid monolayer is heat stable. Further testing using size fractionation showed that the antimicrobial factor was not larger than 10 kDa, that it was sensitive to a Proteinase K treatment, that it was active at physiological salt concentrations, and in the presence of 10% fetal calf serum (FCS). It is possible that the antibacterial factor was produced by the Paneth-like cells or enterocytes that were found to be present in 3D and 2D organoids (Van der Hee, Loonen et al. 2018). The porcine GIT epithelium is known to produce a broad range of antibacterial compounds, with alpha defensins, beta defensins, lysozyme, complement factors, cathelicidins and REG-III as examples. Given the characteristics of the antimicrobial factor found in the conditioned supernatant, the beta-defensins are the most likely candidate, as these are known to be heat-stable peptides, 3-5 kDa in size, active at physiological salt concentrations, and are known to be active against a broad range of microbes (Maisetta, Batoni et al. 2005, Grudlewska, Sysakiewicz et al. 2014). A very similar finding has been previously described, where antimicrobial activity was observed in human airway epithelial cells (Smith, Travis et al. 1996). From the *in vitro* experiments conducted with these human airway epithelial cells, the antimicrobial factor had a broad spectrum of bactericidal activity, was smaller than 10 kDa and was resistant to higher temperatures such as boiling. Future efforts are focused on identifying the antibacterial factor that is produced by porcine ileal organoids. Apart from being a highly interesting finding, the antimicrobial effect of organoid monolayers illustrates how useful such *in vitro* models are to study the interactions between feed components, the gut microbiota, and the epithelium that take place in a living animal.

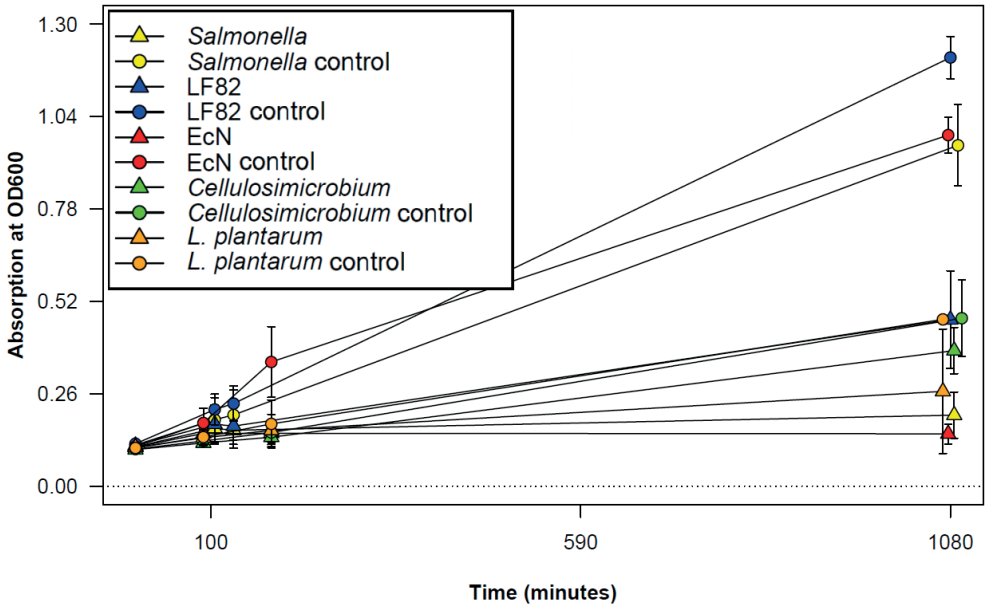

Figure 2. Results of growth experiments performed on conditioned supernatant and untreated organoid growth medium (control) using a number of bacterial strains. Strains were grown overnight at 37°C in LB medium, while shaking at 150rpm. The next day, 1ml of growing culture was combined with 4ml of fresh LB medium and placed back into the stove. Cultures were diluted 50 times when an OD600 of 0.8 was reached and 10µl of this dilution was mixed with 90µl of conditioned supernatant or control medium in a 96-well plate. Resulting growth as absorption at OD600 was measured at several timepoints using a Spectramax® M5 Microplate reader (Molecular devices, San Jose, CA, USA).

*Use of EcN in an **E. coli** challenge trial*

As pathogenic *E. coli* strains are a very important causative agents of post-weaning diarrhoea (PWD), and as we found that EcN successfully lowers Proteobacteria diversity, the next logical step would be to test EcN's competitive efficacy against a pathogenic *E. coli* strain in a challenge trial. During such a trial, it may be useful to administer both the pathogenic strain and EcN during the pre-weaning period, with the administration of EcN prior to the administration of the challenge strain. This way, the taking a foothold and spread of the pathogenic strain during the pre-weaning period in a production environment would be simulated. Given that EcN will likely have the highest presence during the pre-weaning period, its potential efficacy will likely be highest during this period. The strongest impact of EcN will likely be caused by its biofilm formation along the intestinal wall, and its competitive exclusion of close relatives by occupying the environmental niche. The duration of the study should last until at least a few weeks after weaning, as that will provide insight into whether the administration of EcN can prevent or reduce PWD.

The use of feed components with antimicrobial activity

It is widely understood that the use of antibiotic growth promoters (AGPs) in livestock production increases animal performance and feed yields. The mechanisms of action by which AGPs exert these effects are not well understood, and hypotheses that have been proposed for the effectiveness of AGPs vary widely (Broom 2017). Many of these hypotheses

are centred around the modulation of gut microbiota composition, or the modulation of the host's GIT inflammatory status. One study found that the growth-promoting effect of AGPs correlated with a decreased activity of bile salt hydrolase, which is an enzyme produced by gut bacteria that exerts a negative impact on host fat digestion and utilization (Lin 2014). Another study found that the use of AGPs led to a decrease in total bacteria, after which the authors postulated that a reduction in total bacteria may reduce the host's needs for immune responses, thereby being able to divert more energy to growth (Collier, Smiricky-Tjardes et al. 2003). Another explanation could be that a reduction in GIT bacterial load would reduce the fermentation of feed by microbes, leaving more feed components for the host to consume. To the best of my knowledge, it has not yet been determined whether bacterial cell contents are released into the gut lumen after exposure to antibacterial compounds like AGPs. The bacterial cell cytosol may be a rich source of nutrients and metabolites that drives livestock growth performance, or alternatively released bacterial cell components may elicit beneficial effects to the host's local and systemic immune status. If this is an important mode of action of AGPs, then mimicking the effects of AGPs may be possibly with the use of feed components that have antimicrobial properties such as garlic, hops, or plant/herbal extracts. Indeed, inclusion of garlic has shown to improve feed conversion ratio (FCR) in multiple studies (Cullen, Monahan et al. 2005, Samolińska, Grela et al. 2020, Chen, Wang et al. 2021). Additionally, several studies have found that the implementation of herbal extracts can improve FCR (Liu, Wei et al. 2008, Czech, Kowalczuk et al. 2009, Nowak, Kasprowicz-Potocka et al. 2017), thereby offering a range of potential alternatives to the use of AGPs.

The use of fermented feed

With the advancement of fermentation technologies (Sugiharto and Ranjitkar 2019), pig farmers are increasingly applying fermented feed in pig production. Typically these fermentations are started by thoroughly mixing solid feed with water at a ratio of 1:1.5 to 1:4. Sometimes the mixture is inoculated with beneficial microorganisms, and often used microorganisms are strains from the group of lactic acid bacteria such as *Lactobacillus* and *Pediococcus* (Missotten, Michiels et al. 2015), or yeasts such as *Saccharomyces cerevisiae* (Niba, Beal et al. 2009) and *Saccharomyces boulardii* (Zhang, Bao et al. 2020). Fermenting feed provides several advantages, such as enriching feed with vast amounts of live probiotic cells (van Winsen, Urlings et al. 2001). Additionally, fermenting feed enriches feed with lactic acid, SCFAs, other microbial metabolites and enzymes that help digest feed components (van Winsen, Urlings et al. 2001, Zhang, Pan et al. 2021). Additionally, fermenting feed lowers the feed's pH, may make micro-nutrients more available to the host (Samtiya, Aluko et al. 2021) and may reduce ammonia emissions by pigs (Ibrahim, Abdelfattah-Hassan et al. 2020). The lowering of the feed's pH may strengthen the role of the stomach as a first line of defence against pathogens (Missotten, Michiels et al. 2015). In addition, by fermenting feed, one reduces the presence of easily accessible nutrients such as readily digestible sugars (e.g. glucose or fructose) (Bachmann, Michel et al. 2021). This effect would help to select against various gut pathogens that depend on readily digestible sugars (Bachmann, Michel et al. 2021, Rastall, Diez-Municio et al. 2022). In fact, several studies have shown that fermented feed can select against the proliferation of *Salmonella* (Van der Wolf, Wolbers et al. 2001, Yin, Farzan et al. 2014*)* and other members of the *Enterobacteriaceae* (van Winsen, Keuzenkamp et al. 2002, Hong, Thuy et al. 2009). For this effect to take place, the fermented

feed should have a pH below 4.5, should contain sufficient lactic acid bacteria (i.e. > 10^9 CFU/mL) and should contain sufficiently high concentrations of lactic acid (e.g. > 150 mmol/L) (van Winsen, Urlings et al. 2001). Others, however, have reported that 75 mM of lactic acid (Beal, Niven et al. 2002), or 100 mM (Brooks, Beal et al. 2003) of lactic acid suffices. With fermentation of feed, care should, however, be taken that fermentation is taking place in a controlled fashion, so as not to end up with too high levels of acetic acid (i.e. > 30 mmol/L) or the production of biogenic amines, which both adversely affect the palatability of fermented liquid feed. Additionally, a high concentration of yeasts should also be prevented, as growth of detrimental yeast strains can result in off-flavours caused by the production of acetic acid and alcohols (Missotten, Michiels et al. 2015). With a too high concentration of yeasts, the amount of CO_2 gas produced can lead to excessive foaming which can also lower feed intake by pigs (personal communication with pig producer). As more knowledge becomes available to pig producers, and as liquid feed fermentations become more controlled due to advances in fermentation technologies, the use of fermented feed will likely continue to be adopted by increasingly more pig producers in the near future.

The use of microbial consortia

In this thesis' *in vivo* study, a single probiotic strain was assessed for its impact on gut microbiota composition and host health. This impact is influenced by the probiotic's capability to survive and possibly colonize the host's GIT, and by its metabolic repertoire. A consortium of multiple probiotic strains would in theory outperform any single probiotic strain, both in terms of survivability of (at least part of) the probiotic strains, but also in terms of the metabolic repertoire. Fortunately, already a number of *in vivo* studies have been performed with microbial consortia (Stein, Tanoue et al. 2018, Vázquez-Castellanos, Biclot et al. 2019, Pereira, Wasmund et al. 2020), and likely many more will be performed in the near future.

Some microbial consortia have a long history of daily use by humans, such as milk kefir, water kefir, kombucha, boza (Todorov, Botes et al. 2008), tuba, tepache (de la Fuente-Salcido, Castañeda-Ramírez et al. 2015), ginger bug (Canakapalli 2019), Mabisi (Moonga, Schoustra et al. 2019), and many others. In recent years, the scientific community has shown increased interest in these microbial consortia, with several traditionally brewed beverages being studied for their microbial composition (de la Fuente-Salcido, Castañeda-Ramírez et al. 2015, Lynch, Wilkinson et al. 2021). What should be noted, is that the microbial composition of these traditionally brewed beverages ranges widely and is also subject to temporal changes. This makes it a challenge to pinpoint exactly what microbial composition a given traditionally brewed beverage contains. A recent study endeavoured to generate an overview of the microbial composition of water kefir beverages in several countries, and it was reported that there is quite some overlap between kefir beverages that have their origin in different countries (Lynch, Wilkinson et al. 2021). In addition, the water kefir's microbial communities contained several known probiotic species, such as *Lactobacillus brevis, Lactobacillus casei, Lactobacillus plantarum, Streptococcus lactis, Saccharomyces cerevisiae,* and *Bifidobacterium.* Next to the overlap in microbial composition, there seems to also be a functional overlap, since for the 16 different water kefir sources tested, all of them contained multiple lactid acid bacteria species, seven sources contained at least one

acetic acid bacterial species and all contained at least one yeast species (Lynch, Wilkinson et al. 2021).

For each of the mentioned traditionally brewed beverages, there is a long history of use in human consumption, which raises the question whether these microbial consortia can be successfully used to elevate the health status of production animals. Of the traditionally brewed beverages, water kefir may prove to be the most interesting probiotic candidate consortium for use in livestock production, as the used ingredients for production (cane sugar, dried figs and water) are comparatively cheap when compared to many other traditionally brewed beverages, that either require tea extraction, animal milk use or the use of chopped ginger roots. Care should however be taken, that alcohol levels remain below desired levels (especially when administered to young animals) and that the probiotic products remain palatable for animals. Prevention measures should for instance be in place to avoid the build-up of too high levels of acetic acid during fermentation, which in conventional pig feed fermentation occasionally causes the fermented product to be non-palatable to pigs (personal communication with a pig producer). A too high build-up of acetic acid is typically caused by a too high exposure of oxygen during fermentation, meaning acetic acid build-up can be prevented by fermenting in closed containers that have a water lock installed to allow for off-gassing without introducing air from outside. In a recent pilot study with newly weaned piglets, researchers found that piglets did not experience any detrimental effects from the consumption of 1 litre of water kefir per day and that water kefir was palatable to piglets (Gangnat and Kreuzer 2021). In another recent study, researchers found that the use of milk kefir as feed additive during the post-weaning phase enhanced the average daily gain, increased the number of lactic acid bacteria and *Bacillus* spp. in faeces, reduced the number of coliforms in faeces and enhanced a number of indicators of innate immunity (Choi, Son et al. 2021). These results are promising and may lead to the adoption of microbial consortia from traditionally brewed beverages in pig production and other livestock production.

The use of live mushroom spawn

In this thesis' *in vivo* study, yeast-derived β-glucans were assessed for their impact on gut microbiota composition and host health. Alternative sources of β-glucans are cereal grains or mushroom fruiting bodies and mycelial biomass. Especially the latter deserves further attention as a promising candidate for dietary interventions, as mushroom-derived β-glucans are structurally different than those derived from yeast or cereal grains (Zhu, Du et al. 2015), and may elucidate stronger or different effects on host epithelium and immune cells (Borchers, Keen et al. 2004). Mycelial biomass is readily available in the form of mushroom spawn, which is produced in relatively large batches (2-5 tons per production cycle, with several daily cycles per production facility), and is packaged for solid fermentation in filter bags at sizes of 10-30 litres. Several species of grain can be used as raw material for the production of mushroom spawn, with wheat grains, rye grains, millet grains and corn as most often used options. Prepared grains can be inoculated with mycelial sludge from dozens of different mushroom species, of which currently *Agaricus bisporus, Pleurotus ostreatus* and *Lentinula edodes* are the most often used options, as global production is highest for these mushroom species. Other mushroom species that may be highly interesting to use in the production of live mushroom spawn for pig feed, are *Ganoderma lucidum, Cordyceps sinensis, Coriolus versicolor, Grifola frondosa, Agaricus blazei, Hericium erinaceus, Flammulina velutipes* and

Pleurotus eryngii, as these species are renowned for their effects on human health. Duration of fermentation can be extended from the regular four to eight weeks to a duration of up to around four months to increase the amount of mycelial biomass and fermentation end-products. Live mushroom spawn likely has a broad range of effects on animal health, as hundreds of bio-active compounds are produced by mycelium of the mentioned mushroom species. Drying of live mushroom spawn may be an option, but the drying process is likely to lower the presence of numerous bio-active compounds, (Udomkun, Nagle et al. 2015). This introduces a logistical obstacle, as live mushroom spawn typically has a high moisture content (50-70% w/w), which makes it practically impossible to mix it through conventional feed, as it would lower feed shelf-life. An added logistical obstacle is that live mushroom spawn is best stored when refrigerated, as the fermentation process in the filter bag would otherwise continue at ambient temperatures. However, these logistical obstacles do not deter mushroom substrate producers to transport and work with vast amounts of mushroom spawn filter bags, and so pig feed producers and pig farmers should be able to overcome the mentioned logistical obstacles. Although produced in bulk, the cost of live mushroom spawn may still be too high to use during pig fattening, and as such the best use-case may be during the pre-weaning phase as part of creep feeding, until a few weeks after weaning.

Implementation of tracking EcN presence in future studies using high throughput sequencing technologies

The observation that the presence of EcN could, apart from using qPCR, be detected by using the amplicon sequence dataset, was a welcome surprise. This resulted in being able to use two methods to verify the presence of EcN, and resulted in being able to find the presence of EcN in faecal and luminal samples that were not planned to be analysed using qPCR. Since amplicon and metagenomic sequence datasets have been generated for numerous *in vivo* and cohort studies in the past, and since these technologies will frequently be used for future studies, being able to track the presence of EcN by reprocessing such datasets may be an interesting option for researchers. To allow for tracking of EcN presence, custom parameters were used in the data processing pipeline, such as a lower threshold value for the prevalence filter. What should be mentioned, is that the ASV that was found to be specific for EcN in the study related to Chapter 5, does not turn out to be unique when comparing the ASV to large *E. coli* datasets. With the help of Prof. Ulrich Dobrindt and Dr. Christoph Cichon, who both work at the Medical Faculty of the University of Münster, the forward sequence of the EcN-specific ASV was screened against the genomes of 778 strains that belong to ST73, which is an *E. coli* lineage that is often linked to urinary tract infections (de Souza da-Silva, de Sousa et al. 2017, Kallonen, Brodrick et al. 2017). Of the total of 778 strains that were screened, 71 strains were found to possess at least one identical sequence stretch within their 16S rRNA gene(s). None of these 71 strains, nor EcN, was found to have this sequence in all of their 16S rRNA gene copies (which are typically 7). Additionally, the genomes of those 71 ST73 strains do not cluster phenotypically, which means the sequence seems to be caused by random mutation. In summary, the single-nucleotide polymorphism (SNP) that is related to the EcN-specific ASV, is not specific for EcN outside of the *in vivo* study of Chapter 5. This means that the EcN-specific ASV is likely still useful for studies where EcN is administered to individuals, but may not be highly useful for exploratory studies like cohort studies. On the other hand, with the use of the EcN-specific EcN sequence, researchers would be able to

verify that EcN is not present in samples of interest, at least not at cell numbers above the technique's threshold level.

Concluding remarks

The results presented in this thesis contributed to a better understanding of the effects of yeast-derived β-glucans and *E. coli* Nissle 1917 on the gut microbiota, gut epithelium, and the immune system in early life. We demonstrated that conducting *in vivo* studies in combination with the administration of dietary interventions, microbial assays and immunological assays provides new insights and hypotheses for future research. Our results may also motivate livestock farmers to include the administration of dietary interventions into their regular farm practices. The main conclusions of the thesis are summarized below:

1. The effects of the probiotic strain *E. coli* Nissle 1917 on porcine intestinal microbiota composition and immune system are in accordance with its temporal presence in the gut.
2. Early life provides an important and effective window of opportunity to steer the development of the gut microbiome and immune system.
3. *E. coli* Nissle 1917 has a stronger influence on overall porcine gut microbiota than yeast-derived β-glucans. This was evidenced by the effect of the probiotic treatment on microbiota beta diversity being significant, while this was not found for the β-glucan treatment. In addition, EcN-treated pigs showed a reduced diversity of taxa within the phylum Proteobacteria and a lower relative abundance of taxa within the genus *Treponema* during the pre-weaning period.
4. *E. coli* Nissle 1917 has no significant effects on the gene expression of the porcine gut epithelium. In total, 96 genes were tested for their gene expression in both ileum and colon mucosal scrapings, but none were significantly differentially expressed in EcN animals compared to control animals. This suggests that EcN has no large impact on host epithelium, although variation in gene expression among individual animals was high.
5. There is a need for extra precautionary measures and containment in *in vivo* studies that are dealing with live biotherapeutics.

Dietary interventions such as yeast-derived β-glucans and *E. coli* Nissle 1917 have promising effects to improve the development of the weanling pig. With an adequate timing and dosing, dietary interventions may lead to the use of heavy metals and antibiotics in livestock farming becoming obsolete. However, more research is needed to better understand the effects of each dietary intervention, and to better understand what microbial and immunological development lowers the incidence of post-weaning diarrhoea. Here, we have shown that the administration of a single probiotic strain to piglets in early life seems to have a beneficial effect on gut microbiota composition. The next phase would be to start using microbial consortia, with hopefully even more beneficial effects to gut microbiota composition. Effects that may very well empower the livestock farmers of the future to improve animal health and welfare.


A

Appendices

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English Summary

It is well established that abrupt environmental, social and dietary changes associated with weaning can impair animal health. In the past, antibiotic growth promoters (AGPs) were intensively used in the livestock industry to prevent the development of gastro-intestinal disorders and to enhance animal performance. However, the use of in-feed AGPs has been banned in the European Union since 2006 because of the worldwide concern on the emergence of multi-drug resistant bacteria. As a result, alternatives to in-feed AGPs that effectively support animal health and performance received a lot of attention. Researchers have been studying the effects of several promising microbial and non-microbial feed interventions in pigs. Several pre- and probiotics, for example, have been shown to modulate the porcine gut microbiota and to improve gut epithelial barrier function. In addition, it has become evident that the gut microbiota and the immune system are intrinsically linked to each other and are both important determinants of health and disease (**Chapter 1**). However, diet-induced changes in the gastro-intestinal tract (GIT) and immune system are often mild and momentary, which is possibly due to the fact that the resilience of the gut microbiota to modulation increases with age and because most feed additives are administered post-weaning. Since piglets do not have a fully developed gut microbiota and immune system in the first weeks of life, it was hypothesized that dietary interventions administered shortly after birth would have a greater chance to induce significant changes to gut microbiota composition, gut epithelial gene expression, and overall immunological responsiveness. By modulating these systems in early life, we ultimately aim to make pigs more resilient to perturbations around the weaning period.

In Chapter 2, we reviewed the available literature on gut microbiota development in pigs. The review offers a comprehensive understanding of the factors that can influence gut microbiota composition and how they may impair porcine gut health. We also described how the porcine gut microbiota plays numerous beneficial roles. For example, apart from its fermentation activity, the gut microbiota stimulates proper development of gut physiology, host immunity and general well-being of the piglet. The intestinal microbiota of neonatal piglets rapidly develops from a simple to a more stable and mature ecosystem, following a highly dynamic developmental trajectory. Especially around weaning, piglets are highly susceptible to develop gastro-intestinal problems due to weaning-associated perturbations. Conventional methods to prevent gut-related issues include the use of AGPs and the inclusion of zinc oxide in feed. Because these conventional methods have several disadvantages, new methods are being developed to improve gut microbiota development to prevent gut-related issues. Of these new methods, the implementation of fibrous components in feed and the (oral) administration of probiotics to pigs are considered to be the most promising, as several *in vivo* studies have shown the effectiveness of such fibrous components or probiotics on the gut microbiota. Although results are promising in a number of these studies, optimal inclusion rates of fibrous components are still to be determined, and the use of probiotics comes with some practical obstacles that need to be addressed. We also concluded that relatively few studies investigated the effects of dietary interventions during the suckling period. However, administration of dietary components during the suckling period might be more promising in terms of early-life modulation of the

gut microbiota, gut epithelial barrier and mucosal and systemic immune development, and subsequent disease prevention.

The review in **Chapter 2** demonstrated that administration of fibrous components and probiotics to pigs are considered promising strategies to enhance porcine health and performance through modulation of the GIT and immune system. A promising feed additive that has already been widely implemented in the food and feed chain are baker's yeast-derived β-glucans. They have been shown to stimulate immune cells by binding to cell receptors such as Dectin-1 and CR3, which in turn triggers a number of immunological cell signalling pathways. In addition to their immunostimulatory effects, yeast-derived β-glucans are also thought to confer beneficial effects as prebiotic fibres. Because of their molecular structure they are not digested by the host's enzymes, but are fermented by fibre-fermenting bacteria in the hindgut. In **Chapter 3**, we investigated how early life supplementation of yeast-derived β-glucans affects the porcine gut microbiota and immune function during the pre- and post-weaning period. In addition, this study also provided extensive information on how these complex systems develop over time. Yeast-derived β-glucans were orally administered from day two after birth until two weeks after weaning. Faeces, digesta, blood, and mesenteric lymph node tissue samples were collected at different pre- and post-weaning time points. Overall, yeast-derived β-glucans had modest effects on microbiota alpha diversity and the abundance of specific bacterial genera pre-weaning. A similar pattern was observed for the tested immune parameters. These results imply that the pre-weaning period offers an opportunity to modulate the gut microbiota and immune system through diet, but the induction of any long-lasting effects by yeast-derived β-glucans still remain to be elucidated.

Another promising feed additive that has been studied for over 100 years is the probiotic *E. coli* strain Nissle 1917 (EcN). It is known for its ability to protect against pathogenic *E. coli* strains and has been used against gastro-intestinal disorders in humans and animals. However, no studies have investigated its effects in conventional neonatal piglets. In a pilot study described in **Chapter 4**, we investigated the presence, abundance and location of EcN in the porcine GIT after peroral administration of high, medium, or low concentrations of EcN (10^9, 10^8, or 10^7 CFU/mL, respectively). EcN was detected in faeces of all piglets that received EcN, however, EcN was also detected in control animals, albeit at a low abundance. We therefore highly recommended the implication of control measures that aim to prevent cross-contamination in *in vivo* studies that use microbial products such as probiotics. Overall, no major differences were observed between the piglets that received different concentrations of EcN but the amount of EcN present in faeces was highest in piglets that received 10^8 and 10^9 EcN/inoculation. In addition, EcN was detected in the mesenteric lymph node (MLN) of one animal that received 10^9 EcN/inoculation, which implies that EcN is able to translocate to gut-associated lymphoid tissue. Taken together, we demonstrated that EcN is present in faeces, in all segments of the GIT, and in the MLN after peroral administration. This pilot study contributed to a better understanding of EcN's traits and served as a basis for the larger *in vivo* study presented in **Chapter 5**.

In **Chapter 5**, we investigated the spatio-temporal effects of EcN on gut microbiota composition, intestinal epithelial gene expression, and immune system by collecting and

analysing faeces, digesta, blood, mucosal scrapings and mesenteric lymph node tissue at different time points. Piglets perorally received the probiotic strain EcN (10^9 CFU/inoculation) or a control treatment from day two after birth until two weeks post-weaning. Moreover, peroral vaccinations against *Salmonella enterica* serovar *Typhimurium* were given on days 21 and 45 to assess EcN's ability to affect immune responsiveness. In short, piglets that received EcN showed a reduced diversity of taxa within the phylum Proteobacteria and a lower relative abundance of taxa within the genus *Treponema*. Interestingly, this was only observed during the pre-weaning period. Although *Treponema* and Proteobacteria include harmless commensal bacterial species, several members are primarily known as potential pathogens. Thus, the association between EcN and reduced Proteobacteria diversity as well as the potential antagonistic effect against *Treponema* suggest a beneficial effect with respect to porcine gut microbiota composition pre-weaning. In addition, its effect on immune cells (e.g. NK cell activation) and cell responses (e.g. IL-10 production by immune cells) strengthened this conclusion, but should ultimately be confirmed by infection studies. Body weight, gut epithelial gene expression, and immune responses upon vaccination did not differ significantly between the treatment groups, and any differences observed during the post-weaning period were modest. In summary, we concluded that the pre-weaning period offers a 'window of opportunity' to modulate the porcine gut microbiota and immune system through dietary interventions such as EcN.

In **Chapter 6**, I discussed the results of this thesis by addressing different topics and issues related to the porcine gut microbiota and in light of relevant scientific literature. Based on the results presented in this thesis, I concluded that yeast-derived β-glucans and EcN are both able to modulate the porcine gut microbiota (**Chapters 3 & 5**). Importantly, the majority of these effects were solely observed during the pre-weaning period, which is in line with the immunological results presented in these chapters. Post-weaning effects were modest, and any long-term effects induced by these feed additives remain to be elucidated. This confirms our hypothesis that early life provides an opportunity to modulate the porcine gut microbiota and the immune system through diet. In **Chapter 6**, I also discussed the practical implications of our *in vivo* findings, the relevance of conducting a pilot study and the relative importance of dietary interventions. These topics contribute to a better understanding of the impact of my research and how these results can be used in a more applied context. Lastly, I provided future recommendations and perspectives for follow-up research.

Acknowledgements

It is with great pleasure to share with you the results of my PhD thesis. This thesis would not have been the same without the help of numerous colleagues, friends, and family. Clearly, a PhD project is not performed by the individual alone. In the following paragraphs I would like to express my gratitude and would like to thank many people.

First of all, I would like to thank my promotors Hauke Smidt and Jerry Wells for your confidence in me and for giving me the opportunity to start my PhD project at your chair groups. I look back to having received highly capable supervision by both of you and look back to a very positive collaboration. I enjoyed the freedom that you provided for my projects, with in parallel the sharing of in-depth knowledge in your respected fields. Dear Hauke, your structured approach, calmness, and immense motivation have elevated my projects to a higher level. I admire your multitasking capabilities, which I am sure I will never be able to imitate. Dear Jerry, I have enjoyed your contagious enthusiasm, creativity, and in-depth knowledge during our scientific conversations. Always amicable and amusing, is how I like to describe you, with every now and then a brilliant anecdote to spice things up. I wish the best to both of you in your future life and future endeavours.

My PhD project was part of a larger consortium that gave me the opportunity to work together with Huub Savelkoul, Edwin Tijhaar, Aart Lammers, Mirelle Geervliet and Michel Verwoolde. Thank you for all the help and the nice collaborations. The porcine *in vivo* studies and the data analyses would not have been possible without the help of many people from Wageningen University & Research, Wageningen Bioveterinary Research, Vereniging Diervoederonderzoek Nederland (VDN), and the National Veterinary Institute of Denmark (DTU-VET). The staff of the Swine Research Centre (Trouw Nutrition, Nutreco) have really made the *in vivo* studies become a success. I want to thank Carlijn de Bruijn and Hubèrt van Hees for their large contribution regarding the logistics, organization, and execution of the *in vivo* studies. Additionally, I would like to thank the technical staff of the Swine Research Centre, with whom we were able to refine our procedures and who did lots of hard work. Thank you Erik Cuppen for your presentation of leadership at times when this was required, and thank you Luuk Selten for your remark that postponing coffee breaks would not speed things up. These were two beautiful life lessons that I learned while working with you. I would like to thank all others that were involved in sample collection, namely Pieter Roskam, Ralph Kok, Rinie Ernste, Sandra Vreman, Bart van der Hee, Ineke Heikamp-de Jong, Caifang Wen, Natalie Groothuis, Giacomo Antonello, Carmen Sinche Ambrosio, Nico Taverne, Anja Taverne, and Prokopis Konstanti. Special thanks go out to Caifang with whom we have had a fruitful collaboration and with whom I have worked extensively in both the MIB and HMI labs.

After having chosen EcN as one of the dietary interventions, we have had a fruitful collaboration with Rudolf von Bünau and Birgit Klinkert (Ardeypharm GmbH), who provided us with the required quantities of Ponsocol®, but also with relevant literature, in-depth knowledge on molecular detection methods, and extensive microbiological knowledge. Our visits to your company have been a true pleasure. I would like to thank Kerstin Skovgaard

and Karin Tarp from DTU-VET for hosting me at their institute and for doing extensive lab work and data processing.

I also would like to thank all the people from the Laboratory of Microbiology and the people from the Host-Microbe Interactomics Group for always creating such a nice working atmosphere. What I appreciated most was my colleagues' motivation to do things right and having discussions about interesting topics in the life sciences. Being part of the MolEco group got me to join the regular Thursday morning meetings. Even though I was 1 minute late approximately half of the time, nobody ever made a fuss about it, which in my eyes is a testimony to the tolerant attitude that is all-present. The Christmas dinners, summer BBQs, and Lab Trips were all great highlights that made us all get to know each other while sipping on (alcoholic) beverages. Especially the PhD Trips resulted in great memories! I will always remember Aleksandr's Birthday in Hamburg's Cave Club, the beers with Jorrit, Ioannis and Erika in the Reeperbahn, the Carlsberg tour, dining out with Raymond in Boston's Chinatown, the excellent Chinatown hot pot diner with the whole group, and rocking the pinball machine at The Tam together with Thijs and Joep.

The lab work would not have been possible without the kind support of the technicians. Thank you Ineke, Steven, Tom (x2), Ton, Philippe, Sjon, Wim, Nico and Anja for your great support. Jasper, Bart, and Wasin, thanks for your never-ending support on MIB's data processing pipelines, bioinformatics, and data management. Anja, Carolien, Heidi and Loes, thank you for being such competent secretaries. It was a great experience for me to supervise four thesis students. Giacomo Zeni, Iduna Hoefnagel, Panagiotis Zontos, and Giacomo Antonello, thanks a lot for your dedication and hard work. Without your efforts I would probably still be running around the lab to process all those samples. Teaching at the Cell Biology course during my first year as a PhD has been great fun, especially due to everything running so smoothly. It was highly instructive and helpful for my later teaching to see how a handful of people with a combined teaching experience of over 100 years become a well-oiled Cell Biology teaching machine. Thank you, Eddy Smid, and Judith Wolkers-Rooijackers for being such nice colleagues during the teaching of the Advanced Fermentation Science course. Thanks to the positive vibes and the degree of freedom that was present during your course, I look back to many beautiful moments.

Dear Bart, Nico, Anja, Linda, and Joyce, thanks a lot for your kind support and advice on the organoid work. Unfortunately, I was not able to nail down exactly what antibacterial compound is produced by the 2D organoids. Otherwise, I would have had the honour to publish an article on this topic together with you.

I would also like to thank my awesome office mates, Klaudyna, Teresita, Tika, Carrie, Marie-Luise, Kate, Martha, Ying, Hanne, Aleksandr, and Indra. Thanks a lot for the support in and exchange of ideas in statistical analyses, laboratory methods, presentations, and scientific writing. Aleksandr, thanks a lot for the great moments in Barcelona, and the legendary organ diner in France. Indra, I will always remember your bright smile that you showed me in 2012 when we met during a Wageningen bus ride. I directly tried to recruit you for our iGEM team, to no avail. It was a pleasant surprise to find out four years later that we would be colleagues. One day I will visit you in Bali. I have enjoyed the feminine office presence that

came to a climax towards the end of my PhD life. Being surrounded by exclusively women led to a rather lively atmosphere with lots if valuable insights and fun memories. Carrie and Marie-Luise, thanks a lot for being such great successors in the VLAG PhD council.

Working with broadly interested and motivated colleagues opens up opportunities that I gladly capitalized on. To start, I would like to thank Benoit Carreres for his enthusiastic help with building and programming a retro game arcade machine. A machine that I would love to place and enjoy in the middle of my living room, but that somehow always ends up in the attic. I would like to thank Jurre Steens and Thijs Nieuwkoop for introducing me to the amazing worlds of 3D printing and PCB design respectively. Thank you Gerben for having such an analytical mind and thank you Costas for having such an entrepreneurial mindset. Ton van Gelder, thanks a lot for inviting me and Wim to your 101st beer brew and for sharing your advice on pulp fermentation of apples into cider. Whoever rumoured the existence of water kefir cultures (was it you Lot van der Graaf?) during one of our lunch breaks, thanks a lot for sharing this important piece of information.

Beste familie, bedankt voor de steun en interesse in mijn promotie werk en de fijne momenten in mijn vrije tijd. Hopelijk gaan we nog vele mooie momenten beleven, ik ben natuurlijk niet voor niets teruggekomen naar Lierop (voor jullie!). Lieve schoonfamilie, ook al zijn ik en Mirelle neergestreken in Brabant, hoop ik dat we elkaar regelmatig blijven opzoeken. Ik kijk nu al uit naar de wintersport in februari. Beste vrienden van de borrelclub, het dispuut en beluga social club, de safari, biertjes, en karaoke waren enerverend. Beste Paranymphen Bart en Jorrit, jullie zijn geweldig en we moeten samen iets leuks gaan doen in 2023. Dear Phenospex colleagues, thanks for your understanding when I wanted to invest some extra time in finishing my PhD thesis. Let’s conquer the world of digital plant phenotyping together with renewed energy in the new year.

Lieve Mirelle, wat ben ik trots op wat wij samen hebben neergezet. Het was een hele fijne samenwerking omdat we zo goed naar elkaar en naar anderen luisterden. We hebben voor de tweede keer een hele leuke tijd gehad in Wageningen. Je bent een super vrouwke en ook ik kan niet wachten op wat de toekomst ons zal brengen. Ik hou ook van jou!

About the Author

Hugo de Vries was born on April 10th 1989 in Geldrop. Being raised on a farm in the small village of Lierop, he soon developed a strong interest for biology. Collecting stones and frogs were only a few of his many hobbies, and a harbinger of what was about to come. In 2007, he graduated from the St. Willibrord Gymnasium in Deurne and moved to the 'big' city of Wageningen to start his study Biotechnology at Wageningen University & Research. Besides his study, Hugo was an active member of the student association K.S.V. Sint Franciscus Xaverius where he was part of borrelclub 'BFF' and dispuut 'W.H.D. The Knights of the Round Table'. After successfully finishing his Bachelor, he realized there is much more to study and explore and proceeded with the MSc Biotechnology in Wageningen. As part of his MSc study, he moved to the United Kingdom for six months to study the interaction between bacteria and phages at the Coastal Pathogens Group (University of Exeter Medical School) under supervision of Pawel Sierocinski and Michiel Vos. In addition, he participated in the 2012 iGEM competition with a team of 14 students where they worked on a standardized tool for site-specific drug delivery using Virus-Like Particles. At the iGEM European Jamboree he was awarded with 'The Best Presentation Award' and at the finale in Boston his team ended in the top 16 worldwide. After this success, he also had the honour to present this work at the EuroSYNBIO conference in Groningen and at DSM in 2012 and at the 8th CeBiTec Symposium in Bielefeld in 2013. In 2014, Hugo graduated as a Master of Science.

During a short career break Hugo became fascinated with the world of mushrooms. Being a biologist and a son of an entrepreneur, he started a small-scale production of gourmet and medicinal mushrooms. Despite his passion for mushrooms, he was eager to continue his career in science to expand his skills and knowledge. In 2016, he got the opportunity to start as a PhD candidate at the Laboratory of Microbiology and Host-Microbe Interactomics Group to study the effects of dietary interventions on the gut microbiota and the gut epithelial barrier in pigs. Under the supervision of Hauke Smidt and Jerry Wells, Hugo designed, executed, and analysed several *in vitro* and *in vivo* studies, working closely together with other researchers and companies. His scientific work is presented in this PhD thesis. In addition, Hugo was involved in several educational activities, supervised several BSc and MSc students, participated and organized the MIB PhD trip and was member of the VLAG PhD council. Currently, Hugo is working at Phenospex, a company that develops digital solutions for plant phenotyping. Here he works in a diverse team with an interdisciplinary background in biology, agriculture, physics, computer science and engineering. And like a lot of Brabanders, he moved back from the 'big' city of Wageningen to the small village of Lierop where he lives together with his partner (and ex-PhD colleague). Gardening, mushroom foraging and growing mushrooms, brewing fermented drinks (e.g., water kefir), fermenting food, hiking, cooking, 3D printing, and hydroponics are only a few of his weekly sideline activities. These activities that both entail the life sciences and technology not only help Hugo to recreate but are also fundamental for a creative mind that is needed for his current job.

List of Publications

Matthijn C. Hesselman, Dorett I. Odoni, Brendan M. Ryback, Suzette de Groot, Ruben G. A. van Heck, Jaap Keijsers, Pim Kolkman, David Nieuwenhuijse, Youri M. van Nuland, Erik Sebus, Rob Spee, **Hugo de Vries**, Marten T. Wapenaar, Colin J. Ingham, Karin Schroën, Vítor A. P. Martins dos Santos, Sebastiaan K. Spaans, Floor Hugenholtz, Mark W. J. van Passel, A multi-platform flow device for microbial (co-) cultivation and microscopic analysis. *PLoS ONE* 2012, 7(5): e36982. https://doi.org/10.1371/journal.pone.0036982

Marianna Roselli, Robert Pieper, Claire Rogel-Gaillard, **Hugo de Vries**, Mick Bailey, Hauke Smidt, Charlotte Lauridsen, Immunomodulating effects of probiotics for microbiota modulation, gut health and disease resistance in pigs. *Animal Feed Science and Technology* 2017, 233, 104-119. https://doi.org/10.1016/j.anifeedsci.2017.07.011

Jerine A. J. van der Eijk, **Hugo de Vries**, Joergen B. Kjaer, Marc Naguib, Bas Kemp, Hauke Smidt, T. Bas Rodenburg, Aart Lammers, Differences in gut microbiota composition of laying hen lines divergentlyselected on feather pecking. *Poultry Science* 2019, 98:7009-7021. http://dx.doi.org/10.3382/ps/pez336

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Stijn J. J. Schreven, **Hugo de Vries**, Gerben D.A. Hermes, Hauke Smidt, Marcel Dicke, Joop J. A. van Loon, Relative contributions of egg-associated and substrate-associated microorganisms to black soldier fly larval performance and microbiota. *FEMS Microbiology Ecology* 2021, 97(5), fiab054. https://doi.org/10.1093/femsec/fiab054

Mirelle Geervliet, **Hugo de Vries**, Christine A. Jansen, Victor P.M.G. Rutten, Hubèrt van Hees, Caifang Wen, Kerstin Skovgaard, Giacomo Antonello, Huub F.J. Savelkoul, Hauke Smidt, Edwin Tijhaar, Jerry M. Wells, Effects of *E. coli* Nissle 1917 on the porcine gut microbiota, intestinal epithelium and immune system in early life. *Frontiers in Microbiology* 2022, 13:842437. https://doi.org/10.3389/fmicb.2022.842437

Stijn J. J. Schreven, **Hugo de Vries**, Gerben D.A. Hermes, Giacomo Zeni, Hauke Smidt, Marcel Dicke, Joop J. A. van Loon, Black soldier fly larvae influence internal and substrate bacterial community composition depending on substrate type and larval density. *Applied and Environmental Microbiology* 2022, Vol. 88, No. 10. https://doi.org/10.1128/aem.00084-22

Overview of Completed Training Activities

Category A: Discipline specific activities (courses, workshops, symposia, summer schools, conferences etc.)	
Name of the course/meeting	Organizing institute (s)
3rd Wageningen PhD Symposium	Wageningen UR
Symposium "Organoids: disease modeling"	UMC Utrecht
KNVM Scientific meeting	KNVM
Immunoforce Symposium	Wageningen UR
Microbial Ecology Meeting	KNVM
Learning lab techniques (FLUIDIGM) at DTU-VET	DTU-VET
PiGutNet meeting	COST Action
Training School INRA	COST Action/INRA
KNVM Scientific meeting	KNVM
New Frontiers Microbiome	Radboud UMC
Centennial MIB symposium	MIB
The intestinal Microbiome and Diet in Human and Animal Health	VLAG
PiGutNet meeting	Cost Action/WUR
SIAM career event	Soehngen Institute
Healthy Food Design	VLAG
Gut Day	KNVM
Gut Day	KNVM
Workshop Data by Leo Lahti	Radboud UMC
Gut Day	UMC Amsterdam

Category B: General courses (e.g. PhD week, writing and presenting courses, statistics, etc.)	
Name of the course	Organizing institute
VLAG PhD week	VLAG
Basic Statistics with R	PE&RC
PhD Workshop Carousel	Wageningen Graduate School
Big Data	VLAG
Linux Shell course	Wageningen UR
Scientific Writing	In'to Languages

Category C: Assisting in teaching and supervision activities	
Code and name of the course	Year
CBI-10306 Cell Biology	2017
FHM-30806 Advanced Fermentation Science	2016-2018
4x Supervision of thesis student	2017-2019

Category C: Optionals (participation in discussion groups, PhD excursions, MSc courses, etc.)	
Name of the course	Organizing institute
Writing Research Proposal	MIB/HMI
MIB PhD Trip	Lab. of Microbiology
Workgroup meetings MolEco	Lab. of Microbiology
Workgroup meetings HMI	Host-micro inter. Group
VLAG PhD Council	VLAG
MIB PhD Trip	Lab. of Microbiology

Completion of training activities is in fulfilment of the requirements for the education certificate of the Graduate School of Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences (VLAG).

Colophon

The research described in this thesis was financially supported by The Dutch Research Council (NWO) and Vereniging Diervoederonderzoek Nederland (VDN) under grant number 868.15.030. The contents of this article represent the authors' scientific conclusions and neither NWO nor VDN, or any Associated Partners are responsible for any use that may be made of the information contained therein.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design by Judith van Voorst Vader

Layout by ProefschriftMaken | proefschriftmaken.nl

Printed by ProefschriftMaken on FSC-certified paper