EARLY-LIFE FEEDING IN PIGLETS: THE IMPACT ON INTESTINAL MICROBIOTA AND MUCOSAL DEVELOPMENT

RAKA CHOUDHURY

PROPOSITIONS

1. Relatively small amount of fibrous diet intake during early-life accelerates intestinal maturation.
   (this thesis)
2. Allowing a certain degree of experimental noise is important for detection of a reliable and relevant signal.
   (this thesis)
3. Physiological relevance for the biological system studied should be guiding data analysis, because statistical significance as such is irrelevant.
4. Performing interdisciplinary research is often equivalent to being bilingual.
5. Emotional intelligence should be an integral part of the current educational curriculum.
6. The future of *Homo sapiens* is threatened by its own intelligence.

Propositions belonging to the thesis, entitled

Early-life feeding in piglets: the impact on intestinal microbiota and mucosal development

Raka Choudhury

Wageningen, 5 January, 2021

Early-life feeding in piglets: the impact on intestinal microbiota and mucosal development

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This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS)

Early-life feeding in piglets: the impact on intestinal microbiota and mucosal development

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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 Tuesday 5 January 2021
at 4 p.m in the Aula.

Raka Choudhury
Early-life feeding in piglets: the impact on intestinal microbiota and mucosal development, 252 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2021)
With references, with summary in English

ISBN 978-94-6395-606-2
DOI https://doi.org/10.18174/533912

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For all those who encouraged me to fly
towards my dreams,
and for you Dida

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Chapter 1

General Introduction

Introduction

The world population is expected to reach more than 9 billion humans by 2050, thereby imposing global food security challenges. To meet the growing food demand, it is projected that the global meat production would need to rise by over 200 million tonnes in order to reach 470 million tonnes by 2050 (Alexandratos and Bruinsma, 2012). Pork is the most consumed meat in the world accounting for about 36% of total meat consumption (McGlone, 2013; Food and Agriculture Organization of the United Nations, 2014). Post-weaning enteric infection is one of the major concerns related to the gut health of pigs, and is closely associated with economic losses as well as reduced animal health, welfare and growth performance (Fairbrother et al., 2005; Lallès et al., 2007; Aland and Madec, 2009; Gresse et al., 2017). Industrialised pork production systems are challenged by enteric and respiratory diseases affecting the mortality and welfare of post-weaned piglets. For instance, post-weaning diarrhoea (PWD), commonly caused by enterotoxigenic ***Escherichia coli*** (ETEC) is an economically important disease in pig production worldwide, having a piglet mortality rate up to 17-20% (Lallès et al., 2007; Xiong et al., 2019).

In the past, PWD or weaning-associated diarrhoea has been managed and growth performance was optimised by adding antibiotics or mineral compounds like zinc oxide to the feed. However, this poses serious risks to animal health and public health, due to the possible emergence of multidrug-resistant bacterial strains as well as environmental accumulation of zinc (Verstegen and Williams, 2002; Aland and Madec, 2009). This eventually led to the implementation of a full ban on 'in-feed antibiotics' usage in livestock diets by the European Union (EU), from January, 2006, in addition to the gradual phasing out of zinc oxide (as therapeutic use) adopted by European commission (initiated by France and the Netherlands (European Medicines Agency, 2017; European Commission, 2017). This indicates that increasing production is not sufficient to achieve food security, as it must be complemented by sustainable approaches to enhance overall animal health and welfare, and also human health.

Previous studies have indicated gut microbiota (or gastro-intestinal tract colonising bacteria) as one of the potentially important aspect that can be exploited to improve weaning-associated diarrhoea (Lallès et al., 2007; Aland and Madec, 2009; Heo et al., 2013). Besides, the disruption of gut microbiota has emerged as a leading cause of PWD and associated infections in piglets (**Figure 1**) (Gresse et al., 2017). There is, therefore, a growing interest in understanding the mechanisms that determine gastro-intestinal health of young piglets as well as the impact of dietary fibres (or prebiotics) as alternative feed additive(s). Further insight into the interplay between early-life nutrition, microbiota, immunity and intestinal mucosa could be vital in finding novel approaches for improving animal health around weaning, and would be the emphasis of this chapter. The work in this thesis will focus on microbiota and intestinal development with respect to early nutrition (fibrous diet).

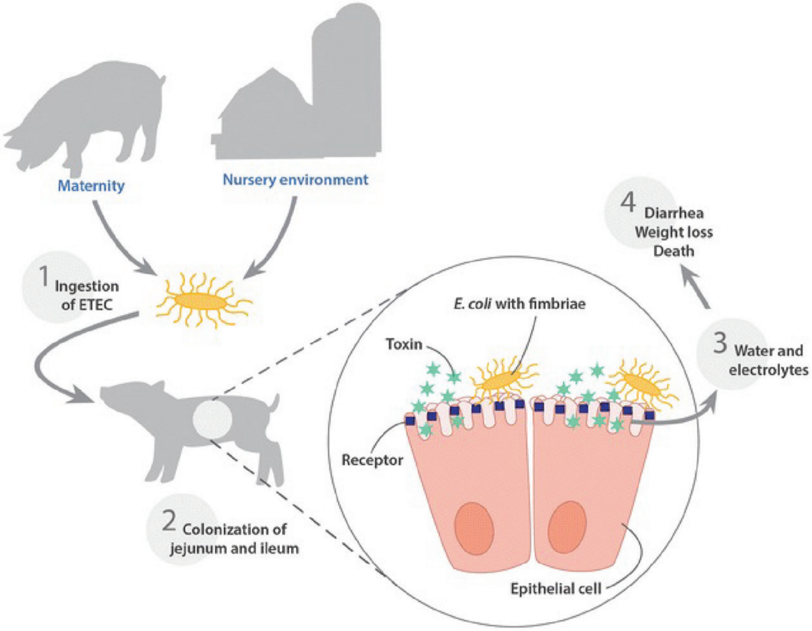


Figure 1: Schematic representation of the steps involved in the pathogenesis of post weaning diarrhoea in pigs. For example, piglets can get exposed to enterotoxigenic Escherichia coli (ETEC) from the environment, upon which the bacteria adhere to the intestinal epithelium, releasing toxins that cause excess secretion of water and electrolytes, eventually leading to dehydration, diarrhoea and possible death (Rhouma et al., 2017).

Weaning and Post-weaning associated problems

Natural vs Commercial weaning

Weaning is a natural process in mammals, which involves gradual transition from complete dependence on the mother's milk to independent feeding (Martin, 1984). However, in commercial (pork production) farming, weaning is an event rather than a process. Piglets are abruptly weaned most often around 3 or 4 weeks of age, which is very different from the natural conditions where they gradually wean up to approximately 20 weeks of age (Petersen et al., 1989; Jensen and Stangel, 1992), allowing slow adjustment to solid feed. Obviously, this early and abrupt weaning practice imposes tremendous stress on the young piglet, whose gastro-intestinal physiology is still developing (Pieper et al., 2006), and is accompanied by adverse physiological changes in the structure and function of the intestine (Pluske et al., 1997; Heo et al., 2013).

Stress and behavioural problems

Weaning stress goes beyond the intestinal tract, as besides the change in diet, weaned piglets have to deal with an additional social stress, due to sudden maternal separation, abrupt relocation to a new environment and often unknown pen-mates (Jensen et al., 1991; Lallès et al., 2007). Weaned piglets can thus develop behavioural problems such as aggressive behaviour directed at their pen-mates after mixing, and oral manipulation of their pen-mates' bodies including ear and tail-biting, leading to stressful conditions (Bolhuis et al., 2005; Colson et al., 2006; Dudink et al., 2006; Widowski et al., 2008). In many countries, the standardised farming practices include tail docking and teeth clipping in piglets to minimise post-weaning damaging behaviour, while overlooking welfare issues. Interestingly, some pre-weaning conditions (e.g. pen enrichment) seem to reduce post-weaning damaging behaviour in piglets (Bolhuis et al., 2005; Oostindjer et al., 2011), indicating the necessity for developing exploration- and foraging-related behaviour for optimal post-weaning adaptation. Whether these behavioural deviations are associated with their gut microbiota, has not been explored yet.

Impact on feed intake and growth

After weaning, piglets have to cope with the sudden withdrawal of mother's milk and adapt to a less digestible, plant-based (solid) diet containing complex protein and carbohydrates (Lallès et al., 2007). Consequently, piglets tend to have a sharp reduction in feed intake immediately after weaning (Pluske et al., 1997), with food neophobia persisting up to 48 hours in approximately 10% of weaned piglets (Brooks et al., 2001). The absence of nutrients in the lumen caused by starvation (or food neophobia), can also explain (partly) the intestinal impairment in piglets post-weaning. Due to this, a transient 'growth check' in the first 7 to 10 days post-weaning (Pluske et al., 1997; Collins et al., 2017) is generally observed, which can potentially have a long-term impact. Due to the multi-factorial (including environmental-, nutritional- and psychological-) stressors piglets are simultaneously exposed to, weaning is associated with a high incidence of diarrhoea.

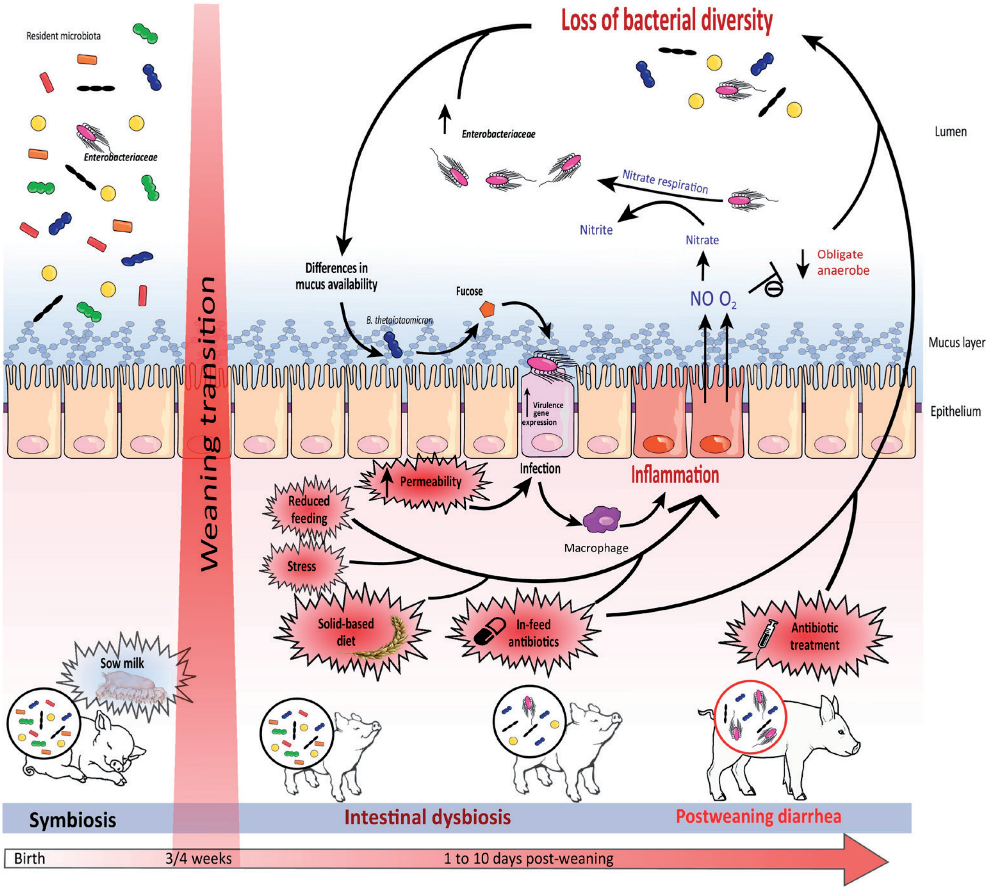
Post-weaning diarrhoea (PWD) and Gut Microbiota Dysbiosis

Post-weaning diarrhoea affects pigs during the first 2 weeks after weaning and is characterised by sudden death or diarrhoea, dehydration, and growth retardation in surviving piglets (Rhouma et al., 2017). The combination of the withdrawal of milk derived passive immunity (maternal antibodies)and the presence of less well-digested food in the gut, results in an increased susceptibility to intestinal colonisation by opportunistic pathogens, leading to gastro-intestinal problems. Post-weaning diarrhoea is usually associated with proliferation of one or more strains of enterotoxigenic *Escherichia coli* (particularly those expressing fimbrial adhesins F4 or F18) (Fairbrother et al., 2005; Nagy and Fekete, 2005), or *Salmonella enterica* serovar *Typhimurium* in the gastrointestinal tract (Gresse et al., 2017). Other pathogens such as *Clostridium perfringens, Lawsonia intracellularis, Brachyspira pilosicoli,* protozoan parasite *Cryptosporidium* and rotavirus have occasionally also been reported to cause post-weaning diarrhoea (Wang and Gänzle, 2019; Zimmerman et al., 2019). This leads to the therapeutic use of antibiotics, most commonly in the suckling and post-weaning period in pig production (Lekagul et al., 2019). The higher susceptibility to infections in young piglets relates to their immature immune system, the developing microbiota and the gastro-intestinal barrier. For instance **(Figure 1**), young piglets can be exposed to ETEC from the environment, especially derived from mammary glands of their mother, the farrowing room or their pen**.** ETEC bacteria adhere to the small intestinal epithelium, releasing enterotoxins, which causes an increased secretion of water and electrolytes into the intestinal lumen in addition to altering the absorptive function of enterocytes (Nagy and Fekete, 2005). Excessive secretion of electrolytes and water eventually leads to dehydration, metabolic acidosis, osmotic diarrhoea and possible death (Rhouma et al., 2017).

Post-weaning diarrhoea has been associated with structural and functional changes to the gastro-intestinal tract (GIT) (Kelly et al., 1991; Pluske et al., 1996), villous–crypt architecture (Spreeuwenberg et al., 2001; Al Masri et al., 2015), GIT barrier function (Kim et al., 2012; Moeser et al., 2017), intestinal inflammation (Pluske et al., 2018) and a disrupted microbiota (Fouhse et al., 2016; Gresse et al., 2017; Guevarra et al., 2018). The temporal change of intestinal mucosal morphology and function post-weaning, could be divided into two periods: an ***acute phase*** (about 5 days) occurring immediately after weaning and an ***adaptive or maturation phase***. The first five days after weaning is usually considered to be the period where piglets experience maximum weaning stress, whereas the adaptive phase (day 5 to 15 post-weaning) corresponds to an adaptation of the gut to the weaning diet (Montagne et al., 2007).

Several studies have reported altered microbial diversity and increase in ***Prevotella spp***., ***Clostridium spp.,*** and ***E. coli*** during the weaning transition (Jensen, 1998; Franklin et al., 2002; Konstantinov et al., 2006; Frese et al., 2015; Bian et al., 2016). However, the results are not always consistent which is possibly due to various confounding variables like health status post-weaning (healthy vs diarrhoeic), weaning age, in-feed antibiotics, sample type as well as chosen method of genomic technology. One former study (Dou et al., 2017) compared the post-weaning microbiota in healthy and diarrhoeic pigs, identifying increased abundance of Enterobacteriaceae in diarrhoeic pigs, contrasting with higher abundance of Prevotellaceae, Lachnospiraceae, Ruminococcaceae and Lactobacillaceae in healthy pigs. Remarkably, the study also proposed that the diarrhoeic pigs (17 days post-weaning) could be discriminated by their pre-weaning microbiota signature, suggesting that there is a possible relation between the pre-weaning microbial colonisation and the post-weaning microbial adaptation.

While several studies (Fouhse et al., 2016; Guevarra et al., 2018; Li et al., 2018b) have shown an association between PWD and a disrupted state of the microbiota or microbial imbalance, commonly referred to as ‘dysbiosis’ (**Figure 2**), the characteristics of such a state are not completely clear. However, a prior review (Gresse et al., 2017) proposes mechanisms for the development of post-weaning dysbiosis by translating information from mice or human studies (**Figure 2**). During the weaning transition, disease susceptibility can increase due to “blooms” of otherwise low abundant and potentially pathogenic bacteria. The loss of bacterial diversity (in weaning transition) potentially makes the glycans in the mucus layer (protecting



Trends in Microbiology

Figure 2: Impact of the weaning transition on the intestinal microbial ecosystem. After weaning, piglets undergo an abrupt change in diet (sow milk to solid feed) as well as environmental and social stress (new environment and pen-mates). The multiple stressors cause disruption of intestinal microbiota composition that can lead to the expansion of enteric pathogens and eventually post-weaning diarrhoea. This figure depicts some potential underlying mechanisms during the weaning transition. In a newly weaned piglet's intestine, the nutritional landscape is modified and the mucus polysaccharides may be more available for commensal bacteria, which can in turn promote the growth of pathogenic species, thereby contributing to the intestinal dysbiosis and inflammation (Gresse et al., 2017).

intestinal epithelium) more available to be utilised by the commensals, releasing sugars such as fucose, galactose, or mannose, which in turn can promote the growth of pathogenic species. In addition, the GI barrier function loss during the weaning transition also facilitates the transmission of pathogens and/or their toxins across the epithelium (Wang et al., 2016). The inflamed gut can provide a favourable environment for 'blooms' of microbial groups like Enterobacteriaceae due to (a) a nitrate-rich environment of the inflamed gut giving advantage to microbes (e.g. *E.coli)* containing nitrate-reductase genes and (b) a higher concentration of oxygen in the inflamed gut (enhanced by higher blood flow) favours bloom of facultative anaerobes (Enterobacteriaceae), decreasing obligate anaerobes, and consequently induce a loss of bacterial diversity. In combination, such processes can contribute to driving the vicious cycle, and ultimately leading to enteric infections (**Figure 2**) (Gresse et al., 2017).

Overall, it is clear that abrupt weaning causes a shift in the intestinal microbiota which can turn into weaning-associated dysbiosis, however not all piglets develop dysbiosis. This shows the importance to better understand microbiota colonisation in healthy piglets and explore strategies to manipulate the gut microbiota to shape a ‘healthy and resilient’ gut. Although a large amount of literature exists about the management of the post-weaning dysbiosis, it has proven to be a complex issue which is still poorly understood. Notably, there is a growing interest in exploring dietary modulation for establishment of a healthy bacterial community, as it can be a preventive approach to handle post-weaning associated problems (Fairbrother et al., 2005; Heo et al., 2013), especially in the early-life of animals (window of opportunity).

The gastrointestinal ecosystem and the “window of opportunity”

The “window of opportunity” refers to the early-life period of an animal, which is characterised by the simultaneous development or programming of the gastrointestinal tract, intestinal microbiota, immune system and the brain. The gastro-intestinal tract (GIT), a vital organ that functions to digest food and absorb nutrients, is a complex ecosystem comprising of intestinal host mucosa, immune cells and the resident microbiota, which have co-evolved together to achieve its normal function and activity (McCracken and Lorenz, 2001; Isaacson and Kim, 2012; Nicholson et al., 2012). Previous *in vitro* and germ free model studies have shown that each of these components can follow a pre-determined developmental pathway even when isolated from the rest, however, the interaction among all the components is vital for a normal physiological development and function (McCracken and Lorenz, 2001). Another component that has recently gained focus is the (brain/enteric) nervous system development, that coincides with the intestine, microbiota and immune development in the post-natal period. The bidirectional communication between the gut and the brain (commonly known as the gut-brain axis) consist of biochemical, neural, immune, and endocrine pathways that can respond to early-life stressors (O’Mahony et al., 2009; Collins et al., 2012). Growing evidence suggests that early-life developmental programming exhibits a high degree of plasticity and results from exposure to external environment (including diet). This also highlights the association of early-life events and later life consequences (Putignani et al., 2014; Pohl et al., 2015; Schokker et al., 2015; Zhuang et al., 2019). Exploring the interplay of the components, especially in early-life, can be crucial to understand and support the host gastrointestinal function (Schokker et al., 2014; Pluske et al., 2018; Guevarra et al., 2019). During the first 12 weeks of postnatal life, the porcine GIT undergoes significant development (Moeser et al., 2017). However, the early weaning practice (~ 4 weeks of age) impacts the GI development and shifts the critical ‘window of opportunity', which could be modulated for potential short- and long-term impact on the overall animal health (**Figure 3**).

Intestinal microbiota development

The gastro-intestinal tract of mammals is colonised by microorganisms that constitute a vast community or ecosystem known as the microbiota, which is often termed as an "organ" in itself due to its functional and metabolic capacity (Nicholson et al., 2012; Clarke et al., 2014). The microbiota is vastly dominated by bacteria, but also contains smaller amounts of archaea and fungal species, as well as a plethora of viruses, which have received lesser attention (compared to bacteria). The gut microbiota is a complex ecosystem increasing throughout the length of GI tract, from 10^1-10^4 bacteria per gram of intestinal content in the stomach to reaching a density of 10^{10}-10^{12} bacteria in the large intestine (Gaskins et al., 2002; Booijink et al., 2007). The host-microbiota interrelationship is considered as a mutualistic symbiosis (sometimes referred as 'super-organism' or 'holobionts'; **Figure 3**), with the host providing an adequate nourishment and physical environment for the microbial populations, while the gut microbes perform functions beneficial for the host such as digesting complex polysaccharides, producing vitamins, and preventing colonisation by pathogens.

The intestinal epithelium acts as a primary barrier to the so-called 'external environment' (or luminal antigens) for the host, protecting underlying tissues from bacterial entry, via a protective mucus layer coated on epithelial surface. The mucus layer is a physico-chemical barrier contributing to the overall mucosal barrier, however the mucosal barrier also encompasses epithelial functions as well as intra-epithelial immune functions. While the small intestine has only one layer of mucus which can be penetrable to bacteria, the colonic mucus layer is organised in two parts: a dense inner layer firmly attached to the gut epithelium that minimizes bacterial-epithelial cell contact, and a loose outer layer that can be broken down by commensal bacteria (Johansson et al., 2011). The microbial metabolic products synthesised in the outer mucus layer can cross the epithelium, reaching the lamina propria and stimulating the host immune system (Caballero and Pamer, 2015; Rooks and Garrett, 2016). The intestinal epithelium, located beneath the mucus layer, is composed of multiple cell types including enterocytes, enteroendocrine cells, goblet cells, and Paneth cells, which contribute to absorption, digestion and secretion, and functions as mucosal barrier (maintained by tight junctions, desmosomes, and adherence junctions). The mucus is secreted by the goblet cells, and comprises of glycosylated proteins such as MUC2, MUC5AC, which are resistant to digestive enzymes and give the mucus its gel-like properties. The antimicrobial products from enterocytes and Paneth cells can infuse with the mucus, thereby generating an antibacterial gradient. Moreover, the "sampling" or "sensing" of luminal antigens by epithelial and immune cells, such as macrophages and dendritic cells, is crucial for avoiding the development of intestinal inflammation and maintaining a healthy

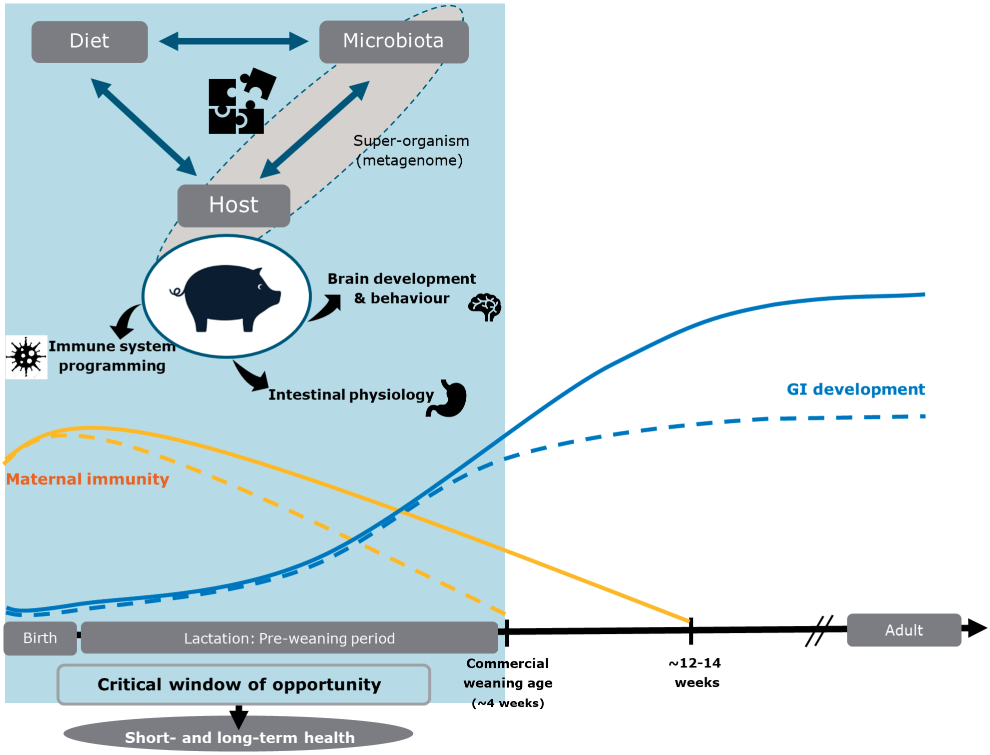


Figure 3: Critical window of post-natal development. Graphical representation of the interaction between diet and the superorganism (microbiota + host), along with the coinciding events in early-life "window of opportunity", which can influence both short- and long-term health of the host. The gastro-intestinal (GI) system in piglets undergoes significant development during the first 12-14 weeks of age. The postnatal period is marked by the developing intestinal epithelial barrier, immune system, microbiota colonisation as well as enteric nervous system. The timing of commercial early weaning (around 4 weeks of age) coincides with a period of exponential GI development as well as declining passive immunity from sow's milk, contributing to the challenges for the piglet. Adapted from (Moeser et al., 2017).

relationship between the commensal microbiota and the host (Haag and Siegmund, 2015; Ribet and Cossart, 2015; Rooks and Garrett, 2016). Enterocytes are adjoined by a paracellular diffusion barrier called tight junctions, which mainly consist of the transmembrane protein complexes (e.g., claudin, occludin, junction adhesion molecule or JAM) and cytosolic proteins (e.g., zonula occludens or ZO-1), which connect them to cytoskeletal actin and adjacent enterocytes. Enteric pathogen and endotoxin translocations are known to increase paracellular permeability (or intestinal barrier dysfunction) through tight junction alterations via dephosphorylation of occludins, redistribution of ZO, and alteration of actomyosin through phosphorylation of myosin light chains (Groschwitz and Hogan, 2009; Kim et al., 2012).

The microbiota of a new-born piglet is less diverse than adult pigs, yet more chaotic with a high individual variation in composition and diversity. Immediately after birth, the GI tract starts to be colonised by maternal and other environmental microbes leading to development of a complex microbial ecosystem. The formation of this ecosystem is a continuous process influenced by various internal and external factors and will continue to develop till it reaches a relatively stable microbiota composition (Putignani et al., 2014). In new-born piglets, the pioneering or early colonisers of the intestine are facultative anaerobes, such as members of the genera ***Escherichia, Fusobacterium, Enterococcus*** and ***Streptococcus***, that can tolerate the relatively high levels of oxygen in the postnatal gut. Within the first few days of life, these early colonisers create an anaerobic environment in the gut lumen which allows the growth of strict anaerobes, such as members of the genera ***Bacteroides, Prevotella*** and ***Ruminococcus*** (Bian et al., 2016; Everaert et al., 2017; Kubasova et al., 2017).

Apart from host genetics, the factors that influence the porcine microbiota composition are age, diet, environment, antibiotics and disease (Isaacson and Kim, 2012; Wang et al., 2019), with age and diet potentially being more important determinants (Bian et al., 2016). Introduction of solid feed during the weaning process, marks an important shift in the gut microbiota composition, favouring the growth of (plant-based) polysaccharide fermenters such as ***Prevotella, Ruminococcus, Faecalibacterium*** (Frese et al., 2015; Moens and de Vuyst, 2017; Guevarra et al., 2019). Several studies have evaluated the microbiota development in pigs over time employing faeces, rectal swabs or colon content (**Table 1**). Due to different confounding variables ranging from experimental design to sequencing technology, it can be challenging to compare the studies. Despite this experimental noise, most of the studies comparing pre- and post-weaning microbiota have found a higher abundance of ***Bacteroides, Fusobacterium*** in suckling piglets whereas ***Prevotella*** and ***Lactobacillus*** were generally prevalent in weaned piglets (**Table 1**). Further, the gut microbial diversity seems to increase post-weaning, reaching a ‘relative stability’ within 2-3 weeks post weaning (**Table 1**), which is probably age-driven.

Immune development in early–life

The immune system is responsible for recognising, responding and adapting to innumerable foreign, non-self, as well as self molecules (development of tolerance) and is therefore of pivotal importance in health and disease. The gastro-intestinal tract (intestinal mucosa) represents the largest surface area which is in contact with antigens from the external environment, and is frequently challenged by pathogens or antigens contained in food and water (Jeon, 2013; Christoforidou et al., 2018). The interplay of diverse antigenic dietary components and varied microbial populations overlying the GIT mucosa facilitates the development of the dynamic and mature intestinal mucosal immune system (Burkey et al., 2009; Sekirov et al., 2010; Honda and Littman, 2016). In early-life, the immune system starts developing in close association with the developing gut microbiota (**Figure 3**). There is significant evidence that the process of immune maturation is influenced by the microbiota that colonise the gut at the early stages of life (Round and Mazmanian, 2009; Schokker et al., 2014; Lewis et al., 2017). Further, microbiota-derived metabolites such as short chain fatty acids (SCFA) have been shown to directly influence immune homeostasis (and maturation) by inducing regulatory T cells (Furusawa et al., 2013; Smith et al., 2013).

The neonatal piglet is immunologically incompetent until about 4 weeks of age (Blecha, 2001) and needs passive immunity from the sow for survival (Bailey et al., 2005). For instance, at birth the Peyer's patches contain very small, primordial follicles and almost no T-cells. The antigen presenting cells appear in the pig intestinal mucosa during the first two weeks of life, whereas the CD4 T-cells appear in the mucosa during 3–4 weeks, and CD8 T-cells start to appear from 4–6 weeks of age (Bianchi et al., 1992; Vega-López et al., 1995; Makala et al., 2000). Moreover, in young piglets the interactions between immune cells may be different from that in adulthood, for example, mucosal CD4 T-cells appear to interact both with resident dendritic cells and with capillary endothelial cells, whereas in adults such interactions are known to be exclusively with dendritic cells (Inman et al., 2010; Everaert et al., 2017). Hence, the early-life period till weaning represents a critical phase, where piglets are required to mount appropriate immune response when exposed to dietary and environmental antigens (Mulder et al., 2009). Previous studies have shown that altered early-life conditions in piglets affect the intestinal mucosal immune system, impacting the expansion of T-cell repertoire (Lewis et al., 2012; Christoforidou et al., 2018) and influence host transcriptome (Mulder et al., 2009; Schokker et al., 2014, 2015). Such evidences indicate the importance of pre-weaning period and the impact of early-life conditions on mucosal immune development.

Early-life brain development and gut-brain axis

Early-life perturbation can impact animal behaviour later in life, influencing the structural and functional plasticity of the brain (Conrad and Johnson, 2015). As previously discussed, the early-life is a critical period for gut microbiota colonisation, intestinal and immune development which coincides with neurodevelopment, that includes the establishment of the stress response system or hypothalamic pituitary adrenal (HPA) axis (De Palma et al., 2014; Davidson et al., 2018; Sherwin et al., 2019). Accumulating evidence indicates that the mammalian microbiome can affect behaviour, with several microbes producing metabolites or neurotransmitters that communicate with the brain via the "microbiota-gut-brain" axis (Collins et al., 2012; Johnson and Foster, 2018). For instance, faecal microbiota transfer in mice caused the behavioural traits of the 'recipient mice' to become more like those of the 'donor mice' (Bercik et al., 2011). Further, microbes such as ***Lactobacillus*** and ***Bifidobacterium*** have been associated with lowered anxiety and depressive-like symptoms in mice and human studies (Desbonnet et al., 2010; Bravo et al., 2011; Messaoudi et al., 2011; Pinto-Sanchez et al., 2017).

Table 1: Studies evaluating porcine intestinal microbiota composition studied over time in experimental set ups. This table extracts information highlighting the pre- and post-weaning period.

Reference	Antibiotics	Weaning day	Sampling time-points	Sample type	Method*	Main results	
						Microbiota relative abundance (↑)	**Other findings**
(Alain et al., 2014)	No	28	At weaning, 2 weeks post-weaning	Faeces	V1-V3, Roche 454, EzTaxon-e	**Pre-weaning:** *Bacteroides, Fusobacteria, Escherichia* **Post-weaning:** *Prevotella, Clostridium, Roseburia, Blautia; Lactobacillus* and *Oscillibacter* remained constant throughout the weaning transition	Increasing diversity during the weaning transition
(Zhao et al., 2015)	No	30	1, 2, 3, 6 months	Faeces, colon, Jejunum, ileum	V4, Illumina Miseq, RDP**	**Pre-weaning:** Proteobacteria **Post-weaning:** Firmicutes	Firmicutes to Bacteroidetes ratio increase 10 fold post-weaning; Colon microbiota more similar to faeces than the small intestine
(Mach et al., 2015)	No	28	14, 28, 36, 48, 60 and 70 days	Faeces	V3-V4, Roche 454, Greengenes	**Pre-weaning:** *Bacteroides, Oscillibacter, Escherichia/Shigella, Lactobacillus and unclassified Ruminococcaceae genera* **Post-weaning:** *Acetivibrio, Dialister, Oribacterium, Succinivibrio and Prevotella*	Two enterotype clusters identified: Ruminococcaceae cluster pre-weaning and *Prevotella* cluster post-weaning
(Niu et al., 2015)	No	28	28, 60, 90 and 150	Faeces	V4, Illumina Miseq, RDP	**Pre-weaning:** *Actinomyces, Akkermansia, Cloacibacillus, Collinsella, Desulfovibrio, Sharpea* **Post-weaning:** *Lactobacillus, Treponema*	Firmicutes and Bacteroidetes–the most dominant phyla in all samples. Increasing diversity over time
(Frese et al., 2015)	Yes (chlortetracycline and tiamulin in weaning feed)	21	1, 3, 5, 7, 14, 21, 28, 35, and 42 days	Rectal swab	V4, Illumina Miseq, RDP	**Pre-weaning:** Bacteroidaceae, Clostridiaceae, Lachnospiraceae, Lactobacillaceae, Enterobacteriaceae **Post-weaning:** Lactobacillaceae, Ruminococcaceae, Veillonellaceae, Prevotellaceae	Increasing alpha diversity over time, plateauing after weaning.
(Slifierz et al., 2015)	No	21	1, 3, 7, 14, 21, 28, 35, 42, and 49.	Rectal swab	V4, Illumina Miseq, RDP	**Pre-weaning:** *Clostridium, Escherichia* **Post-weaning:** *Megasphaera, Lactobacillus*	Relative degree of stability evident at 2–3 weeks post-weaning. Richness and diversity increased with age.

Continue

Continued

Reference	Antibiotics	Weaning day	Sampling time-points	Sample type	Method*	Main results	
						Microbiota relative abundance (↑)	**Other findings**
(Chen et al., 2017)	No	NA	10 days before weaning, at weaning, 10 and 21 days post-weaning	Faeces	V3-V4, Illumina Miseq, RDP	**Pre-weaning:** *Fusobacterium, Lactobacillus, Bacteroides, Escherichia/Shigella, and Megasphaera* **Post-weaning:** *Clostridium sensu stricto, Roseburia, Paraprevotella, Clostridium XIVa, and Blautia*	Reached relatively stable level in 10 days after weaned. Increasing alpha diversity with age.
(Guevarra et al., 2018)	No	21	21 and 28 days	Faeces	V5-V6, Illumina MiSeq, Greengenes	**Pre-weaning:** *Bacteroides* **Post-weaning:** *Prevotella and Lactobacillus*	Functional prediction (weaning transition): heat shock and oxidative stress during the weaning transition.
(Li et al., 2018b)	No	NA	At weaning and 3 days post-weaning	Colon	V3-V4, Illumina MiSeq, GreenGenes	**Pre-weaning:** Porphyromonadaceace, *Alloprevotella, Barnesiella, Oscillibacter* **Post-weaning**: Lachnospiraceae, Negativicutes, Selenomonadales, Campylobacterales	Functional capacity (prediction) of microbes during weaning transition: Phenylalanine metabolism, Citrate cycle (TCA cycle), Glycolysis or Gluconeogenesis, Propanoate metabolism, Nicotinate and nicotinamide metabolism pathways
(Ke et al., 2019)	No	28	25, 80, 120, 240 days	Faeces	V3-V4, Illumina MiSeq, GreenGenes	**Pre-weaning:** *Fusobacterium, p-75-a5* **Post-weaning:** *Prevotella, Treponema*	Enterotype-like clustering found in pre- and post-weaning period
(Wang et al., 2019)	No	20	Multiple time-points encompassing the lactation, nursery, growing and finishing stages (0–174 days)	Rectal swab	V4, Illumina MiSeq, GreenGenes	**Pre-weaning:** *Prevotella stercorea, Oscillospira, Bacterroides* **Post-weaning:** *Prevotella copri, Lactobacillus, Blautia, Faecalibacterium prausnitzii, Megasphaera, Roseburia*	Alpha diversity showed an overall increasing trend

* Method details the 16S rRNA gene variable region amplified, sequencing platform and the reference database employed for taxonomic identification.
** Ribosomal Database Project (RDP) classifier
NA = Not applicable

Most of the studies exploring the 'microbiota-gut-brain axis' have been limited to rodent species. It is increasingly accepted that pigs have a closer resemblance to humans with respect to brain development, diet, physiology, and gastrointestinal function as compared to rodent models (Gieling et al., 2011; Conrad and Johnson, 2015; Nguyen et al., 2015). Pigs can be particularly useful to evaluate the host-microbe interactions, as the gut microbiota is substantially more stable over time in both pigs and humans compared with rodent models; apart from both being outbred animal species, having considerable inter-individual variability (Merrifield et al., 2016). The timing of brain growth and development can vary among species, and therefore should be considered while conducting translational research (Dobbing and Sands, 1979). **Figure 4** shows a comparison of brain development in different mammalian species, indicating that the 'window of brain development' coincides for human and pig species (Dobbing and Sands, 1979), compared to other species. Further, like humans, pigs have a gyrencephalic brain (cerebral cortex with convolutions), whereas rodents have lissencephalic brain (smooth brain with no gyri or sulci), indicating the similarities in human-pig brain anatomy and development.

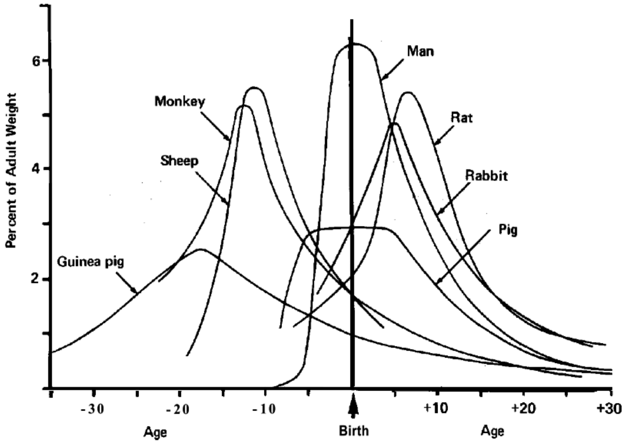


Figure 4: Brain growth spurt of different mammalian species. The brain in all species appears to grow in a sigmoid curve when its weight is plotted against its age. The transient period of rapid growth is commonly known as the 'brain growth spurt'. The non-human primate brain develops prenatally (76% adult brain at birth) whereas the rat seems to be inclined towards post-natal development with only 12% of adult brain at birth. The pig brain shows comparable state (25%) with the human brain (27%) (Dobbing and Sands, 1979).

Modulating microbiota by dietary (fibres) strategies

Dietary fibres (DF) are plant-based carbohydrates that cannot be digested by host enzymes and are known to influence intestinal microbiota and host physiology. Based on glyosidic linkages, carbohydrates can be divided into sugars, oligosaccharides and two broad classes of polysaccharides, starch and non-starch polysaccharides. DF consists of non-starch polysaccharides and other plant components such as cellulose, hemicellulose, resistant starch, inulin, chitin, pectin, β-glucan, and oligosaccharides (Lindberg, 2014; Jha et al., 2019). Based on their ability to be fully dispersed when mixed with water, DF can also be classified into soluble and insoluble fibres, although their functionality might be more related to their fermentability rather than their solubility. The fermentability of DF can be attributed to its impact on microbial biomass, faecal bulking, fermentation end-products, energy for colonocytes as well as selective growth of specific microbes and colonisation resistance to pathogens (Williams et al., 2019).

DF escape digestion by host endogenous enzymes in the proximal small intestine and are fermented by the residing microbiota in the distal small intestine and large intestine (colon). As the major energy source for hindgut microbiota, DF are known to have significant impact on the microbiota composition. The microbial fermentation products include short chain fatty acids (SCFAs) such as butyrate, acetate and propionate, which are recognised to influence physiological functioning of the GIT such as formation and protection of intestinal barrier as well as host defence and inflammatory responses (Den Besten et al., 2013; Furusawa et al., 2013; Tan et al., 2014; van der Beek et al., 2017; Xiong et al., 2019). SCFAs are also known to have neuroactive properties that can modulate brain and behaviour via the gut-brain axis (Russell et al., 2013; Dinan et al., 2015).

Prebiotics are a subset of dietary fibres which act as “a substrate that is selectively utilised by host microorganisms conferring a health benefit” (Verbeke et al., 2015; Gibson et al., 2017; Carlson et al., 2018). Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin, beta-glucan, and resistant starch are a few examples of prebiotic fibres, which have been increasingly associated with intestinal ‘health benefits’ (Carlson et al., 2018; Tran et al., 2018; Davani-Davari et al., 2019). Broadly, prebiotics are known to have favourable impact on the gut microbiota and intestinal homoeostasis directly and/or indirectly, consequently inhibiting pathogenic infections (Tran et al., 2018). Although DF is a broader term compared to prebiotics, the underlying mechanism of both is potentially similar (**Figure 5)**. Prebiotics can inhibit pathogen adhesion by several mechanisms: (1) modulating resident microbiota by selectively stimulating beneficial colonic bacteria, which can form bio-films on the intestinal epithelial cells, thereby (2) blocking the adhesion of pathogens to the host’s epithelial cells. Furthermore, the microbial fermentation products like SCFA can inhibit the expression of adhesion factors or the invasion genes (Sun and Riordan, 2014) in addition to the growth inhibition of the pathogens due to lower luminal pH. The commensal microbes can also exhibit an acute (3) antimicrobial action against invading pathogens by producing receptor analogues such as exopolysaccharides that inhibit the attachment of pathogens. For example, ***Lactobacillus reuteri*** strains have been shown to produce exopolysaccharides (reuteran), which inhibits ETEC adhesion to intestinal mucosa in weanling piglets (Yang et al., 2015). In addition, antibiotic-like bacteriocin compounds can be produced by these beneficial bacteria which can selectively eliminate pathogens (Gresse et al., 2017; Tran et al., 2018). Prebiotics can (4) reinforce the host immune system and support the gut barrier function (paracellular permeability through tight junctions) to maintain intestinal homeostasis. Intriguingly, the products of prebiotics fermentation can also influence brain-derived neurotrophic factors, neurotransmitters and synaptic proteins, thereby impacting cognition, anxiety and exploratory behaviour (Savignac et al., 2013; Williams et al., 2016; Cerdó et al., 2017; Fleming et al., 2019).

Numerous studies (Ivarsson et al., 2012; Haenen et al., 2013; Dicksved et al., 2015; Umu et al., 2015, 2018; Burbach et al., 2017; Liu et al., 2018; Soler et al., 2018; Chen et al., 2020) have evaluated the impact of DF on intestinal microbiota composition, however, focussing mostly on weaned or growing pigs. It is important that such nutritional intervention is started as early as possible, and not only around weaning, to be able to shape the intestinal microbiota of young piglets. **Table 2** summarises the studies which employed DF or prebiotics during the early-life “window of opportunity” in suckling piglets and assessed their impact on microbiota composition and other host physiological parameters, although only a handful employ current sequencing technology. Overall, they report alteration in microbiota composition due to the pre-weaning dietary intervention, especially focussing on increased abundances of genera like *Lactobacillus* and *Bifidobacterium*, and/or decreased abundances of potentially pathogenic species like *Escherichia coli*, *Streptococcus suis*. It is evident that DF have the potential to impact gut microbiota and intestinal development, however, there is an obvious gap in the literature (mostly employ single time-point and outdated technology) during the lactation or pre-weaning period in pigs.

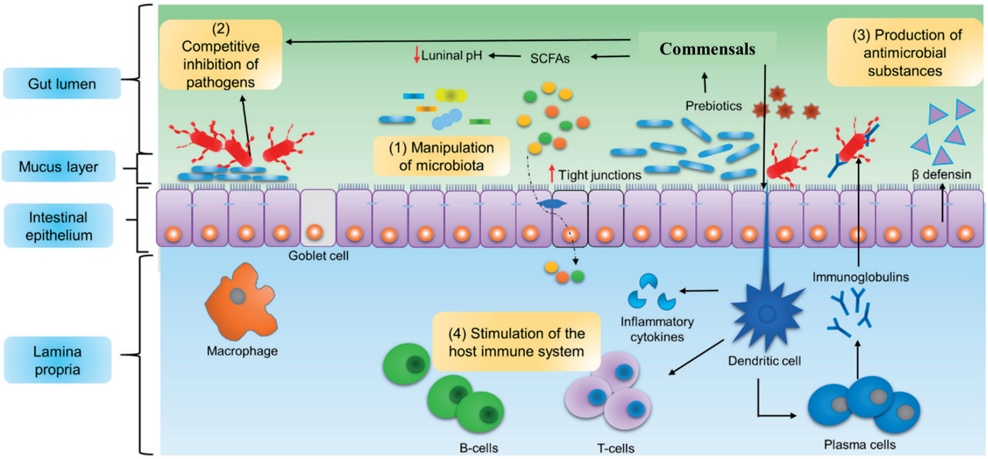


Figure 5: The potential mechanism of action for prebiotics, adapted from (Guevarra et al., 2019). Prebiotics can act by manipulating the microbiota composition, competitive inhibition of pathogens, production of antimicrobials as well as stimulation of the host immune system.

Table 2: Impact of pre-weaning dietary (fibres) intervention on intestinal microbiota composition and related phenotypic markers in suckling piglets

Reference	**Dietary source**	**Intervention period (days)**	**Weaning day**	**Sampling time-points**	**Sample type**	**Methods**	**Main results**	
							Microbiota relative abundance	**Other findings**
(Shim et al., 2005)	Oligo-fructose (0.2%), probiotics (0.3%), synbiotics (0.5%)	21 days	21	1	Colon	CFU	Probiotics and synbiotics diet: ↑ Bifidobacteria population and ↓ total coliform bacteria	Oligofructose diet: ↑ duodenal crypt depth
(Zhang et al., 2016)*	Alfalfa, wheat bran and cellulose	7 – 22 days	NA	1 (day 23)	Caecum, proximal and distal colon	Pig Intestinal Tract Chip analysis	Alfalfa diet: ↑ *Coprococcus eutactus*, ↓ *Streptococcus suis* Wheat bran diet:↑ Dorea, ↓*Lactobacillus paracasei* Pure cellulose diet: ↑ *Prevotella ruminicola* ↓ *Eubacterium pyruvativorans*	NA
(Berding et al., 2016)	A mixed diet including Galacto-oligosaccharides (3.5% GOS)	30 days	NA	31	Ileum, colon, faeces	V3-V5, Pyrosequencing	↑ *Parabacteroides, Clostridium IV, Lutispora* ↓ *Mogibacterium, Collinsella, Klebsiella, Escherichia-Shigella, Eubacterium, and Roseburia*	↑ Jejunal lactase ↑ Ileal expression of vasoactive intestinal peptide
(Alizadeh et al., 2016)	Galacto-oligosaccharides (59% GOS)	3 – 26 days	2	2 (day 3, 26)	Duodenum, jejunum, ileum, caecum, colon, faeces,	qPCR	↑ *Lactobacillus, Bifidobacterium* (Bifidogenic effect)	↓caecal pH ↑ caecal SCFA ↑ Duodenal villus height and ratio villus:crypt ↑ Occludin protein levels (Colon)
(Mu et al., 2017)*	Alfalfa, wheat bran and cellulose	7 – 22 days	23	1 (day 23)	Caecum, Colon	qPCR	↑*Clostridium cluster XIVa*	↑ genes encoding proteins in butyrate production (butyryl-CoA:acetate CoA-transferase, butyrate kinase)

Continue

Continued

Reference	Dietary source	Intervention period (days)	Weaning day	Sampling time-points	Sample type	Methods	Main results	
							Microbiota relative abundance	**Other findings**
(Li et al., 2018a)	Inulin (0.5 – 3 g/day)	4 weeks	28	2 (day 28 and 49)	Colon	qPCR	↓ ***Escherichia coli,*** Enterobacteriaceae (day 28)	↑ SCFA ↑ Villus height, ratio villus height: crypt depth (jejunum and ileum) ↑ Body weight IL-8, TNF-a, TLR-4
(Tian et al., 2019)	Galacto-oligosaccharides (GOS;1g/kg body weight)	0 – 7 days	NA	8, 21	Ileum	16S, RDP	↑ ***Lactobacillus, Bifidobacterium*** (Bifidogenic effect in colon)	
(Schokker et al., 2018)	Fructooligo- saccharides (FOS)	2 – 14 days	26	14, 25	Jejunum, Colon	V3, Illumina Miseq, Greengenes	↑ Firmicutes, ***Lactobacillus;*** ↓ Proteobacteria, ***Escherichia***, ***Clostridium sensu stricto, Streptococcus***	↑ Crypt depth (jejunum; trend) Altered gene expression in jejunum ↑ Extra cellular matrix ↓ Immune-related process, Cell cycle-related process
(Van Hees et al., 2019)	long-chain arabinoxylan (lc-AXOS) and non-fermentable purified cellulose (CELL)	Day 2 – 25 (liquid diet gradually replaced by solid feed)	25	Before weaning	Mid- Colon	V3-V4, Ilumina Hiseq, RDP	CELL: ↓ ***Escherichia-Shigella***	↑ SCFA (caecum, colon) lc-AXOS: ↑ Colon with digesta weight CELL: ↑Small intestine, Colon length and colon with digesta weight

*Same study but repeated with a different method and analysis

Rationale of the thesis

This thesis evaluates the potential of early-life feeding of dietary fibres as a preventive approach to post-weaning associated problems, by utilising the 'window of opportunity'. It is known that early nutrition can have both nutritional and non-nutritional roles in piglet development. In natural conditions, young piglets go out on 'foraging trips' with the sow from a few days of age, gradually familiarising them with solid food. This has a positive impact on the pig's intestinal and behavioural development, by offering a source of minerals and vitamins, enhancing feed intake, promoting stabilisation of gut microbiota, stimulating oral tolerance, as well as developing foraging- and explorative behaviour. Therefore, 'mimicking nature' in the form of providing pre-weaning access to fibrous feed can be an attractive strategy to prepare young piglets for the stressful weaning period, which has been assessed in this thesis. Apart from the applied part of the research, the thesis also aims to explore some fundamental aspects involved in the 'early-life development', which may provide important insights on the host-microbe interactions in pigs.

Aims and outline of this thesis

The overall aim of this thesis was to understand the molecular effects of early-life feeding, associated with the pattern of intestinal microbiota colonisation and gut maturation. We hypothesised that early feeding of a fibrous diet containing both soluble and insoluble fermentable fibres will affect the microbiota and physiological development of neonatal or suckling piglets, facilitating a better preparation for the weaning transition. The specific objectives addressed in each chapter are discussed below (**Figure 6**). An effective and consistent sampling method is a prerequisite for reliable microbiota analysis. Faeces are commonly used to study gut microbiota, however, this might not be feasible in all circumstances, especially involving neonatal piglets. In order to assess whether rectal swabs provide an alternative sample type (to faecal samples) for studying porcine microbiome development, we **first aimed** to evaluate microbiota composition at 7 and 20 days of age, as described in **Chapter 2**. The success rate of faeces collection in neonatal piglets was first assessed, followed by a comparative analysis of age-related microbiota in three sample types i.e., pre-swab (or pre-defecation swab), post-swab (or post-defecation swab) and faecal samples. The results of this study supported rectal swabs as a suitable alternative to study the porcine microbiome development in early-life.

Although microbiota modulation via dietary fibres has been previously studied, very few studies have focussed at the pre-weaning period (early-life "window of opportunity") to modulate microbiota colonisation in neonatal piglets. Our **second aim** was to evaluate the impact of (mixed) fibrous feed provision in suckling piglets, focussing on their microbiota colonisation and intestinal physiology. **Chapter 3** is a longitudinal study that followed the gut microbiota

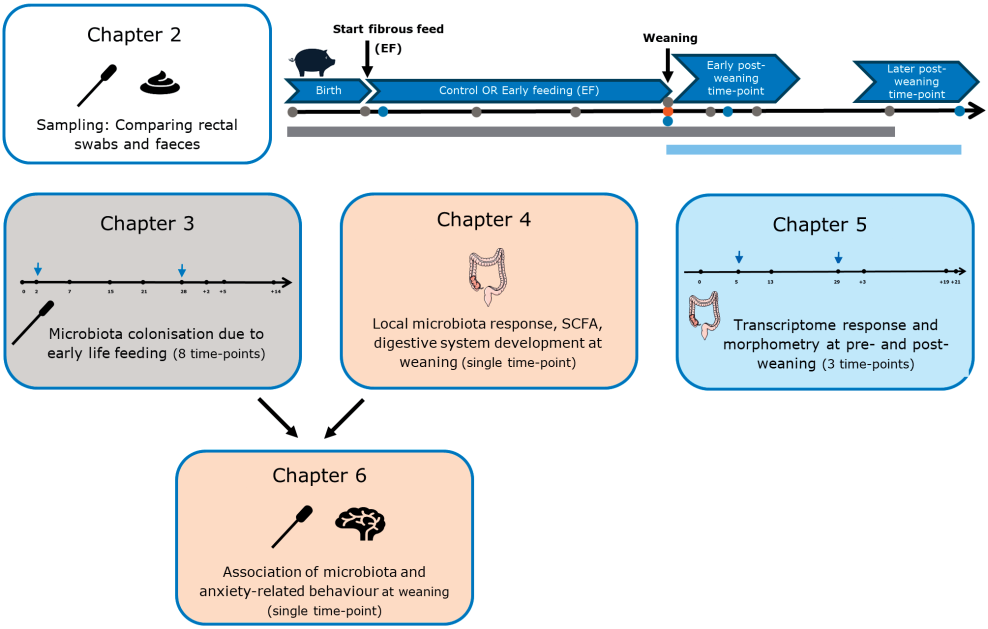


Figure 6: Schematic overview of the experiments and the chapters of this thesis

development over time (employing rectal swabs), thus allowing a comprehensive assessment of individual microbial colonisation in piglets. 16S rRNA gene sequencing analyses identified distinct pre- and post-weaning microbiota signatures and clearly highlighted an accelerated microbiota colonisation in early-fed (EF) piglets, compared to the control (CON) piglets which consumed mother's milk exclusively. 'Acceleration' or 'maturation' of the intestinal microbiota was characterised by the expanded colonisation of typical post-weaning associated microbial groups at pre-weaning stages. In addition, we observed a quantitative relation between the eating behaviour (video scores) of EF piglets and their microbiota signature, suggesting that the piglets who spent more time at the feeding trough had a higher abundance of 'accelerated' microbial groups. Subsequently, in **Chapter 4**, we focussed on the local (small- and large-) intestinal microbiota, comparing their composition in the CON and EF group. Furthermore, the luminal pH, SCFA (distal gut) as well as the host macroscopic and microscopic intestinal measurements were estimated. We showed that quantified eating behavioural scores could significantly explain the variation in microbiota composition of EF piglets and hence classified individual piglets into good, moderate and bad eaters based on their eating behaviour. This enabled an individual level assessment of eating behaviour and its (quantitative) relation with other significantly altered parameters (observed at group level).

After establishing the impact of early feeding on microbiota colonisation, we moved to our **third aim** that was to investigate the host mucosa response due to pre-weaning fibrous feed. Previous results from this thesis showed that early feeding had maximum effect just before weaning, which was the focus in **Chapter 5**. In this chapter, we assessed the mucosal transcriptome response in jejunum and colon just before weaning (~4 weeks of age), 3 days after weaning (early post-weaning time-point) and 21 days after weaning (later post-weaning time-point). As mentioned before, maximum weaning stress is observed in the first few days after weaning, followed by subsequent adaptation leading to a stabilised microbiota. The early post-weaning time-point was evaluated in order to check the persistence of the EF treatment during the 'post-weaning dip'. The later post-weaning time-point acted as a reference for a 'more mature' state, which aided in comparing the treatment groups. Transcriptome analysis, microbiota profiling as well as histological measurements were employed to enable multivariate analysis of the effect of early feeding. In line with our expectations, we found that the mucosal transcriptome converges post-weaning in both groups, re-affirming the relatively 'stable' or 'convergent' state. Corresponding to the microbiota results, the colon transcriptome showed the maximum impact of early feeding just before weaning, with altered gene expression related to cellular energy metabolism and immune pathways. This chapter clearly demonstrates that the early-fed piglets have a closer proximity to later post-weaning 'relatively mature' time-point compared to the control piglets, thus indicating accelerated or matured transcriptome.

It is well known that the impact of gut microbiota goes beyond the gut and can influence the behaviour of the host. However, the relation between microbial communities and behavioural characteristics remain poorly studied in pigs. Therefore, our **fourth aim** was to explore the associations between the intestinal microbiota and anxiety-related and explorative behaviour in suckling piglets in a novel environment. In **Chapter 6**, we used a multivariate approach by employing redundancy analysis (RDA) that was further re-validated in individual spearman correlation and Multivariate Analysis by Linear Models (MaAsLin). A few microbial groups such as *Coprococcus, CAG-873, Atopobium* and *Prevotella* were identified to be associated with anxiety- and/or exploration-related behaviour, although these results are premature and need further validation for their biological relevance. However, this chapter can provide an inspiration towards future studies that are more dedicatedly designed to investigate these associations.

Finally, **Chapter 7** summarises the main findings of the thesis and discusses its contribution to the current knowledge of microbiota colonisation and host-microbe interactions in piglets, with an emphasis on future directions.

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2

Chapter 2

Legitimate and reliable determination of the age-related intestinal microbiome in young piglets; rectal swabs and faecal samples provide comparable insights

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Frontiers in Microbiology 2019, 10:1886

Abstract

A prerequisite for reliable microbiota analysis is having an effective and consistent sampling method. Faecal sampling, commonly used to study the intestinal microbiome, might not be suitable in all situations, especially considering the potential difficulties in obtaining fresh faeces from young animals. Indeed, this study shows that the success rate of collecting faecal samples from young piglets (< 2 weeks of age) was very low. Therefore, we evaluated rectal swabs as an alternative sample type (to faeces) for studying porcine microbiome development, and performed a comparative analysis of microbiome composition obtained from fresh faecal samples and rectal swabs in fifteen healthy piglets at seven (6 piglets) and twenty (9 piglets) days of age. Three samples (fresh faeces, rectal swab before and after defecation) were collected from individual piglets and microbiome composition was assessed by 16S rRNA gene sequencing. The results demonstrated that rectal swabs and faecal samples provide similar microbiome composition profiles, with samples clustering predominantly by individual animal rather than sample type. Furthermore, regardless of the sample type, the biological interpretation with respect to microbiota colonisation patterns associated with different ages (7 and 20 days) was found to be comparable. Independent of sample type, we observed age-related changes like increasing microbiota diversity and alterations in relative abundances of the phyla Firmicutes, Bacteroidetes and Fusobacteria, which was also reflected in consistent family- and genus level microbiota changes. This study establishes that rectal swabs are a suitable alternative sample type to study the porcine microbiome development in early-life, when faecal sampling is challenging.

Keywords: rectal swab, faeces, gut microbiota, early-life, pig

Introduction

Early-life is considered critical in terms of host (immune and metabolic) development and may offer a unique 'window of opportunity' for dietary microbiome modulation (Putignani et al., 2014; Pohl et al., 2015; Schokker et al., 2015). This is supported by the observation that early-life perturbations have been linked to the onset of health consequences like irritable bowel syndrome (IBS), inflammatory bowel disease (Pohl et al., 2015) and neuro-behavioural disorders like attention deficit hyperactivity disorder (ADHD) and depression (O'Mahony et al., 2009; Cenit et al., 2017) in adulthood. It is therefore crucial to accurately determine early-life development of the gut microbiome in relation to intestinal (mucosal) development, to eventually unravel microbiome effects on immune-, metabolic- and neuro-development. Such knowledge could serve to design and evaluate intervention strategies that are intended to modulate early-life gut microbial colonisation, and consequently support healthy development of the host organism.

Animal models are often used to study disease aetiology, drug discovery and to investigate fundamental processes. Porcine models have been proposed as a translational model for the study of developmental consequences of early-life perturbation, and/or nutritional studies in humans (Conrad and Johnson, 2015; Mudd and Dilger, 2017). Such proposition is based on anatomical- (cardiovascular and urinary system, skin, brain) and functional-similarities (gastrointestinal and immune system) (Guilloteau et al., 2010; Nguyen et al., 2015; Wang and Donovan, 2015; Merrifield et al., 2016) and has been driven by the availability of relevant porcine disease models (diabetes, atherosclerosis, gastric ulcer, wound healing) (Gieling et al., 2011). However, apart from its value as a translational model for human diseases and fundamental processes, the study of early-life development in pigs is also highly valuable in veterinary and animal sciences that aim to improve animal health and welfare.

Previous studies have demonstrated the relevance of early-life manipulation (via nutrition, pre/post-natal antibiotics and stress) and its effect on porcine microbiome and intestinal development (Zhang et al., 2014; Schokker et al., 2015, 2018; Leblois et al., 2017; Lewis et al., 2017; Mu et al., 2017; McCormack et al., 2018). In the porcine gut microbiome, Firmicutes and Bacteroidetes are known to be the pre-dominant phyla, regardless of age (Kim et al., 2011; Chen et al., 2017; Lu et al., 2018). Aging has been associated with increased abundance of Firmicutes and decreased abundance of Proteobacteria, Fusobacteria and Actinobacteria (Slifierz et al., 2015; Chen et al., 2017). In commercial pig husbandry, weaning is an abrupt event comprising of a dietary shift from sow milk to usually solid-feed based diets, which poses a challenge to piglets during early-life development. During the pre-weaning phase, microbiome composition is dominated by milk associated microbial families like Bacteroidaceae and Lactobacillaceae, which rapidly changes after weaning when a (largely) plant-based diet is introduced. For instance, ***Prevotella***, having a very low abundance in suckling piglets, dramatically increases post-weaning due to availability of plant substrates in the feed (Frese et al., 2015; Mach et al., 2015). The rapidly changing microbiome of young piglet seems to increase in microbial diversity and richness in the suckling phase and gradually stabilises post-weaning (Kim et al., 2011; Frese et al., 2015; Slifierz et al., 2015; Chen et al., 2017).

For comprehensive analyses of microbiota colonisation patterns, a longitudinal time-series study with a consistent, easy-to-obtain sampling method is required. Faecal sampling has been commonly used to study the intestinal microbiome of humans and animals. However, a few papers have pointed out the practical challenges and confounders during faeces collection and analysis in human microbiome studies. This can range from the potential risk of contamination during sample handling, time-lag in cryopreservation, intermediary sample thawing (e.g. during transport to laboratory), to stool consistency (Budding et al., 2014; Bassis et al., 2017; Vandeputte et al., 2017). Varying storage conditions (4ºC, -20ºC or -80ºC), preservation buffer (e.g. TE Buffer, Stabilisation buffer) have been known to induce variation in the outcomes of microbiota composition analyses of the samples. In a few clinical studies, rectal swabs and rectal mucosal biopsies were investigated as an alternative (reproducible) sampling method for studying the intestinal microbiome, but these have mainly focused on the consistency of methods for storing and preserving samples in a human clinical setting (Araújo-Pérez et al., 2012; Budding et al., 2014; Bassis et al., 2017; Jones et al., 2018). For studies focused on non-human subjects, microbiome samples are commonly obtained from intestinal content or scrapings collected from euthanized animals. While this method of sample collection evades microbial composition differences between intestinal content and faecal samples, it is incompatible with repeated sampling schemes from the same individual animal and increases the number of animals needed (Ingala et al., 2018). A few studies have been conducted to decipher early-life porcine gut colonisation patterns in which either rectal swabs or faeces were used to analyse the microbiome composition (Frese et al., 2015; Mach et al., 2015; Slifierz et al., 2015; Chen et al., 2017; Lu et al., 2018). However, there is limited information available about the consistency of the biologically relevant information obtained from rectal swab or faecal sampling regimes. Burrough et al., 2017 have addressed comparability of porcine microbial profiles from intestinal lumen content and mucosal scrapings from the colon of pigs inoculated with pathogenic bacterial strains that causes swine diarrhoea (Burrough et al., 2017). Although this study described differences in microbial profiles deduced from those sample types, the microbiota composition consistently clustered by disease phenotype, illustrating that the overall biological interpretation is conserved in both sample types.

The aim of the present study was to investigate whether rectal swabs are a legitimate alternative sampling method relative to faecal samples in studies addressing age-related alterations (early-life microbiota development) in piglets. In addition, we aimed to evaluate whether the timing of the swab collection relative to the moment of defecation had an influence on the microbiome composition detected. We anticipated that collecting faecal samples in young suckling piglets might pose a practical challenge (Pluske et al., 2007). Therefore, we evaluated the success rate of obtaining faecal samples from piglets during the pre-weaning period and analysed whether rectal swabs can serve as a reliable alternative sample type. We found that despite rectal stimulation, it is not practically feasible to consistently obtain faecal samples from young piglets, especially during the first weeks of life. However, the readily-obtained rectal swabs are a suitable alternative sample type, and the moment of swab collection relative to defecation, does not have a major impact on the microbiome composition detected. Thereby, this study validates rectal swabs as a suitable sample type compared to faeces, and in addition illustrates that early-life changes in microbiome colonisation patterns in piglets can be studied irrespective of the sample type employed.

Materials and methods

Rectal Stimulation and Swab, Faecal Sampling

The Animal Care and Use committee of Wageningen University & Research (Wageningen, The Netherlands) approved the protocol of the experiment. Animals (Top Pi x Topigs 20, both sexes, born from 9 multiparous sows ranging from 1-7 parity) were housed in the research facilities of Cargill Animal Nutrition Innovation Center, Velddriel, the Netherlands in conventional (2.2 × 2.0 m) farrowing pens with sows confined in a farrowing crate. Within two days after birth, litter size was standardized to 13-15 piglets by cross-fostering. The piglets were reared on the sow and were provided ad libitum access to water and creep feed throughout the suckling period. Details about animal housing and standard procedures has been reported previously (Middelkoop et al., 2018)

To evaluate the success rate of rectal stimulation for obtaining faecal samples in suckling piglets, we randomly selected 84 piglets in total from six different pens and rectally stimulated them at different time-points (day 3 (n = 26), day 7 (n = 36), day 13 (n = 43) or day 20 (n = 41)) after birth. Rectal stimulation was performed by inserting the tip of a cotton swab 20-30 mm into the rectum and making small, gentle movements (circular and back- and forward) for up to a maximum of 2 minutes.

To compare faecal and rectal swab samples, ten piglets were randomly selected from three pens. Rectal swabs and faecal samples were collected at two time-points at 7 and 20 days old. From each animal, three samples were sequentially collected, a pre-swab (rectal swab before defecation), faeces (faecal material collected after rectal stimulation), and a post-swab (rectal swab after defecation). Rectal swabs were obtained by inserting a sterile cotton swab (Puritan Medical, Guilford, ME USA; Cat Number-25-3306-U) 20-30 mm into the rectum and rotating the swab against the bowel wall. Swabs were withdrawn and placed in 5ml eppendorf tubes pre-filled with 500 μl of phosphate buffered saline (pH 7.0). Faecal material was collected in a sterile container (Sarstedt, Nümbrecht, Germany; Cat number-80.734.311), kept on ice during transport to the laboratory, where they were stored at -20ºC until further processing.

DNA extraction

DNA was extracted from rectal swabs and faeces using the repeated bead beating method (Yu and Morrison, 2004) and the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. This method combines lysis by bead beading, DNA precipitation, RNA and protein removal and DNA purification by QIAamp DNA Stool Mini Kit to obtain the maximum yield from faecal material samples. 0.1g of faecal samples and 500 μl of rectal swab solution was used as starting material for DNA extraction. The cells were lysed in Lysing Matrix B tubes prefilled with 0.1 mm silica beads (MP Biomedicals, Santa Ana, California, USA) using FastPrep-24™ (MP Biomedicals, Santa Ana, California, USA) at 5.5 m/s for 3 min (with intermittent cooling on ice in between after every minute). Following the protocol of Yu and Morrison, RNA and protein were removed from the samples by DNase-free RNase (10mg/ml, Qiagen, Hilden, Germany) and Proteinase K (Qiagen, Hilden, Germany) treatment. DNA was subsequently purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as previously described (Yu and Morrison, 2004). DNA integrity and quantity were determined using a Nanodrop DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE USA) according to manufacturer's instructions.

Amplification and Sequencing of 16S rRNA gene

The V3-V4 region of the bacterial 16S rRNA gene was PCR-amplified using V3F primer (5'-CCTACGGGNGGCWGCAG-3') and V4R primer (5'-GACTACHVGGGTATCTAATCC-3'), 5'-extended with adapter sequences 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3' respectively, which are required for sequencing purposes (see below). PCR reactions were carried out in in a Bio-Rad C1000 thermal cycler (Bio-Rad Laboratories, Veenendaal, The Netherlands) using a 50 μl total volume, consisting of 5 μl 10x KOD buffer (Toyobo, Japan), 3 μl MgSO4 (25 mM) 5 μl dNTPs (2 mM each), 1,5 μl V3F primer (10 μM (Eurogentec, Luik, Belgium)), 1,5 μl V4R primer (10 μM, (Eurogentec, Luik, Belgium), 1,0 μl (0.02U/μl) KOD hot start DNA polymerase (Toyobo, Japan) and 10ng (minimum) of template DNA. PCR cycles were programmed with a single initiation cycle of 95 °C for 2 minutes, followed by 25 amplification cycles encompassing denaturation at 95 °C for 20 seconds, annealing at 55 °C for 10 seconds, and elongation at 70 °C for 15 seconds, and was completed by a single elongation step at 72 °C for 5 minutes. Amplicon concentrations were measured by DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE USA) and amplicon size (~550 bp) was verified by agarose (1%) gel electrophoresis. Subsequently the amplicons were purified from the PCR reaction mixture using MSB Spin PCRapace (STRATEC Molecular, Germany).

Purified amplicons were subjected to extension PCR using barcoded Illumina universal index sequencing adapters prior to sequencing. The samples were sequenced (paired-end) using the Illumina MiSeq system (performed by BaseClear BV, Leiden, The Netherlands), generating FASTAQ sequence files by Illumina Casava pipeline version 1.8.3. Quality assessment was based on Illumina Chastity filtering and FASTQC quality control tool version 0.10.0. A BaseClear in-house filtering protocol was applied for removal of reads containing adapters and/or PhiX control signal.

Phylogenetic composition analysis

The 16S rRNA gene sequencing data was processed and analysed using CLC Genomics Workbench version 10.1.1 and CLC Microbial Genomics Module version 2.5 (CLC bio, Arhus, Denmark). The paired end reads were merged into one high quality representative by default settings of CLC Workbench (Mismatch cost = 1, Minimum score = 40, Gap Cost = 4, Maximum unaligned end mismatches = 5). The CLC pipeline was used for primer and quality trimming (Trim using quality scores = 0.05; Trim ambiguous nucleotides: maximum number of ambiguities = 2; Discard reads below length = 5). Greengenes 13.5 reference database (http://greengenes.lbl.gov/Download/) was used for sequence alignment and sequences were binned into operational taxonomic unit (OTUs) based on 97% similarity. The OTU table is further filtered by removing OTUs with low abundance (Minimum combined count = 10), to get a final abundance table for each sample. The phylogenetic tree was constructed using Maximum Likelihood Phylogeny tool based on a Multiple Sequence Alignment of the OTU sequences (100 most abundant OTUs) generated by MUSCLE (Multiple Sequence Comparison by Log- Expectation) tool (Edgar, 2004) in the workbench. The Maximum Likelihood Phylogeny tool determines the probability of the sequences in the tree, using Neighbor Joining as construction method and Jukes Cantor as Nucleotide substitution model. The OTU table was normalized by rarefaction to an even sequencing depth (20,000 reads) in order to remove sample heterogeneity. The rarefied OTU table was used to calculate alpha diversity indices like number of OTUs and Chao1 indices.

Comparative microbiota analysis

To determine diversity shared between two communities (beta diversity), UniFrac metric was used. Weighted UniFrac distance was calculated in CLC workbench and significance was measured by PERMANOVA analysis (permutational multivariate analysis of variance) with 99,999 number of permutations. Log transformation was used as a normalization method for downstream analyses including univariate analysis of taxa relative abundance (Dhariwal et al., 2017) (www.microbiomeanalyst.ca/)], redundancy analysis and heat maps. Principal Component Analysis (PCA; unsupervised) and Partial Redundancy analysis (pRDA; supervised) was performed using CANOCO 5 (Microcomputer Power, Ithaca, NY, USA). The pRDA procedures were performed to analyse the effect of intrinsic experimental variables (age, pen, gender) separately by removing remaining co-variates in the data from the ordination as described in Canoco 5 manual (Braak and Smilauer, 2012). In an RDA plot, arrows pointing in the direction of environmental variable (or confounding factors) correspond to species (OTU/genus/family) that are predicted to be positively correlated with the values for that environmental variable. The key microbial families correlating to 7 and 20 days of age in faecal samples were selected by their response scores in the biplot, obtained by projecting the species points perpendicular to the axes of environmental variables. The same microbial families were visualized in heat maps for rectal swabs to assess sample type differences. To characterize the age-related dynamics of microbial community, heat maps were constructed by hierarchical clustering of microbial families (selected from Redundancy analysis) in Perseus software (http://www.maxquant.org/) where relative abundance values were log2 transformed and subsequently normalized by z-score transformation. Euclidean distance was utilized to measure the distance and clustering was conducted using the average linkage method. The pen-related effects on the microbiota were analysed using only the data obtained at 20 days of age, to obtain a more equal pen-distribution and to avoid the confounding age-related effect. To perform comparative analysis of OTUs among sample types, OTUs less than 10 reads in pre-swab, faeces or post-swab were discarded. Differential taxa abundance (cut off ≤ 0.01%) between groups were determined by t-test and Mann Whitney test t- test in GraphPad Software 5.03 (California, USA, www.graphpad.com). Univariate analysis (Kruskal-Wallis test) was used to compare the microbial composition among sample types and Benjamini-Hochberg FDR value (< 0.05) was used to correct the multiple testing. Linear discriminant analysis Effect Size (LEfSe) analysis (Segata et al., 2011) was used to identify microbial groups which were enriched in sample types. The null hypothesis for all statistical tests was rejected at $P < 0.05$.

Results

Rectal stimulation and faecal sampling

Faeces are the most commonly collected sample type for intestinal microbiota studies. However, sometimes it might be practically challenging to collect such samples, particularly from very young animals. With the aim of understanding early-life microbiota development in young piglets, we evaluated the success rate of obtaining faecal samples using rectal stimulation on randomly selected piglets from six different pens at four time-points after birth (average 2, 7, 13 and 20 days of age). Rectal stimulation of suckling piglets did not provide faecal material in all cases, and particularly failed in piglets younger than 2 weeks of age, with a success rate of only 8% and 31% at 2 and 7 days of age, respectively (**Figure 1**). At a later age, rectal stimulation was increasingly successful with success rates increasing to 70% and 88% at 13 and 20 days of age, respectively.

Faecal and swab sample collection

As mentioned above, rectal stimulation and faecal sample collection success rate in very young (< 2 weeks) piglets was low, indicating that an alternative sampling method is required

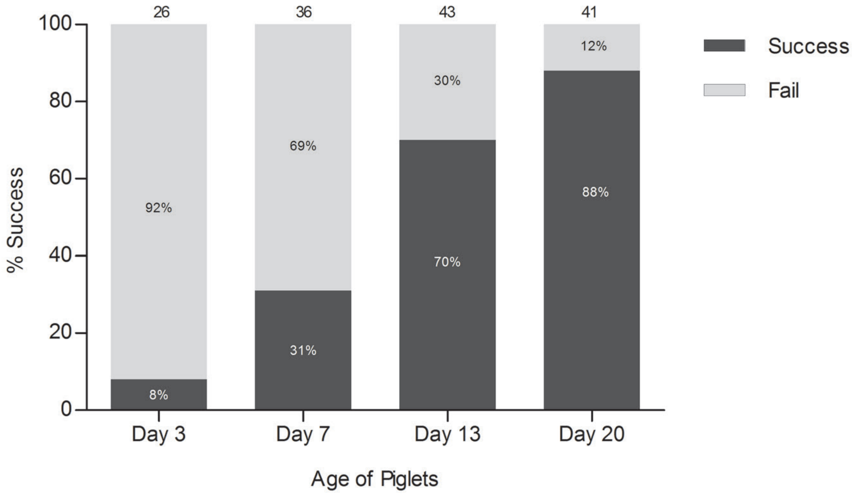

Figure 1: Success rate of rectal stimulation. The numbers at the top of each bar represent the total number of piglets that were stimulated rectally at each time-point.

to reliably obtain longitudinal samples for the investigation of early-life porcine microbiome development. Rectal swabs may serve as an alternative sample type for this purpose, and we investigated the comparability of microbiota composition data in rectal swabs and faecal samples. Additionally, we investigated the impact of the timing of rectal swab sampling relative to defecation (before or after), which may also influence the microbiome composition data obtained from the rectal swabs. To this end, we collected a rectal swab before defecation (pre-swab), followed by collection of a faecal sample upon rectal stimulation, and subsequently a rectal swab after defecation (post-swab) from the same piglet to compare microbiota composition results between these sample types. These combined samples were collected from 10 piglets of three pens at 7 (sample 1-10) and 20 (sample 11-20) days of age (**Supplementary table 1**). Only complete sample sets (pre-swab, faeces, post-swab) that consistently yielded sufficient DNA to amplify the V3-V4 variable region of the 16S rRNA encoding gene, were analysed by sequencing (45 samples in total from fifteen piglets). This restriction led to the exclusion of four and one sample set (s) collected at 7 and 20 days of age, respectively.

Microbiota composition analysis

Illumina sequencing of the 16S rRNA amplicons generated more than 1.8 million high-quality reads that were classified into OTUs with an average of 41,020 ± 5620 (SD) reads per sample (**Supplementary table 1**). The average number of OTUs per sample (after filtering) was 516 ± 22.

Since faecal samples are the most commonly used sample type for microbiota composition analysis we first analysed the data derived from these samples, focusing first on potential explanatory variables. This analysis revealed a highly significant age effect on the microbiota, an expected pen effect, and a small-sized influence of gender (**Figure 2A**). Since gender was found to have a limited and non-significant effect in our experiment (seen in RDA analyses; **Supplementary figure 2A**), it was excluded as a co-variate in further analysis. In contrast, the effect of pen in the microbial composition was significant (**Supplementary figure 2B**), which has also been observed in other studies (Mach et al., 2015; Yang et al., 2017). The impact of age on the microbiota was reflected in a significant increase of the microbiota diversity at 20 relative to 7 days of age (**Figure 2B**). As expected, bacteria classified among the Firmicutes and Bacteroidetes phyla were the most abundant in all faecal samples, followed by members of the Proteobacteria and Actinobacteria (**Supplementary figure 1**). Comparison of the faecal samples revealed that the relative abundance of the Firmicutes increased (52.5% to 69.7%, $P = 0.010$) and Bacteroidetes decreased (24.2% to 13.2%, $P = 0.014$) when the piglets aged from 7 to 20 days after birth (**Figure 2C**). Notably, bacteria classified among the Fusobacteria were relatively abundant at 7 days of age, but were not detected (absent) at 20 days of age ($P = 0.025$), whereas Actinobacteria displayed high relative abundance variation due to a single animal having a high relative abundance of this phylum at 20 days of age (**Figure 2C**).

Pen and age-related changes in the microbiota were further investigated by redundancy (RDA) and partial redundancy (pRDA) analyses. The RDA analyses revealed a significant pen effect on the microbiome composition at genus level (**Supplementary figure 2B**). The pRDA for age (corrected for pen) revealed a significant association of specific bacterial families with 7 or 20 days of age. The microbiota associated with 7 days of age displayed a higher relative higher abundance of the Actinomycetaceae, Fusobacteriaceae, Tissierellaceae, Alcaligenaceae, Clostridiaceae, Pasteurellaceae and Bacteroidaceae, whereas the microbiota at 20 days of age appeared enriched for Synergistaceae, Methanobacteriaceae, Spirochaetaceae, Methanomassiliicoccaceae, Christensenellaceae, Pirellulaceae and Paraprevotellaceae (**Figure 2D**). Hierarchical cluster analysis performed on the bacterial families associated with age-related microbiota changes, clearly separated 7 and 20 days of age associated microbiota (**Figure 2E**), although not all families appeared to be equally discriminating.

These analyses demonstrate that substantial microbiota changes can be detected in faecal samples from piglets during early-life pre-weaning stages (7 and 20 days of age), and that these changes can be associated with changes in specific bacterial phyla and families. In particular, the family-based analysis led to the detection of a microbial family-signature that clearly discriminates the two ages analysed here.

Sample type analysis

An important aim of this study was to investigate whether rectal swabs recapitulate the microbiota composition differences detected in faecal samples. Unsupervised clustering of all samples, corroborated that clustering by age is predominant in the data irrespective of the sample type (**Figure 3**). Comparative analysis of the OTUs identified in each sample type

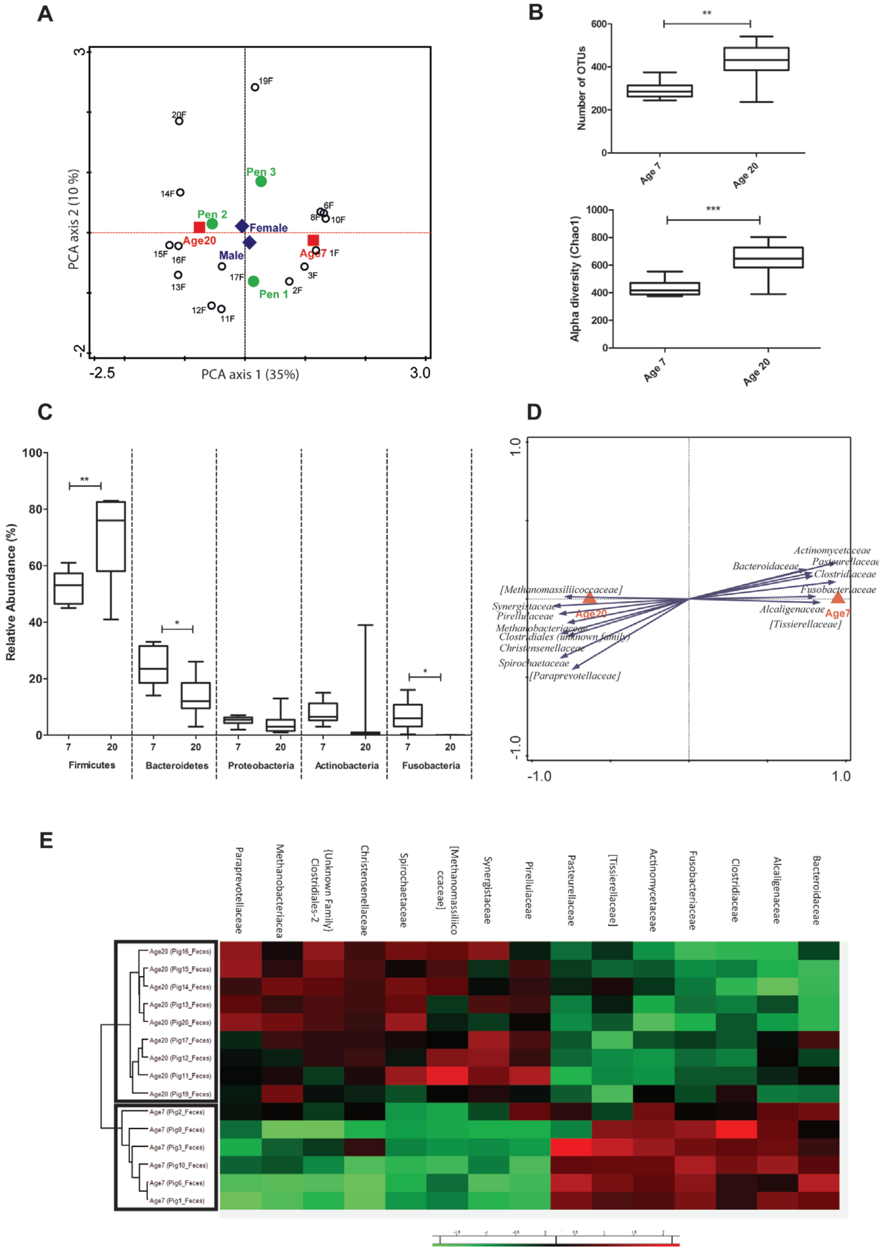

Figure 2: Microbial composition and explanatory variables of faecal samples. (**A**) Principal Component Analysis (PCA) plot showing variation in terms of explanatory variables in the data. Testing single variables by Weighted UniFrac PERMANOVA supports clear separation by age ($P < 0.001$) and pen ($P = 0.013$) but not by gender ($P = 0.948$). (**B**) Alpha diversity (Number of OTUs and Chao1 bias corrected) showing increased diversity at 20 compared to 7 days of age. (**C**) Relative abundance of five phyla with highest relative abundance at two ages in faecal samples (Mann Whitney test, *: $P < 0.05$; **: $P < 0.01$). (**D**) Partial redundancy analysis (pRDA) for the explanatory variable age (corrected for pen) at family level (RD1 = 40.23% and RD2 = 11.36%). (**E**) Heat map showing relative abundance of 15 most discriminative bacterial families for age.

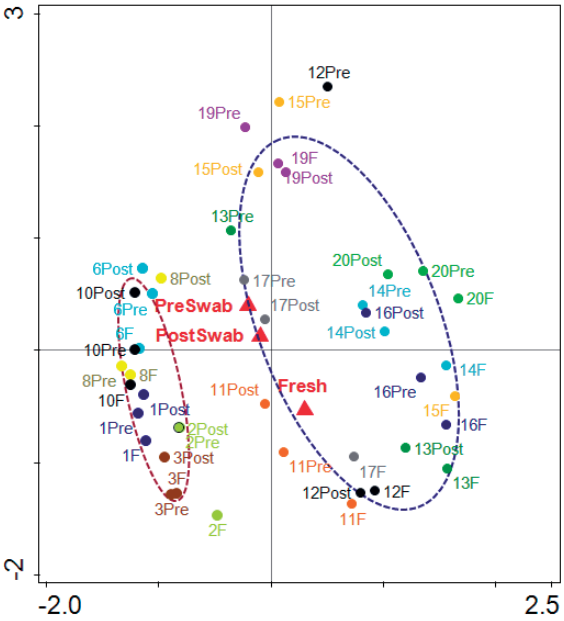

Figure 3: Principal component analysis (PCA) based on OTU level microbiota composition of all samples (PC1=26.02% , PC2=10.49%). Pre-swab (Pre), Faeces (F), Post-swab (Post) are indicated by red triangles, dotted clouds indicate 7 (brown) and 20 days (blue) of age separating age-related microbiota composition irrespective of sample type.

revealed an overlap of 51.42% shared OTUs among the sample types. Importantly, these shared OTUs captured the vast majority of the total microbial population (93-98%) in the different sample types (**Supplementary figure 3**). Global analysis of alpha and beta diversity failed to detect significant differences between sample types (**Supplementary figure 4**). Moreover, weighted UniFrac distance of different sample types (faecal versus pre- and post-swab) obtained from individual animals revealed a substantially higher similarity 'between sample types' (intra-individual) as compared to 'between animals' (inter-individual) (**Figure 4**, $P < 0.0001$). In other words, microbiota differences of individual animals is substantially larger than differences between sample types. This inference is also supported by principal component analysis at both ages, showing that samples cluster predominantly by individual animal and not by sample type, although the clustering of three animals/samples was less clear (**Figure 5A, B**). These disparities in the PCA analysis for these three animals (number 12, 13 and 15) could be explained by a higher relative abundance (although not significant) of a few microbial groups (**Figure 5B**, **Supplementary figure 5**).

Analysis of the rectal swab derived microbiota data (including both pre- and post-swab) established that similar age-related microbiota development changes (phylum-level relative abundance changes) could be detected in the swab samples as compared to the faecal samples (**Figure 6A**). Analogous to the faecal microbiota changes over time, the swab samples

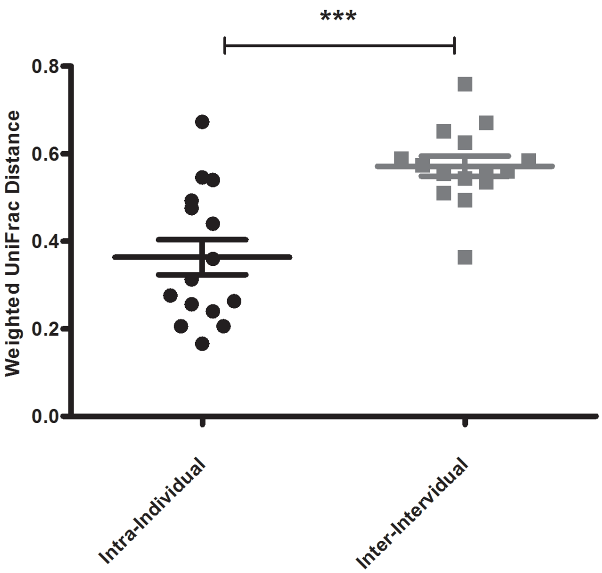

Figure 4: Mean pairwise Weighted UniFrac distance reflecting sample to sample variation within animal (Intra-individual) and between animal (inter-individual). Averaged weighted UniFrac distances indicated that inter-individual variation is greater than intra-individual variation (***: $P < 0.0001$).

identified increasing Firmicutes and decreasing Bacteroidetes abundance at day 20 relative to day 7. Similarly, Fusobacteria were detected at much higher abundance at day 7 relative to day 20, although they did not disappear completely at this later time point according to swab-derived microbiota. Finally, the abundance of Actinobacteria also displayed a high variability between animals at 20 days of age. A notable difference between the swab and faecal sample derived microbiota data was a higher abundance of Proteobacteria (averaging above 10% relative abundance) in swab samples as compared to faeces (averaging below 5% relative abundance) (**Figure 6A**).

To further investigate whether swab-sample derived analyses lead to similar biological conclusions as faecal samples, we repeated the analyses that were performed for faecal samples using the swab-derived microbiota data, focusing on age-related changes. Importantly, the swab samples also displayed higher alpha diversity at day 20 compared to day 7 (**Figure 6B**), and the pRDA for age (corrected for pen) identified most (but not all) of the bacterial families and genera that were associated with day 7 or day 20 in faecal samples (**Figure 6C, Supplementary figure 6**). Furthermore, hierarchical cluster analysis performed on these bacterial families separated day 7 and day 20 microbiota samples similar to what was observed with the faecal samples (**Figure 6D**). However, the bacterial families of Pasteurellaceae, Tissierellaceae, Clostridiaceae, Paraprevotellaceae were not identified as discriminatory variables (apparently less discriminating) in swab analyses, and were included in the hierarchical clustering (**Figure 6D**) for reasons of comparison with the faecal analyses.

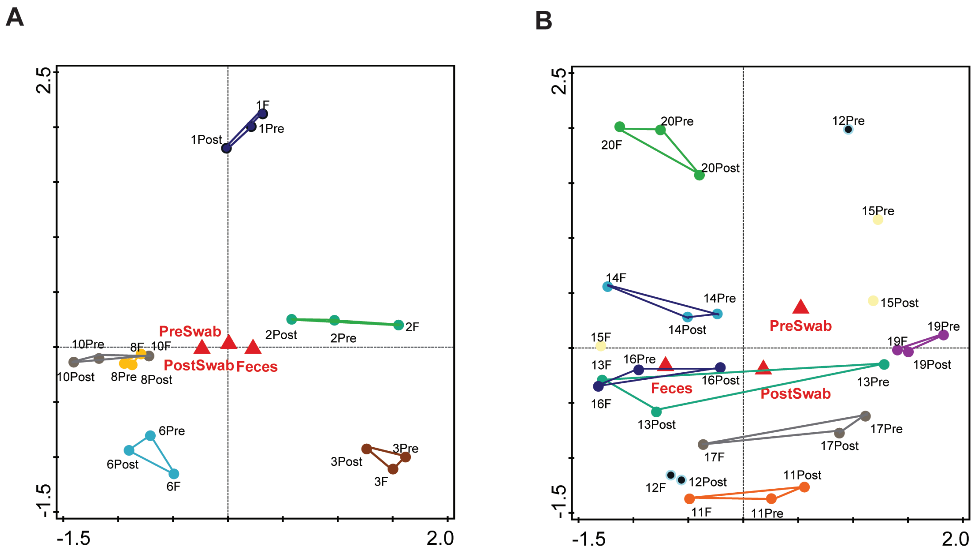

Figure 5: Principal component analysis (PCA) based on OTU level microbiota composition of sample types. Pre-swab (Pre), Faeces (F) and Post-swab (Post) are indicated by red triangles (**A**) at day 7 (**B**) at day 20.

Comparison of different sample types revealed no significant dissimilarities (in relative abundance) at 7 days of age, but detected several differences in the samples obtained at 20 days of age. The relative abundance of the Proteobacteria phylum was higher in both pre- and post-swab samples compared to faeces, in almost all animals (**Figure 7A, B**), whereas the post- and pre-swab samples obtained at 20 days of age detected higher and lower relative abundances of Firmicutes and Fusobacteria, respectively (**Supplementary figure 7**). Importantly, the high-level of variation in Actinobacteria relative abundance was detected by each of the sample types and was consistently associated with high Actinobacteria in a single animal (**Figure 7C, D**). These analyses illustrate a high level of congruency of the biological conclusions drawn from microbiota analyses using these different sample types.

To refine this conclusion, we performed univariate tests at phylum, family and genus-level relative abundances to compare the three sample types. Only ***Trueperella*** (genus level) and Pasteurellaceae (family level) were significantly more abundant in pre-swab compared to faeces and post-swab (**Figure 7E, F, G**), although relative abundance of Pasteurellaceae was very low (< 0.01%). Other microbial families like Staphylococcaceae and Aerococcaceae were found to be higher in pre-swab samples of few animals, although this was not significant (**Supplementary figure 5**). These observations suggest that the microbiota composition in post-swab samples is slightly more comparable to faecal samples, than pre-swab samples. Finally, we assessed the rectal pre- and post-swabs separately to investigate whether the timing of the swab relative to defecation had an effect on rectal swab microbiota composition. This analysis revealed that pre- and post-swab samples generate highly comparable age-related microbiota composition

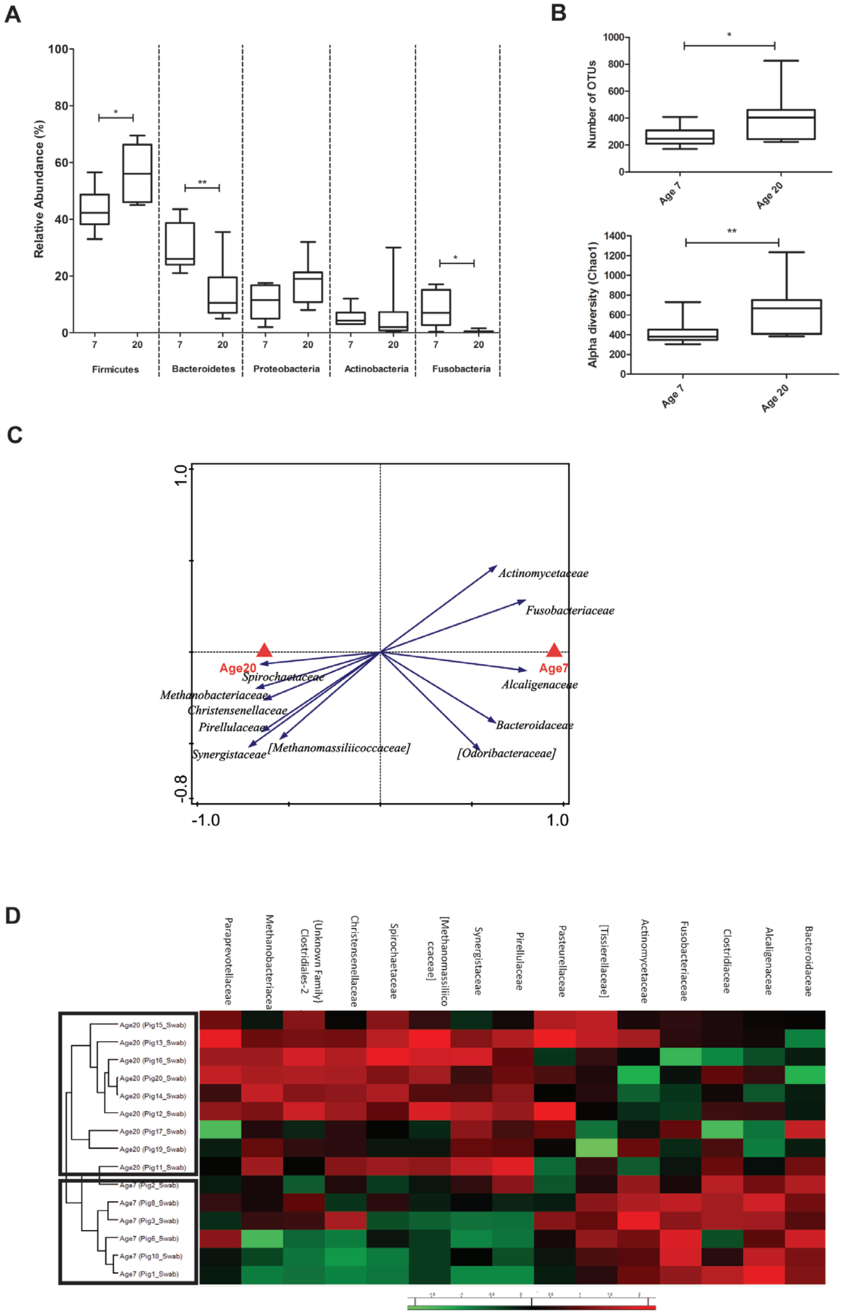

Figure 6: Microbial community composition of rectal swab samples. (**A**) Relative abundance of five phyla with highest relative abundance at two ages in rectal swabs (Mann Whitney U-test, *: $P < 0.05$; **: $P < 0.01$). (**B**) Alpha diversity (Chao1 bias corrected) display changing diversity between the two ages. (**C**) Redundancy analysis (partial RDA) of explanatory variable age (corrected for pen) at family level of both the swabs (RD1 = 20.05% and RD2 = 16.79%). (**D**) Heat map showing relative abundance of most discriminative bacterial families of age (same as faecal samples).

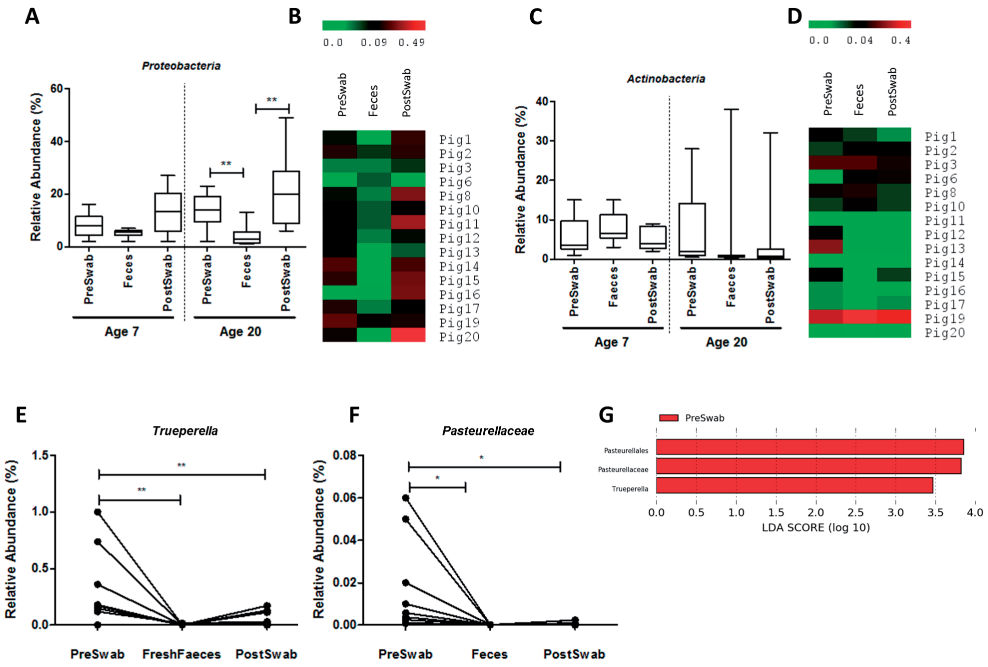

Figure 7: Microbial community composition of sample types. (**A**)(**C**): Relative abundance of Proteobacteria and Actinobacteria at day 7 and day 20. (**B**)(**D**): Heat map of relative abundance of Proteobacteria and Actinobacteria at day 20 of all animals. (**E**)(**F**): Relative abundance of the *Trueperella* genus and Pasteurellaceae family at day 20 among sample types. (**G**) LEfSe analysis identified *Trueperella* genus and Pasteurellaceae family as the most differentially abundant taxa among sample types (*: $P < 0.05$; **: $P < 0.01$).

information (**Supplementary figure 8, 9**) and cannot be distinguished on basis of community richness, beta diversity, or differential taxa abundance (**Supplementary figure 4, 7**).

Discussion

Increased insight in early-life intestinal microbiota colonisation patterns in piglets is crucial in the design of intervention strategies that aim to modulate developmental processes to improve health of the host organism at later stages of life. Reliable and standardized sampling procedures are required to expand our insights in microbiome colonisation during the pre-weaning stage of life of piglets. The purpose of this study was mainly to evaluate sample types, with a secondary objective to verify whether the age-related differences can be identified irrespective of the sampling procedure. To address this, the present study explored three questions: 1) What is the success rate of obtaining fresh faeces from young piglets?; 2) Do rectal swabs provide a reliable alternative sample type for the canonically used faecal samples to study age-related microbiota composition and diversity?; 3) Does the timing of obtaining the rectal swab sample, relative to defecation influence the microbiota profile detected in the rectal swabs?

Rectal stimulation was used to assess the success rate of obtaining faeces in young piglets, showing that faecal sample collection from piglets younger than 2 weeks of age is unreliable. Therefore, to obtain a comprehensive overview of microbiota colonisation from an early age onwards would be difficult if one would rely on faecal sampling. Rectal swabs can provide an alternative sampling choice, although we also observed a higher frequency of technical problems like low DNA yield in swab samples. This is possibly due to the relatively low amount of intestinal material obtained by rectal swab sample at a younger age. However, this hurdle can be overcome by reducing the volume of buffer (PBS) in which the swabs are collected (data not shown) to maintain the maximum possible microbiota biomass concentration and thus increase success rate in downstream microbiota analysis.

The primary aim of this paper was to evaluate the similarities and dissimilarities among the sample types (pre-swab, faeces and post-swab) obtained from individual animals at two time points (7 and 20 days of age). Although several differences were observed between faeces and rectal swabs, these differences were limited to only few microbial groups/taxa. Our comparisons did not reveal differences in microbial richness between the swab and faecal samples. Importantly, our analyses clearly establish that the individualized microbial signature detected in each animal was reproducibly observed and independent of the sample type. Only a few studies have compared microbiome composition of sample types, like rectal swabs, faeces and/or mucosal biopsies and/or scrapings (Araújo-Pérez et al., 2012; Budding et al., 2014; Bassis et al., 2017; Jones et al., 2018) in humans. The findings of these studies are largely in agreement with our finding that the microbiome composition can vary (slightly) between sample types (faecal versus swab samples), but the individual animal signature is detected consistently over time. Intriguingly, the swabs we collected prior to defecation (pre-swab) were significantly enriched in taxa belonging to the ***Trueperella*** and Pasteurellaceae, while these bacterial groups were also enriched in the swab samples collected after defecation (post-swab), albeit not significantly. These microbial groups have been associated with mucosal adherence and can thrive in both anaerobe and aerobic conditions (Jacques and Paradis, 1998; Machado and Bicalho, 2014). This observation suggests that swab samples (particularly those that are taken prior to defecation) include a representation of the mucosa-adherent microbiota, with higher abundance of several oxygen-tolerant microbial taxa compared to the microbes residing in the anaerobic intestinal lumen that are detected in faecal material (Jones et al., 2018). This may imply that although faecal samples are considered the ‘gold-standard’ for microbiota analysis, rectal swabs may more adequately represent both luminal and mucosa adherent microbes.

Our analyses illustrated the conservation of the age-dependent microbiota composition changes in faecal and rectal swab samples. The prominent differences in microbiota composition at

7 and 20 days of age, clearly established age-related changes that included an increasing community diversity over time and alterations in relative abundance of several phyla e.g. Firmicutes, Bacteroidetes and Fusobacteria. These changes in microbiome development are consistent with previous studies (Slifierz et al., 2015; Chen et al., 2017; Lu et al., 2018) where these bacterial groups rapidly changed in developing piglets. In our study, a higher relative abundance of Proteobacteria was observed in the rectal swab samples compared to the faecal samples. Remarkably, a similar observation has been reported when comparing swab and faecal samples obtained from koala (Alfano et al., 2015). Notably, it has been proposed that the observed abundance of Proteobacteria may be influenced by the method used for sample storage (Tris-EDTA buffer or faecal collection tubes with stabilising medium) (Vandeputte et al., 2017), which suggests that the difference in collection methods for faecal and rectal swab samples could have contributed to the difference in observed Proteobacteria relative abundance.

Besides the prominent age-related microbiota effects reported here, pen was an additional environmental variable that was associated with specific microbiota differences. Pen-associated effects could be related to (a combination of) various undistinguishable differences, including a direct environmental effect (same pen), a maternal effect (same uterus, milk, sow), or a host-genetics effect. Our study did not detect a gender-associated microbiota difference, which may not be expected in piglets of this young age where gender-specific development is not yet very pronounced (Reiland, 1978; Medland et al., 2016). Importantly, our study was not designed to address the influence of other environmental variables like pen or gender, and as a consequence the sample distribution over different pens and gender was not very comparable. Study designs to investigate pen and gender-associated microbiota effects require a different design with probably larger numbers of animals to reach significant and meaningful conclusions.

Overall, our study illustrates that despite some differences among the sample types, the biological conclusions drawn are conserved in the three sample types we collected. Furthermore, multivariate analyses (PCA and hierarchical clustering) shows that clustering of samples occurs predominantly per animal rather than sample type, illustrating that sample type is not a major source of variation and is overruled by microbiota signatures of the individual animals. Despite the limited sample size and the unequal distribution of samples over the two time points (6 versus 9) we did succeed to detect consistent and significant age related microbiota signatures that were independent of the sample type used. Thereby this study underpins the reliability of early-life microbiota colonisation analyses using rectal swabs. This is particularly relevant because obtaining fresh faecal samples from young animals can be challenging. Future studies with increased numbers of animals and including specific interventions can expand these findings of intestinal colonisation in early-life and may answer questions related to its susceptibility to modulation, for example by diet, and how such modulation can affect development of the host.

Acknowledgements

This study is part of the research program ‘Genetics, nutrition and health of agricultural animals’ with project number 868.15.010, which is financed by the Netherlands Organization for Scientific Research, Cargill Animal Nutrition (CAN) and Coppens Diervoeding. We would like to acknowledge CAN for the use of their research facilities and thank Fleur Bartels for her help during sample collection and personnel of CAN Innovation Center in Velddriel for their help with the experiment.

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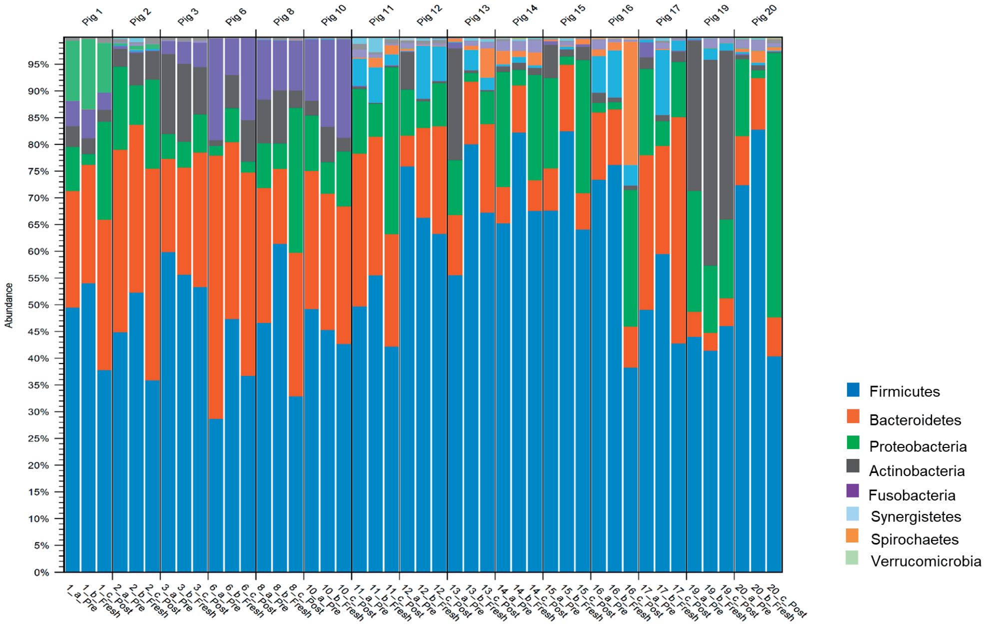
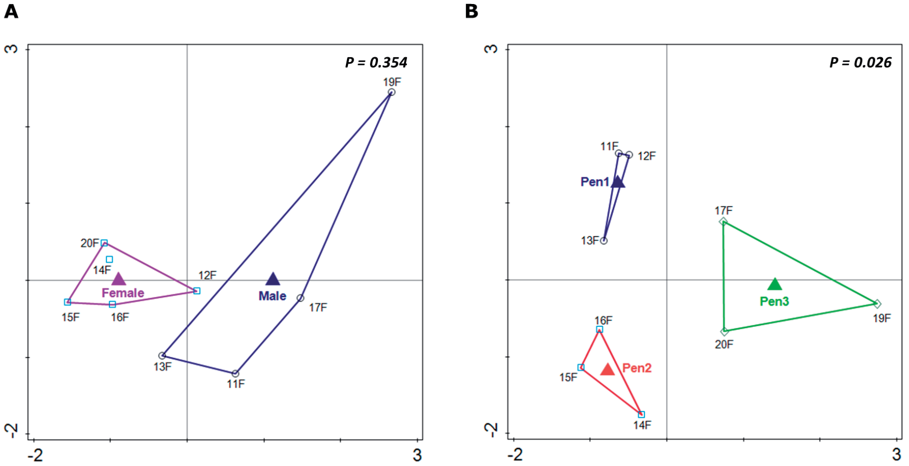
Supplemental information

Supplementary table 1: Table with sample information, read and OTU counts. Number of reads after quality control and removal of chimeric sequences. Number of OTUs at an identity threshold of 97%. Pre = Pre-Swab; Fresh = Faeces, Post = Post-Swab.

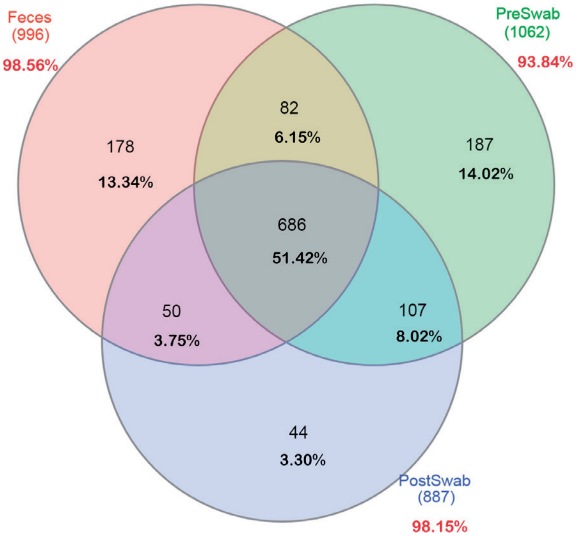
Animal	Age	Sample	Total Number of reads	Reads in OTU	Number of OTUs
Pig 1	7 days	1_a_Pre	64856	46461	400
Pig 1	7 days	1_b_Fresh	51335	35088	370
Pig 1	7 days	1_c_Post	61620	47897	330
Pig 2	7 days	2_a_Pre	61549	47488	505
Pig 2	7 days	2_b_Fresh	68932	42785	538
Pig 2	7 days	2_c_Post	50107	36861	358
Pig 3	7 days	3_a_Pre	60780	39727	435
Pig 3	7 days	3_b_Fresh	49371	32034	393
Pig 3	7 days	3_c_Post	57895	43054	463
Pig 6	7 days	6_a_Pre	53275	42209	302
Pig 6	7 days	6_b_Fresh	63850	40993	358
Pig 6	7 days	6_c_Post	58549	44955	386
Pig 8	7 days	8_a_Pre	61275	40263	377
Pig 8	7 days	8_b_Fresh	56143	34577	379
Pig 8	7 days	8_c_Post	61297	47089	660
Pig 10	7 days	10_a_Pre	63996	41900	350
Pig 10	7 days	10_b_Fresh	64251	38930	411
Pig 10	7 days	10_c_Post	58679	43788	338
Pig 11 (Pig1_Age20)	20 days	11_a_Pre	64456	48163	471
Pig 11 (Pig1_Age20)	20 days	11_b_Fresh	52314	32978	525
Pig 11 (Pig1_Age20)	20 days	11_c_Post	63502	49349	520
Pig 12 (Pig2_Age20)	20 days	12_a_Pre	54479	31076	847
Pig 12 (Pig2_Age20)	20 days	12_b_Fresh	59355	35360	572
Pig 12 (Pig2_Age20)	20 days	12_c_Post	53176	33136	579
Pig 13 (Pig3_Age20)	20 days	13_a_Pre	67458	45113	395
Pig 13 (Pig3_Age20)	20 days	13_b_Fresh	53850	32569	658
Pig 13 (Pig3_Age20)	20 days	13_c_Post	59725	43014	641
Pig 14	20 days	14_a_Pre	48248	30951	593
Pig 14	20 days	14_b_Fresh	61313	40682	672
Pig 14	20 days	14_c_Post	53789	36363	628
Pig 15	20 days	15_a_Pre	59364	41193	805
Pig 15	20 days	15_b_Fresh	55591	33321	615
Pig 15	20 days	15_c_Post	60832	47610	705
Pig 16 (Pig6_Age20)	20 days	16_a_Pre	63236	39146	651
Pig 16 (Pig6_Age20)	20 days	16_b_Fresh	67263	37811	594
Pig 16 (Pig6_Age20)	20 days	16_c_Post	57183	44139	659
Pig 17	20 days	17_a_Pre	65898	52736	420
Pig 17	20 days	17_b_Fresh	57385	37528	603
Pig 17	20 days	17_c_Post	62514	48299	422
Pig 19	20 days	19_a_Pre	60277	43478	373
Pig 19	20 days	19_b_Fresh	75951	50056	380
Pig 19	20 days	19_c_Post	55105	38751	360
Pig 20 (Pig10_Age20)	20 days	20_a_Pre	69700	46683	785
Pig 20 (Pig10_Age20)	20 days	20_b_Fresh	61750	38903	716
Pig 20 (Pig10_Age20)	20 days	20_c_Post	56498	41380	695



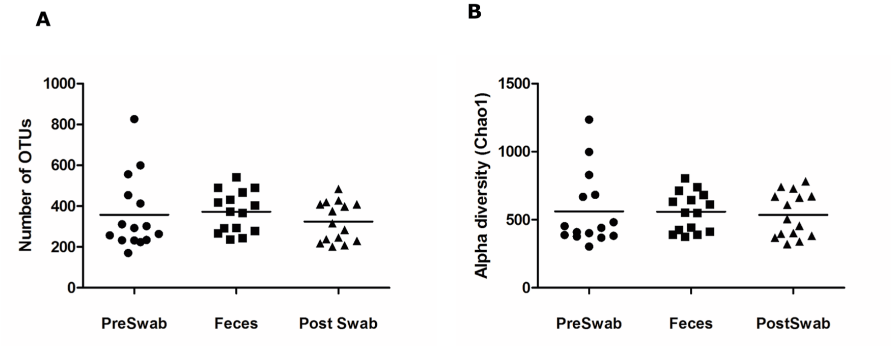
Supplementary figure 1: Taxonomic composition of the 16S rRNA samples. Stacked bar plot depicting relative microbial abundances of all samples at phyla level. Pig 1- 10 are 7 days old (time-point 1) and Pig 11–20 are 20 days old (time-point 2); Pre = Pre-Swab; Fresh = Faeces, Post = Post-Swab. The figure highlights individual variation captured irrespective of the sample type.



Supplementary figure 2: Biplot of redundancy analysis (RDA) of environmental variables in faecal samples (day20 only) at genus level. (**A**) Gender (**B**) Pen. Constrained explanatory variables are indicated by triangles. Top right shows the *P*-value of Monte Carlo Permutation testing. F = faecal sample, Number = animal ID.



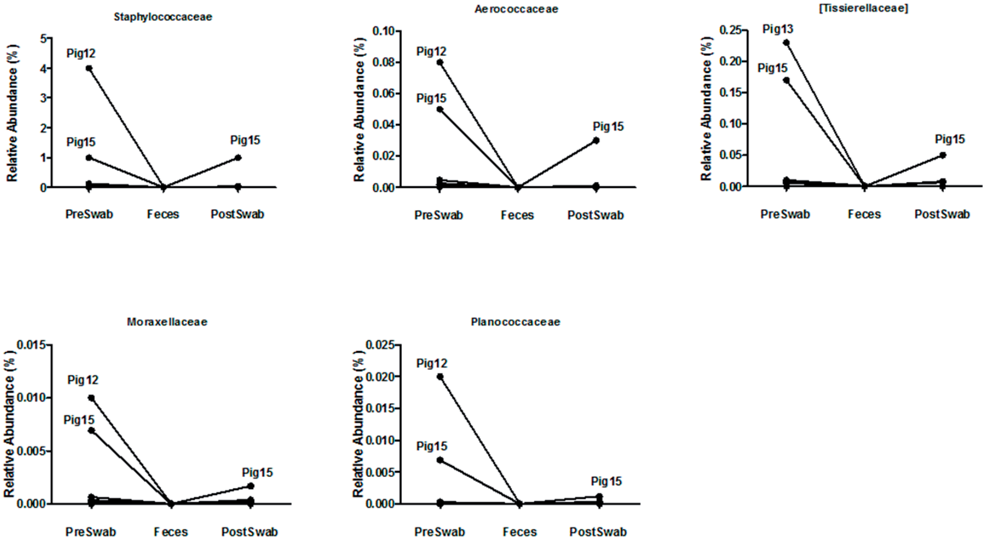
Supplementary figure 3: Venn diagram of bacterial communities with shared and unique operational taxonomic units with at least 10 reads (total OTUs found 1334) in faeces, pre-swab or post-swab samples. The percentage within the diagram represents the percentage of the total OTUs unique and shared among the sample types. The percentages (in red) shown outside the diagram represent the relative abundance captured by the 686 shared OTUs.



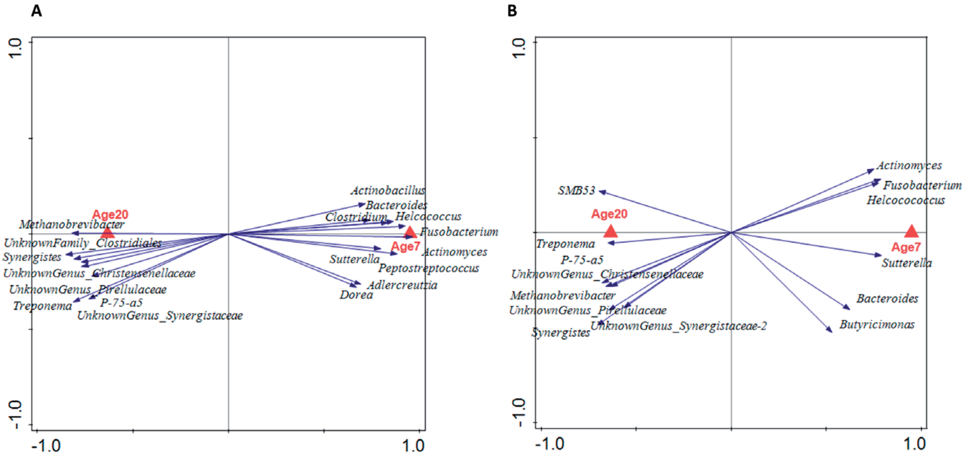
C

PERMANOVA of Weighted UniFrac	Age d7	Age d20
Pre-Swab vs Faeces	P=1.00	P=0.317
Faeces vs Post-Swab	P=1.00	P=1.00
Pre-Swab vs Post-Swab	P=1.00	P=0.32

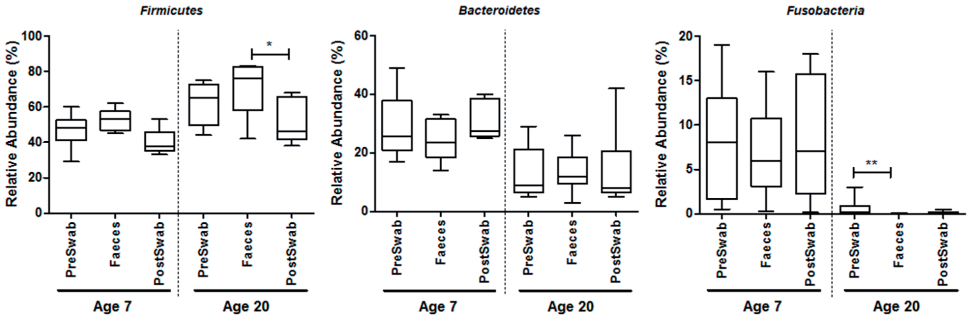
Supplementary figure 4: Alpha and Beta diversity of sample types (**A**) Number of species and (**B**) Chao1 bias corrected. (**C**) PERMANOVA analysis of Weighted UniFrac of sample types at two time-points.



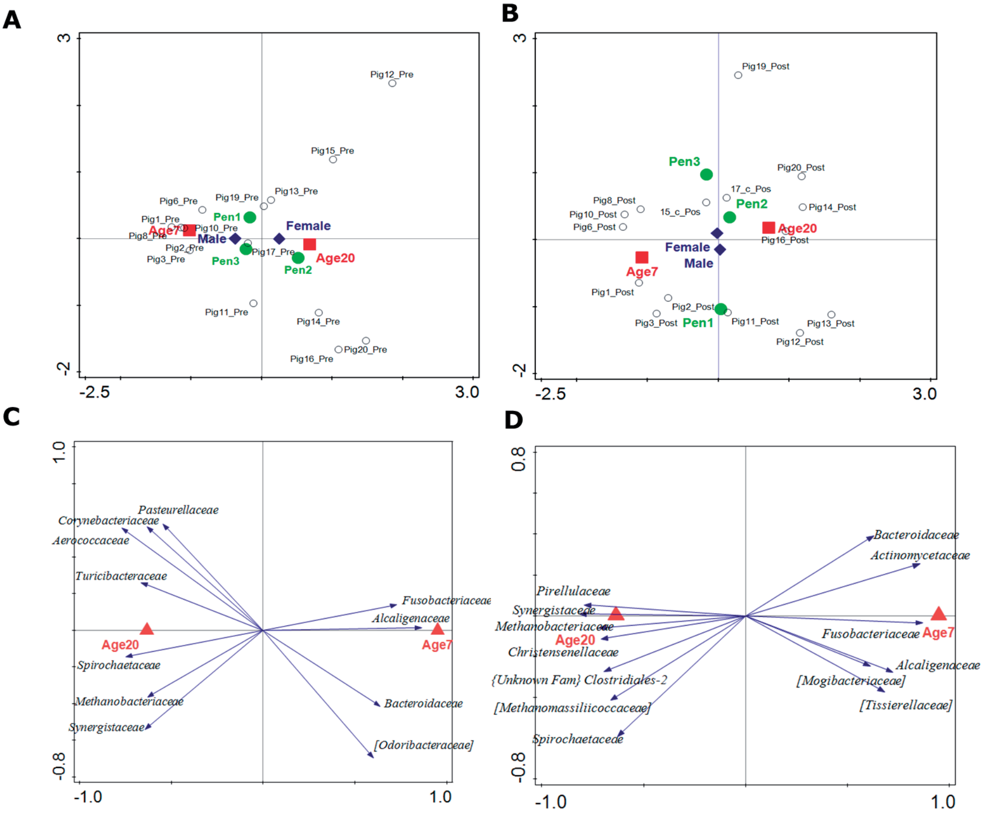
Supplementary figure 5: Differential abundance of microbial families in three animals (not significant).



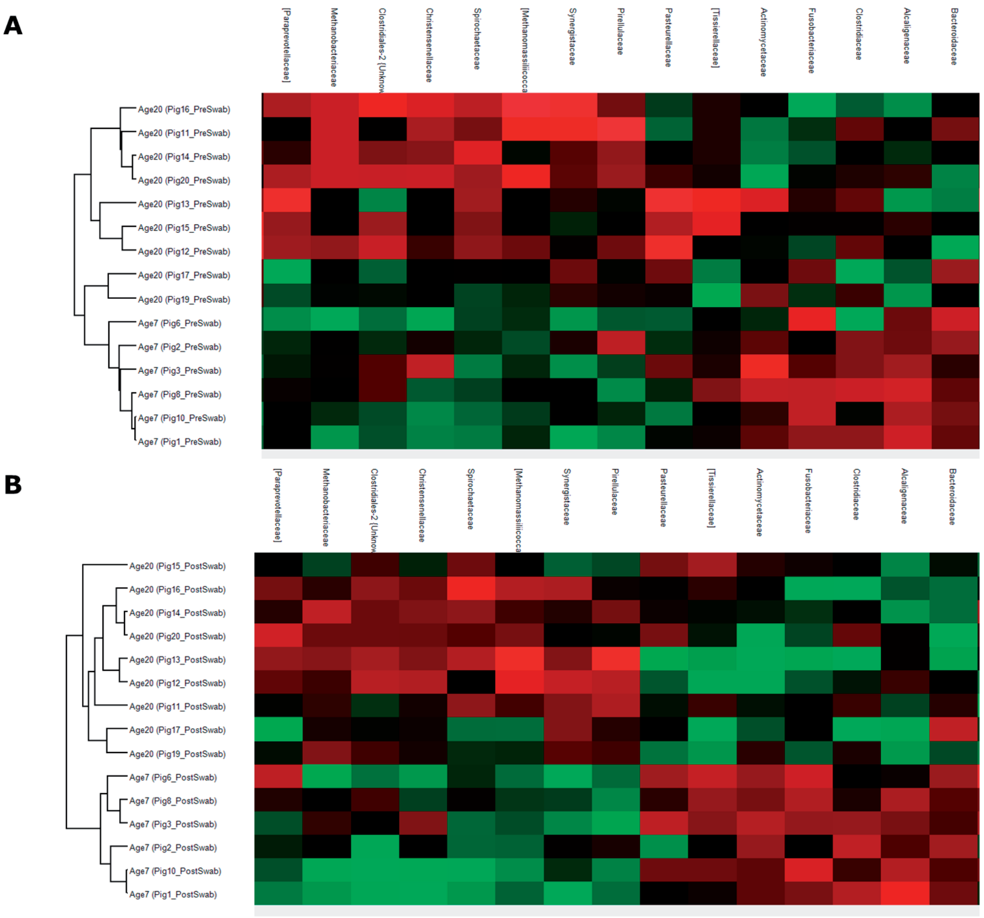
Supplementary figure 6: Partial redundancy analysis (pRDA) for the explanatory variable age (corrected for pen) at genus level. (**A**) Faeces (RD1 = 38.85% and RD2 = 10.83%). (**B**) Rectal swabs (RD1 = 16.64% and RD2 = 15.59%)



Supplementary figure 7: Relative abundance of sample types (phylum level) at both time-points (the age is indicated in "days after birth").*: *P* <0.05; **: *P* <0.01.



Supplementary figure 8: Principal component analysis of explanatory variables and Redundancy analysis (partial RDA) of age variable (corrected for pen) for (**A**)(**C**) Pre-swab samples and (**B**)(**D**) Post-swab Samples.



Supplementary figure 9: Heat map showing relative abundance of most discriminative bacterial families for age variable (corrected for pen) for (**A**) Pre-swab and (**B**) Post-swab samples

3

Chapter 3

Early–life feeding accelerates gut microbiome maturation in piglets

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Submitted for publication

Abstract

Early-life microbiome perturbations have been suggested to have important effects on host development, physiology, and behaviour, which can persist throughout life. We hypothesise that early feeding (access to a pre-weaning fibrous diet) can affect gut microbiome colonisation and development in neonatal piglets. In this study, a customised fibrous diet was provided to early-fed piglets (EF; 6 litters) starting two days after birth until weaning (28 days of age) in addition to sow's milk, whereas control piglets (CON; 4 litters) suckled mother's milk only. From 10 piglets per treatment, rectal swabs were collected at multiple timepoints until six weeks of age (i.e., 2 weeks post-weaning) to investigate intestinal microbiota composition development over time using 16S rRNA gene profiling. We observed a dynamic intestinal microbiota colonisation pattern during the pre-weaning period in both EF and CON groups, which rapidly stabilised and was closely related within individual animals at 2 weeks post-weaning. The microbial (alpha) diversity increased with age and seemed to reach a plateau in the early post-weaning time-point (day+5). The homogenous post-weaning microbiota was represented by microbial groups including ***Prevotella, Roseburia, Faecalibacterium, Ruminococcus, Megasphaera, Catenibacterium and Subdoligranulum,*** that are mostly fibre-degraders and/or SCFA producers. Remarkably, early feeding of neonatal piglets resulted in accelerated maturation of the intestinal microbiota at pre-weaning time-points, characterised by increased rate of microbial diversity and expanded colonisation of typical post-weaning associated microbial groups (mentioned before) at pre-weaning stages. The acceleration in EF piglets was illustrated by the simultaneous emergence of typical post-weaning-associated microbial groups and a more rapid decline of typical early-life/pre-weaning microbial genera. In addition, the individual eating behaviour scores of the piglets quantitatively correlated with the accelerated change of their microbiome. Furthermore, the EF piglets showed a tendency of higher relative body weight at 5 days post-weaning and lower coefficient of variation in body weight in the early post-weaning period. Overall, these findings show the importance of early-life nutritional strategies to influence the gut microbiota maturation in neonatal piglets.

Keywords: early-life, development, fibre, feeding strategies, microbiota, neonatal, pig, suckling, weaning

Introduction

The mammalian gastrointestinal tract (GIT) is inhabited by complex and diverse microbial communities that influence host health and disease. Rapid advances in next generation sequencing have facilitated the understanding of the factors that shape this complex community. The complex ecosystem development initiates by microbial groups starting to assemble shortly after birth (derived from the mother and environment) and gradually stabilises over time (Schokker et al., 2015; Timmerman et al., 2017). In early-life, the influence of colonising gut microbiota on intestinal development is crucial as in this period, the microbiota is considered essential for appropriate development and programming of the mucosal immune response (Schokker et al., 2015; Zhuang et al., 2019). Based on the plasticity of the mucosal system during this early-life development phase, it has been proposed as a “window of opportunity” where perturbations may have long-lasting impacts on health and welfare (Putignani et al., 2014; Nowland et al., 2019).

The transition from mother’s milk to solid food, commonly known as weaning, is normally a gradual process in young mammals. In nature, the weaning process of piglets approaches completion between 17–20 weeks of age approximately (Newberry and Wood-Gush, 1985; Jensen and Stangel, 1992). However, in modern swine industry practice, piglets are weaned abruptly around 3–4 weeks of age, a time-period that coincides with the developmental changes of the gastrointestinal system (Moeser et al., 2017). Evidently, early weaning is a highly dynamic and stressful event as the piglets deal with sudden changes in diet and environment, including the separation from their mother and littermates. Weaning stress is typically associated with low feed intake, sub-optimal weight gain, diarrhoea episodes and maladaptive behaviour, leading to compromised animal health and welfare, increased piglet mortality and economic losses (Le Dividich and Sève, 2000; Bruininx et al., 2002; Heo et al., 2013; Everaert et al., 2017; Gresse et al., 2017; Pluske et al., 2018). Gut microbiota dysbiosis (characterised by microbial imbalance, intestinal inflammation, reduced gut barrier function, and increased abundance of potential pathogens) is one of the factors that could contribute to the weaning transition problems, and is considered as one of the leading causes of post-weaning diarrhoea and associated GIT infections in piglets (Gresse et al., 2017; Pluske et al., 2018). The two major pathogens, identified as causative agents in post-weaning diarrhoea of piglets, are ***Salmonella enterica serovar Typhimurium*** and especially enterotoxigenic ***Escherichia coli*** (ETEC), causing high pig mortality rates worldwide (Gresse et al., 2017; Tran et al., 2018). Although weaning transition has been associated with intestinal dysbiosis, the role of the microbiota changes in the overall health-risks at weaning remains elusive. This highlights the need for further research to decipher the impact of early-life perturbations on gut microbiota colonisation and its consequences during the weaning transition as well as later life in pigs.

In neonatal piglets, the microbiota colonisation is a dynamic process with rapid microbial shifts from the initial pioneering microbial groups (present during the first weeks of life) to the changing microbial populations eventually reach a climax, adult-like microbial community (Guevarra et al., 2019). Several studies (Kim et al., 2011; Alain et al., 2014; Frese et al., 2015; Mach et al., 2015; Niu et al., 2015; Slifierz et al., 2015; Zhao et al., 2015; Chen et al., 2017; Ke et al., 2019; Wang et al., 2019) have evaluated the microbiota development in pigs, analysing the age-related microbial shifts, employing either faecal samples or rectal swabs. For instance, Chen and colleagues (Chen et al., 2017) characterised longitudinal changes in faecal microbiota of suckling piglets at four different ages and described a quickly shifting intestinal microbiota around weaning that stabilized in a period of approximately 10 days post-weaning, with microbial groups like ***Bacteroides, Escherichia/Shigella*** group enriched during pre-weaning ages, and ***Prevotella*** predominating the post-weaning ages where it accumulated to approximately 25% of the overall gut microbiota community.

It is well established that diet can be considered a major factor that can shape the intestinal microbiota in mammals. Dietary fibres, abundant in common plant-based feedstuffs, pass through the small intestine in an undigested form and are fermented in the distal ileum and colon, stimulating the growth of microbes. Microbial fermentation of undigested fibres usually takes place in the distal part of the gastrointestinal tract, resulting in the formation of short-chain fatty acids (SCFA), that are known to influence physiological functioning of the intestines such as formation and protection of intestinal barrier as well as host defence and inflammatory responses (Den Besten et al., 2013; Furusawa et al., 2013; van der Beek et al., 2017; Xiong et al., 2019). Several studies have evaluated the impact of dietary fibres on intestinal microbiota composition, focussing mostly on weaned or growing pigs (Yao, 2008; Ivarsson et al., 2012; Liu et al., 2012, 2018; Haenen et al., 2013; Dicksved et al., 2015; Umu et al., 2015, 2018; Burbach et al., 2017; Zhao et al., 2018; Soler et al., 2018; Yin et al., 2019; Chen et al., 2020). However, only a handful of studies have modulated the early-life “window of opportunity” using dietary treatments in neonatal piglets and evaluated the impact of fibres on microbiota composition (Shim et al., 2005; Berding et al., 2016; Zhang et al., 2016; Mu et al., 2017; Schokker et al., 2018; Van Hees et al., 2019). Most of these studies assessed the effect of specific dietary fibres on the intestinal microbiota at a single time-point, and employed outdated methods of analysis like microbiota cultivation, 16S DNA-microarray technology, and/or qPCR analysis of selected microbial groups. Overall, they report alteration in microbiota composition due to the pre-weaning dietary intervention, especially focussed on increased abundances of genera like ***Lactobacillus and Bifidobacterium,*** and/or decreased abundances of potentially pathogenic species like ***Escherichia coli, Streptococcus suis*** or ***Clostridium perfringens.***

In the present study, we provided a customised fibrous diet to a group of suckling piglets from two days of age, intending to familiarise them with the consumption of solid feed and to investigate the impact on their gut microbiota compared to control piglets that did not receive pre-weaning solid feed. Both groups of piglets revealed substantial dynamics of the gut microbiome during the pre-weaning period, characterized by the appearance and disappearance of specific microbial genera over time. Additionally, our findings establish that pre-weaning fibrous feed can accelerate gut microbiome colonisation towards a more 'mature' microbiome, that resembles that of post-weaning microbial composition. Moreover, the magnitude of this acceleration effect appeared to be quantitatively related to the amount of pre-weaning feed intake as deduced from observational studies. Finally, the pre-weaning provision of fibrous feed reduced variability of post-weaning weight gain, and resulted in a trend towards higher body weight shortly after weaning.

Materials and methods

Animals and experimental design

The Animal Care and Use committee of Wageningen University & Research (Wageningen, The Netherlands) approved the protocol of the experiment (AVD104002016515). The protocol is in accordance with the Dutch law on animal experimentation, which complies with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Ten multiparous Topigs-20 sows (parity 3-5) housed and inseminated at research facility Carus (Wageningen University & Research, The Netherlands), were divided into two groups (n=6 litters for early-fed or EF group; and n=4 litters for control or CON group) based on sow's parity, body weight and genetic background. Within two days after birth, the litter size was set to maximum 14 piglets per litter (Tempo x Topigs-20) with no cross-fostering. The new-born piglets were cohoused with sow and littermates till weaning (28 days of age) and received ear tags for individual identification and an iron injection, standard to pig husbandry practice. From two days of age, the neonatal piglets belonging to the EF group were given access to customised fibrous feed (**Supplementary Table 1**) *ad libitum* in addition to suckling sow's milk whereas the CON group suckled sow's milk only. Briefly, the feed contained 26% dietary non-starch polysaccharides, mainly originating from sugar beet pulp (4%), oat hulls (4%), inulin (4%), galacto-oligosaccharides (5%) and high amylose maize starch (4%) as fibrous ingredients. A subset of piglets (n=64; 32 for each group, EF and CON) were weaned at four weeks of age and followed for two weeks post-weaning. At weaning, piglets were mixed within the same treatment group and housed in separate pens with four unfamiliar piglets per pen (i.e., 4 piglets per pen originating from different litters within treatment). After weaning, all piglets had *ad libitum* access to commercial weaner diet (Inno Speen Pro, Coppens Diervoeding, Helmond, The Netherlands). Additional information about the housing and management have been described in detail in a previous study (Middelkoop et al.).

Eating behaviour by video observation

The eating behaviour of piglets was assessed by video recordings from two days of age till weaning. For recognition during behavioural observations, piglets were individually numbered on their back using dark permanent hair dye. Eating frequency of individual EF piglets was determined daily from 07:00 to 19:00 hours via video observations as an estimate for pre-weaning solid feed intake. From the video observations, the amount of time spent eating or "eating time" was evaluated. When an EF piglet placed its snout into the trough for a minimum of 5 seconds (s), the behaviour was scored as eating (Pajor et al., 1991; Adeleye et al., 2014). The eating time was categorised into short (5–9s), medium (10–29s) and long (≥ 30s) feeding bouts. The feeding bout ended when the snout of the piglet was out of the trough for a minimum of 5s. Exploratory behaviour towards the feed trough such as chewing the trough was not scored as eating. Daily/weekly eating activity per piglet was (semi-) quantified by summing the (minimum) number of seconds spent eating (where short, long and medium bouts counted as 5, 10 and 30 seconds respectively) from 2 days of age to weaning (at 28 days of age). All the observers were trained and instructed with the evaluating criteria to obtain homogeneous and accurate quantification of eating behaviour. Some parts of the videos were double checked by more than one person to evaluate consistency and observer dependent variations. This is to note that the eating behaviour measurements may have some degree of subjectivity and were taken as an "estimate" for the amount of eating per piglet and used as an indicative quantification of eating.

Intestinal microbiota sampling and microbiota metataxonomic analysis

To investigate intestinal microbiota colonisation patterns, rectal swab samples were obtained from piglets by inserting a sterile cotton swab (Puritan Medical, Guilford, ME USA; Cat Number-25-3306-U) 20–30 mm into the rectum and rotating the swab against the bowel wall for a minute before placing it into a 5ml eppendorf tube. The samples were kept on ice during transport to the laboratory and stored at −20°C until further processing. The selection of piglets (n=10 per treatment group) were made by the following criteria: (a) no antibiotic treatment (b) no pre-weaning diarrhoea (c) close to average weight of the treatment group (d) equal male to female ratio. A total of 160 swab samples were collected, repeatedly from the same piglets at five time-points pre-weaning (2, 7, 15, 21, 28 days of age) and three time-points post-weaning (+2, +5 and +14 days of age). At 2 and 7 days of age, samples were collected fixed to their birth date, thereafter all samples were taken on the same day for all piglets fixed to the day of weaning. However, one sample (from EF group at 7days of age) was unsuccessful at the sequencing step and therefore could not be included in the analysis. The EF piglets selected for microbiota analysis belonged to four pens (or litters) before weaning (2-3 piglets selected per pen), and two pens had to be omitted from microbiota analysis due to uterine infection of the sow and subsequent antibiotic treatment during the suckling phase.

DNA extraction from rectal swabs was performed by the repeated bead beating method (Yu and Morrison, 2004) using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 500 µl of lysis buffer was added to the 5ml eppendorf tube (holding swab) to obtain swab solution, which was used as a starting material for DNA extraction. The quality and quantity of extracted DNA samples were checked by gel electrophoresis (only representative samples) and Nanodrop DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE USA) respectively. The DNA template was used for amplifying the V3-V4 region of the bacterial 16S rRNA gene using V3F primer (5′-CCTACGGGNGGCWGCAG-3′) and V4R primer (5′-GACTACHVGGGTATCTAATCC-3′), 5′-extended with extension-PCR-adapters 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′ respectively. PCR Amplification (Bio-Rad C1000 thermal cycler, Bio-Rad Laboratories, Veenendaal, The Netherlands) of the 16S rRNA V3-V4 region was completed in a 50 µl reaction volume consisting of 5 µl 10× KOD buffer (Toyobo, Japan), 3 µl MgSO4 (25 mM) 5 µl dNTPs (2 mM each), 1.5 µl V3F primer [10 µM (Eurogentec, Luik, Belgium)], 1.5 µl V4R primer [10 µM, (Eurogentec, Luik, Belgium)], 1.0 µl (0.02 U/µl) KOD hot start DNA polymerase (Toyobo, Japan) and 10 ng (minimum) of template DNA. The amplification conditions included a single initiation cycle of 95°C for 2 min, followed by 25 amplification cycles encompassing denaturation at 95°C for 20 s, annealing at 55°C for 10 s, and elongation at 70°C for 15 s, and was completed by a single elongation step at 72°C for 5 min. Amplicons were purified using MSB Spin PCRapace (STRATEC Molecular, Germany) and were sequenced at BaseClear BV (Leiden, The Netherlands) using (paired-end) Illumina MiSeq system. Purified amplicons were subjected to extension-PCR using barcoded Illumina universal index sequencing adapters prior to sequencing. The Illumina MiSeq system generated FASTAQ sequence files using the bcl2fastq2 version 2.18 and these sequences were subjected to quality control based on Illumina Chastity filtering and FASTQC quality control tool version 0.11.5. Subsequently, a BaseClear in-house filtering protocol was applied for removal of reads containing adapters (up to minimum read length of 50bp) and/or PhiX control signal, to generate the FASTAQ data file used for microbiota analysis.

Illumina reads were imported into the CLC Genomics Workbench version 11.01 and were processed using the CLC Microbial Genomics Module version 2.5.1 (CLC bio, Arhus, Denmark). The paired end reads were merged into one high quality representative by CLC Workbench (Mismatch cost = 1, Minimum score = 40, Gap Cost = 4, Maximum unaligned end mismatches = 5). The CLC pipeline was used for primer and quality trimming (Trim using quality scores = 0.05; Trim ambiguous nucleotides: maximum number of ambiguities = 2; Discard reads below length = 5). The remaining high quality sequences were clustered into operational taxonomic unit (OTUs) at 97% identity threshold using SILVA database v132 (released on Dec 13, 2017) (Quast et al., 2013). OTUs lower than 2 reads (Minimum combined count = 2) were excluded from the analyses. To achieve even sequencing depth between samples, the OTU table was rarefied to 14,000 reads for calculation of alpha and beta diversity indices. Principal component analysis (PCA; unsupervised), redundancy analysis (RDA; supervised) and partial redundancy analysis (pRDA; supervised) were performed using CANOCO 5 (Microcomputer Power, Ithaca, NY, USA) according to manufacturer's instructions (Braak and Smilauer, 2012). To evaluate the impact of environmental variables like age, pen, gender and treatment separately, RDA and pRDA analyses were performed, the latter analyses (pRDA) allowing us to correct for other covariates in the data as described in Canoco 5 manual (Braak and Smilauer, 2012). The pen-related impact was assessed separately per time-point and in pre- and post-weaning phases to avoid confounding age and weaning-related differences. The microbial groups detected by RDA analyses were selected by their response scores in the biplot (obtained by perpendicular projection of the taxa arrows on the X axis) and were further filtered by the condition of having ≥ 0.1% average relative abundance in at least one of the time-points (for biological relevance). The median relative abundance of selected microbial groups was calculated for each of the time-points in 20 piglets (assessing age) or 10 piglets (assessing treatment groups). Relative abundance of age-related microbial groups were visualised by heat maps by normalising by the highest median of all timepoints (scaling from 0 to 1), in Perseus software (Tyanova et al., 2016). Euclidean distance was utilized to measure the distance and clustering was conducted using the average linkage method. Time dependent treatment effect were assessed by using a linear ordination method known as principal response curves (PRCs) in Canoco 5, which is based on redundancy analysis (RDA), adjusted for overall changes in microbial community response over time (Van Den Brink and Ter Braak, 1999). The principal component is plotted against time, yielding a principal response curve of the community for each treatment. The PRC method also shows microbial response scores by a 1-dimensional plot, thereby enabling quantitative interpretation of treatment effect towards the microbial species level. Statistical significance was evaluated by Monte Carlo permutation procedure (MCPP) with 499 permutations. Response-variable based case scores (CaseR) was extracted from redundancy analysis of age, which was defined as their 'microbiome age' as they correspond to the individual sample position in the progressing-age-ordination plot. To evaluate the quantitative relationship between observed eating behaviour and magnitude of the microbiota change, a non-parametric spearman correlation was performed between eating scores (in seconds) and squared Bray Curtis distance of piglets or 'microbiome age' between individual time-points and their corresponding day+14 time-point in GraphPad Software 8.1.1 (California, USA, www.graphpad.com). Comparison of the specific taxa relative abundance and diversity indices between treatments were performed by Mann Whitney U-test whereas comparison among time-points were assessed by one-way ANOVA (Kruskal-Wallis statistic) using a Dunnett's test for multiple comparison using GraphPad Software 8.1.1. The level of statistical significance was set at $P < 0.05$ with a trend defined as $0.1 > P \geq 0.05$.

Performance data analysis

Piglets were weighed individually in all microbiota-sample-collection time-points pre- and post-weaning. Relative body weights of piglets (normalised to their weaning weights at day28) were calculated in the early post-weaning period to evaluate their body weight development during the weaning transition. To assess differences between the groups, Mann Whitney U-test and coefficient of variation (CV%) were calculated in GraphPad Software 8.1.1.

Results

Dynamics of gut microbiome development in early-life of piglets

In this study, we first investigated the dynamics of intestinal microbiota development by analysing rectal swab samples collected from two days after birth (day2) to two weeks post weaning (day+14). All rectal swab samples were used to assess the microbiota development over time, irrespective of their allocation to the intervention group receiving pre-weaning fibrous feed (EF), or not receiving any additional feed (CON). 16S rRNA Illumina sequencing produced 5,013,336 reads after quality filtering, having a mean depth of 27,009 ± 5019 reads per sample.

Irrespective of the treatment, the porcine gut microbiota showed typical microbiota colonisation patterns with Firmicutes being the predominant phyla (60.01%), followed by Bacteroidetes (23.5%), Proteobacteria (8.04%), Actinobacteria (2.77%) and Fusobacteria (1.56%), together capturing 95.9% of total microbiome. Firmicutes and Bacteroidetes together accounted for 64-97% of total sequences across all ages. While the piglets aged from day2 to day+14 post-weaning, the relative abundance of Firmicutes increased (44% to 67%; $P < 0.0001$) and Bacteroidetes fluctuated pre-weaning eventually increasing post-weaning (19.9% to 29.2%; $P < 0.0001$) (**Supplementary figure 1**). The relative abundance of Fusobacteria significantly dropped from around 10% at day2 to less than 1% after 15 days of age ($P < 0.05$) almost disappearing over time ($P < 0.0001$). During the same time-period, the microbial (alpha) diversity strongly increased, especially during the pre-weaning period (**Figure 1A, Supplementary figure 2A)** and reached an apparent plateau post weaning. This is reflected by both the Chao 1 index (richness) that increased significantly from 2 days after birth (day2) up to day 5 post-weaning (day+5) as well as the Shannon index (richness and evenness) that increased gradually during the weaning period (day28) and remained stable afterwards.

The impact of variables in the experimental set-up such as age, pen, gender and weaning, on the microbiota composition were investigated by redundancy (RDA) and partial redundancy (pRDA) analyses. The pen effect on microbiota was assessed by partial RDA (pRDA) corrected for other variables (age, treatment, gender) and revealed the expected (Frese et al., 2015) small but significant impact of pre-weaning pens on the intestinal microbiota (explained variation = 4.32%, $P = 0.002$). The effect of gender was evaluated by pRDA analyses for each time-point separately and did not reveal any gender-related influence on microbiota composition in the present experiment, which is analogous to what has been previously reported (Mach et al., 2015; Ke et al., 2019). A strong age-related effect (corrected for variables pen, gender, and

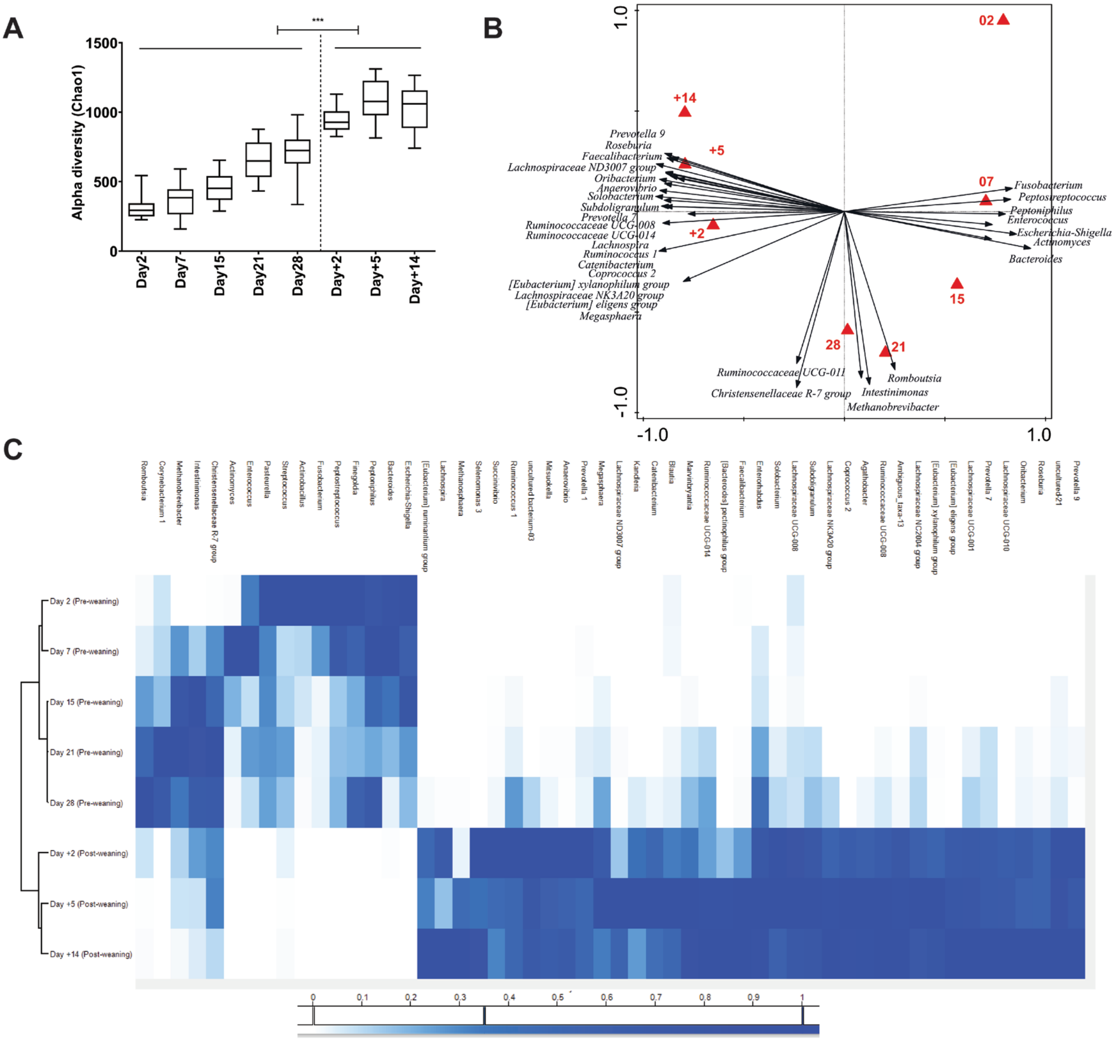

Figure 1: Age-associated intestinal microbiota dynamics pre- and post-weaning. **(A)** Alpha diversity (Chao1 bias corrected) display shifting diversity over time. **(B)** Redundancy analysis of age at genus level (explained variation = 28.22%, $P = 0.002$) with associated microbial groups at different ages. The age-related microbial groups visualized have minimum 35% fit on horizontal axis with a response score ≥ 0.7 in the biplot (obtained by projecting the taxa points perpendicular to the axes). **(C)** Heat map showing normalised relative abundance of the discriminative bacterial genera identified in redundancy analysis of age. Significant differences between time-points were assessed by student t test or Mann-Whitney U test (***: $P < 0.001$).

treatment) on microbiome composition at genus level was observed (**Figure 1B**; explained variation = 28.22%, *P* = 0.002). The microbiota composition development as a function of age from day2 to day+14 displayed a horse-shoe shaped progression curve over time (**Figure 1B;** biplot with red triangles indicating different ages). Moreover, it allowed the identification of dominant microbial genera (black arrows) associated with certain time-points or parts of the overall timespan of the experiments. These microbial genera provided the markers for the time-dependent colonisation pattern in these piglets, irrespective of their treatment (EF or CON). For example, during the pre-weaning period, microbial groups such as *Actinomyces, Fusobacterium, Enterococcus, Peptostreptococcus, Bacteroides, Escherichia-Shigella* were abundant during the first few weeks of age as shown in heatmap representation (**Figure 1C**). On the other hand, genera like *Romboutsia, Intestimonas, Methanobrevibacter, Christensellaceae group R-7* emerged during the third or fourth weeks of age, but strikingly disappeared again post-weaning (**Figure 1C**). As expected, a sharp distinction was observed between the pre- and post-weaning microbiota, with several bacterial groups like *Prevotella, Faecalibacterium, Lachnospira, Roseburia, Subdoligranulum, Catenibacterium, Succinivibrio, Megasphaera, Coprococcus* particularly associated with post-weaning microbiota composition (**Figure 1B, 1C, Supplementary figure 2B**). Hierarchical clustering of age-related microbial genera (detected by RDA analyses) clearly separated the pre- and post-weaning associated microbiota, which is most probably governed by the sudden dietary shift, from pre-weaning mother's milk to post-weaning solid (plant-based) feed. We observed a homogenising effect on the microbiota during the post-weaning period, which was characterised by comparable alpha diversity scores **(Figure 1A)**, closely clustered post-weaning time-points (**Figure 1B)** and lower Bray Curtis (PCoA) distance between post-weaning samples (**Supplementary figure 2C**).

Impact of early–life (fibrous) feed intervention on microbiome development

Video observation shows eating behaviour increasing with time

Four pens (EF group) were provided with fibrous feed from two days of age in addition to suckling mother's milk. We observed their eating behaviour around the feed trough using video recordings (0700 to 1900 hrs daily; eating time measured in seconds) for four weeks pre-weaning. The quantified eating behaviour scores for the EF piglets gradually increased as the piglets became older, reaching a clear amplified level of eating in the last week before weaning (**Figure 2A**). Eating behaviour scores during the first week of life were almost negligible, with piglets spending no- or only a very limited time exploring and eating the feed. However, during the second week of life, all the piglets started exploring and consuming the provided feed, and this behaviour increased during the third and increased strongly in the fourth week just before weaning. This individual eating behaviour was also supported by the previously reported (Middelkoop et al.) increase in feed intake at pen-level during the 4 weeks pre-weaning. However, despite this universal and prominent increase in eating behaviour over time, the

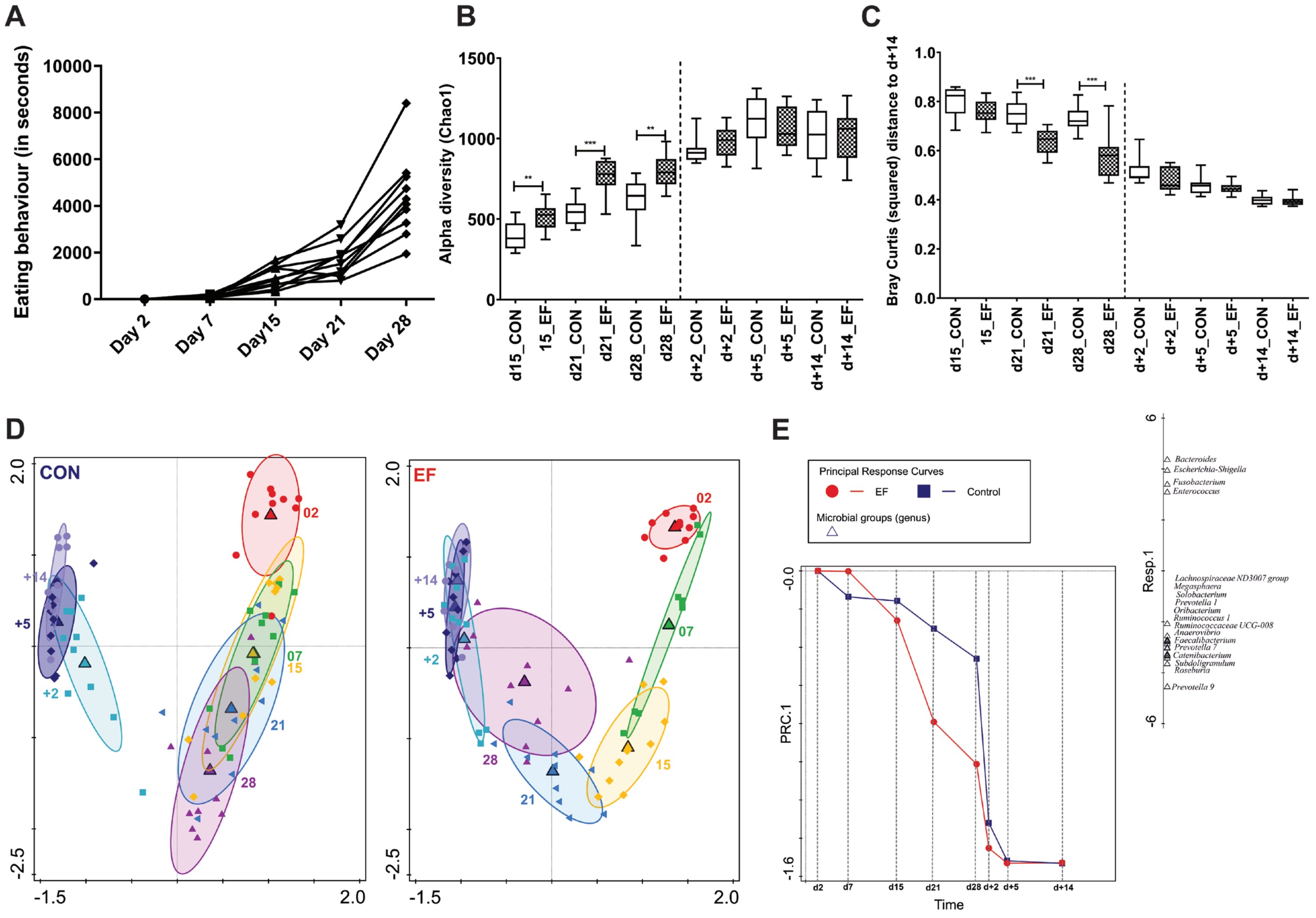
A
Eating behaviour (in seconds)
10000
8000
6000
4000
2000
0
Day 2
Day 7
Day15
Day 21
Day 28
B
Alpha diversity (Chao1)
1500
1000
500
0
**
***
**
d15_CON
15_EF
d21_CON
d21_EF
d28_CON
d28_EF
d+2_CON
d+2_EF
d+5_CON
d+5_EF
d+14_CON
d+14_EF
C
Bray Curtis (squared) distance to d+14
1.0
0.8
0.6
0.4
0.2
0.0
***
***
d15_CON
15_EF
d21_CON
d21_EF
d28_CON
d28_EF
d+2_CON
d+2_EF
d+5_CON
d+5_EF
d+14_CON
d+14_EF
D
CON
2.0
-2.5
-1.5
2.0
02
07
15
21
28
+14
+5
+2
EF
2.0
-2.5
-1.5
2.0
02
07
15
21
28
+14
+5
+2
E
Principal Response Curves
● — EF
■ — Control
Microbial groups (genus)
△
-0.0
PRC.1
-1.6
d2
d7
d15
d21
d28
d+2
d+5
d+14
Time
6
Resp.1
-6
Bacteroides
Escherichia-Shigella
Fusobacterium
Enterococcus
Lachnospiraceae ND3007 group
Megasphaera
Solobacterium
Prevotella 1
Oribacterium
Ruminococcus 1
Ruminococcaceae UCG-008
Anaerovibrio
Faecalibacterium
Prevotella 7
Catenibacterium
Subdoligranulum
Roseburia
Prevotella 9

Figure 2: Microbiota colonisation development in early-fed (EF) and control (CON) group. **(A)** Eating behaviour (total eating seconds per week; n = 10) in EF litters over time pre-weaning. **(B)** Alpha diversity (Chao1 bias corrected) display diversity differences between the two groups from 15 days onwards. **(C)** Comparison of squared Bray Curtis index (distance between different samples with their d+14 time-point in individual piglets) between two groups. **(D)** Principal component analysis of microbiota in all ages (PC1 = 42.4%, PC2 = 12.3%), shown for the CON and EF group separately. **(E)** Principal response curve (PRC) analysis presenting changes in microbiota composition (genus level) across time for EF (red) and CON (green) group. The horizontal axis represents time and the vertical axis denotes PRC score values. Microbial response scores are shown on the right in a 1-dimensional plot. The combination of PRC score values and microbial response scores offers a quantitative interpretation and direction of change at different time-points. The effect of treatment (including its interaction with time) was significant according to Monte Carlo permutation test (*P* = 0.002). Significant differences between groups were assessed by student t test or Mann-Whitney U test (**: *P* < 0.01; ***: *P* < 0.001).

quantified estimates of eating of individual piglets displayed considerable variation, analogous to the variation of feed-intake measurements per pen (Middelkoop et al.).

Early-life feeding accelerates microbiome colonisation maturation

One of the objectives of our study was to evaluate the impact of early-life feeding of fibrous feed on the microbiota composition, compared to the control group of piglets that did not receive any pre-weaning feed. Therefore, we investigated this effect using the microbiota composition information from 15 days of age onwards, because at the timepoints prior to day15, the piglets spent very little time eating solid feed (see above). Remarkably, the period with significant observed eating behaviour (day15 and onward) coincided consistently with higher levels of richness of the microbiota determined in the EF group relative to the CON group (**Figure 2B)**. Moreover, in EF piglets, squared Bray Curtis distance (**Figure 2C**) of individual samples (day21 and day28) was significantly lower from their corresponding day+14, indicating proximity to a more “matured” state. The closeness of the EF group to the post-weaning microbiome, was also reflected in the increased ‘microbiome age’ at pre-weaning time-points (**Supplementary figure 3A**). However, this difference was no longer observed post-weaning, indicating that the microbiota diversity in the CON group rapidly caught-up with that of the EF piglets. These differences between EF and CON groups could be recapitulated in group-specific (EF or CON) PCA analysis (**Figure 2D**), which clearly demonstrated a considerable overlap of pre-weaning time-points day28, and to a smaller extent day21, with the post-weaning timepoints in EF piglets, whereas such overlap was completely absent in the CON piglets (**Figure 2D**). The EF treatment response over time was further investigated by principal response curve (PRC) analysis revealing a significant interaction of early feeding with time on the first constrained axis (*P* = 0.002; **Figure 2E**). In comparison to the CON group, the microbiota composition of EF group was enriched for several microbial genera (**Figure 2E**), including *Prevotella 9, Roseburia, Subdoligranulum, Catenibacterium, Faecalibacterium*, which are among the genera that are also typically among the microbial groups associated with the post-weaning microbiota in comparison to the pre-weaning microbiota (**Figure 1C, Supplementary figure 2B**). Obviously, day28 and day21 emerged as interesting time-points to be explored further to evaluate accelerated microbiome colonisation in the EF group, which is in agreement with the increased eating scores observed during these later pre-weaning time-points. The strongest discrimination between the EF and CON group was detected by RDA analysis at day28 (*P* = 0.002, **Figure 3A**), followed by day21 (*P* = 0.002; **Supplementary figure 3B**) and day15 (*P* = 0.04; **Supplementary figure 3C**). The microbial groups identified as main discriminants between the EF and CON group on day28 included those identified by PRC analysis, e.g., *Prevotella 9, Roseburia, Faecalibacterium, Megasphaera, Succinivibrio, Subdoligranulum, Catenibacterium, Coprococcus*, corroborating the enrichment of the post-weaning associated microbial genera in the EF group pre-weaning relative to the CON group. The maturation is also illustrated in hierarchical clustering of age-related microbial genera (detected by RDA analyses), where the EF group at day28 is clearly separated from the rest of the pre-weaning clusters (**Supplementary figure 4**). These microbial groups start colonising EF piglets from 21 and/or 28 days of age (**Figure 3C, Supplementary figure 5**). For example, *Prevotella 9*, the dominant genus of the post-weaning phase, started appearing from day21 in the EF group and continued to have significantly higher abundance at day28 (**Figure 3B**), compared to the CON group. RDA analyses at earlier time-points like day15 and day21 (**Supplementary figure 3B, C**) demonstrate the emergence of some microbes like *Succinivibrio*, *Ruminoccus 2* (day15 onwards) and *Prevotella 9*, *Megasphaera, Lachnospira, Subdoligranulum, Coprococcus* (day21 onwards) which persisted post-weaning.

Furthermore, the EF group also displayed an accelerated loss of pre-weaning (day2-day7-day15-day21-day28)-associated microbial genera, like *Fusobacterium, Finegoldia, Intestimonas, Bacteroides* (**Figure 3C**), that is mostly found in the CON group (data not shown). Taken together, these results show that early feeding of fibrous diet accelerates the porcine microbiota colonisation, characterised by the earlier appearance and subsequent expansion of post-weaning associated microbial groups, in parallel with the more rapid disappearance of pre-weaning associated microbes. The pre-weaning microbiota differences induced in the EF group appeared to persist somewhat until five days post-weaning (**Supplementary figure 6A, B**), but disappeared and became undetectable two weeks post-weaning (day+14).

Quantitative relation between eating behaviour and microbiota

Since the accelerated microbiota colonisation patterns in the EF group coincided with the increased eating behaviour of individual piglets after two weeks of age (> 15 days), we investigated the quantitative relation between observed eating behaviour and the microbiota composition within the EF group.

Spearman correlation analysis was performed between eating behaviour (summed score per time-point) and squared Bray Curtis distance of individual piglets between a certain time-point and their day+14 "matured" time-point. It revealed a strong and significant negative correlation between these parameters, for both short term (last day; **Supplementary figure 7A**, last two

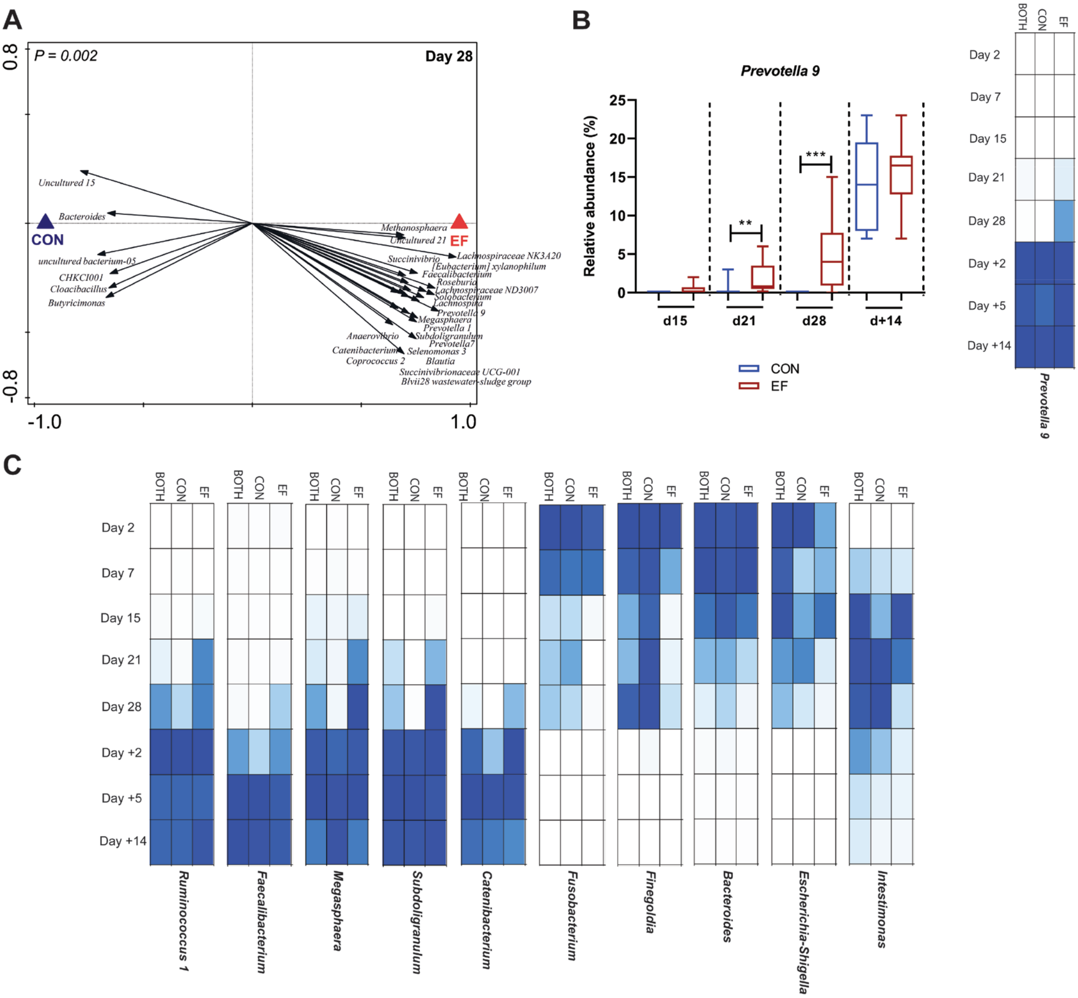

Figure 3: Accelerated microbiota maturation in early-fed (EF) group illustrated by emergence of post-weaning associated microbial groups in pre-weaning period. **(A)** Redundancy analysis of treatment at day28 (PC1 = 24.1%, PC2 = 19.7%; *P* = 0.002) with associated microbial groups in EF and CON groups (response score ≥ 0.65 on horizontal axis). **(B)** Changes in relative abundance of *Prevotella 9 in* EF and CON groups at pre- and post-weaning time-points. **(C)** Heat map of normalised relative abundance for representative individual genera at different ages showing appearance of post-weaning associated microbial groups and rapid loss of pre-weaning associated microbes, simultaneously. Significant differences between groups were assessed by student t test or Mann-Whitney U test (**: *P* < 0.01; ***: *P* < 0.001).

days; r =–0.798, *P* < 0.0001; **Figure 4**) and longer term (last week; **Supplementary figure 7B)** eating scores prior to the microbiota analysis timepoint. This indicates the consistency of eating behaviour in individual piglets, which is reflected in its association with both short-term and long-term scores. Since eating behaviour increased with age, the correlation analysis was

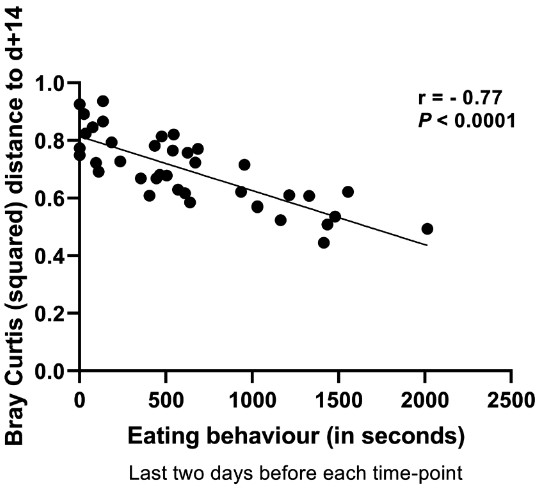

Figure 4: Spearman correlation between eating scores of individual piglets and squared Bray Curtis distance to their d+14 "matured" time-point. The last two days eating behaviour before each corresponding time-point was employed (r =–0.77, *P* < 0.0001).

done using different eating scores (ranging from last day – last week prior to the microbiota analysis timepoint). We found that inclusion of only 'recent eating scores' already quantitatively correlated with the magnitude of microbiota adaptation. Similarly, there was a strong positive association between microbiome age and eating behaviour (last two days; **Supplementary figure 7C**), fortifying the quantitative relation of eating behaviour and maturing microbiota.

Impact of early feeding on post-weaning body weight

To assess the impact of early feeding on post weaning performance, body weight of piglets was measured at three time-points day+2, day+5 and day+14 post weaning. The EF piglets tend to have a higher relative body weight compared to CON piglets at day+5 post weaning (**Figure 5**). Notably, we also observed a "smoother" transition for EF piglets in terms of relative body weight gain within the first few days post weaning, which was further supported by lower coefficients of variation and a consistent lack of body weight loss in piglets belonging to the EF group. However, the difference in relative body weight disappears at a later post-weaning time-point (day+14).

Discussion

It is known that early-life perturbations can impact the intestinal microbiota, however only a few studies have utilised this "window of opportunity" to improve the weaning transition. In this study, we have assessed the age-associated microbiota development in young piglets

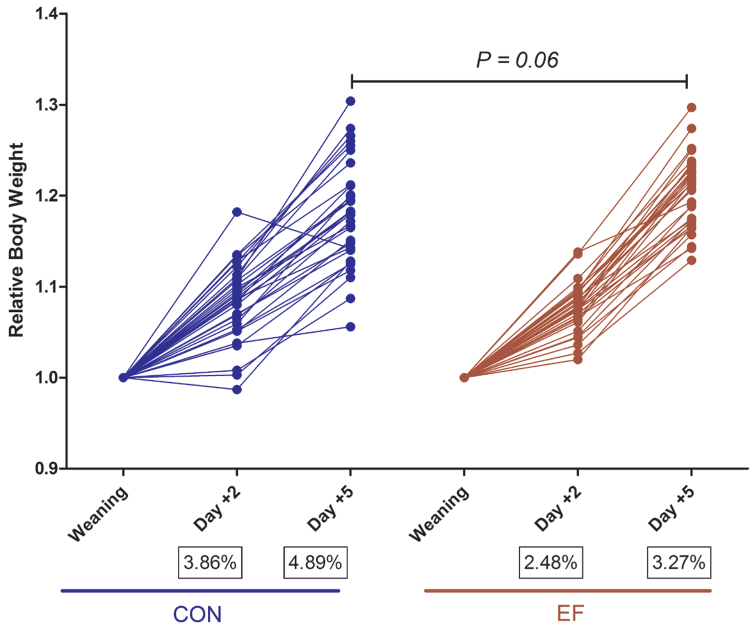

Figure 5: Comparison of relative body weight between the groups (n=32 per group) during the weaning transition (till 5 days post-weaning). Statistical comparisons between the groups were assessed by student t test or Mann-Whitney U test in GraphPad Software 8.1.1. The coefficient of variation (CV%) is indicated in black boxes below the labels.

and evaluated the impact of early-life feeding (pre-weaning access to a fibrous feed) on the gut microbiota colonisation and maturation over time (until 2 weeks post-weaning). Our results show that exposure to a fibrous diet in early-life accelerates the intestinal microbiota maturation pre-weaning, which is characterised by an increased microbial diversity, the earlier emergence and expansion of post-weaning-associated microbial groups and an enhanced loss of pre-weaning associated microbes. Importantly, analysis of individual piglets allowed us to establish that the magnitude of these microbiota compositional changes corresponds strongly with the individualized quantified eating behaviour during the pre-weaning period.

We observed a dynamic, age-related microbiota development with varied microbial groups associated at different time-points, and a discrete pre- and post-weaning associated microbiota. This is in line with multiple studies (Alain et al., 2014; Frese et al., 2015; Mach et al., 2015; Niu et al., 2015; Slifierz et al., 2015; Zhao et al., 2015; Guevarra et al., 2018; Li et al., 2018; Ke et al., 2019; Wang et al., 2019) which assessed bacterial communities in early-life of piglets, showing age as well as weaning as the driving factors in influencing microbiota development. The sudden shift in microbial communities after weaning is likely due to the abrupt dietary change from a highly palatable milk diet to a not-as-easily digestible plant-based solid feed. Firmicutes followed by Bacteroidetes were found to be the dominant phyla across all experimental time-points, and were particularly abundant post-weaning, where they accounted for more than 90% of all microbes detected. Although this is consistent with the majority of earlier studies (Kim et al., 2011; Alain et al., 2014; Mach et al., 2015; Niu et al., 2015; Chen et al., 2017; Holman et al., 2017; Ke et al., 2019; Wang et al., 2019), there are a few reports with Bacteroidetes (Lu et al., 2014; Guevarra et al., 2018) or Proteobacteria (Slifierz et al., 2015; Zhao et al., 2015) as the pre-dominant phyla. Analogously, the decreasing abundance of Fusobacteria observed during the first weeks of life has also been reported by several other studies (Alain et al., 2014; Niu et al., 2015; Slifierz et al., 2015; Ke et al., 2019), although there are also studies where this bacterial group was not detected at all (Frese et al., 2015; Guevarra et al., 2018). Such differences in taxonomic identification could, to some extent, be due to the different primers and variable regions of the 16S rRNA gene that were targeted in these metataxonomic investigations, which has been shown to capture different community structures (Albertsen et al., 2015; Holman et al., 2017), disallowing an accurate comparison between different studies. It should be noted that the other phyla we detected in this study, like Actinobacteria, Spirochaetes, Synergistetes, were consistently present at low relative abundance levels, which may contribute to their large fluctuations among studies. Despite these variations among studies, and the various confounding factors among study designs and conditions, including pig genetics, environmental conditions, piglets age at weaning, sampling procedures, sample processing and sequence analysis methods, there is a striking congruency in the biological interpretation of differences between pre-and post-weaning associated microbes in most studies (Alain et al., 2014; Frese et al., 2015; Mach et al., 2015; Slifierz et al., 2015). This is largely in agreement with our observation in the present study and pinpoints the early intestinal colonisers belonging to ***Bacteroides, Escherichia-Shigella*** and exemplifies ***Prevotella*** as a prominent microbe in the typical post-weaning microbiota together with species belonging to ***Roseburia, Faecalibacterium, Blautia, Subdoligranulum.*** The drastic increase in relative abundance of ***Prevotella*** post-weaning (from less than 3% to more than 18% of the total community) is likely due to the established capacity of the members of the genus to produce enzymes that can degrade the plant polysaccharides (Flint and Bayer, 2008; Ivarsson et al., 2014), which are prominent constituents of the plant-based weaner diet. Similarly, the current observation of the increasing microbial diversity (Chao and Shannon) with age, which eventually reached a plateau post-weaning (within 2 weeks), is in good accordance with several previous studies (Alain et al., 2014; Frese et al., 2015; Niu et al., 2015; Slifierz et al., 2015; Chen et al., 2017; Ke et al., 2019; Wang et al., 2019), although opposite diversity development patterns have been reported in a few studies as well (Guevarra et al., 2018; Li et al., 2018). This discrepancy might be due to variations in weaning age (e.g., the last two studies employed 21 days as weaning age) as well as the post-weaning sampling time-point (e.g., the last two studies had a shorter post-weaning sampling time-point; within a week). Microbiota metrics like alpha diversity, Bray Curtis distance, as well as hierarchical clustering clearly separated samples taken pre- and post-weaning, with the shorter-term post-weaning samples (2 and 5 days post-weaning) being remarkably close to the last post-weaning timepoint (14 days post-weaning). This suggests that the porcine microbiota rapidly evolves post-weaning, towards an apparently homogeneous and stable microbiome structure within 2 weeks post-weaning, reflecting the crucial role of diet in dictating the microbiome adaptation during the weaning transition. These diet driven effects clearly overrule various pre-weaning factors that contribute to the higher microbiome variation observed prior to weaning, such as litter-specific genetic diversity, distinct consequences of vertical transmission from the sow, and/or variations in sow milk composition. Taken together, the present study confirms and refines various earlier observations of the microbiome development during early-life stages in piglets and exemplifies the major impact of the weaning transition that leads to the establishment of a homogenous, rich and stable microbiota composition post-weaning.

The central aim of the present study was to investigate whether provision of pre-weaning fibrous diet would impact the microbiome development in suckling piglets. Quantitative eating scores of individual early-fed (EF) piglets rapidly increased over time, reaching the highest eating score in the last week prior to weaning. This is in agreement with creep feeding studies (Fraser et al., 1994; Bruininx et al., 2002) which provided solid feed preweaning and found that piglets consumed about 60-80% of total feed in the last week before weaning (4 weeks of age). Unlike traditional creep feeding diets, in this study, the pre-weaning diet was designed to contain a mixture of soluble and insoluble fibres, that can potentially stimulate the large intestinal microbiota. We could show that piglets which consumed this fibrous diet pre-weaning displayed an accelerated microbiota maturation compared to the control (CON) piglets suckling sow's milk only. Acceleration of the microbiota development in the EF piglets was observed by a higher alpha diversity compared to CON piglets from 15 days of age until weaning, coinciding with the observation of increased eating scores from 15 days of age, thereby suggesting the relation between feed consumption and these changes. Moreover, the EF piglets' microbiota resemblance to the post-weaning microbiota increased during the last two weeks of pre-weaning period, an effect that was not noticeable in the CON piglets. This effect in the EF piglets was most prominently reflected by the emergence of increasing relative abundances of typical post-weaning associated genera like ***Prevotella, Roseburia, Faecalibacterium, Ruminococcus, Megasphaera, Catenibacterium and Subdoligranulum.*** Additionally, these effects coincided with the accelerated disappearance of typical pre-weaning associated microbes such as ***Fusobacterium, Finegoldia, Bacteroides, Eschechichia-Shigella,*** which did occur at a much slower rate in the CON piglets. Previous studies have occasionally found similar microbial groups on consumption of solid feed or specific dietary fibres in suckling piglets (Berding et al., 2016; Zhang et al., 2016; Schokker et al., 2018; Wang et al., 2019). Importantly, the degree of resemblance observed was quantitatively correlated to the amount of feed consumed pre-weaning, strengthening the relation between feed-consumption and the acceleration in microbiota development.

Furthermore, in the present study, the EF piglets showed a tendency of higher relative body weight at 5 days after weaning, compared to CON piglets. Intriguingly, we observed a smoother transition post-weaning in EF piglets, with a lower variation (CV) and lack of negative trendlines in body weight development from weaning to day+2, thus reflecting consistency in post-weaning performance. Microbial fermentation of undigested fibres results in SCFA formation, creating a niche for bacterial groups that possess saccharolytic properties. For example, ***Prevotella*** that represents a group of strictly anaerobic bacteria, is reported as the dominant genus in the large intestine of pigs (Leser et al., 2002; Liu et al., 2012; Wang et al., 2019), especially after the introduction of fibrous solid feed post-weaning. The discriminating microbial groups that we observed in EF piglets are mostly fibre-degrading, anaerobic, SCFA producers. One of the expected differences between the two groups is that the EF piglets may have higher SCFA concentrations in distal part of the intestine, contributing to more SCFA exposure in intestinal mucosa, thereby stimulating intestinal development towards adaptation for better handling of post-weaning diet by properly digesting and acting on nutrients. This could potentially explain the smoother transition during the first five days post-weaning. Further, the CON group quickly 'catches up' post-weaning after exposure to weaner diet, towards a homogeneous microbiota observed in both groups at day+14 post-weaning. However, they made a 'longer distance' in that short time period, which is possibly reflected in their less smooth and higher variation in body weight development. In future studies, enlarging sample size could lead to detection of significant post-weaning weight development effect, which was observed as a trend in the current study.

In conclusion, this study confirms age-related microbiota dynamics in early-life stages (first weeks of life) and their progression over time in neonatal piglets. The present study illustrates the impact of early-life feeding of fibrous diet on gut microbiota, showing an increased microbiome maturation or higher "microbiome age" in early-fed piglets at pre-weaning stages. Further, we observed a strong quantitative association between eating behaviour and microbiota, suggesting that the piglets who spent more time at the feeding trough had higher abundance of 'accelerated' microbial groups. This indicates the importance of developing pre-weaning strategies (fibrous solid diet) to enhance eating behaviour in suckling piglets for increased eating and more matured microbiota. Overall, these findings emphasises the importance of early-life "window of opportunity" for modulation of the gut microbiota development and could aid in the development of optimal nutritional strategies to support the timely gut colonisation of relevant microbial taxa in the gradually diversifying neonatal gut.

Acknowledgements

This study is part of research programme "Genetics, nutrition and health of agricultural animals" with project number 868.15.010, which is financed by the Netherlands Organization for Scientific Research, Cargill Animal Nutrition and Coppens Diervoeding. The authors want to thank the technicians and students of the Host-Microbe Interactomics group and Adaptation

Physiology group (Wageningen University & Research) who helped during the animal experiment, sample processing and video observations. We would like to acknowledge the personnel and trainees of the animal research facilities in Wageningen (Carus) for taking care of the animals and for their technical assistance. The authors are grateful to Tamme Zandstra for producing the experimental feed and to FrieslandCampina DOMO (Amersfoort, The Netherlands) for provision of Vivinal GOS. We also want to thank Jos Boekhorst for his inputs in microbiota analysis employing Canoco software.

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Supplemental information

Supplementary table 1: Ingredients and calculated nutrient composition of the pre-weaning fibrous feed[1].

	Pre-weaning feed
Ingredients, %	
Wheat	21.9
Barley	15
Maize	15
Soy protein concentrate	7
Soybeans (heat treated)	5
Galacto-oligosaccharides[2]	**5**
Potato protein	4
Sugar beet pulp	**4**
Oat hulls	**4**
Inulin[3]	**4**
Resistant starch[4]	**4**
Soybean oil	3
Blood meal (spray dried)	2
Dicalcium phosphate	1.7
Sucrose	1.5
Calcium carbonate	1.0
Sodium chloride	0.5
Premix[5]	0.5
Potassium bicarbonate	0.3
L-lysine hydrochloride	0.3
DL-methionine	0.2
L-threonine	0.04
L-tryptophan	0.04
Calculated nutrient composition[6], g/Kg	
Dry matter	891
Starch	290
Non-starch polysaccharides, NSP[7]	261
Crude protein	195
Crude fat	61
Crude fibre	44
Crude ash	57
Calcium	9.1
Phosphorus	6.1
Sodium	2.2
Standardized ileal digestible lysine	11.9
Standardized ileal digestible methionine	4.8
Standardized ileal digestible threonine	7.1
Standardized ileal digestible tryptophan	2.4
Net energy, MJ/kg	11.8

[1] Feed was mixed by Research Diet Services (Wijk bij Duurstede, The Netherlands), and extruded using a co-rotating double screw extruder (M.P.F. 50, Baker Perkins, Peterborough, United Kingdom).

[2] Source: Vivinal® GOS powder (Friesland Campina, Amersfoort, The Netherlands) containing 69% galacto-oligosaccharides.

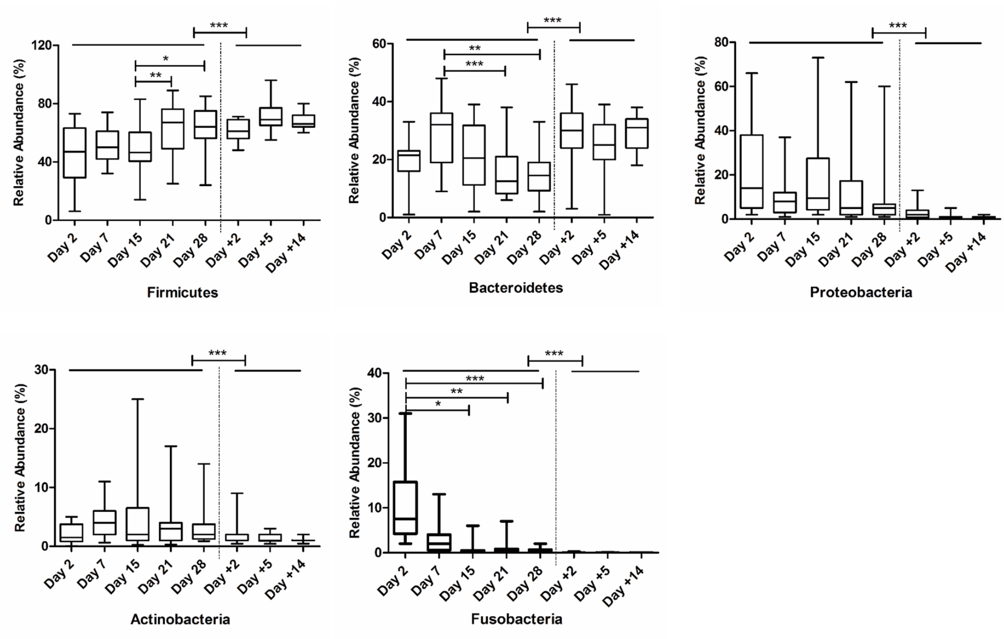
[3] Source: Prebiofeed 95 inulin powder (Cosucra group, Belgium) containing 85% inulin.

[4] Source: AmyloGel® Native Starches (Cargill, Wayzata, USA) derived from high amylose maize with 75% amylose content.

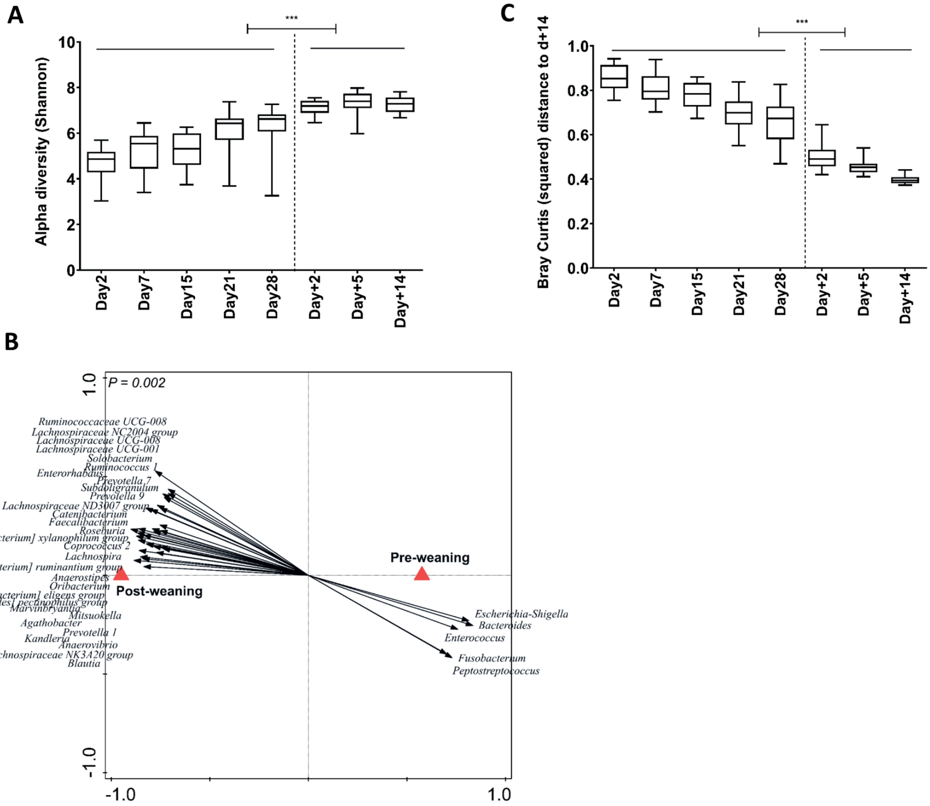
[5] Vitamin and mineral premix (per kg of feed): vitamin A: 10000 IU, vitamin D3: 2000 IU, vitamin E: 40 mg, vitamin K: 1.5 mg, vitamin B1: 1 mg, vitamin B2: 4 mg, vitamin B6: 1.5 mg, vitamin B12: 0.02 mg, niacin: 30 mg, D-pantothenic acid: 15 mg, choline chloride: 150 mg, folate: 0.4 mg, biotin: 0.05 mg, iron: 100 mg, copper: 20 mg, manganese: 30 mg, zinc: 70 mg, iodine: 0.7 mg, selenium: 0.25 mg, anti-oxidant: 125 mg.

[6] According to CVB (2007), nutrients are presented in g/kg dry matter, except for dry matter (g/kg) and net energy (MJ/kg).

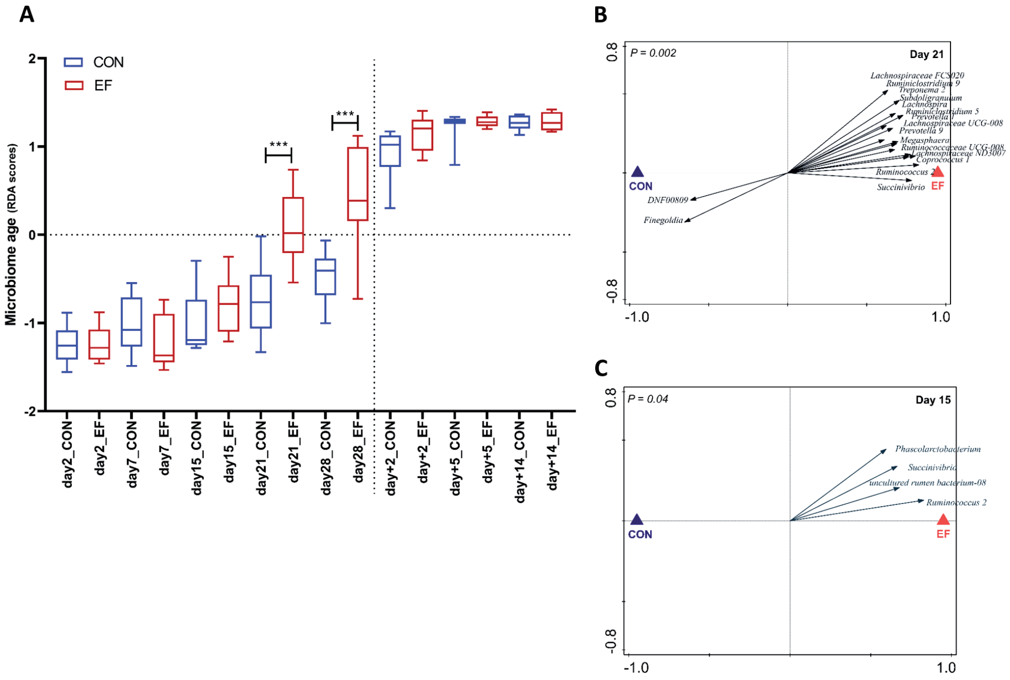
[7] Non-starch polysaccharides: Calculated as the difference between dry matter and the sum of starch, sugars, crude protein, crude fat, and crude ash.



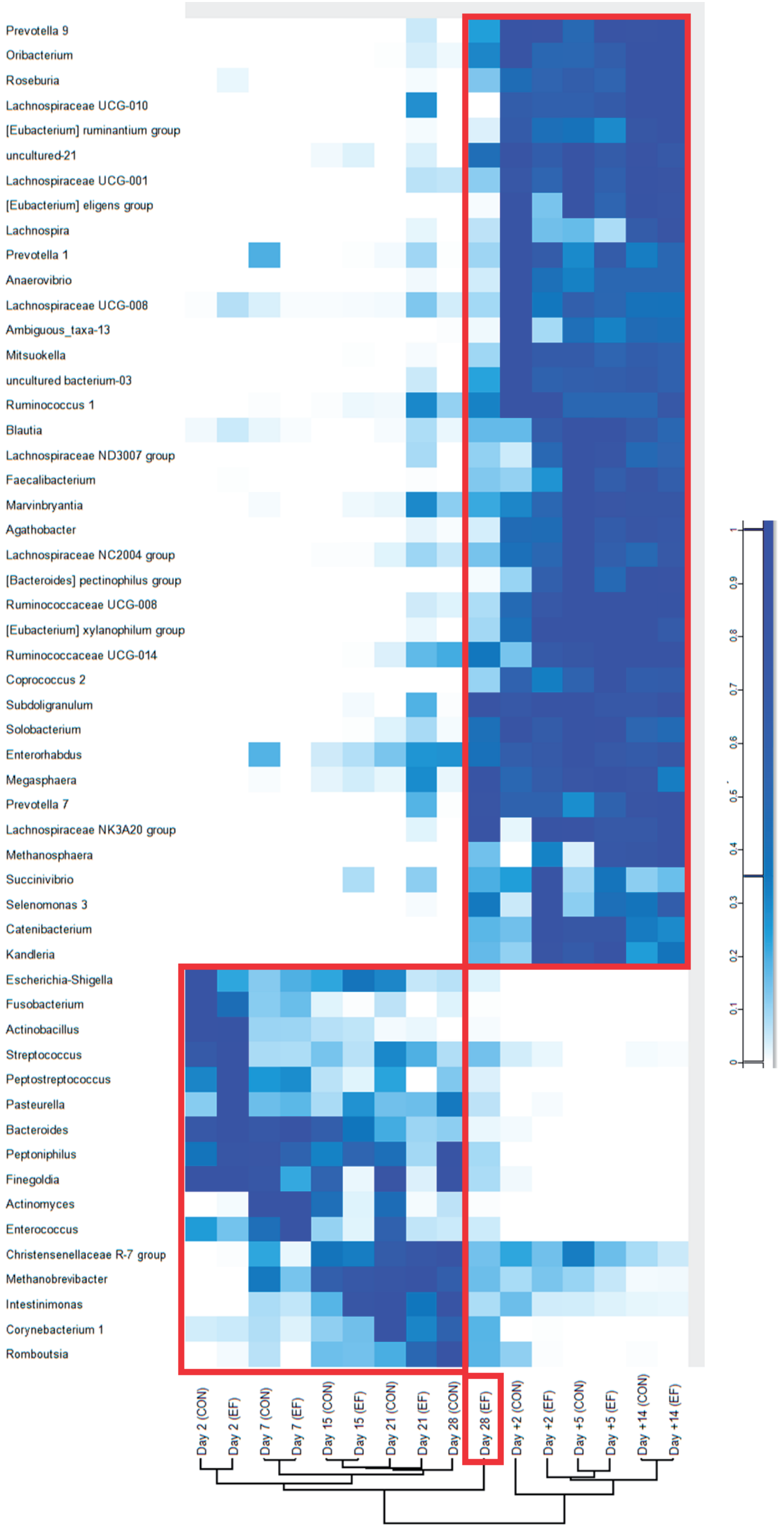
Supplementary figure 1: Age-related microbiota dynamics showing relative abundance of taxa (phyla level) at pre- and post-weaning time-points. Statistical differences were assessed by Mann Whitney U-test or one-way ANOVA (Kruskal-Wallis statistic) using a Dunnett's test for multiple comparison. (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).



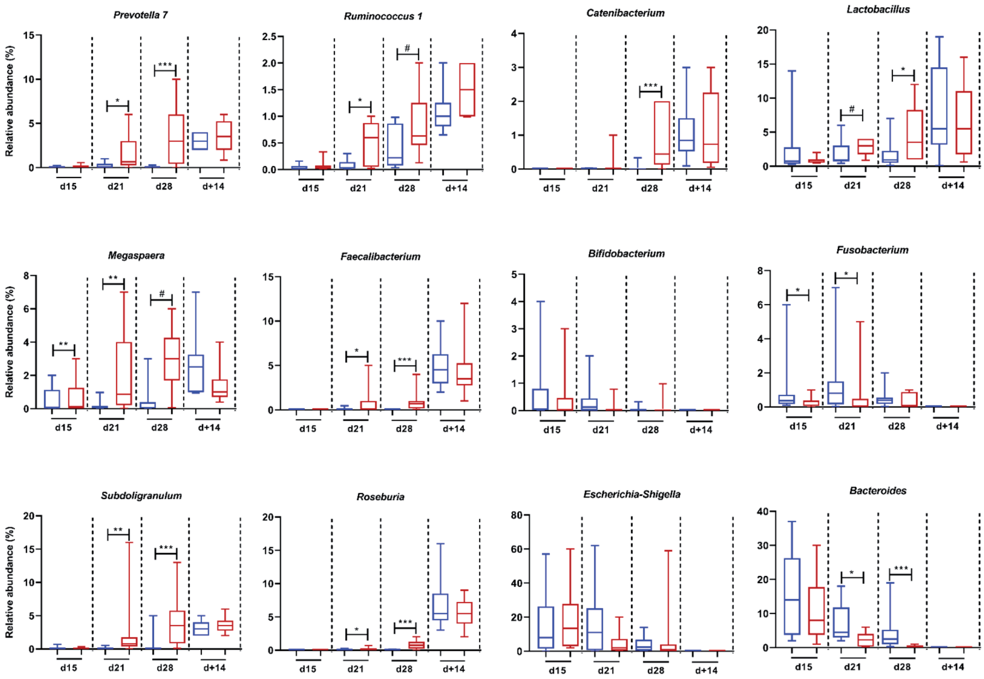
Supplementary figure 2: Age-driven intestinal microbiota progression in pre- and post-weaning period. **(A)** Alpha diversity (Shannon index) showing increasing diversity over time. **(B)** Redundancy analysis of weaning (explained variation = 32.4%; *P* = 0.002) showing both pre- and post-weaning associated microbiome. The microbial groups visualized have minimum 40% fit on horizontal axis with a response score ≥ 0.7 in the biplot (obtained by projecting the taxa points perpendicular to the horizontal axis). **(C)** Bray Curtis (squared) distance between pre- and post-weaning time-points. Significant differences between groups were assessed by student t test or Mann-Whitney U test (***: *P* < 0.001).



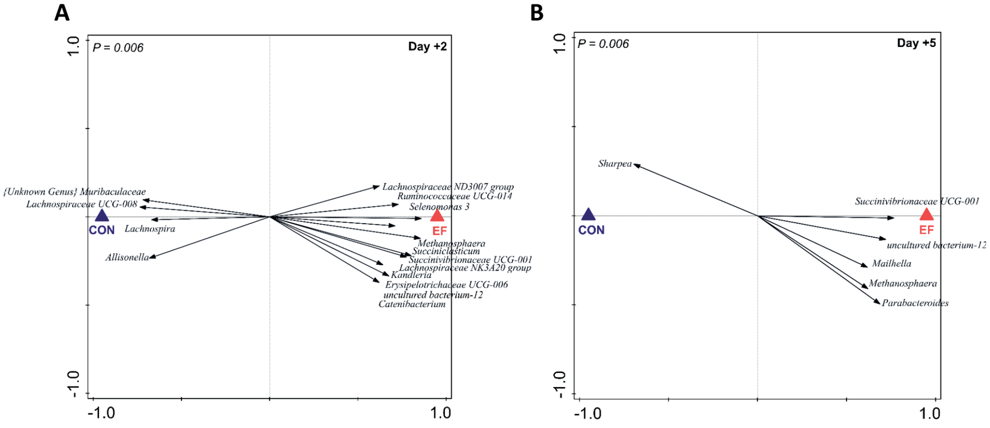
Supplementary figure 3: (A) Comparison of the developing 'microbiome age' between early-fed (EF) and control (CON) piglets. Response-variable based case scores (CaseR) that was extracted from redundancy analysis of age, was defined as 'Microbiome age' as they correspond to the individual sample position in the progressing-age-ordination plot. Significant differences between groups were assessed by student t test or Mann-Whitney U test (***: $P < 0.001$). Redundancy analysis of treatment at pre-weaning time-points: **(B)** day21 (PC1 = 15.6%, PC2 = 18.8%; $P = 0.002$) and **(C)** day15 (PC1 = 8.7%, PC2 = 24.3%; $P = 0.04$), with associated microbial groups in EF and CON groups (response score ≥ 0.6 on horizontal axis).



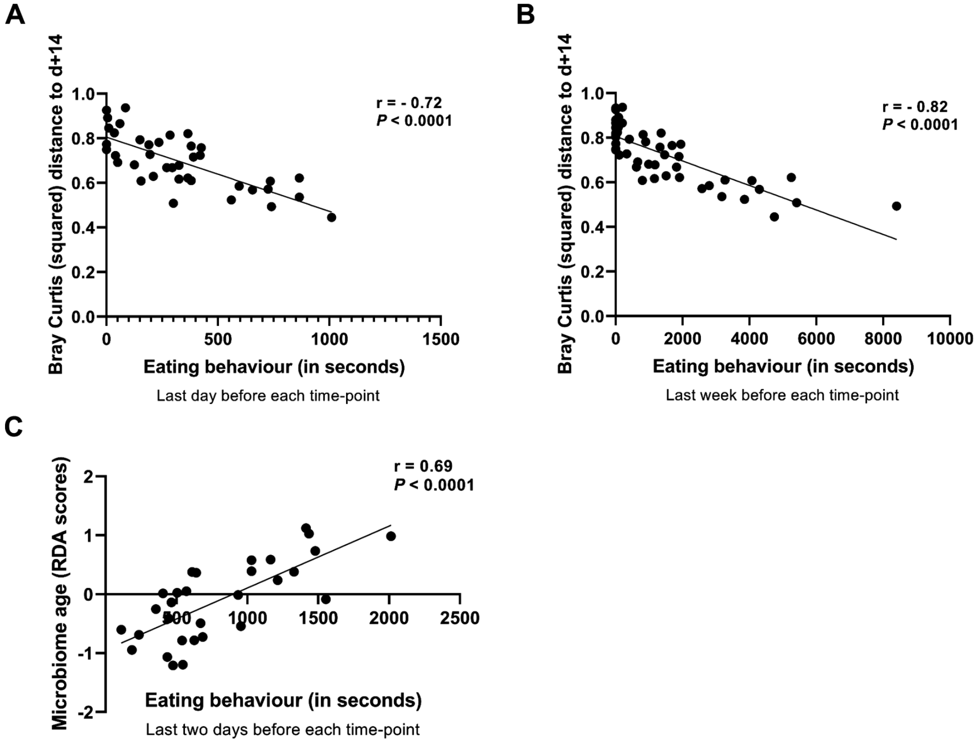
Supplementary figure 4: Hierarchical clustering of early-fed (EF) and control (CON) groups at different ages, using normalised relative abundance of the discriminative microbial genera (identified in redundancy analysis of age). The EF group at day28 clearly separates from the other pre-weaning groups and is closer to the post-weaning microbiome.



Supplementary figure 5: Changes in relative abundance of representative microbial groups (shown in box plots) in early-fed (EF; red) and control (CON; blue) groups at pre- and post-weaning time-points**.** Statistical differences were assessed by Mann Whitney U-test (non-parametric) or t test (#: 0.1 > *P* ≥ 0.05; *: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001).



Supplementary figure 6: Redundancy analysis of treatment at post-weaning time-points **(A)** day+2 (PC1 = 16.01%, PC2 = 25.6%; *P* = 0.006) and **(B)** day+5 (PC1 = 10.8%, PC2 = 20.8%; *P* = 0.006) with associated microbial groups in early-fed (EF) and control (CON) groups (response score ≥ 0.6 on horizontal axis).



Supplementary figure 7: Spearman correlation between eating scores of individual piglets (n = 10) and squared Bray Curtis distance to their day+14 “matured” time-point: **(A)** last day eating score before each corresponding time-point (r =–0.72, *P* < 0.0001) and **(B)** last week before each corresponding time-point (r =–0.82, *P* < 0.0001). **(C)** Spearman correlation between eating scores (last two days from day15 timepoint) and their ‘microbiome age’ (r =–0.69, *P* < 0.0001).

4

Chapter 4

Impact of early-life feeding on local intestinal microbiota and digestive system development in piglets

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Submitted for publication

Abstract

Early-life gut microbial colonisation is known to influence host physiology and development, shaping its phenotype. Suckling piglets with a developing gastro-intestinal tract, provide a “window of opportunity” for programming their intestinal microbiota composition and corresponding intestinal development. Here, we investigated the impact of early feeding on jejunum and colon microbiota composition, and intestinal maturation in suckling piglets. From two days of age, early-fed (EF; n = 6 litters) piglets had access to solid feed containing a mixture of fibres till weaning (day29) in addition to sow’s milk, whereas the control (CON; n = 6 litters) piglets exclusively fed on sow’s milk. Early feeding elicited a significant impact on the colon microbiota, whereas no such effect was seen in the jejunal and ileal microbiota. Quantified eating behavioural scores could significantly explain the variation in microbiota composition of EF piglets and support their classification into good, moderate, and bad eaters. Members of the Lachnospiraceae family, and the genera ***Eubacterium, Prevotella***, and ***Ruminococcus*** were quantitatively associated with eating scores. EF piglets were found to have a decreased pH in caecum and colon, which coincided with increased short-chain fatty acid (SCFAs) concentrations. Moreover, they also had increased weights and lengths of several intestinal tract segments, as well as a decreased villus-crypt ratio in jejunal mucosa and an increased abundance of proliferative cells in colon mucosa. The approaches in this study indicate that early feeding of a mixed-fibre (pre-weaning) diet changes the microbiota composition, pH, and fermentation products in the distal gut of piglets, while it also alters both macroscopic and microscopic intestinal measurements. These results exemplify the potential of early feeding to modulate intestinal development in young piglets.

Key-words: early-life, fibrous feed, gut microbiota, intestinal development, pre-weaning**,** suckling piglet

Introduction

In natural conditions, very young piglets begin to forage food items already when a few days old (Gundlach, 1968; Meynhardt, 1980; Petersen, 1994), familiarising with solid food and preparing for the weaning transition gradually over a period of 20 weeks of age approximately (Newberry and Wood-Gush, 1985; Jensen and Stangel, 1992). In contrast, weaning in commercial pig farms is an abrupt process that commonly takes place between 3-4 weeks of age, where piglets are exposed to various simultaneous stressors, including separation from their sow and littermates, new housing conditions, unknown pen-mates, and a sudden change of diet. These abrupt changes are often accompanied by a transient low feed intake, poor growth, intestinal dysbiosis and diarrhoea post-weaning, thus compromising animal health and welfare, increasing piglet mortality and causing economic losses (Le Dividich and Sève, 2000; Bruininx et al., 2002; Heo et al., 2013; Everaert et al., 2017; Gresse et al., 2017; Pluske et al., 2018). Creep feeding, a method of supplementing suckling piglets with solid feed, is prevalent in modern pig farming to ease the weaning transition as well as stimulate post-weaning eating (or feed intake) (Bruininx et al., 2002; Pluske et al., 2005). However, traditional creep feed is highly palatable, easily digestible and mainly based on milk proteins (Okai et al., 1976; Heo et al., 2018), which is distant from fibrous solid feed exposure in both natural and post-weaning conditions.

At the time of weaning, the gastro-intestinal tract of a young pig is still developing (McCance, 1974; Everaert et al., 2017) and undergoing rapid changes in gut microbiota colonisation, digestive system and immune development (Alain et al., 2014; Frese et al., 2015; Pluske et al., 2018; Xiong et al., 2019). Importantly, the gut microbiome has been recognised to play a crucial role in overall animal health and development, especially in early-life (Mulder et al., 2009; O'Mahony et al., 2009; Schokker et al., 2014; Merrifield et al., 2016). The early-life microbial colonisation with potentially beneficial and diverse gut microbes can influence the maintenance of intestinal homeostasis and prevent gut dysbiosis (Pluske et al., 2018; Guevarra et al., 2019; Nowland et al., 2019). Dietary fibres can modulate the gut microbiome, and they are widely recognised as food/feed components that influences gut health positively (Jha et al., 2019). Notably, dietary fibres have also been implicated in gastrointestinal tract development and mucosal changes in pigs (Bach Knudsen et al., 2012; Van Hees et al., 2019). These fermentable fibres pass through the small intestine undigested and act as a substrate for the distal gut microbiota, stimulating microbial fermentation and short chain fatty acid (SCFA) production in the colon. The predominant SCFAs formed (approximately 95%) are acetic, propionic and butyric acid, although some other organic acids can be detected as well, such as lactic, succinic, isovaleric, and isobutyric acid (Ríos-Covián et al., 2016). Absorbed SCFA can provide up to 15% of the maintenance energy requirement of growing pigs and 30% in gestating sows (Varel and Yen, 1997). However, special attention is commonly given to butyric acid since it serves as a major source of energy for colonic epithelial cells, and has been proposed to exert several (additional) effects that are considered pivotal in establishment and maintenance of homeostasis in the colon mucosa, including colonocyte growth and proliferation (Hamer et al., 2008; Berni Canani et al., 2012; Leonel and Alvarez-Leite, 2012; Den Besten et al., 2013; van der Beek et al., 2017; Xiong et al., 2019). The SCFAs, particularly butyric acid, can modulate the expression of genes involved in gut motility, host defence and inflammatory responses, contributing to formation and protection of intestinal barrier as well as stimulating differentiation and regulation of T cells (Furusawa et al., 2013; Smith et al., 2013). Although the exact mechanisms by which microbial SCFAs influence mucosal physiology remain to be resolved, a few effects of SCFAs and the underlying mechanisms have been revealed, including the function of SCFAs as ligands for G protein-coupled receptor pair GPR41, GPR43 in epithelial or immune cells, and their inhibition of histone deacetylases (HDAC) activity (Mingming Sun, Wei Wu, Zhanju Liu, 2017; Xiong et al., 2019).

Although previous studies have characterised porcine gut microbiota in relation to dietary fibre intervention, most of them have focussed on the post-weaning period, assessing the impact on weaned piglets (Dicksved et al., 2015; Umu et al., 2015, 2018; Kraler et al., 2016; Burbach et al., 2017). Currently, a handful of studies have assessed how early-life (pre-weaning) feeding might influence the gut microbiome (Shim et al., 2005; Zhang et al., 2016; Shi et al., 2018; Brink et al., 2019) and contribute to the intestinal development of suckling piglets (Alizadeh et al., 2016; Yeruva et al., 2016; Li et al., 2018; Van Hees et al., 2019). In our previous study (**Chapter 3**), we established that early feeding with a fibre enriched pre-weaning feed from 2 days of age, accelerates pre-weaning microbiota colonisation patterns towards those that resemble a typical post-weaning microbiome. In the present study, we evaluated the impact of this early-life feeding strategy on the intestinal microbiota composition in different regions of the intestinal tract, and investigate its consequences for intestinal development and maturation. We hypothesised that the pre-weaning consumption of solid mixed-fibre feed would result in an increased level of undigested substrate in the colon, and investigated its impact on local microbiota composition, SCFA production, macroscopic development of the digestive system, as well as its microscopic consequences on mucosal morphology. Suckling piglets show large variation in solid feed intake before weaning [between (Kuller et al., 2007) and within litters (Pajor et al., 1991)], and therefore we exploited the quantified variation in eating behaviour of piglets to assess the impact of early feeding at an individual piglet level.

Materials and methods

Animals and experimental design

The Animal Care and Use committee of Wageningen University & Research (Wageningen, The Netherlands) approved the protocol of the experiment (AVD104002016515). The protocol is in accordance with the Dutch law on animal experimentation, which complies with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The experiment was conducted with 12 multiparous Topigs-20 sows (range parity: 3-5), housed and inseminated at research facility Carus (Wageningen University & Research, The Netherlands). Within two days after birth, the litter size was set to a maximum of 14 piglets per litter (Tempo x Topigs-20) with no cross-fostering. The new-born piglets were cohoused with their respective sows and littermates until weaning (~29 days of age). They received ear tags for individual identification and an iron injection, standard to pig husbandry practice. The litters were divided into two experimental groups, early-fed or EF group (n=6) and control or CON group (n=6) based on sow's parity, farrowing date, body weight (of the litter at birth and 2 days of age) and genetic background. From 2 days onwards, piglets belonging to the EF group were given the opportunity to forage on customised mixed-fibre feed (**Supplementary table 1**) *ad libitum* in addition to suckling sow's milk whereas the CON group nursed on sow's milk only. Briefly, the diet contained 26% non-starch polysaccharides including sugarbeet pulp (4%), oat hulls (4%), inulin (4%), galacto-oligosaccharides (5%) and high amylose maize starch (4%) as fibrous ingredients. Additional details about the housing and management have been described previously (Middelkoop et al.).

Eating behaviour by video observation

The eating behaviour of piglets was assessed by means of video recordings, as described previously (**Chapter 3**). Briefly, eating frequency of individual EF piglet (identified by back numbers) was determined daily (2 to 29 days of age) from 07:00 to 19:00 hours via video observations as an estimate for pre-weaning solid feed intake. From the video observations, the amount of time spent eating or "eating time" was evaluated. When an EF piglet placed its snout into the trough for a minimum of 5 seconds (s), the behaviour was scored as eating (Pajor et al., 1991; Adeleye et al., 2014). Daily/weekly eating activity per piglet was (semi-) quantified by summing the (minimum) number of seconds spent eating from 2 days of age to weaning (at 29 days of age). Of note, the eating scores were taken as an "estimated quantification" of the amount of eating per piglet.

Intestinal microbiota sampling and microbiota metataxonomic analysis

At the end of the suckling period (just before weaning), a subset of piglets was sacrificed (n=28; n=14 per treatment, seven males and seven females, distributed over two consecutive sampling days) at 29 days of age. Piglets were euthanised by intravenous injection of 20% sodium pentobarbital (EUTHASOL®, 500 mg/ml, AST Farma B.V., Oudewater, The Netherlands). The selection of sacrificed piglets were made by the following criteria: (a) no antibiotic treatment (b) close to mean body weight of the litter (c) close to average weight of the treatment group (d) one to three piglets per litter (e) equal male to female ratio. For each piglet, the gastrointestinal tract was removed from the abdominal cavity and dissected immediately to collect 20 cm of intestinal segments from different intestinal locations within 25 mins after sacrifice, i.e., jejunum (1.5 metres from duodenal-jejunal flexure), ileum (50 cm upstream from ileocaecal valve) and colon (mid-spiral colon). Luminal contents were collected from the intestinal tissues segments, immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Approximately 300 mg of luminal content (wet weight) from jejunum, ileum and colon samples was used for microbial DNA extraction. Total genomic DNA was extracted by the repeated bead beating method (Yu and Morrison, 2004) using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The quality and quantity of extracted DNA was checked by gel electrophoresis and Nanodrop DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE USA) respectively. The V3-V4 region of 16S rRNA gene was sequenced and the raw reads were processed in CLC Genomics Workbench version 11 (CLC bio, Arhus, Denmark) as described previously (**Chapter 3**). Briefly, the PCR amplified 16S rRNA gene was purified, extended by adaptors prior to sequencing using the Illumina MiSeq system (BaseClear BV, Leiden, The Netherlands) which generated FASTAQ sequence files, which were subjected to a BaseClear in-house quality control and filtering protocol. Subsequently, the CLC pipeline was utilised for merging the paired-end reads into one high quality representative sequence, primer and quality trimming and binning the sequences into operational taxonomic unit (OTUs) at 97% identity threshold using SILVA database v132 (released on Dec 13, 2017)(Quast et al., 2013). To evaluate alpha and beta diversity indices, OTUs were rarefied to minimum library size (11,000 reads) attaining even sequencing depth between samples. Alpha diversity was evaluated using microbial species richness (Chao1 bias corrected) and evenness (Shannon) indicators. Relationship between microbial groups and intestinal location or treatment groups was determined by principal component analysis (PCA; unsupervised), partial redundancy analysis (pRDA; supervised) and redundancy analysis (RDA; supervised) using CANOCO 5 (Microcomputer Power, Ithaca, NY, USA), according to accompanying instructions (Braak and Smilauer, 2012). Statistical significance was evaluated by Monte Carlo permutation procedure (MCPP) with 499 permutations. The discriminative microbial families (identified in RDA analysis of colon microbiota) were visualised in a heat map of microbial relative abundance to assess consistency of the EF treatment at individual animal level. Heat maps were constructed by hierarchical clustering of microbial groups (selected from Redundancy analysis; microbial genera below 0.01% relative abundance in less than 10% of individual samples were not included) in Perseus software (Tyanova et al., 2016), where relative abundance values were log2 transformed and subsequently normalized by z-score transformation. Euclidean distance was utilized to measure the distance and clustering was conducted using the average linkage method. The online tool "MicrobiomeAnalyst" (Dhariwal et al., 2017) for comprehensive statistical, visual, and meta-analysis of microbiome data was also used for detecting microbial taxa which were differentially abundant among different locations/treatments. Low abundance OTUs were removed, where OTUs with less than two counts in <10% of the samples. The OTU table was rarefied to minimum library size and transformed using trimmed mean of M-values (TMM) which was used to evaluate differentially abundant taxa ("Classical Univariate analysis" with multiple correction). To assess the beta diversity (Bray Curtis distance) between intestinal segments, PERMANOVA test was performed in MicrobiomeAnalyst.

pH and SCFA measurement

After euthanasia, the gastro-intestinal tract was removed and the contents of the stomach, small intestine, caecum and colon were collected by gently squeezing the digesta from the different parts of the intestine. Immediately after sampling, the pH was recorded by inserting a pH electrode (pH 300, HANNA Instrument, Padova, Italy) in homogenized digesta.

SCFAs (acetic, propionic, butyric acid) along with succinic acid and lactic acid, were quantified in colon digesta samples using an Ultimate 3000 HPLC equipped with an autosampler, a RI-101 refractive index detector (Shodex, Kawasaki, Japan), and an ion-exclusion Aminex HPX-87 H column (7.8 mm × 300 mm) with a guard column (Bio-Rad, Hercules, CA, USA). Samples weighing ~200 mg were taken in a 2ml eppendorf tube, filled with milliQ water to have a final weight of 1 gram, vortexed followed by centrifugation (10 mins, 30,000*g). The supernatant (10 uL) was injected onto the column and eluted with 5 mM H2SO4 at a flow rate of 0.6 mL/min at 65°C oven temperature. Calibration curves of each acid were prepared in a range of 0.01 – 1 mg/ml. Chromeleon 7.1 software (Dionex, Sunnyvale, CA, USA) was used for data analysis.

Macroscopic intestinal parameters

During sacrifice, intestinal organ weights and lengths were determined. Weights of the stomach, small intestine, caecum, and colon (full and empty), as well as length of the small intestine and large intestine (caecum plus colon) were recorded for each piglet. Empty weights of the intestinal segments were determined after removal of digesta by gently squeezing the intestine, followed by rinsing of the intestine in saline solution and removal of excess rinsing fluids using paper towels.

In addition, adrenal gland, gallbladder, pancreas, spleen, and liver were also weighed. The statistical analyses were performed with linear (MIXED) mixed models in the statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Organ measurements were compared with a model including treatment (EF vs. CON) as a fixed factor and using body weight at sacrifice as the covariate in the model; untransformed data are presented as means ± SEM, differences at $P < 0.05$ were considered statistically significant and differences at $0.05 \leq P < 0.10$ were considered as trend. For correlation analysis with the other measurements in the study, the organ-weight and -size values were converted to body-weight-normalized values by dividing absolute values by the scaled “body weight factor” (body weight of an animal divided by the smallest body weight in the group; scaled between 1 – 1.9) to obtain “normalized organ-weight and -size values” per animal.

Histological morphometric measurements and immunohistochemical staining of intestinal proliferating cells

At weaning, piglets were sacrificed and their intestinal sections (about 2cm) from proximal jejunum and mid colon (n=16, 8 per treatment) were fixed with 4% paraformaldehyde (PFA), and then dehydrated and embedded in paraffin blocks. 5 μm sections were cut with a Accu-Cut® SRM™ 200 Rotary Microtome (Sakura Finetek Europe B.V., Alphen aan de Rijn, The Netherlands), deparaffinized, hydrated and stained with Haematoxylin-eosin (H&E). Slides were examined using a Leica DM6 B microscope (Leica Microsystems Ltd. CH9435 Heerbrugg) and images (5x magnification) were processed with LAS X software (Leica Microsystems Inc., Buffalo Grove, IL, USA). Intestinal (histo-)morphometric parameters like villus length and crypt depth (μm) were measured from jejunal sections and crypt depth was measured from colonic tissue sections. These parameters were measured on 90 well-formed villi and their corresponding crypts per animal (n=8 per group; three intestinal sections per animal). In jejunum, the villus length was defined from the tip of a villus to their base and the crypt depth was measured as a distance from the base of the villus (i.e, villus-crypt transition) to muscularis mucosa. Subsequently, the ratio of the villus length to crypt depth (V:C) was calculated.

For immunohistochemical staining of proliferating cells in intestinal tissues, 5 μm sections were deparaffinized, rehydrated and treated for antigen retrieval in citrate buffer (pH 6.0) at 95°C for 20 min, followed by cooling in tris-buffered saline and tween 20 (TBSt) buffer for 20 min at room temperature (RT). Non-specific staining were blocked using 10% goat serum for 30 minutes at RT. To detect proliferating cells, sections were incubated with primary antibody (anti-PCNA antibody, PC10 mouse anti-rat IgG2a monoclonal antibody, Merck-millipore, Darmstadt, Germany, MAB424R; 1:200) overnight at 4˚C, followed by TBSt washing and incubation with secondary antibody (Goat anti-Mouse IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 555, ThermoFisher Scientific, Waltham, Massachusetts, USA; 1:300) for 1 hour at RT. Nuclei were stained with Hoechst 33342 Solution (Invitrogen, ThermoFisher; 1:1000 dilution). For quantification of proliferative cells, 10 high quality 16bit grayscale images were captured per animal for both locations (80 representative images//treatment group/location) at 20X magnification using Leica DM6b microscope fitted with appropriate fluorescence filters along with their corresponding nuclei images. Image analysis was performed using Cell Profiler 3.1.8 (Broad Institute, Cambridge Massachusetts USA; www.cellprofiler.org) and FCS Express 6 Flow plus Image (De Novo Software, CA, USA, www.denovosoftware.com), as described in **Supplementary file1**. PCNA is a nuclear stain and thus the number of proliferating cells was normalized by the total number of Hoechst positive nuclei in an image, to obtain ratio of PCNA:Hoescht identified nuclei/cells.

Association of eating scores in EF piglets with multiple readouts

We first investigated the impact of early feeding on microbiota composition (colon), pH (caecum/colon), SCFA (colon), macroscopic and microscopic intestinal measurements. Subsequently, we evaluated whether these parameters are quantitatively associated with individualised quantification estimates of eating behaviour. To evaluate the relationship between eating behaviour and other measured parameters, a non-parametric spearman correlation matrix was calculated using GraphPad Software 8.1.1 (California, USA).

Statistical analyses

Data analyses were performed in Graph Pad Prism 8.1.1 (GraphPad Software, California USA). Normality of data (Shapiro-Wilk test) and statistical differences were checked with a limit of significance set at $P < 0.05$. Comparative analysis of the diversity indices, pH, SCFA concentrations, histological morphometric measurements and proliferating cells were performed by Mann Whitney U-test (for non-parametric) or Student's t-test (for parametric).

Results

Gut microbiome composition in different intestinal segments

We assessed the microbiota composition in jejunum, ileum, and colon of 28 piglets sacrificed at the end of the pre-weaning phase (day29). Illumina Miseq 16S rRNA gene sequencing of the V3-V4 region generated 1,211,527 number of reads after quality filtering, with a mean sample depth of 16,596 ± 3844 reads.

Prominent (intestinal) location-specific differences in microbiota composition were observed. Principal component analysis showed two distinct clusters (**Figure 1A**), reflecting different early-life microbial colonisers in the small and large intestine. For example, microbial families like Lactobacillaceae, Peptostreptococcaceae, Clostridiaceae 1 were found to be dominant in the small intestinal (jejunal and ileal) samples whereas Rikenellaceae, Prevotellaceae, Ruminococcaceae, Lachnospiraceae were more abundant in large intestine (**Figure 1B; Supplementary figure 1A**). This was further demonstrated by microbiota compositional (redundancy) analysis as a function of 'intestinal location' which identified dominant microbial groups associated with each intestinal segment (**Figure 1B**). The small difference between jejunum and ileum microbiota was recognised by the limited explained variation (4.76%) encompassed by the second principal component (PC2), as compared to the first principal component (PC1; explained variation 66.66%), which separates small and large intestinal microbiota (**Figure 1A**). The large intestine had microbiota with a higher microbial richness (**Figure 1C**) and evenness (**Supplementary figure 2A**) compared to the small intestine and clustered separately when assessed by Bray Curtis distance (**Supplementary figure 2A**; PERMANOVA, $P < 0.0001$). Comparing jejunal and ileal microbiota, Aerococcaceae,

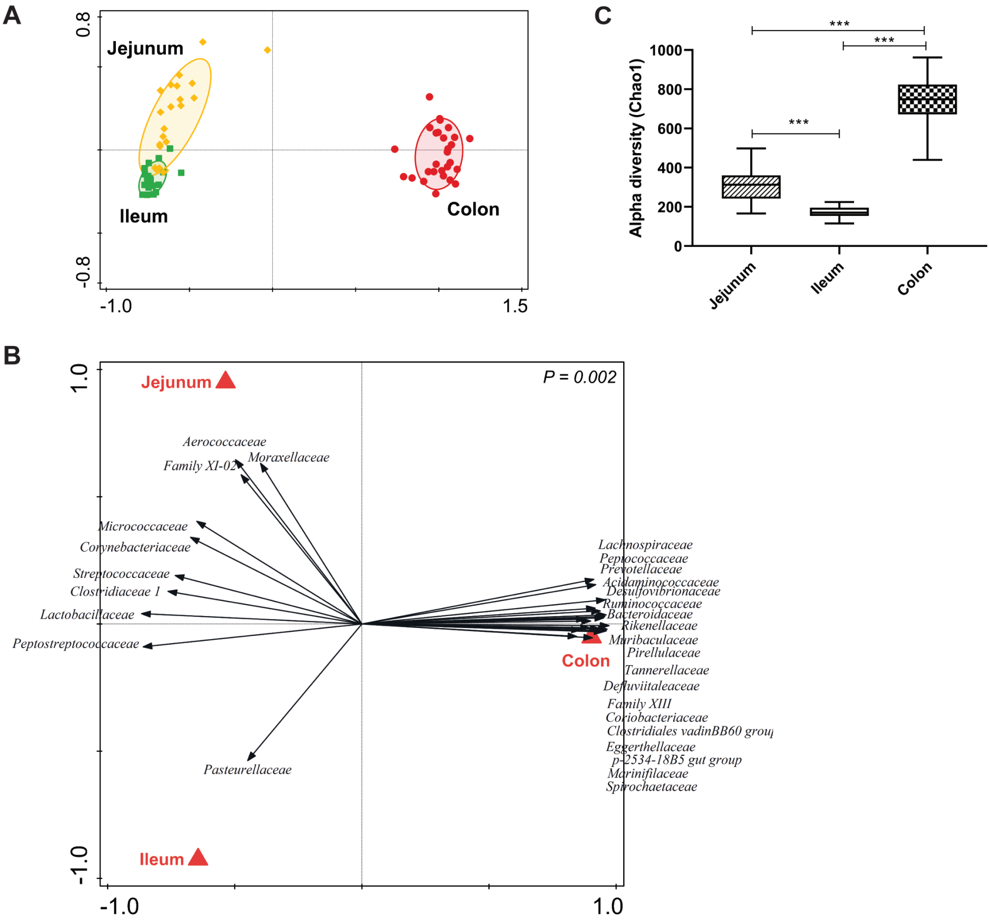

Figure 1: Microbiota composition along the intestinal tract. **(A)** Principal component analysis of jejunum, ileum and colon microbiota (PC1 = 66.66%, PC2 = 4.76%) at genus level. **(B)** Redundancy analysis (explained variation = 67.8% *P* = 0.002) of intestinal location with associated microbial groups at family level. Microbial groups visualized in this figure have a minimum fit value of at least 40% on the horizontal axis and a response score > 0.63 and > 0.80 for jejunum/ileum and colon, respectively. Specific microbial families which are differentially abundant in jejunum/ileum were enforced in this graph). **(C)** Alpha diversity (Chao1 bias corrected) comparison among jejunum, ileum and colon. Significant differences between groups were assessed by student t test or Mann-Whitney U test (***: *P* < 0.001).

Fusobacteriaceae, Moraxellaceae were found to be more abundant in jejunum, while only Pasteurellaceae was found to be more abundant in ileum (**Supplementary figure 1B**), which corroborated the redundancy analyses for intestinal location (**Figure 1B**). Remarkably, we observed significant differences in alpha and beta diversity between jejunal and ileal microbiota (**Supplementary figure 2B**). Jejunum microbiota was found to have a higher richness (**Figure 1C**) compared to ileum, although there was no difference observed in evenness (**Supplementary figure 2B**). These findings established prominent differences in microbiota composition in small and large intestinal locations, and underpin the high relatedness between the microbiota of the jejunum and ileum regions of the small intestine, which was also supported by the partial separation of jejunal and ileal samples in hierarchical clustering (**Supplementary figure 3**).

Effect of early feeding on local intestinal microbiota

One of the main objectives of this study was to gain insight into the impact of early-life feeding on jejunal, ileal and colon microbiota. We did not find any impact on small intestinal (jejunal and ileal) microbiome composition or diversity due to early (pre-weaning) feeding of piglets (**Supplementary figure 4**). In contrast, the colon microbiome was found to be significantly altered due to early feeding, and RDA analyses identified several microbial groups (such as *Ruminococcus 2, Lachnospira, Lachnospiraceae group ND3007, Roseburia, Papillibacter, Eubacterium, Prevotella 1)* associated with this difference (**Figure 2A**). Notably, some of these microbes (**Figure 2C;** marked in bold) were also detected to be enriched in rectal swab samples taken pre-weaning from early-fed piglets in a previous study (**Chapter 3**). Those microbial groups represent typical post-weaning associated microbes, demonstrating that early feeding with a fibrous diet accelerates the 'maturation' of the microbiota towards a post-weaning composition. Due to the absence of post-weaning microbiota samples in this study (piglets sacrificed before weaning), this correlation with the post-weaning microbiota cannot be confirmed, but the association of the same microbial groups with early feeding supports the similarity of microbiota impact. To further evaluate the impact of early feeding on microbiota composition, beta diversity was assessed by Bray Curtis distance, which revealed significant dissimilarity between the EF and CON groups (PERMANOVA, $P = 0.04$; data not shown), although no significant difference was observed in alpha diversity (**Figure 2B**).

To assess the impact of early feeding at individual animal level, hierarchical clustering of all samples was performed based on EF/CON group-associated microbes (detected in RDA analyses; **Figure 2A**). Partial separation of EF and CON piglets was observed at individual animal level (**Figure 2C**). Overall, the hierarchical clustering divided the piglets into three main clusters: (1) five EF piglets clustering clearly separate, (2) seven EF piglets clustering together but less distant from the next cluster (3) encompassing all the CON piglets and the remaining two EF piglets. The genera comprising of *Prevotella 1, Roseburia, Lachnospiraceae ND3007 group, Lachnospira, Selenomonas 3, Roseburia, Eubacterium xylanophilum, CAG-352 and Ruminococcus 2* were found to be most abundant in the first cluster whereas the second cluster had a more variable abundance of these groups. Cluster 3 was characterised by higher abundance of other microbial groups, including *Ruminococcaceae (UGC-004, UGC010), Tyzzerella, CAG-873, Pyramidobacter, Synergistes, Butyricimonas and Intestimonas*. Intriguingly, some of these microbes such as *Butyricimonas, Pyramidobacter, Intestimonas* and *Synergistes* were also found associated with the CON group in our previous study (**Chapter 3**).

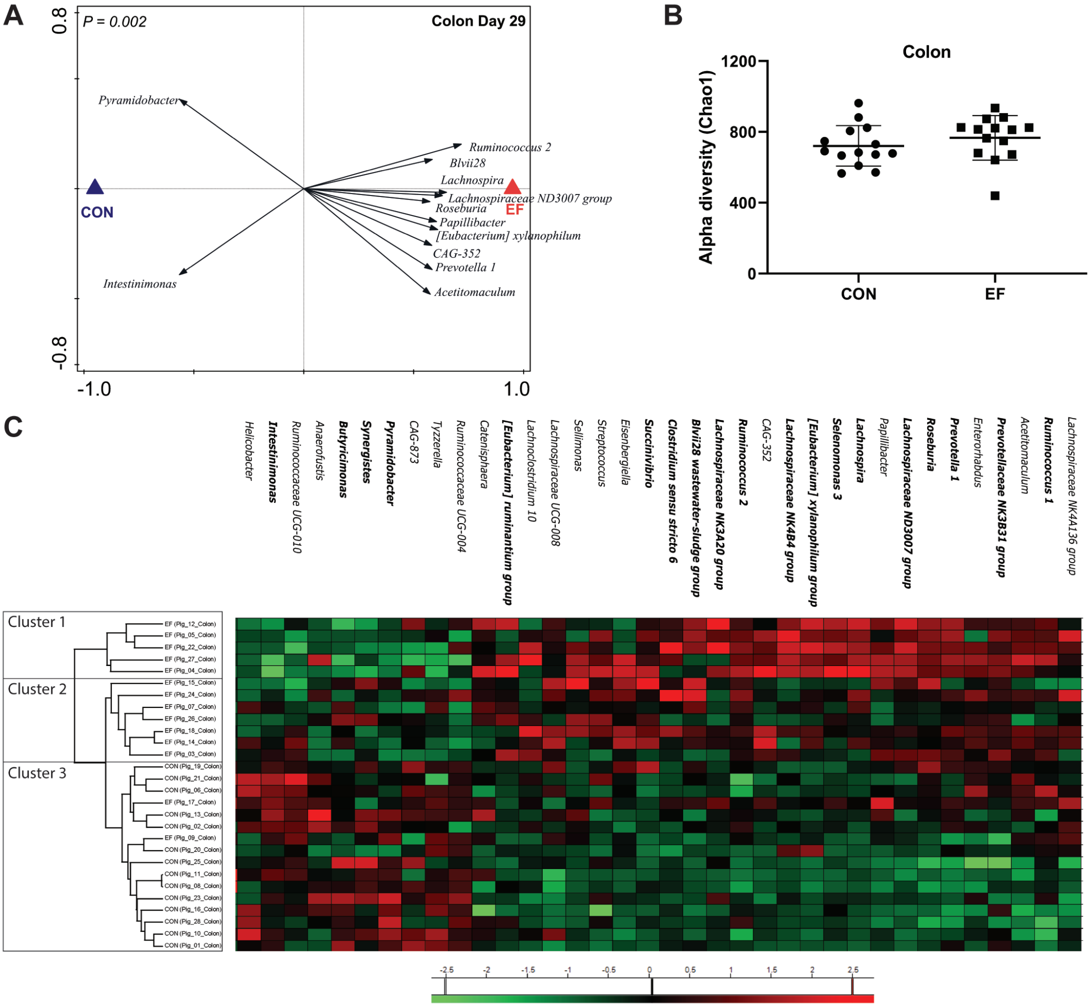

Figure 2: Colonic microbiota composition in early-fed (EF) and control (CON) group. **(A)** Redundancy analysis at genus level (PC1 = 8.75%, PC2 = 16.12%; *P* = 0.002) with associated microbial groups shown (minimum fit value of at least 30% and > 0.55 response score on horizontal axis). **(B)** Alpha diversity (Chao1 bias corrected) comparison between the two groups. **(C)** Heat map showing relative abundance of discriminative bacterial genera (≥ 0.40 response score in x axis) as found in redundancy analysis. RDA identified microbes that were also detected in a previous study with similar design (**Chapter 3**) are shown in bold.

Quantitative estimate of eating behaviour

To estimate the eating time per individual piglet, video recordings of the six EF litters (12 hours per day) were observed and eating bouts were recorded during the four-week pre-weaning period. The eating scores were employed as a quantitative indication of eating. The eating behaviour of the EF piglets gradually increased over time, reaching the highest eating score in the last week pre-weaning (**Figure 3A**), which is similar to our previously reported observations (**Chapter 3**). However, the quantification of eating behaviour in the present study was more variable and on average lower as compared to our previous study of a similar design (**Supplementary figure 5**). Nevertheless, the substantial variation in the estimated eating among EF piglets in the present study provides the opportunity to evaluate the relation between the individualised quantification of eating behaviour and the piglet-specific microbiota composition and intestinal measurements.

Previously it was shown that eating quantification observed shortly before (rectal swab) microbiota sampling is strongly and quantitatively related to the microbiota changes, that are driven by consumption of the fibre-enriched feed (**Chapter 3**). Analogously, in this study, we employed the summed eating scores of the last two days prior to sacrifice to investigate their relation with the microbiome signatures observed in individual EF piglets. Based on the eating scores in the last two days, we can classify individual EF piglets into good (> 2 times the median; green), moderate (between 0.8 and 2 times the median; blue) and bad (below 0.8 times the median; red) eaters (**Figure 3B**). The RDA analyses show that these individual eating scores (cumulative eating scores from the "last two days") could significantly explain the colon microbiota composition (**Figure 3C**). Notably, all periodical eating scores ("Total seconds", "Last week", "Last two days" and "Last day") were significantly and positively correlated with each other (**Supplementary figure 8**), and were all significantly reflected by the microbiota RDA scores (**Supplementary figure 8, 9**). Moreover, the microbiota composition supported the classification of EF piglets into good (green), moderate (blue) and bad (red) eaters on the basis of discriminatory microbial groups that were either more abundant in good eaters (e.g., *Lachnospiraceae ND3007 group, Lachnospiraceae NK3A20 group, Kandleria, Eubacterium xylanophilum, Lachnospira, Prevotella1, Faecalibacterium, Roseburia*) or more abundant in the bad eaters (e.g., *Odoribacter, Butyricimonas*). However, since the overall group of EF piglets in this study includes a substantial amount of bad eaters, significant correlations were observed between only a few individual microbial groups and eating scores (**Figure 3D**, **Supplementary figure 6**). Nevertheless, the individualised eating behaviour substantiated the partial separation of EF piglets in hierarchical clustering (**Figure 2C**) where some EF piglets (classified as bad eaters) were clustering closely to the control piglets. Taken together, these findings underpin the microbiota compositional changes in response to individualised eating behaviour.

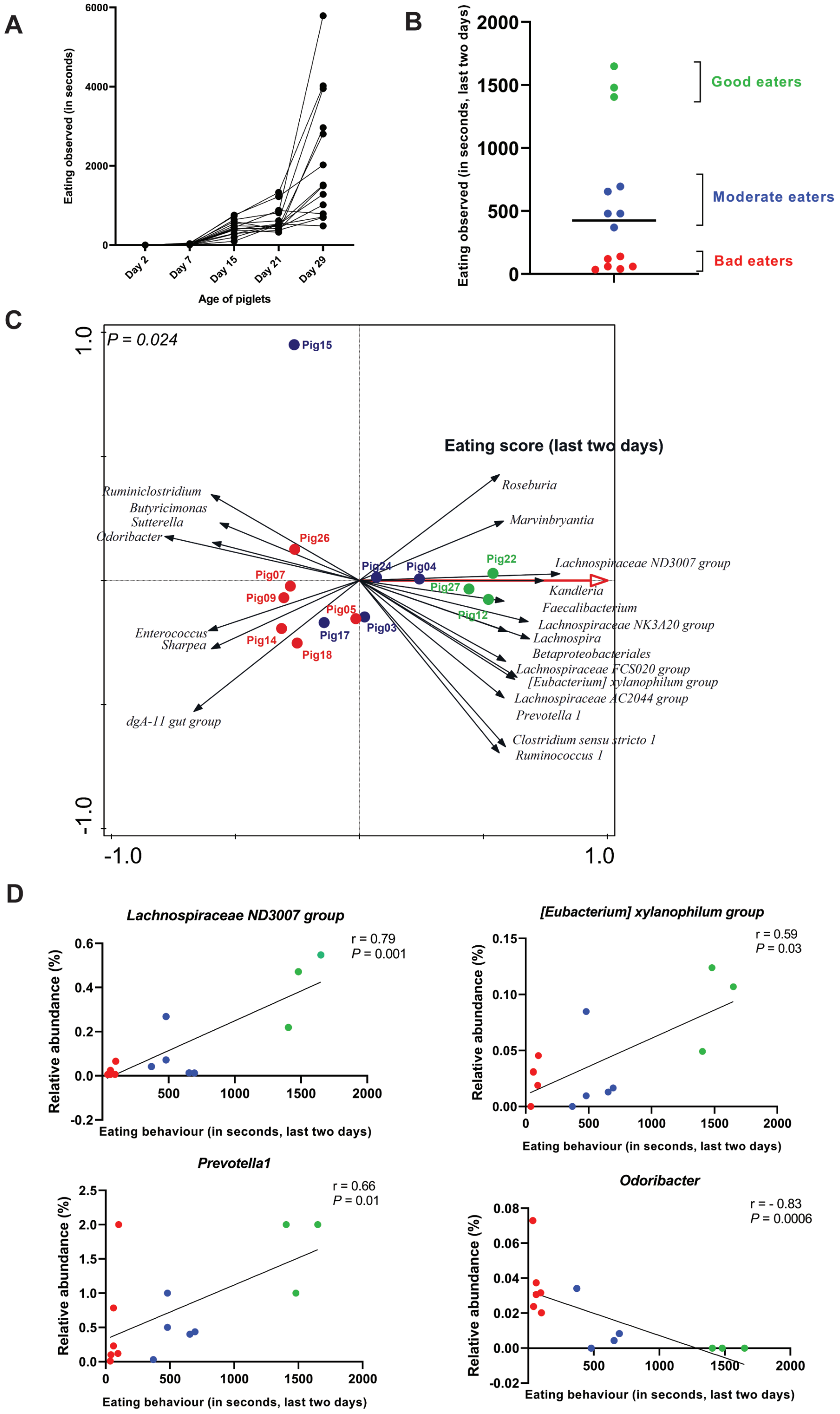
A
6000
4000
2000
0
Eating observed (in seconds)
Day 2
Day 7
Day 15
Day 21
Day 29
Age of piglets
B
2000
1500
1000
500
0
Eating observed (in seconds, last two days)
Good eaters
Moderate eaters
Bad eaters
C
1.0
-1.0
P = 0.024
Pig15
Eating score (last two days)
Roseburia
Marvinbryantia
Ruminiclostridium
Butyricimonas
Sutterella
Odoribacter
Pig26
Pig22
Lachnospiraceae ND3007 group
Pig24
Pig04
Pig07
Pig27
Kandleria
Pig09
Pig12
Faecalibacterium
Pig05
Lachnospiraceae NK3A20 group
Pig03
Enterococcus
Pig17
Lachnospira
Sharpea
Pig14
Betaproteobacteriales
Pig18
Lachnospiraceae FCS020 group
[Eubacterium] xylanophilum group
Lachnospiraceae AC2044 group
Prevotella 1
dgA-11 gut group
Clostridium sensu stricto 1
Ruminococcus 1
-1.0
1.0
D
Lachnospiraceae ND3007 group
r = 0.79
P = 0.001
0.6
0.4
0.2
0.0
-0.2
Relative abundance (%)
500
1000
1500
2000
Eating behaviour (in seconds, last two days)
[Eubacterium] xylanophilum group
r = 0.59
P = 0.03
0.15
0.10
0.05
0.00
Relative abundance (%)
0
500
1000
1500
2000
Eating behaviour (in seconds, last two days)
Prevotella1
r = 0.66
P = 0.01
2.5
2.0
1.5
1.0
0.5
0.0
Relative abundance (%)
0
500
1000
1500
2000
Eating behaviour (in seconds, last two days)
Odoribacter
r = - 0.83
P = 0.0006
0.08
0.06
0.04
0.02
0.00
-0.02
Relative abundance (%)
500
1000
1500
2000
Eating behaviour (in seconds, last two days)

Figure 3: Classification of early-fed (EF) piglets into good (green), moderate (blue) and bad (red) eaters. **(A)** Individual piglet's eating behaviour quantification (14 EF piglets; total eating seconds per week) for four weeks pre-weaning by video observation. **(B)** Good (> 2 x median; green), moderate (between 0.8 x and 2 x median; blue) and bad (below 0.8 x median; red) eaters, grouping based on eating observed in the "last two days" before weaning. **(C)** Redundancy analysis based on eating scores from "last two days" before weaning (explained variation = 5.42% , $P = 0.024$), establishing the microbiota discrimination between the "good", "moderate" and "bad" classification within the EF piglets (minimum fit value of at least 30% and > 0.55 response score on horizontal axis). **(D)** Spearman correlation of individual microbial genera with the eating score from "last two days". ***Lachnospiraceae ND3007, Eubacterium xylanophilum, Prevotella 1 and Odoribacter*** were identified in redundancy analysis.

Changes in pH and SCFA concentration

To assess the intestinal fermentation as a result of early feeding, the intraluminal pH in different segments of the intestinal tract and short chain fatty acid (SCFA; i.e., succinic-, lactic-, acetic, propionic- and butyric-acid) concentrations in the colon content were determined. The digesta pH of both caecum and colon significantly decreased due to early feeding of piglets ($P < 0.05$), whereas no differences in stomach or small intestinal digesta were detected (**Figure 4A**). Subsequently, the relation between eating scores and digesta pH in individual EF piglets was investigated, revealing that caecum pH tended to be negatively correlated with eating behaviour, whereas no such association was observed for the colon (**Figure 4B).** Nevertheless, the levels of colonic SCFAs were significantly impacted by early feeding, and relative to the CON group, concentrations of acetic acid, butyric acid and total SCFAs were significantly higher whereas propionic acid tended to be higher ($P = 0.06$) in the EF group (**Figure 4C**), indicative of increased carbohydrate fermentation in the distal gut. As anticipated, caecum and colon pH negatively correlated with SCFAs acetic acid, propionic acid and butyric acid. In contrast, no differences were observed in lactic- and succinic-acid between the CON and EF piglets (**Supplementary figure 7**). Notably, SCFA (acetic-, propionic- and butyric acid) concentrations in individual piglets were found to be significantly correlating with each other (**Supplementary figure 8**). However, the distribution of the differential eating classifications (good, moderate and bad eaters) did not appear to be significantly related to the levels of SCFAs observed, although a trend ($P = 0.08$) of positive correlation was observed between propionic acid and eating scores (**Supplementary figure 8**, **9**). Remarkably, the caecal pH appeared to be significantly reflected by the levels of acetic, propionic and butyric -acid (and total SCFAs) measured in the colon, whereas colonic pH values only correlated with the level of butyric acid (**Supplementary figure 8**).

Digestive organ measurements (macroscopic)

Macroscopic measurements such as digestive organ weight and lengths were determined to check the effect of early feeding on the intestinal development of piglets at weaning **(Table 1)**. EF and CON piglets did not differ in organ weights of the adrenal gland, gallbladder, spleen, liver, stomach, and caecum. However, EF piglets tended to have a heavier pancreas ($P = 0.05$) compared to CON piglets. The small intestine ($P = 0.096$) and the total intestinal tract ($P = 0.05$)

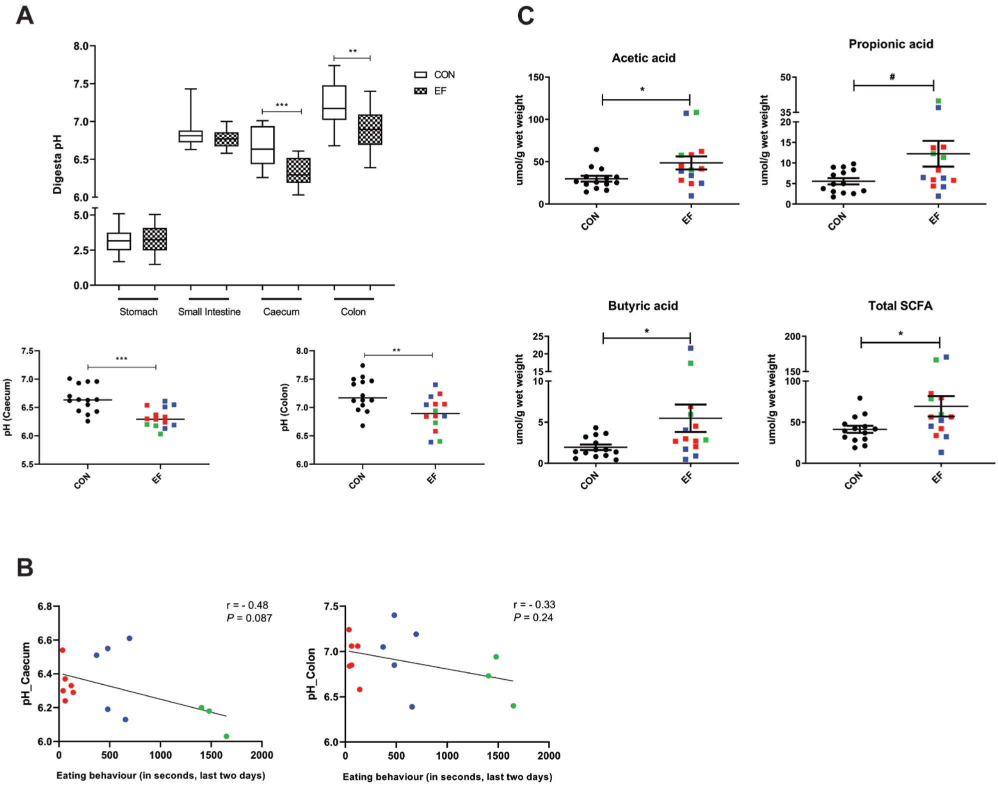

Figure 4: Impact of early feeding on pH and short chain fatty acids (SCFA). **(A)** Digesta pH in different parts of the GIT for EF and CON and an expansion of the two segments where significant difference in pH was detected, illustrating the distribution of individual piglets in both groups (using the EF group classification of good (green), moderate (blue) and bad (red) eaters; see **figure 2B**). **(B)** Correlation of pH caecum/colon of individual EF piglets with the quantified eating score from last two days (Caecum: r =–0.48, $P = 0.087$; Colon: r =–0.33, $P = 0.24$). **(C)** Group level comparison for colonic SCFA concentration (µmol/g wet weight of digesta) in piglets. Significant differences between groups were assessed by student t test or Mann-Whitney U test (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$). EF = early-fed group; CON = control group. Green = good eaters; Blue = moderate eaters; Red = bad eaters.

showed a tendency to be longer in EF piglets, while the weights of the small intestine and the total intestinal tract were higher ($P < 0.05$), both with (full) and without (empty) digesta in EF piglets. Further, the large intestine (including caecum and colon) was significantly longer ($P < 0.05$) and tended to be heavier with digesta ($P = 0.08$) in EF piglets.

The intestinal segment lengths showed a significant positive correlation with each other, however, organ and intestinal weights did not show such correlation (**Supplementary figure 8)**. Notably, the weight of the small intestine (without digesta; empty) as well as the whole gastrointestinal tract (with or without digesta; full or empty) significantly correlated ($P = 0.01$; **Supplementary figure 8, 9**) with the eating scores (“total seconds”), whereas small intestine (with digesta; full) tended to show the same correlation (*P* = 0.06; **Supplementary figure 8, 9**). Taken together, these results indicate that early eating has (moderate) effects on some macroscopic measurements of the digestive system (**Table 1**), but not all these effects appear to significantly correlate with the quantified estimate of eating.

Mucosal morphometry and epithelial proliferation in jejunum and colon

Intestinal morphometry (microscopic) measurements were assessed using histological staining (n=8; randomly selected piglets from each treatment group) of jejunum and colon mucosa. No significant alteration was observed in the villus length or crypt depth in the jejunal mucosa, nor in the colonic crypt depth of tissue samples from EF compared to CON group (**Figure 5**). However, due to a combination of modestly increased villus length and decreased crypt depth in jejunal mucosa, we observed a tendency for a reduced villus length : crypt depth ratio (V:C ratio) in the EF compared to the CON animals (*P* = 0.06). However, no quantitative

Table 1: Intestinal weights of early-fed (EF) and control (CON) piglets at weaning (day29), mean ± SEM. *P*-values are based on linear mixed model using body weight at sacrifice as covariate.

Item	CON	EF	*P*-value
Body weight, kg	8.2 ± 0.3	8.5 ± 0.4	0.57
Organ weight, g			
Adrenal gland	0.5 ± 0.02	0.5 ± 0.03	0.70
Pancreas	13.5 ± 0.7	15.5 ± 0.8	**0.05**
Spleen	19.4 ± 1.0	19.4 ± 1.0	0.70
Liver	213.6 ± 7.2	219.4 ± 11.0	0.79
Gallbladder	3.0 ± 0.4	2.5 ± 0.3	0.39
Intestine weight, g			
Stomach, full	146.2 ± 10.7	160.7 ± 11.0	0.47
Stomach, empty	43.2 ± 1.6	44.7 ± 1.7	0.76
Small intestine, full	421.7 ± 17.8	482.5 ± 31.3	**0.02**
Small intestine, empty	328.5 ± 10.5	370.3 ± 20.3	**0.03**
Cecum, full	47.9 ± 2.5	51.0 ± 3.5	0.67
Cecum, empty	18.8 ± 1.1	19 ± 0.9	0.89
Colon, full	80.4 ± 4.2	96.8 ± 7.5	**0.08**
Colon, empty	47.2 ± 2.4	50.3 ± 3	0.54
Total gastro-intestinal tract, full	696.2 ± 27.3	790.9 ± 45.9	**0.02**
Total gastro-intestinal tract, empty	437.7 ± 14.3	484.3 ± 23	**0.03**
Intestine length, m			
Small intestine	7.8 ± 0.2	8.3 ± 0.2	**0.096**
Large intestine	1.2 ± 0.02	1.3 ± 0.03	**0.046**
Total gastro-intestinal tract	9 ± 0.2	9.6 ± 0.2	**0.05**

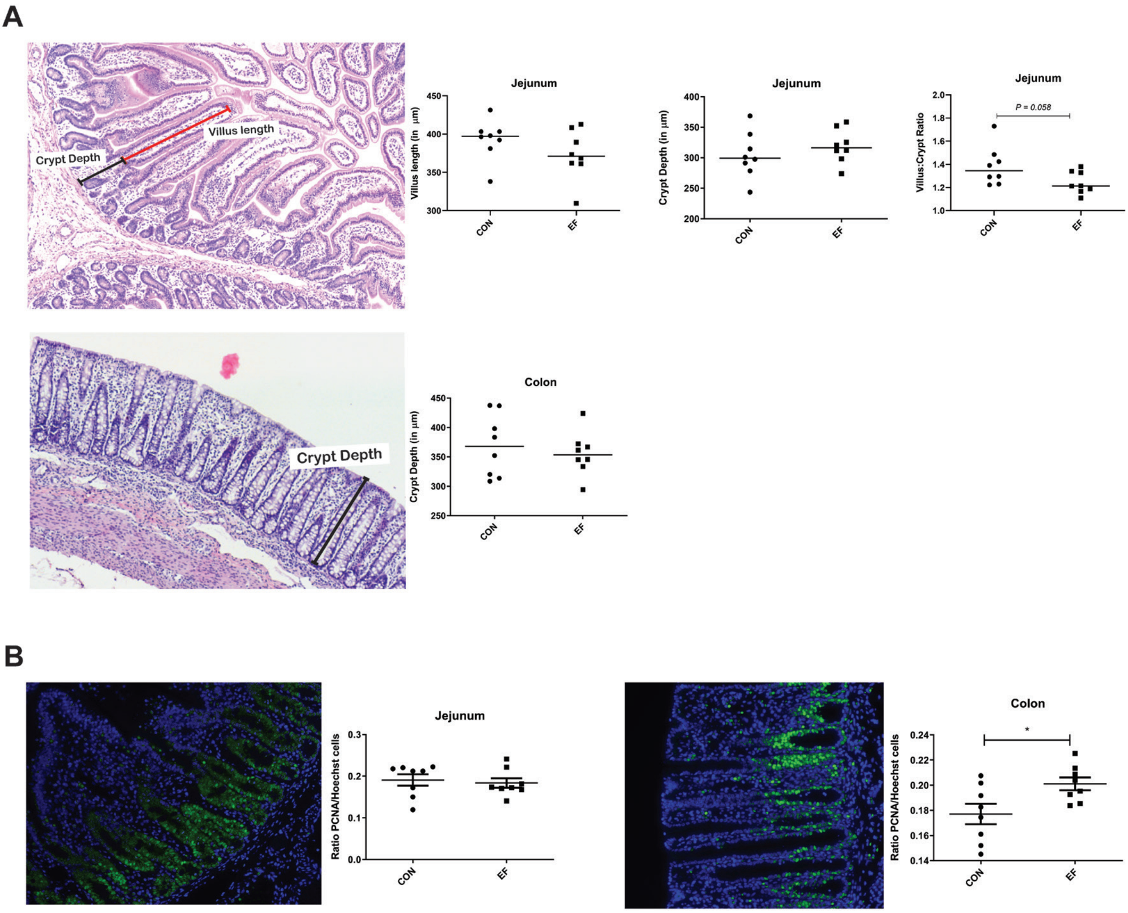

Figure 5: Effects of early feeding on **(A)** intestinal morphometry and **(B)** PCNA$^+$ proliferating cells in jejunum and colon (representative image) at weaning (day29). Hoechst and PCNA positive cells are represented in blue- and green-coloured cells respectively. EF = early-fed group; CON = control group. Differences between groups were assessed by student t test or Mann-Whitney U test (*: $P < 0.05$).

association was found between these mucosal morphometric measurements and eating scores (**Supplementary figure 8, 10**), although it should be noted that the animals selected for this analysis were not evenly distributed over the good-, moderate- and bad-eaters classification within the EF group. For example, the inclusion of only a single piglet of the "good eater" group may have led to an underestimation of the relatedness of the eating scores and the mucosal morphometric differences.

Epithelial proliferation was evaluated using immuno-histochemistry in combination with a standardised quantification pipeline in jejunum and colon mucosa. To determine the relative amount of proliferative epithelial cells in the jejunum and colon mucosa samples, tissues were stained with nuclei stain PCNA and Hoechst (n=8; randomly selected piglets from each treatment group). There was an increase in the number of colonic proliferating cells in EF group compared to CON (ratio of PCNA:Hoechst positive cells), however, no such difference was observed in jejunal tissue (**Figure 5**). Notably, eating scores (based on last two days and last day) correlated with proliferating cells in jejunum (**Supplementary figure 8, 10),** although the effect size is very low (Cohen's d ≤ 0.2) indicative of substantial individual variation, raising questions about the reliability of this observation.

Discussion

Early-life conditions are known to have a key influence on the developing gut microbial ecosystem and have been shown to have long lasting consequences for the microbiota as well as the host (Schokker et al., 2015; Fouhse et al., 2016; Everaert et al., 2017). The present study was aimed to investigate whether early-life feeding (pre-weaning access to a mixed-fibre feed) has an impact on the intestinal microbiota composition and digestive system maturation at weaning. The hypothesis was that early feeding (of mixed-fibre feed) would modulate the microbiota composition in the intestine of piglets and would support digestive system development. Our results show that early feeding impacts the intestinal microbiota and its metabolism (short chain fatty acids), as well as digestive system development, determined at both macroscopic and microscopic level. Importantly, we observed the microbiota compositional changes only in the colon and their magnitude responds proportional to the individual piglet's quantified eating behaviour during the pre-weaning period. In contrast, many of the macro- or microscopic digestive system changes associated with early feeding did not appear to be related with the eating quantification, suggesting that these changes could be driven by feed exposure (and/or its associated intestinal microbiota) per se, rather than the amount of feed consumed.

Early feeding did not appear to elicit significant changes in the small intestinal microbiota which was very different in composition and diversity as compared to the colon microbiota (irrespective of the intervention). Substantial differences between the microbiota in these different regions are in agreement with earlier studies (Zhao et al., 2015; Holman et al., 2017; Mu et al., 2017a; Crespo-Piazuelo et al., 2018). Our analyses detected a significant difference in richness (Chao1) between jejunal and ileal microbiota samples. However, previous studies have reported conflicting conclusions related to similar analyses (Kraler et al., 2016; Yang et al., 2016; Holman et al., 2017; Mu et al., 2017a; Crespo-Piazuelo et al., 2018), which may be due to a high dynamics of the small intestinal microbiota that was suggested to be driven by many (nutritional and environmental) factors (Booijink et al., 2010; Zoetendal et al., 2012) that differ within and between studies and thereby intrinsically prohibit consistent conclusions.

The impact of early feeding (of fibrous feed) on the colon microbiota is in accordance with previous studies (Shim et al., 2005; Zhang et al., 2016; Mu et al., 2017b), and corroborates the impact of dietary fibres on the distal regions of the intestine. Various studies have reported the influence of fibres on the microbiota composition, mostly focussing on weaned or growing pigs (Umu et al., 2015, 2018; Kraler et al., 2016; Burbach et al., 2017; Fouhse et al., 2019). In the present study, a customised mixed-fibre feed was formulated especially for suckling piglets, with inclusion of both soluble (GOS, inulin) and insoluble (oat hulls, sugarbeet pulp, resistant starch) dietary fibres. In line with our previously described observations (**Chapter 3**), early feeding with this diet elicited higher relative abundances of fibrolytic and/or butyrate-producing bacterial groups, like ***Ruminococcus, Lachnospira, Roseburia, Eubacterium,*** and ***Prevotella***, which reflect the accelerated pre-weaning microbiota development towards a "post-weaning-resembling" microbial-ecosystem.

Consistent with previous studies (Pajor et al., 1991; Delumeau and Meunier-Salaün, 1995; Huting et al., 2019), a large variation in eating behaviour was observed among the EF piglets. In addition, the eating behaviour of the EF piglets in the present study, may have been compromised by a diarrhoeic episode during the third week (spreading between day 16-24) in almost all litters, the cause of which is unknown. Bruininx and coworkers (Bruininx et al., 2004) concluded that there was considerable within-litter variation in creep-feed intake, and designated piglets as good, moderate and non-eaters based on the colour of their faeces using chromium oxide as a marker. Here we used a similar classification system on eating behaviour video-observation scores and it should be noted that a relatively large proportion of the dissected piglets was classified as bad eaters (42%), which is substantially more than in our previous study (**Chapter 3**) that employed a similar design. The higher degree of feed-intake variation in this study, was exploited to correlate the eating scores with the microbiota changes per individual piglet, demonstrating that the eating time is strongly reflected in the magnitude of the colon microbiota changes. Importantly, this reflection appeared independent of the eating score (time-period) employed, supporting the robustness of the observation that the eating time is a key-driver of the microbiota adaptation. This is in good agreement with our previous study (**Chapter 3**), where it was shown that the microbiota analysed in rectal swabs at different stages during the pre-weaning period accurately reflects the eating behaviour quantification in piglets.

It is well established that dietary fibre reaches the large intestine, due to their indigestibility by the host digestive enzymes that are released in the proximal small intestine. In the colon, these fibres are fermented by the colonic microbiota into short chain fatty acids (SCFA) (Jha et al., 2019). Prior studies have shown elevated levels of SCFAs after fibrous diet intake in piglets, which was associated with lower pH values in distal gut (Alizadeh et al., 2016; Li et al., 2018; Van Hees et al., 2019). Analogously, in the present study EF piglets had higher levels of the canonical SCFAs (acetic, propionic and butyric acid) in their colon, which was reflected by a lower pH of both caecal and colonic digesta. Notably, although eating time tended to quantitatively correlate with caecal pH in our study, it did not associate with colonic pH or colonic SCFA levels. A possible explanation for this might be that luminal SCFAs production and their absorption by the colonic epithelium is highly dynamic (Von Engelhardt et al., 1989), whereby colonic SCFA levels poorly represent the flux of SCFA production and absorption (Verbeke et al., 2015). Thereby, the caecum, where the fermentative capacity of the microbiota is considered to be at its maximum (Cummings et al., 1987), and where mucosal absorption rates of the luminal SCFAs may be lower as compared to the colon, might have provided a more appropriate intestinal region to assess SCFA concentrations changes in relation to dietary intake, compared to the colon.

Increased weight of digestive organs, including the weight of the intestine itself as well as its length have been associated with solid food consumption (Jørgensen et al., 1996; Al Masri et al., 2015; Van Hees et al., 2019). Our study reached related conclusions, where early feeding (with mixed-fibre feed) was associated with (relatively modest, but significant) changes in macroscopic digestive organ measurements, including a heavier small intestine (empty and full) and complete gastro-intestinal tract (empty and full), as well as an extended large intestine and complete gastrointestinal tract length. However, only a few of these changes appeared to have an association with the quantified feed-intake estimates within the early-feeding group, although the relatively small number of animals may have obscured such relationships, and to definitely disqualify them would require the analysis of a larger amount of EF animals. For example, eating behaviour was associated with the weights of the small intestine and total gastro-intestinal tract (with or without digesta). Increase in gut fill may be due to the typical water-retention activity and ‘bulking agent’ capacities of the (insoluble) fibres (Morris, 1992; Tungland and Meyer, 2002) that are present in pre-weaning diet (Bach Knudsen et al., 2012). These results suggest that early feeding of a fibre-enriched feed stimulates expansion of the intestinal size as well as digesta. This notion was further supported by a significant positive correlation between the changes in microbiota (microbiota scores RDA), colonic SCFA concentrations and the weight of the small intestine, colon and gastrointestinal tract with or without its digesta, indicating their interrelatedness. Intriguingly, some measurements significantly correlated, for example, SCFAs associated with a few macroscopic measurements such as weight of gall bladder and colon (with digesta). However, the correlations were mainly depending on EF (good and moderate) piglets with relatively extreme SCFA-level values compared to other piglets, which is most likely a chance event in the intestinal dynamics of SCFA production and absorption. Thereby the limited numbers of animals in this study and the observation that these observations are largely determined by a small proportion of these animals may indicate that these conclusions are biologically less reliable and would require further studies that include larger numbers of animals.

Presence of luminal nutrients in the gut can cause changes to the structure and function of the intestinal mucosa (Diamond and Karasov, 1983; Pluske et al., 2018). Feed intake has been positively associated with adaptation of mucosal architecture, i.e., altered villus length or V:C ratio, which has in particular been reported in relation to post-weaning intestinal adaptations

(Kelly et al., 1991; Pluske et al., 1996; Dong and Pluske, 2007; Muns and Magowan, 2018). However, these effects have not been unambiguously established in the literature and include contradicting inferences. For example, Bruininx et al. (Bruininx et al., 2004) reported that morphometric measurements (villus length, crypt depth, V:C ratio) were not affected by pre-weaning (commercial) creep feed consumption. On the other hand, a recent study (Alizadeh et al., 2016) reported morphological changes in the intestinal mucosa (thicker and extended villi in the jejunum) of piglets that were separated from their mother after 48 hours and fed with milk replacer diet, supplemented with or without 0.8% galacto-oligosaccharides for 26 days. In the present study, we detected moderate changes in the mucosal architecture, which were only apparent in jejunal V:C ratio of early-fed piglets. Remarkably, decreased villus length, increased crypt depth and the corresponding decrease in V:C ratio have been suggested to be indicative of intestinal mucosa maturation in un-weaned piglets (Hampson, 1986; Al Masri et al., 2015). Importantly, post-weaning modulations of the intestinal mucosal architecture were determined in a separate study showing progressively reducing V:C ratios in jejunal mucosa post-weaning (**Chapter 5**), which would support an "accelerated maturation" of the mucosal architecture in EF piglets in this study. Likewise, it has been reported that colonic epithelial proliferation and crypt depth increase post-weaning (Brunsgaard, 1997). In the present study, we used PCNA immune-histochemistry (a universal nuclear marker of proliferative cells) to assess the epithelial proliferation in jejunum and colon, showing that early-fed piglets had an increased colonic proliferation, although this difference was not reflected in the coinciding increase of colonic crypt depth. This observation would also support accelerated maturation of mucosal development by early feeding of fibrous feed. However, though both V:C ratio and colonic PCNA staining were considered to have a large effect size (Cohen's d > 0.8), the actual fold changes in the V:C ratio (0.89) and colonic PCNA staining (1.13) were relatively small. Taken together, these are intriguing observations that would support accelerated maturation, but their actual physiological relevance would require further studies.

Overall, our study illustrates that early feeding with fibre-enriched feed influences the colonic microbiota composition, increases microbial fermentation products in the colon and modulates intestinal development at weaning. Importantly, the EF-associated changes in colonic microbial signatures were concluded to be strongly associated with the amount of eating of a piglet, which corresponded with increased weights of the small intestine and total gastro-intestinal tract (with or without digesta). Although the estimated feed intake was relatively low and highly variable among EF piglets in this study, eating behaviour quantification and the classification of individual piglets into good, moderate and bad eaters enabled a reliable and consistent evaluation of eating-behaviour consequences in piglets at an individual level.

Acknowledgements

This study is part of research programme “Genetics, nutrition and health of agricultural animals” with project number 868.15.010, which is financed by the Netherlands Organization for Scientific Research, Cargill Animal Nutrition and Coppens Diervoeding. The authors thank the technicians and students of the Host-Microbe Interactomics group and Adaptation Physiology group (Wageningen University & Research). Special thanks to Pieter Roskam, Soumya Kanti Kar, Sandra Vreman, Anja Taverne-Thiele, Bjorge Laurenssen and Ilona van den Anker-Hensen for assisting in the dissection procedure of the piglets. We would like to acknowledge the personnel and trainees of the animal research facilities in Wageningen (Carus) for taking care of the animals and for their technical assistance. The authors are grateful to Tamme Zandstra for producing the feed and to FrieslandCampina DOMO for provision of Vivinal GOS. We also want to thank Prof. Henk Schols and Christiane Rösch (Food Chemistry group, Wageningen University and Research) for assisting in the SCFA analysis of colon digesta samples.

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Supplemental information

Supplementary table 1: Ingredients and calculated nutrient composition of the pre-weaning fibrous feed[1].

	Pre-weaning feed
Ingredients, %	
Wheat	21.9
Barley	15
Maize	15
Soy protein concentrate	7
Soybeans (heat treated)	5
Galacto-oligosaccharides[2]	**5**
Potato protein	4
Sugar beet pulp	**4**
Oat hulls	**4**
Inulin[3]	**4**
Resistant starch[4]	**4**
Soybean oil	3
Blood meal (spray dried)	2
Dicalcium phosphate	1.7
Sucrose	1.5
Calcium carbonate	1.0
Sodium chloride	0.5
Premix[5]	0.5
Potassium bicarbonate	0.3
L-lysine hydrochloride	0.3
DL-methionine	0.2
L-threonine	0.04
L-tryptophan	0.04
Calculated nutrient composition[6], g/Kg	
Dry matter	891
Starch	290
NSP[7]	261
Crude protein	195
Crude fat	61
Crude fibre	44
Crude ash	57
Calcium	9.1
Phosphorus	6.1
Sodium	2.2
Standardized ileal digestible lysine	11.9
Standardized ileal digestible methionine	4.8
Standardized ileal digestible threonine	7.1
Standardized ileal digestible tryptophan	2.4
Net energy, MJ/kg	11.8

[1] Feed was mixed by Research Diet Services (Wijk bij Duurstede, The Netherlands), and extruded using a co-rotating double screw extruder (M.P.F. 50, Baker Perkins, Peterborough, United Kingdom).

[2] Source: Vivinal® GOS powder (Friesland Campina, Amersfoort, The Netherlands) containing 69% galacto-oligosaccharides.

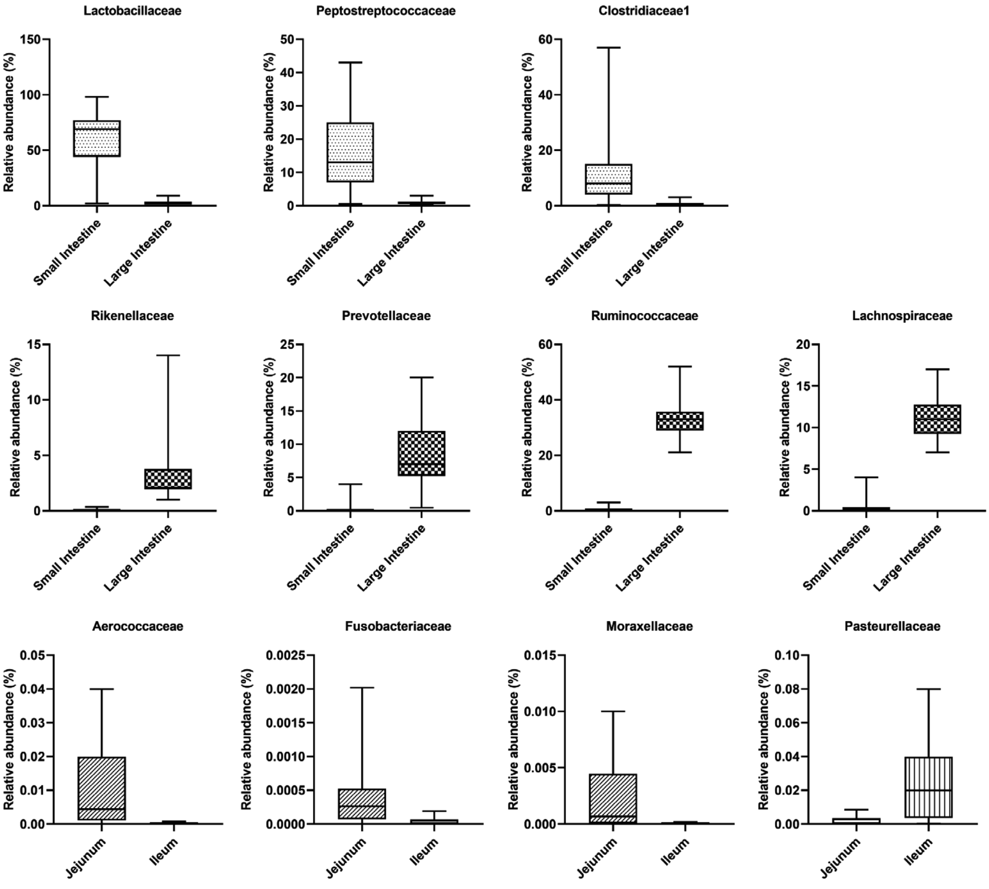
[3] Source: Prebiofeed 95 inulin powder (Cosucra group, Belgium) containing 85% inulin.

[4] Source: AmyloGel® Native Starches (Cargill, Wayzata, USA) derived from high amylose maize with 75% amylose content.

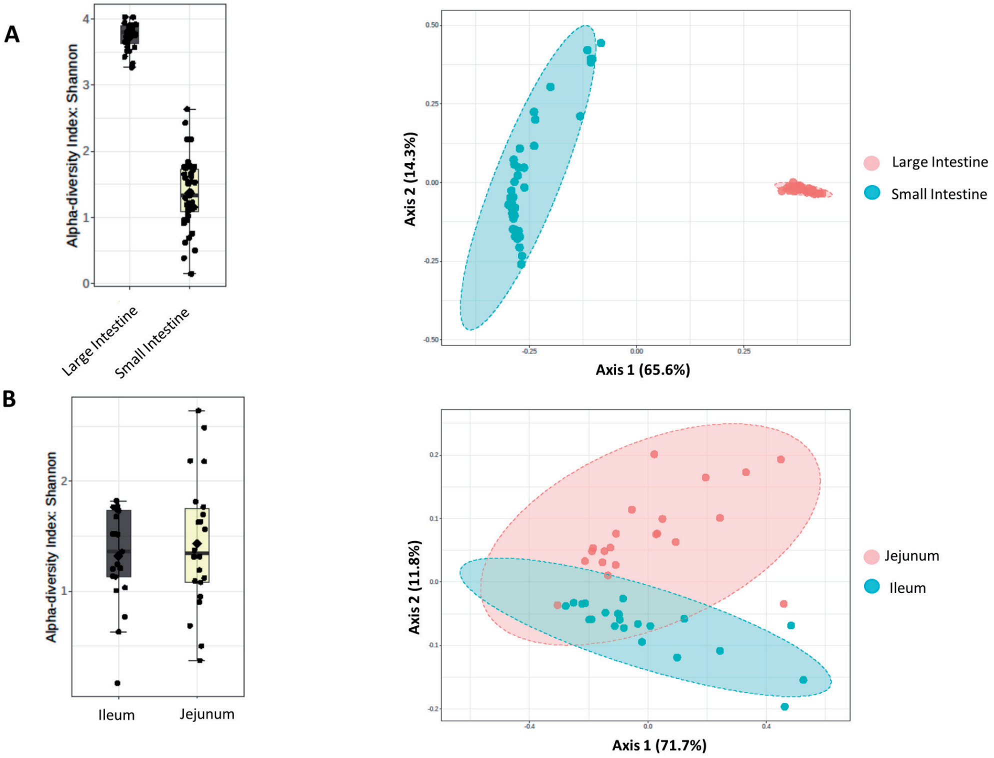
[5] Vitamin and mineral premix (per kg of feed): vitamin A: 10000 IU, vitamin D3: 2000 IU, vitamin E: 40 mg, vitamin K: 1.5 mg, vitamin B1: 1 mg, vitamin B2: 4 mg, vitamin B6: 1.5 mg, vitamin B12: 0.02 mg, niacin: 30 mg, D-pantothenic acid: 15 mg, choline chloride: 150 mg, folate: 0.4 mg, biotin: 0.05 mg, iron: 100 mg, copper: 20 mg, manganese: 30 mg, zinc: 70 mg, iodine: 0.7 mg, selenium: 0.25 mg, anti-oxidant: 125 mg.

[6] According to CVB (2007), nutrients are presented in g/kg dry matter, except for dry matter (g/kg) and net energy (MJ/kg).

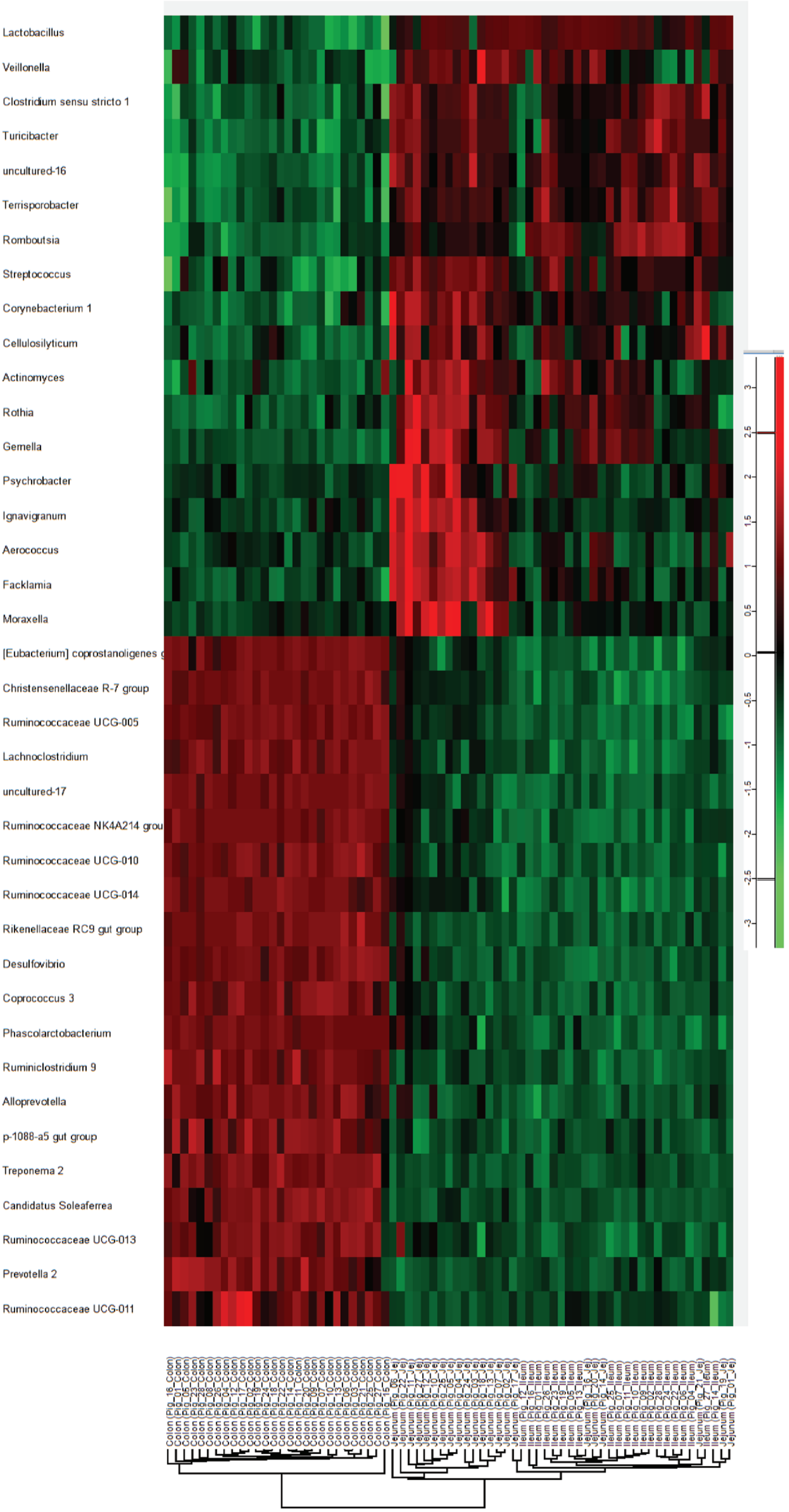
[7] Non-starch polysaccharides: Calculated as the difference between dry matter and the sum of starch, sugars, crude protein, crude fat, and crude ash.



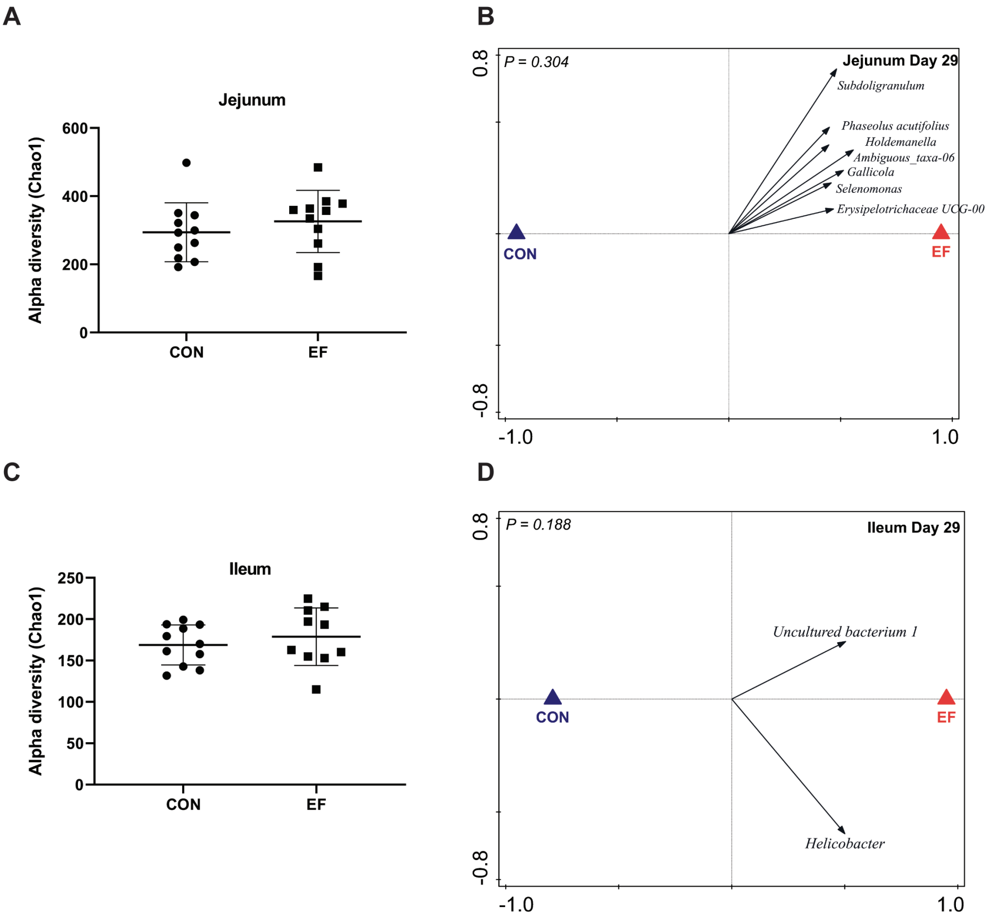
Supplementary figure 1: Box plots of the relative abundance of microbial families at different intestinal locations. **(A)** Comparing small and large intestine. **(B)** Comparing jejunum and ileum. Representative families which have significant (FDR corrected P value < 0.0001) differential relative abundance are shown in this figure.



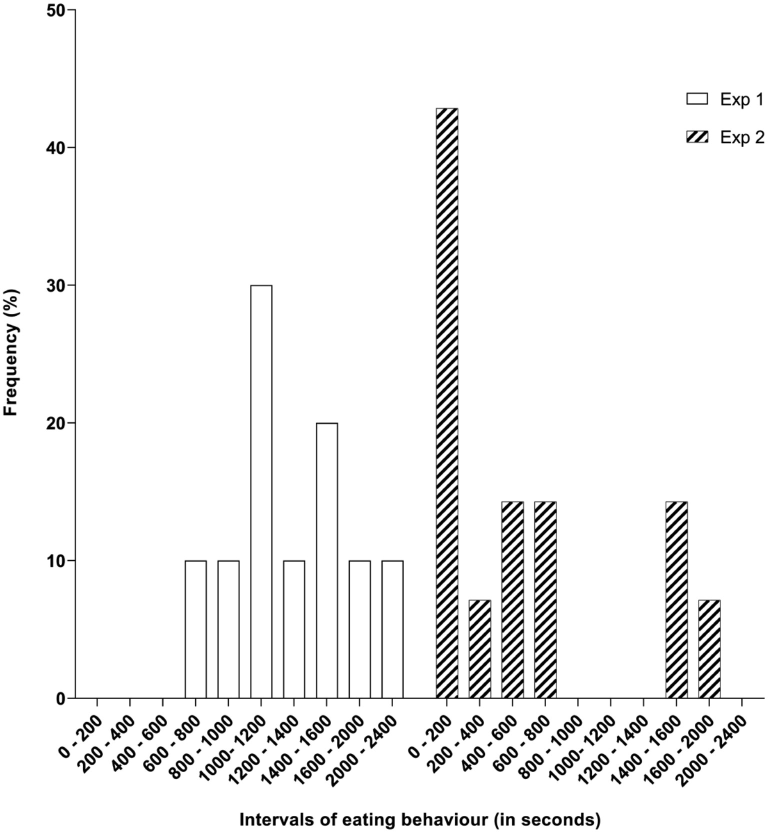
Supplementary figure 2: Comparison of diversity metrices among different intestinal locations at genus level. **(A)** Comparison between small and large intestine using alpha diversity (Shannon: $P < 0.0001$) and beta diversity (PERMANOVA of Bray Curtis distance as depicted in PCoA 2D plot; $P < 0.001$). **(B)** Comparison between small intestinal locations jejunum and ileum using alpha diversity metrices (Chao1: $P = 8.1931e\text{-}071$; Shannon: $P = 0.49$) and beta diversity (PERMANOVA of Bray Curtis distance as depicted in PCoA 2D plot; $P < 0.052$). All diversity comparisons were performed after rarefying the reads to minimum library size.



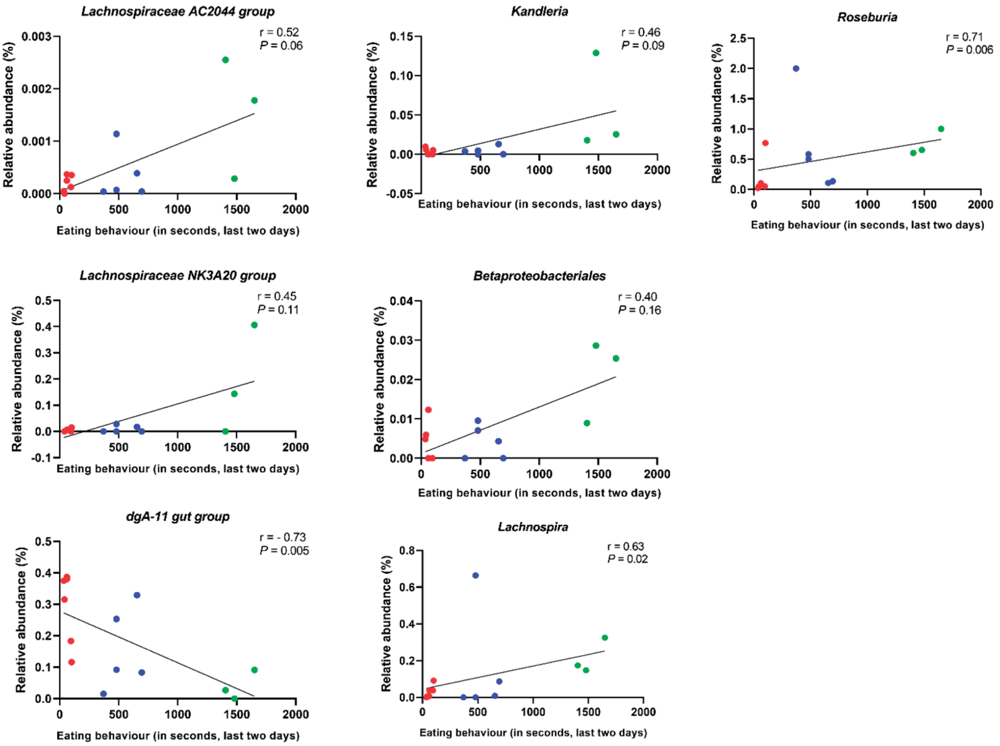
Supplementary figure 3: Heat map (of individual animals) showing relative abundance of discriminative bacterial groups as identified in redundancy analysis as a function of "intestinal location" at genus level.



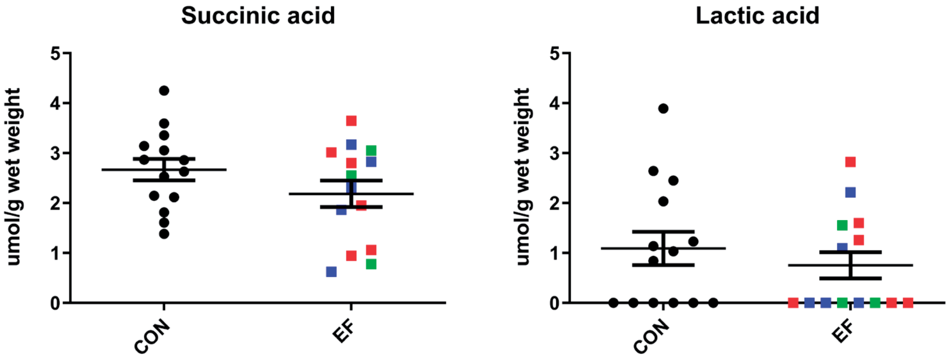
Supplementary figure 4: Jejunal and Ileal microbiota composition in early-fed (EF) and control (CON) group. **(A)** Alpha diversity (Chao1 bias corrected) comparison between the two groups. **(B)** Redundancy analysis at genus level in jejunum (Explained variation = 0.65%; PC1 = 5.38%, PC2 = 27.85%; *P* = 0.304) and ileum (Explained variation = 1.27%; PC1 = 5.97%, PC2 = 18.36%; *P* = 0.188) with associated microbial groups shown (20% minimum fit on horizontal axis).



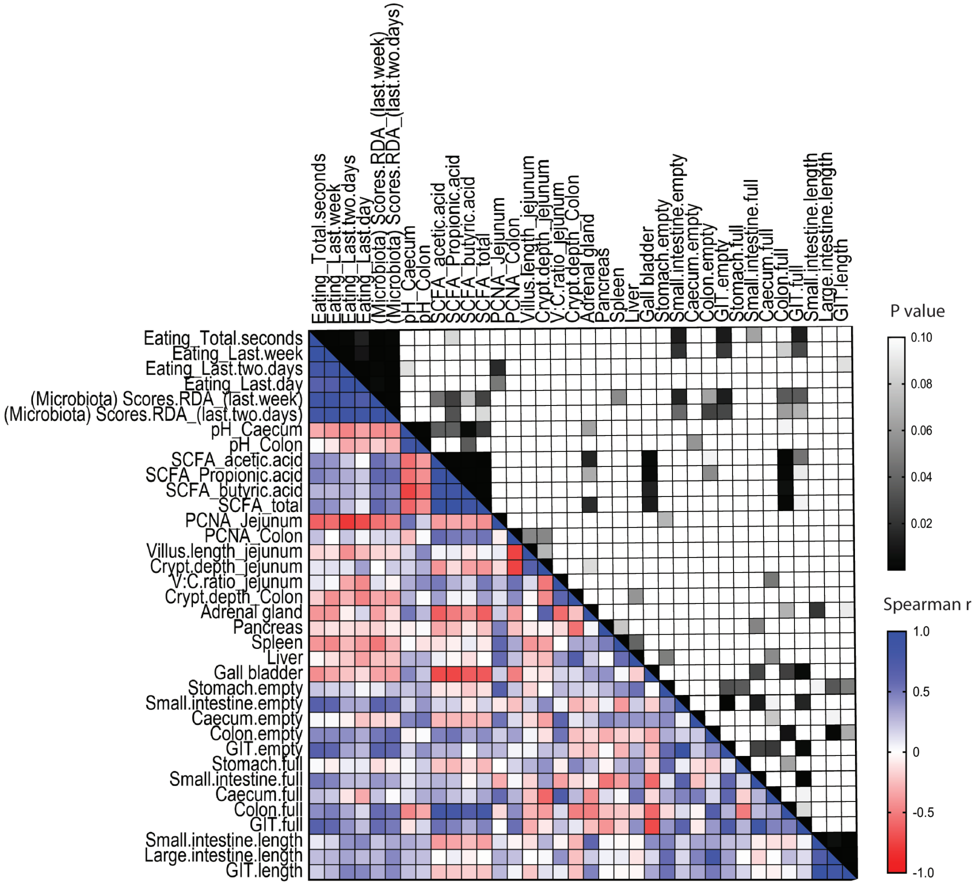
Supplementary figure 5: Frequency of eating scores in last two days before weaning, comparing two experiments (exp1 = previous study; exp2 = this study) with respect to eating behaviour. Frequency distribution of EF piglets at various intervals (200 seconds considered per interval) of eating behaviour two days before weaning. The total eating score was also lower in the present study (3204 ± 1810 seconds) compared to the previous one (8032 ± 3387 seconds).



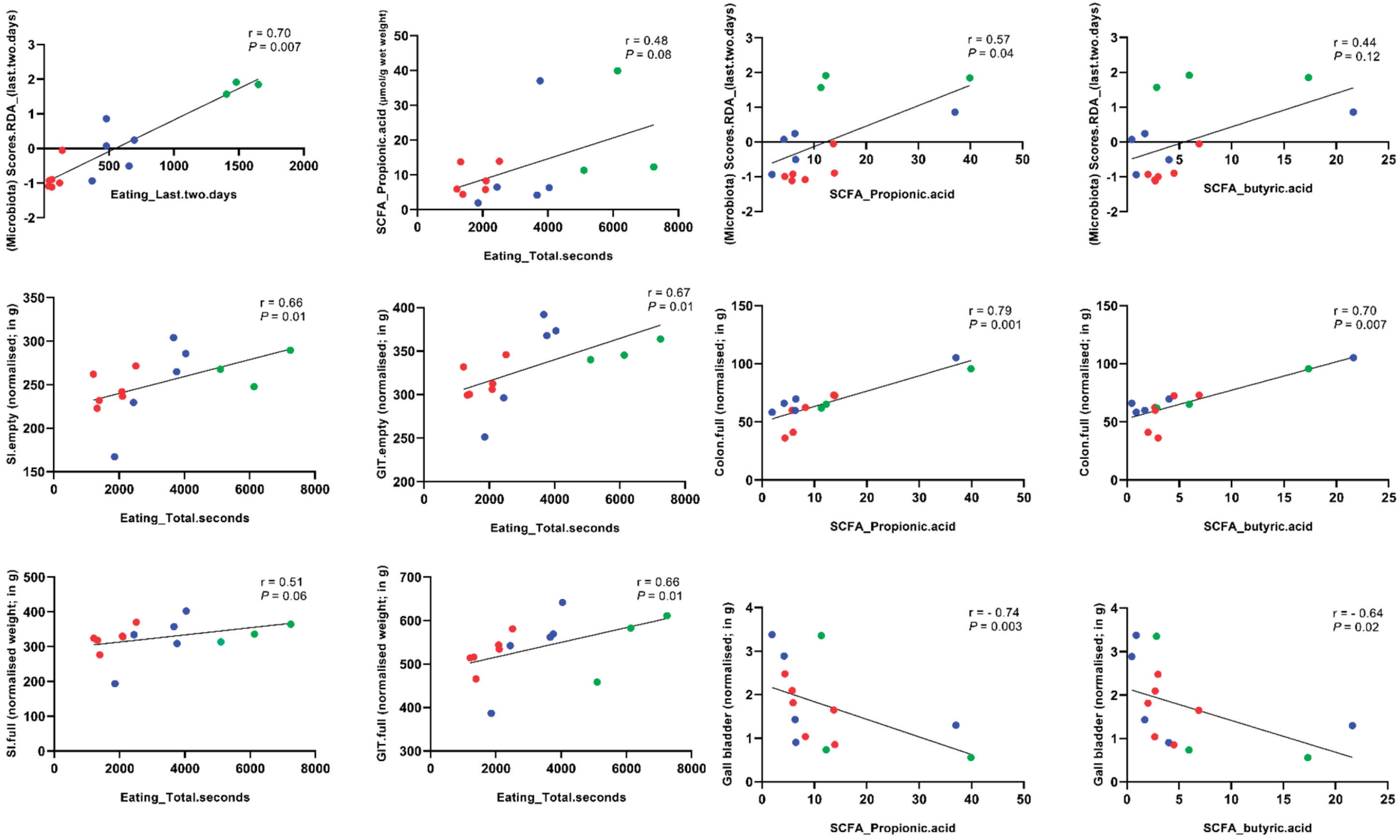
Supplementary figure 6: Correlation of individual microbial genera with the "classified" eating scores per individual during the last two days before weaning identified in redundancy analysis. The early-fed (EF) piglets are marked with colours of EF classification based on eating observed in the "last two days" before weaning. Green = good eaters; Blue = moderate eaters; Red = bad eaters.



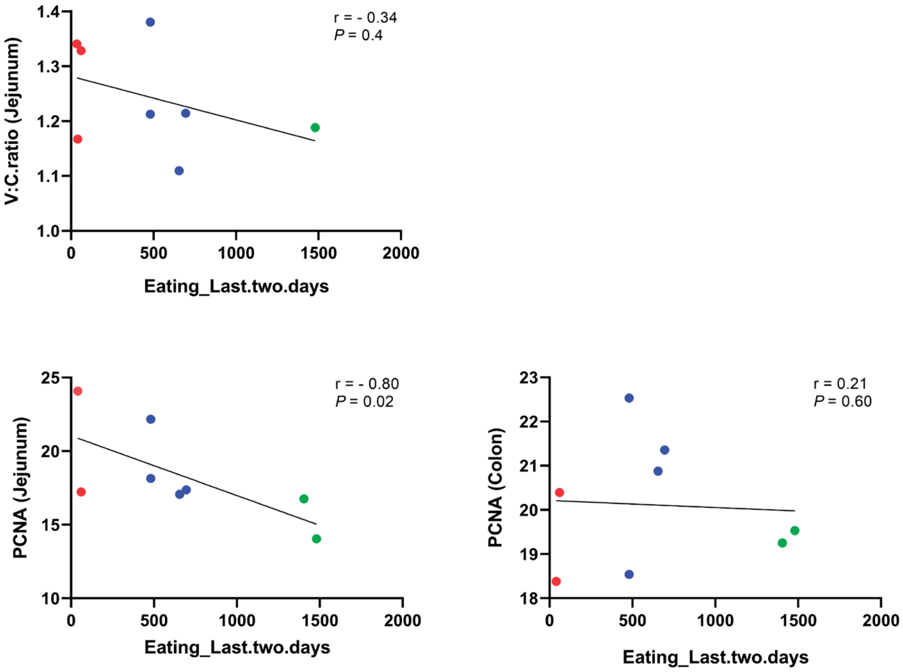
Supplementary figure 7: Concentrations of short chain fatty acids (SCFA) succinic and lactic acid in control (CON) and early-fed (EF) piglets. The EF piglets are marked with colours of EF classification based on eating observed in the "last two days" before weaning. Green = good eaters; Blue = moderate eaters; Red = bad eaters.



Supplementary figure 8: Correlation matrix (spearman) of different measured parameters of individual early-fed (EF) piglets including eating scores, microbiome RDA score, pH, SCFA, PCNA, intestinal morphometry and intestinal macroscopic measurements.



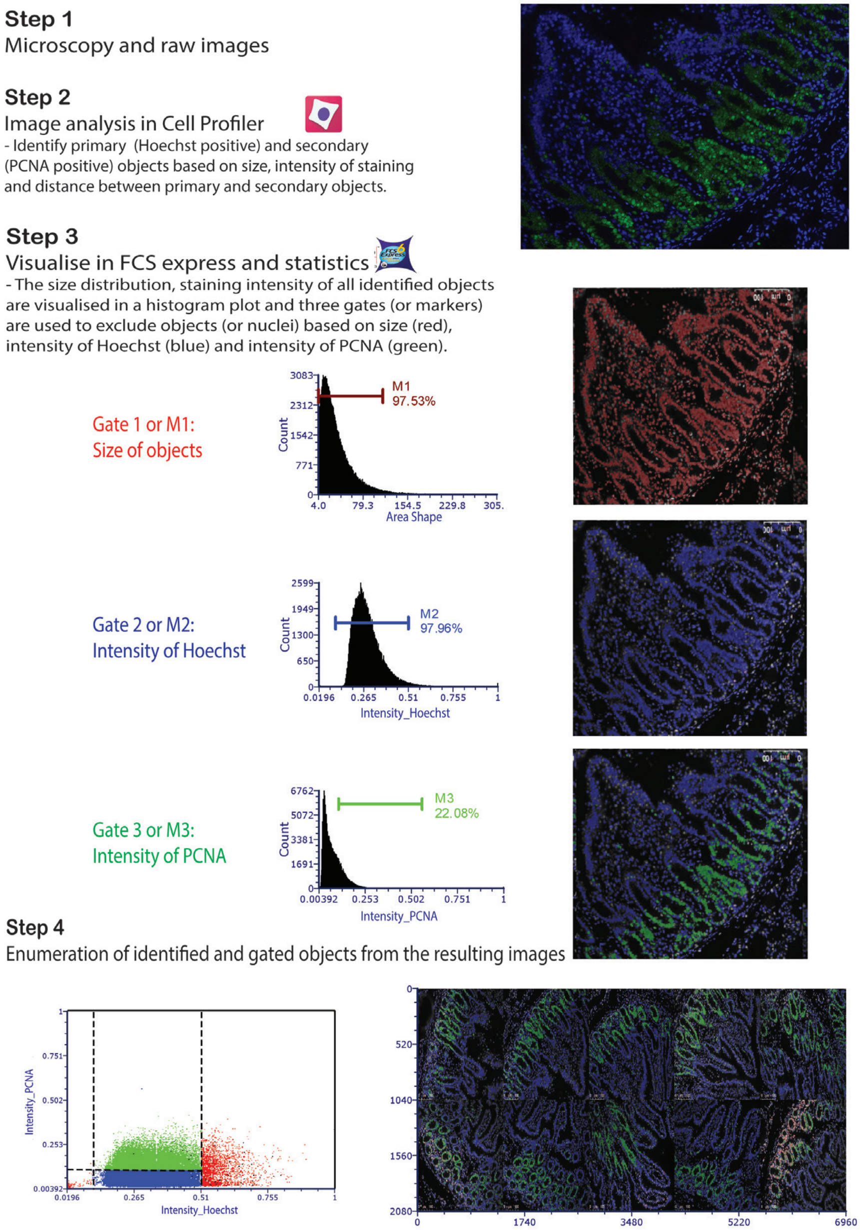
Supplementary figure 9: Representative parameters identified in the correlation matrix visualised in separate (spearman) correlation plots. The early-fed (EF) piglets are marked with colours of EF classification based on eating observed in the "last two days" before weaning (Green = good eaters; Blue = moderate eaters; Red = bad eaters). The piglets classified as good eaters are also the best eaters when "total seconds" are considered, but the moderate/bad eaters classification is less reliable, with two moderate piglets categorised as bad eaters in "total seconds".



Supplementary figure 10: Spearman correlation of microscopic measurements (V:C ratio and PCNA) with eating score. Green = good eaters; Blue = moderate eaters; Red = bad eaters.

Supplementary file 1: Quantitative immunostaining

The image analysis pipeline employed Cell Profiler 3.1.8 (Broad Institute, Cambridge Massachusetts USA; www.cellprofiler.org) to identify PCNA/Hoechst positive nuclei FCS Express 6 Flow plus Image (De Novo Software, CA, USA, www.denovosoftware.com) to visualise and enumerate the identified nuclei. The raw (16-bit grayscale) images were analysed in a pipeline developed in Cell Profiler, consisting of different modules, which carry out automated mining of cellular features in every image. The Hoechst positive nuclei (primary objects) are identified in the first module with nuclei ranging between 2 to 20 pixels in diameter and applying an adaptive threshold strategy in combination with the two-classes Otsu algorithm. In the second module, PCNA positive nuclei (secondary objects) are identified by using primary objects as a reference for guided detection. The method uses the propagation algorithm (regularisation factor, $\lambda = 0.05$), which assigns (a) dividing lines between secondary objects that touch each other and (b) dividing lines between the secondary objects and the background of the image, taking into account both the distance to the nearest primary object and intensity gradients. The subsequent modules extract features such as mean intensity, size from each object and converts identified objects back into images. The output derived from Cell profiler (.cpout file), containing the raw data associated with individual objects and analysis, was used to visualise and enumerate the number of ide ntified primary and secondary objects in FCS Express 6 Plus. The size distribution, intensity of all identified objects was visualised in a histogram and three gates (or markers) were utilized to include objects (i.e., nuclei) based on size-limits (red; gate 1), minimal Hoechst intensity (blue; gate 2) and minimal intensity of PCNA staining (green; gate 3). The flow chart below depicts the gate-settings and the corresponding images that display the included nuclei in each gating step. Gate 1 (or M1) is based on size distribution with a lower bound of Median-SDV (SDV = Standard deviation) and a upper bound of Median+3SDV, leading to inclusion of the red-stained objects (i.e., nuclei; 97 ± 0.2% of total nuclei) in the corresponding image, and exclusion of white-stained objects that mostly represent overlapping or over-exposed nuclei. Gate 2 (or M2) is based on Hoechst-staining intensity with a lower bound of Median-2SDV and a upper bound of Median+3SDV, leading to inclusion of blue-stained objects (98 ± 0.5% of the nuclei that passed gate 1). Gate 3 (M3) is based on PCNA intensity with Median+SDV and Median+10SDV as lower and upper bound, leading to inclusion of high-intensity PCNA objects (displayed as green-stained nuclei; 18 ± 3.32% of the nuclei that passed gate 2). The objects passing all three gating steps represent nuclei that are within the nucleus-size boundaries and are considered positive in Hoechst and PCNA staining. The gating values were based on the highest (visual) resemblance of the processed images (picture plot showing patterns of identified objects/nuclei in the tissue, marked by different gates) with the original microscopic overlayed-images of Hoechst and PCNA staining. From the processed images, the number of Hoechst and PCNA positive objects were obtained and the ratio of PCNA:Hoechst positive nuclei was calculated to assess the relative fraction of proliferating cells in the mucosal tissue of individual animals.



Flowchart outlining the steps for quantitative immunostaining of intestinal proliferative cells. **Step 1**: Raw microscopic image with Hoechst (blue) and PCNA (green) staining. **Step 2**: Identification of primary and secondary objects in Cell Profiler based on size, intensity of staining and distance to corresponding primary object. **Step 3**: Identified objects or nuclei were visualised and analysed in FCS Express. Three gates (or markers) were utilized to include objects (or nuclei) based on size-limits (red), minimal intensity of Hoechst staining (blue) and minimal intensity of PCNA staining (green). The intensity of Hoechst and PCNA identified objects (gated by M1, M2 and M3) is shown in a dot plot. **Step 4**: The resulting images provide the number of Hoechst and PCNA positive objects and then the ratio of PCNA:Hoechst positive nuclei was calculated.

5

Chapter 5

Early feeding leads to molecular maturation of the gut mucosa in suckling piglets

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Manuscript in preparation

Abstract

Diet-microbiota-host interactions are increasingly studied to comprehend their implications in host metabolism and overall health. Keeping in mind the importance of early-life programming in shaping intestinal mucosal development, the pre-weaning period can be utilised to understand these interactions in suckling piglets. The objective of this study was to investigate the consequences of early-life feeding on the time-resolved mucosal transcriptional program as well as mucosal morphology. A customised fibrous feed was provided to piglets (early-fed or EF group; 7 litters) from five days of age until weaning (29 days of age) in addition to sow’s milk, whereas control piglets (CON; 6 litters) suckled mother’s milk only. Rectal swabs, intestinal content and mucosal tissues (jejunum, colon) were obtained pre- and post-weaning for microbiota analysis (16S amplicon sequencing) and host transcriptome analysis (RNA sequencing). Early feeding accelerated both microbiota colonisation as well as host transcriptome, towards a more “mature state”, with a more pronounced response in colon compared to jejunum. Early feeding elicited the largest impact on the colon transcriptome just before weaning (compared to post-weaning time-points), exemplified by the modulation of genes involved in cholesterol and energy metabolism and immune response. The transcriptional impact of early feeding persisted during the first days post-weaning, and was highlighted by a stronger mucosal response to the weaning stress, via pronounced activation of barrier repair reactions, which is a combination of immune activation, epithelial migration and “wound-repair” like processes, compared to the CON piglets. Our study demonstrates the potential of early-life nutrition in neonatal piglets as a means to support their intestinal development during the suckling period, and to improve adaptation during the weaning transition.

Keywords: early-life, dietary fibre, fibrous feed, microbiota, mucosal, pig, transcriptomics

Introduction

In commercial pig farming, after weaning piglets have to deal with multiple stressors including separation from the sow, a sudden dietary shift from milk to solid feed as well as a novel environment and often unfamiliar pen-mates, leading to a transient low feed intake or anorexia, intestinal inflammation, and an unbalanced gut microbiota or dysbiosis (Lallès et al., 2007; Montagne et al., 2007; Heo et al., 2013; Gresse et al., 2017). Weaning stress in piglets is thus characterised by a multitude of changes in the intestinal physiology and function, and is often accompanied by post-weaning diarrhoea. Several studies have reported decreased intestinal digestive enzyme activities, damaged tight junction proteins, impaired immune response and barrier function, as well as altered mucosal morphometry, especially during the first week after weaning (Hampson, 1986; Pluske et al., 1997; Pié et al., 2004; Moeser et al., 2007, 2017; Campbell et al., 2013). For instance, alterations in intestinal mucosal architecture typically occurs post-weaning, often characterised by lowered villus length and deepened crypt depth (Lallès et al., 2007; Al Masri et al., 2015). Furthermore, the process of weaning triggers transcriptional changes in the intestinal mucosa of pigs, mostly related to oxidative stress and immune activation (Bauer et al., 2011; Yang et al., 2016; Xiong et al., 2019). It is therefore important to employ appropriate nutrition and management strategies to minimise the adverse effects of weaning stress, aiming to improve animal health and welfare during the weaning transition, which may also have a positive health-impact later in life.

At the time of weaning in commercial pig-husbandry systems (around 3-4 weeks of age), the gastro-intestinal tract of the piglets is still developing (Blecha, 2001), including drastic adaptations in microbiota colonisation and the mucosal immune system (Bailey et al., 2005; Moeser et al., 2017). The pre-weaning period in early-life, thus provides a “window of opportunity” to modulate and support the host gastrointestinal function of the young piglet (Pluske et al., 2018; Guevarra et al., 2019). Inclusion of fibres in pre-weaning diet (instead of milk-based creep feed) may positively influence gut function and animal health, and thus can be used as a contemporary pig management strategy to change the structure of the gut microbial communities (Jha et al., 2019). Dietary fibres are known to influence gut microbiota via microbial fermentation of fibres leading to production of short chain fatty acid (SCFAs) such as butyrate, acetate and propionate (Hamer et al., 2008). Moreover, SCFAs are recognised to influence intestinal functions such as the enhancement of the intestinal barrier function, as well as mucosal immune system development (Den Besten et al., 2013; Furusawa et al., 2013; Xiong et al., 2019).

Studies reporting on the host mucosal transcriptome response to dietary fibres are scarce (Schokker et al., 2018), especially in piglets during the pre-weaning period. The studies that assessed the dietary fibre-microbiota-host interaction mostly employed growing pigs or were performed in alternative animal models, like mice (Rodenburg et al., 2008; Haenen et al., 2013b; Hugenholtz, 2015; Lange et al., 2015; Li et al., 2019; Chen et al., 2020). Previously we have reported that early feeding of fibrous feed leads to acceleration of the intestinal microbiota composition towards a more mature microbiome in suckling piglets (**Chapter 3**). In the present study, we expand this work to evaluate the influence of early feeding and the coinciding microbiota changes on the development of the host mucosa. We therefore provided fibrous feed pre-weaning (from five days of age) and studied its impact on the host mucosal transcriptome patterns, mucosal morphology and proliferation at the moment of weaning (29 days of age). In addition, similar analyses at two time-points post weaning (3 and 21 days after weaning) enabled us to investigate the post-weaning development and evaluate the persistence of the effects of early-life feeding. Our findings establish that early feeding can accelerate mucosal transcriptome profiles towards a more "mature" state that is more closely resembling the post-weaning transcriptome profiles. Moreover, the data also demonstrate that early-fed piglets display an improved responsiveness during the weaning transition compared to the control piglets.

Materials and methods

Animals, experimental design and sampling

The Animal Care and Use committee of Wageningen University & Research (Wageningen, The Netherlands) approved the protocol of the experiment (AVD104002016515). The protocol is in accordance with the Dutch law on animal experimentation, which complies with the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

The experiment was conducted with thirteen multiparous Topigs-20 sows (Parity 2-8) and their new-born piglets (Tempo x Topigs-20), housed at research facility Carus (Wageningen University & Research, The Netherlands) (**Figure 1)**. The litters were divided into two treatment groups, early-fed or EF group (n=7 litters) and control or CON group (n=6 litters), balanced for sow parity, body weight and genetic background. Within two days after birth, the litter size was set to a maximum of 15 piglets per litter with no cross-fostering. The new-born piglets were cohoused with their mother and littermates till weaning (29 days of age), and received ear tags for individual identification and an iron injection, standard to pig husbandry practice. Piglets belonging to the EF group were given access to customised fibrous feed ***ad libitum*** (**Supplementary Table 1**) from 5 days of age in addition to suckling sow's milk, whereas the CON group suckled sow's milk only. Briefly, the feed included sugarbeet pulp (4%), oat hulls (4%), inulin (4%), galacto-oligosaccharides (5%) and resistant starch (4%) as fibrous ingredients. A subset of piglets (n=72; 36 per treatment group) were weaned at 29 days of age and followed until 21 days post-weaning. At weaning, piglets were mixed within the same treatment group and housed in separate pens, having three piglets per pen. After weaning, all piglets had ***ad*** *libitum* access to a standard weaner diet (**Supplementary Table 2**), mixed and pelleted by Research Diet Services (Wijk bij Duurstede, The Netherlands).

Rectal swabs were collected at pre- (13 days of age [day13]; n = 32) and post-weaning (19 days after weaning [day+19]; n = 16) time-points for microbiota analysis. This was done by inserting a sterile cotton swab (Puritan Medical, Guilford, ME USA; Cat Number-25-3306-U) 20–30 mm into the rectum and rotating the swab against the bowel wall for a minute before placing it into a 5ml eppendorf tube. The samples were put on ice immediately after collection, transported to the laboratory and stored at −20°C until further processing. Intestinal content and mucosal tissue samples were collected at three time-points (just before weaning [day29], 3 days after weaning [day+3] and three weeks after weaning [day+21]) following the sacrifice of a subset of piglets (10 piglets per treatment per time-point). The selection of sacrificed piglets at pre- and post-weaning time-points was made by the following criteria: a) no antibiotic treatment b) close to mean body weight of the litter c) moderate to good eaters (scan sampling for EF piglets; see below) d) no pre-weaning diarrhoea e) gender balanced for treatment. Instantaneous 1-min scan sampling (6 hours per day) on three time-points pre-weaning (15, 22 and 26 days of age), was used to determine “eaters”, allowing the selection of EF piglets that were sacrificed for bio-sampling. All piglets were scanned once each minute and the observer recorded whether piglets were eating or chewing feed from the trough or floor. Just before weaning, the data from all three observation days (60*6*3 = 1080 scans) were combined and subsequently piglets were classified into “good”, “moderate” and “bad” eaters (i.e., found to be eating or chewing feed) at least once, in 3, 2, 1 day (s) out of 3 days respectively (data not shown).

Piglets were euthanised by intravenous injection of 20% sodium pentobarbital (EUTHASOL®, 500 mg/ml, AST Farma B.V., Oudewater, The Netherlands). After euthanasia, the gastro-intestinal tract was removed and dissected immediately to collect intestinal segments (~25cm) from jejunum (1.5 metres from duodenal-jejunal flexure) and colon (mid-spiral colon). For host mucosal tissue transcriptome analyses, tissue samples (2-3 cm) were collected from the intestinal segments in RNAlater™ and snap frozen in liquid nitrogen. For microbiota analysis, jejunal and colon luminal contents were collected from the adjacent part of the segment (approximately 10cm) in a 5ml eppendorf tube and immediately frozen in liquid nitrogen. Lastly, the adjacent posterior tissue part (whole tissue; 2 cm) was collected in 4% paraformaldehyde (PFA) for histology and immunohistochemistry. All intestinal samples were stored at -80°C until further processing.

DNA extraction and 16S rRNA gene based amplicon sequencing

DNA was extracted from rectal swabs and intestinal content by the repeated bead beating method (Yu and Morrison, 2004) using QIAamp PowerFecal® DNA Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. PowerBead solution (750 μl) was added to the 5ml eppendorf tube (containing rectal swab) and mixed well to obtain the extracted swab solution that was used as a starting material for DNA extraction. For jejunum and colon samples, approximately 200 mg of luminal content (wet weight) was used for microbial DNA extraction. The quality and quantity of extracted DNA samples were checked by gel electrophoresis (only representative samples) and Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific, Wilmington, DE USA), respectively.

Library construction of the V3-V4 hypervariable region (from 16S rRNA gene) followed by sequencing on an Illumina HiSeq 2500 platform (paired end reads; 2*250 bp) were performed at Novogene (Novogene Co. LTD, China). Amplicons of the V3-V4 hypervariable region of the 16S rRNA gene, were generated using the primer set 341F/806R (341F: 5′-CCTAYGGGRBGCASCAG-3′, 806R: 5′-GGACTACNNGGGTATCTAAT-3′). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs Ltd., Ipswich, USA), according to standard protocols at Novogene. The PCR products were mixed with the same volume of 1x loading buffer (contained SYBR green) and were detected by electrophoresis on 2% agarose gel. Samples with a bright band between 400–450 bp were used for library construction. Prior to library preparation, the PCR products were mixed in equimolar ratio and purified using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing libraries were constructed using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina Inc., San Diego, USA), according to the manufacturer's instructions. Subsequently, after in-house quality check at Novogene, the sample-specific barcodes and primer sequences were trimmed from the Illumina raw reads.

16S sequencing data analysis

The trimmed paired end reads were imported into the CLC Genomics Workbench version 11.01 and were processed using the CLC Microbial Genomics Module version 2.5.1 (CLC bio, Arhus, Denmark). The paired end reads were merged into one high quality representative sequence using CLC default parameters (Mismatch cost = 1, Minimum score = 40, Gap Cost = 4, Maximum unaligned end mismatches = 5). The sequences were then clustered into operational taxonomic unit (OTUs) at 97% identity threshold, followed by taxonomic annotation using SILVA database v132 (released on Dec 13, 2017) (Quast et al., 2013). Multivariate redundancy analysis (RDA) was employed to identify microbial signatures in different time-points or treatment groups using CANOCO 5 (Microcomputer Power, Ithaca, NY, USA) according to manufacturer's instructions (Braak and Smilauer, 2012). Additionally, the linear discriminant analysis effect size (LEfSe) algorithm (Segata et al., 2011) was used to characterize the microbial differences between groups. Principal coordinate analysis (squared Bray Curtis distance) was employed to assess microbiota maturation in colon (day29 and day+3) by comparing to the relatively 'mature' post-weaning time-point (day+19) in rectal swabs.

RNA–seq library preparation and sequencing

Total RNA was extracted from the jejunal and colonic tissue samples using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. RNA quantity and quality were assessed using Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific, Wilmington, DE USA), and the Agilent 2200 TapeStation system (Santa Clara, CA, USA). All RNA samples were found to be suitable for RNA sequencing, based on the minimum requirements that the sample showed intact 18S and 28S ribosomal RNA bands, and had a RIN (RNA Integrity Number) above 8.0.

The RNA samples were transferred to Novogene (Novogene Co. LTD, China) for library preparation and RNA sequencing. Briefly, 1 μg total RNA in 50 μl was used for library preparation using NEBNext® Ultra Directional RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, US) following the manufacturer's protocol. After RNA quality verification, mRNA was enriched using NEBNext Oligo d(T), fragmented randomly and first strand cDNA was synthesised using random primers. Second Strand cDNA was synthesised using dNTPs (with dUTP replacing dTTP). The double-stranded cDNA was then purified using Agencourt AMPure XP Beads (Beckman Coulter, Beverly, MA, USA), followed by end repair of cDNA library and adaptor ligation. The adaptor ligated DNA was PCR enriched and the PCR products were purified by Agencourt AMPure XP Beads. The library quality was assessed by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), Qubit 2.0 fluorometer (Thermo Fisher Scientific,Waltham, MA USA) and qPCR (iCycler, BioRadLaboratories, Hercules, CA, USA). The libraries were sequenced on Illumina Novaseq6000 (Illumina, San Diego, CA, USA) by Novogene (Novogene Co. LTD, China) at 6 Gb raw data/sample with 150 bp paired-end reads. The raw reads (in fastq format) were first processed using in house scripts. Reads with adapter sequences, poly-Ns, and low quality were removed to obtain a clean-read dataset. Read counts and bases were summarized for both raw reads and clean reads. Additionally, Q20, Q30, error rate, and GC-content of clean data were also calculated.

Differential expression analysis and biological interpretation of transcriptome data

Clean sequencing reads were imported in the CLC Genomic Workbench (QIAGEN, Aarhus, Denmark), mapped to the *Sus scrofa* 11.1 reference genome, resulting in read counts per gene which was used for downstream analysis. Differential gene expression (DGE) analyses of the treatment groups were performed using 'Empirical analysis of DGE' tool in CLC workbench, that employs the 'Exact test' (Robinson and Smyth, 2008) incorporated in the EdgeR bioconductor package (Robinson et al., 2010). Lowly expressed genes (< 5 counts per million [CPM], in more than 80% of the samples) were excluded from further analysis according to EdgeR pipeline (Robinson and McCarthy, 2010). The web-based galaxy platform (https://usegalaxy.org/; RNAseq tool in the EdgeR package) was used to obtain the normalised counts (logCPM) of the filtered gene list (~12000 genes). Further, the unknown Ensemble gene IDs (*Sus scrofa*) in the list were converted into gene names using g:Profiler (g:Convert tool) (Raudvere et al., 2019), with 435 (colon) and 416 (jejunum) out of 1332 of these unknown IDs attaining gene annotation. Using this (filtered, normalised) gene table, principal component analysis (PCA), principal co-ordinate analysis (PCoA, squared Bray Curtis distance) and principal response curve (PRC) analyses were performed in CANOCO 5 (Microcomputer Power, Ithaca, NY, USA)

(Braak and Smilauer, 2012). Principal response curve analyses detected temporal changes in transcriptome and its interaction with early feeding treatment. In addition, the significance of the interaction was tested using MonteCarlo permutation test (499 permutations). Among the total of 120 samples, two were classified as outliers (Crosby et al., 1994; Rousseeuw and Hubert, 2011), employing modified Z scores of summed PC components (**Supplementary figure 1A**) and removed from further analysis.

To identify canonical pathways associated with up- and down-regulated genes in the EF group relative to the CON group, Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood city, CA, USA) was employed, which uses curated information from the Ingenuity Knowledge Base (www.ingenuity.com). IPA provides statistical assessment (based on Fisher's exact test) of biological pathway enrichment, by determining whether there are non-random associations. The statistical test calculates the probability of genes associated with a pathway (from the dataset) relative to the total number of genes that define the canonical pathway within the IPA knowledge base. The list of differentially expressed genes was uploaded into the IPA software, containing gene identifiers and corresponding fold change (FC; absolute FC > 1.2) and P values (< 0. 05). Canonical pathways identified in IPA, having logP value ≥ 1.3 (or $P < 0.05$; enrichment score from Fisher's exact test) and an absolute Z score ≥ 2 (assessing the match of observed and predicted up/down regulation patterns) (Qiagen, 2014), were subsequently visualised in GraphPad Software 8.1.1 (California, USA). Following primary DGE analysis (i.e., the convergence of post-weaning transcriptome at day+21; see results), the gene expression changes over time were evaluated by normalising expression values (logCPM; per group per time-point) by scaling to the mean expression value of day+21 (irrespective of treatment), and visualised in Multiple Experiment Viewer (MeV) version 4.9.0 (http://mev.tm4.org/#/welcome) (Saeed et al., 2003). In addition, unsupervised hierarchical clustering (euclidean distance) of the identified genes was performed in MeV, for group comparison over time.

To assess the functional interactions among pathway-associated genes, the Reactome FIviz (functional interaction) application (Wu et al., 2014) in Cytoscape 3.7.1 (Shannon et al., 2003) was employed. The genes corresponding to enriched pathways in EF group (identified in IPA; unique to one or more time-point(s)) were used to build the functional networks ('Gene Set Analysis' tool) utilising information from the Reactome FI database (Croft et al., 2014), which is an expert-curated, peer reviewed database of human biological pathways. Prior to building the network, the porcine gene IDs needed to be converted into their human homologues using g:Profiler (g:Orth tool) (Raudvere et al., 2019).

Histology analysis and immunohistochemical staining of colonic proliferating cells

Intestinal tissues (jejunum and colon) collected at three time-points day29, day+3 and day+21 were fixed in 4% paraformaldehyde (PFA), and then dehydrated and embedded in paraffin blocks

(8 animals per treatment per time-point per location). Histological and immunohistochemical staining were performed as previously described (**Chapter 4**). Briefly, 5 μm sections were cut with a Accu-Cut® SRM™ 200 Rotary Microtome (Sakura Finetek Europe B.V., Alphen aan de Rijn, The Netherlands), deparaffinized, hydrated, stained with Haematoxylin-eosin (H&E) and examined using a Leica DM6 B microscope (Leica Microsystems Ltd. CH9435 Heerbrugg). Images (5x magnification) were processed with LAS X software (Leica Microsystems Inc., Buffalo Grove, IL, USA) to measure villus length and crypt depth (μm) from tissue sections (60 measurements per animal: 3 sections per animal*20 measurements per section). For immunohistochemical staining of colonic proliferating cells, 5 μm sections (from 8 animals per treatment per time-point) were deparaffinized, rehydrated and treated for antigen retrieval in citrate buffer (pH 6.0) at 95°C for 20 min, followed by cooling in tris-buffered saline and tween 20 (TBSt) buffer and blocking with 10% normal goat serum (Invitrogen™). To detect proliferating cells, sections were incubated with primary antibody (anti-PCNA antibody, PC10 mouse anti-rat IgG2a monoclonal antibody, Merck-millipore, Darmstadt, Germany, MAB424R; 1:500) and secondary antibody (Goat anti-Mouse IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 555, ThermoFisher Scientific, Waltham, Massachusetts, USA; 1:300). Nuclei were stained with Hoechst 33342 Solution (Invitrogen, ThermoFisher; 1:1000 dilution). 10 high quality 16bit grayscale images were captured per animal at 20X magnification (80 representative images/treatment) using Leica DM6b microscope fitted with appropriate fluorescence filters along with their corresponding nuclei images.

Image analysis was done using a semi-automated in-house workflow that we described previously (**Chapter 4**), encompassing image data-extraction by Cell Profiler 3.1.8 (Broad Institute, Cambridge Massachusetts USA; www.cellprofiler.org) and analysis in FCS Express 6 Flow plus Image (De Novo Software, CA, USA, www.denovosoftware.com). The relative number of proliferating cells was obtained by normalising with the total number of Hoechst positive nuclei (PCNA:Hoescht ratio) in each image.

Other statistical analysis

Normality of data (Shapiro-Wilk test) and statistical differences were checked with a limit of significance set at $P < 0.05$ in GraphPad Software 8.1.1. Comparison of the squared Bray Curtis distance, histological morphometric measurements and proliferating cells between treatments were performed by Mann Whitney U-test (non-parametric) or t-test (parametric), whereas comparison among different time-points (histological morphometric measurements and proliferating cells) were assessed by one-way ANOVA (parametric) or Kruskal-Wallis test (non-parametric) using a Dunnett’s test for multiple comparisons.

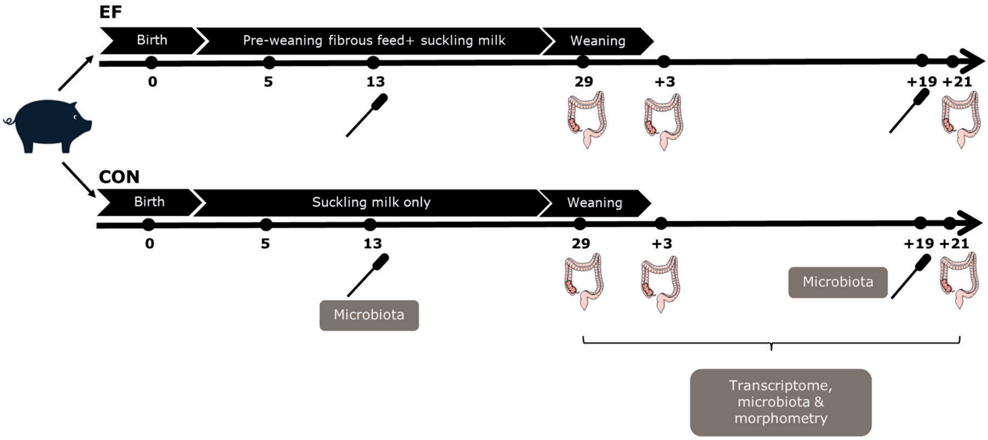

Figure 1: Schematic experimental design and sampling. Rectal swabs were collected at day 13 and d+19 for pre-and post-weaning microbiota analysis. Jejunal and colon samples were collected from the sacrificed piglets to perform transcriptome, microbiota and intestinal morphometry analyses at the time of weaning (day29), 3 days post-weaning (day+3) and 21 days post-weaning (day+21). Notably, luminal samples at day+21 were not included for microbiota analysis, instead the rectal swab samples at day+19 were used as a proxy, to reflect the 'relatively mature' post-weaning microbiota composition.

Results

Pre-weaning fibrous diet accelerates gut microbiota maturation in early-fed piglets

We have previously shown that pre-weaning consumption of fibrous feed accelerates the development of the intestinal microbiota in suckling piglets (**Chapter 3**), which is underpinned by a pre-weaning microbiome that is more adapted towards a typical post-weaning microbiota composition. As the present study employed a similar intervention design, our initial analyses were geared to confirm the anticipated impacts of early feeding with fibrous feed on the colonisation pattern and maturation of the gut microbiota.

The typical pre- and post-weaning associated microbiota compositions were analysed and compared using rectal swabs obtained at 13 days of age to reflect the pre-weaning microbiota (preceding substantial eating behaviour of the fibrous feed) and 19 days post-weaning (day+19) as a reflection of the typical post-weaning microbiota (**Chapter 3**). Specific microbial groups were associated with the pre- and post-weaning stages irrespective of the early feeding treatment (**Figure 2A**), which was corroborated by analysis of the individual time-points day13 and day+19 that did not reveal any treatment-associated differences (**Supplementary figure 2A, B**). Notably, the microbial groups that were most discriminant between the pre-weaning (day 13) and post-weaning (day+19) microbiota composition strongly resembled those found in our previous study (**Chapter 3**), which is exemplified by more than 50% overlap in the microbial genera identified.

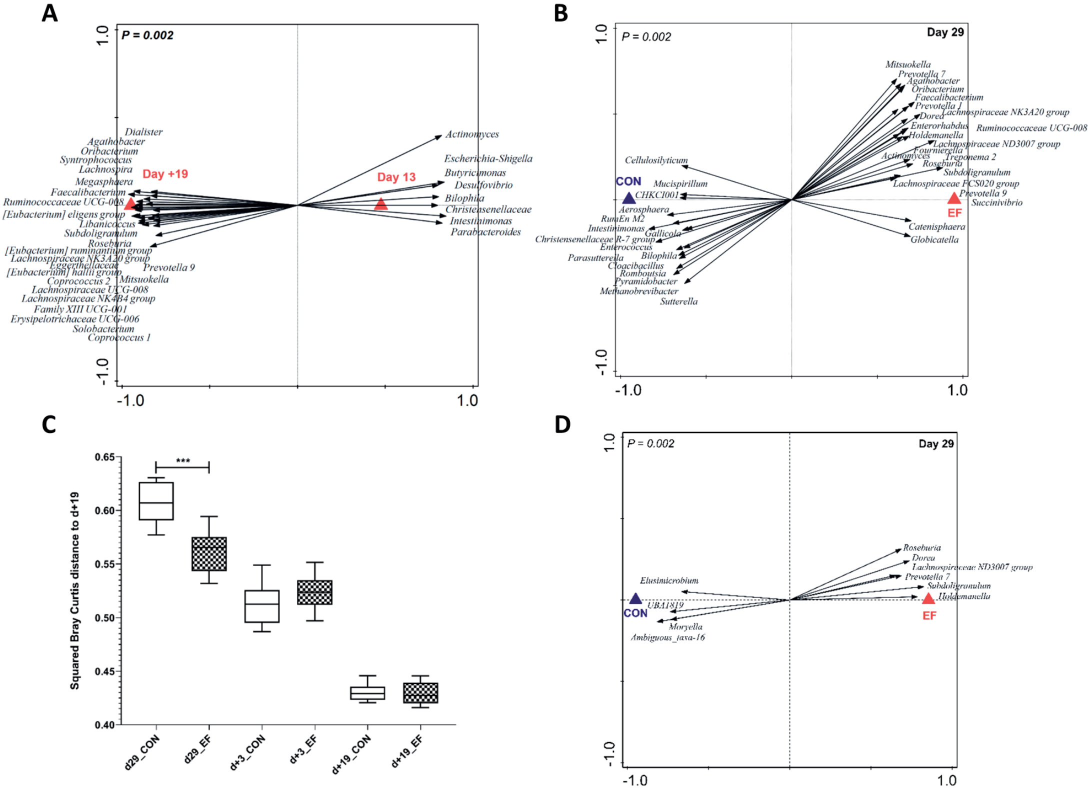

Figure 2: Pre-and post-weaning microbiota composition (irrespective of treatment) and pre-weaning acceleration in the early-fed (EF) group as compared to the control (CON) group (genus level). (A) Redundancy analysis of pre- (d13) and post-weaning (d+19) time-points (explained variation = 41.81%; *P* = 0.002) with associated microbial groups. Microbial groups visualized have a minimum response score of > 0.80 on horizontal axis. **(B)** Redundancy analysis (colon, d29) of the EF and CON group (explained variation = 16.1% ; *P* = 0.002) with discriminating microbial groups (response score > 0.60). **(C)** Comparison of squared Bray Curtis index (distance to d+19 time-point) over time, between the two groups. **(D)** Redundancy analysis (jejunum, d29) of the CON and EF group (explained variation = 11.6% ; *P* = 0.002) with discriminating microbial groups (response score > 0.65) at genus level. Significant differences between groups were assessed by student t tests or Mann-Whitney U tests (***: *P* < 0.001).

Colon microbiota analysis of sacrificed piglets at weaning (29 days of age) revealed that early-fed (EF) piglets had significantly expanded microbial genera (**Figure 2B**; **Supplementary figure 2C**) that are typically associated with the post-weaning microbiota (**Figure 2A**), including *Prevotella, Subdoligranulum, Faecalibacterium, Roseburia* and *Megasphaera*. Conversely, colon samples obtained from control (CON) piglets appeared to be enriched in microbes belonging to *Enterococcus*, *Intestimonas, Christensenellaceae R-7 group, Romboutsia*, *Methanobrevibacter* (**Figure 2B**; **Supplementary figure 2C**) which are associated with the pre-weaning microbiome (**Figure 2A**). Besides evaluating “accelerated-maturation” at weaning, we also assessed the “persistence of the impact of early feeding” 3 days post-weaning (day+3).

We observed that the pre-weaning microbiota differences in the colon (day29) persisted in a modest way to the first time-point analysed after weaning (day+3), which is reflected by the higher relative abundance of the genera ***Coprococcus, Marvinbryantia, Selenomonas, Prevotella, Prevotellaceae NK3B31 group, Eubacterium xylanophilum, Catenisphaera*** and ***Agathobacter*** in the colonic microbiota of EF piglets versus ***Peptostreptococcus*** and ***uncultured-39*** found in the CON group (**Supplementary figure 2D**). In contrast, and according to our anticipation, the pre-weaning treatment effects on the colonic microbiota were no longer detectable at the later post-weaning time-point (day+19) (**Supplementary figure 2B**). The accelerated microbiome development in EF piglets was further underpinned by the significantly lower Bray Curtis distance of the EF samples at 29 days of age, relative to the 'mature' post-weaning time-point (day+19) (**Figure 2C**), where microbiota convergence was observed.

In the early-life intervention study presented here, we also detected a significant effect of early feeding on the jejunal microbiota on day29 (**Figure 2D**), which contrasts with a previous study using a similar design (**Chapter 4**). Remarkably, the distinctive microbial groups were previously identified to reflect the early feeding intervention in the colon microbiota (i.e., EF-associated) including the increased relative abundance of ***Roseburia, Lachnospiraceae ND3007 group, Prevotella, Subdoligranulum, Holdemanella, Dorea***. Notably, the jejunal microbiota differences elicited by early feeding were no longer detectable post-weaning (day+3), which could reflect a more modest impact of early feeding observed in this intestinal region. Although the impact of early feeding on the jejunal microbiota is moderate compared to the colon microbiota, these findings are suggestive of a similar influence of early feeding on small and large intestinal locations, although with a varying degree of strength.

Early feeding leads to "accelerated maturation" in intestinal mucosal transcriptome

To explore the consequences of early feeding on the time-resolved mucosal transcriptional program, we conducted transcriptome analyses (in jejunal and colonic tissue) on the day of weaning (day29), and two time-points post weaning (day+3 and day+21). At each time-point 10 animals from both groups (CON and EF) were sacrificed to harvest jejunal and colonic tissue samples, which were processed for different purposes (see M&M for details, and see below), including tissue transcriptome analysis using RNA sequencing on an Illumina Hiseq platform, generating an average of 25 million PE reads per sample.

Global comparison of both colonic and jejunal transcriptome profiles by principal component analysis (PCA; **Figure 3A, B**) revealed a clustering of samples based on age, irrespective of the pre-weaning treatment (EF versus CON). To investigate the impact of early feeding on the jejunal and colonic transcriptome, differentially expressed genes (DEG; EdgeR test) were assessed at each time-point separately. These analyses revealed that day29 has the most DEG in colon tissue, and these differences between treatment groups decrease over time or

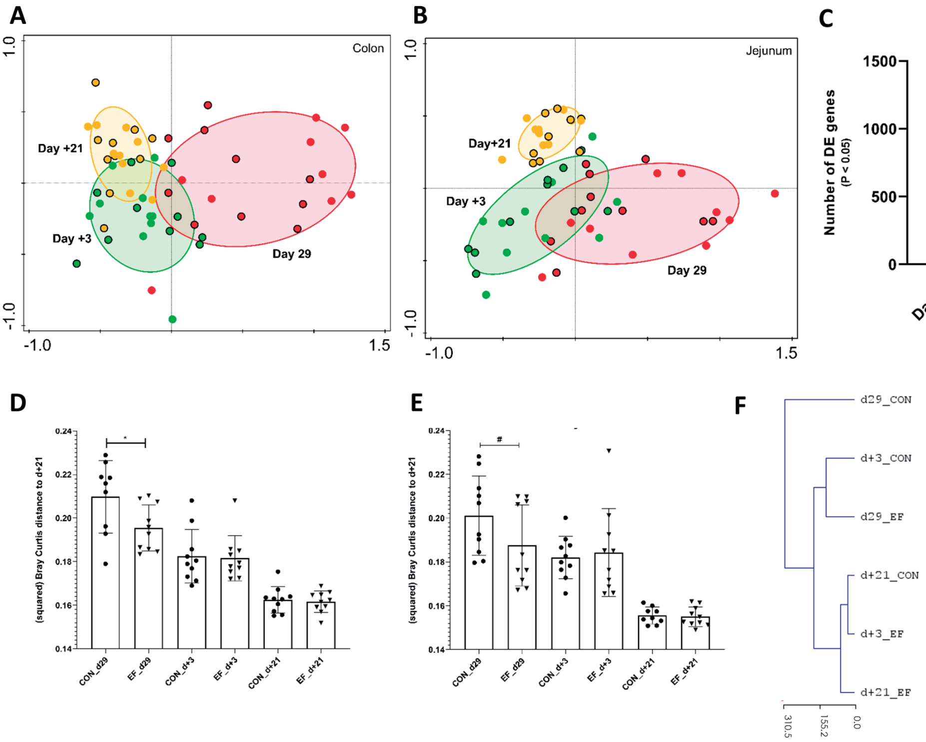

Figure 3: Jejunum and Colon transcriptome alterations over time (pre- and post-weaning). **(A)** Principal component analysis of whole colon transcriptome (PC1 = 26.6%, PC2 = 10.4%) at day29 (pre-weaning; red), day+3 (post-weaning; green) and day+21 (post-weaning; yellow) denoted by circles with (Early-fed or EF piglets) and without (control or CON piglets) black border. **(B)** Principal component analysis of whole jejunal transcriptomics (PC1 = 21.1%, PC2 = 11.6%) at day29, day+3 and day+21. **(C)** Number of differentially expressed genes (DEG; EdgeR test: *P* value < 0.05) at day29, day+3 and day+21 in colon (red) and jejunal (black) samples. **(D)** CON and EF group comparison of squared Bray Curtis distance at day+21 time-point in **(D)** colon and **(E)** jejunum. **(F)** Unsupervised hierarchical clustering (euclidean distance) of whole colon transcriptome using normalised (logCPM) expression values (averaged per group per time-point) and scaled by the mean value of day+21 gene expression (irrespective of treatment).

"converge" post-weaning (**Figure 3C)**. The number of DEG (*P* < 0.05) decreased from 1366 genes (at day29) to 573 (at day+3) and 270 (at day+21) genes, respectively, however, the number of differential genes post-weaning became negligible when the *P* value was corrected for multiple testing (False discovery rate or FDR < 0.1; **Supplementary figure 1B**). Much less DEG were observed in jejunal tissue (compared to colon), and the number of DEG remained more or less similar over time (*P* < 0.05; **Figure 3C**), although FDR correction (**Supplementary figure 1B)** somewhat refined this conclusion, demonstrating a declining number of DEG also in the jejunal transcriptome, analogous to the colon. To evaluate the development of gene expression over time, we compared the treatment groups in relation to the last post-weaning time-point (day+21), where we observed the convergence of the transcriptome data in EF and CON groups.

Strikingly, at day29 the colon transcriptome profile of the EF group had a significantly smaller distance (Bray Curtis) to the "convergent transcriptome average of both groups" (day+21) compared to the CON group (**Figure 3D**). Similarly, the day29 jejunal transcriptome profile of the EF group tended to resemble the day+21 "convergent jejunal transcriptome of both groups" more closely as compared to the CON group ($P = 0.06$, **Figure 3E**). These observations were also evident in PCA analyses (**Figure 3A, B**) that illustrate the EF day29 transcriptomes (red circles with black border) clustering in closer proximity to the day+21 transcriptome profiles relative to the CON day29 transcriptome (red circles). This was further corroborated by principal response curves (PRC) analysis which assessed the temporal effects of the EF treatment and detected the biggest impact of early feeding at day29 in the colon (5.4% of total variation; 499 permutations; $P = 0.01$), as well as by unsupervised hierarchical clustering of the colon transcriptome, positioning the day29 CON group transcriptome farthest from the post-weaning time-point and highlighting its closer resemblance to the day29 EF group transcriptome (**Figure 3F**). Remarkably, the EF group transcriptome determined on day+3 appeared to be more similar to the average day+21 transcriptome profile, as compared to the CON transcriptome. Unlike colon, we did not detect a significant impact of early feeding over time in jejunum (PRC; 4.8% of total variation; $P = 0.12$), thereby reflecting the relatively moderate impact of early feeding on the jejunum. The unsupervised hierarchical clustering of the jejunal transcriptome positioned the day+3 CON farther from the post-weaning (day+21) time-point compared to the day+3 EF (**Supplementary figure 3A**), which might indicate the impact of EF on jejunum more relevant at day+3. Overall, these results illustrate that the largest effect of early feeding is detected at the end of the pre-weaning stage (day29), and that this effect rapidly diminishes after weaning to converge to indistinguishable transcriptome profiles three weeks post-weaning (day+21). Moreover, this also reveals that early feeding appears to induce an acceleration of the molecular development of the intestine mucosa, particularly in the colon mucosa.

Functional analysis of the DEG due to early feeding

To further explore the transcriptional response due to early feeding, pathway analysis was performed on the DEG at individual time-points. Analogous to the larger number of DEG identified in the colon transcriptome at day29, more up- and down-regulated canonical pathways were significantly enriched at day29 (32 canonical pathways) compared to the post-weaning time-points day+3 (10 canonical pathways) and day+21 where no pathways appeared to be enriched (**Supplementary figure 3B**). These analyses re-affirmed the convergence of the colon transcriptome within 3 weeks post-weaning in the treatment groups. On the other hand, reflective of the lower and more consistent numbers of DEG identified in the comparison of the jejunal transcriptomes, pathway analysis at all three time-points (day29, day+3, day+21) identified only 3 and 7 significantly enriched canonical pathways (in EF as compared with CON piglets) at day29 and day+3, respectively (**Supplementary figure 3C**).

In the EF group, oxidative phosphorylation and cholesterol biosynthesis pathways were significantly upregulated in the colon mucosa at day29 (**Figure 4A**). In addition, oxidative stress related pathways such as glutathione redox reactions, ketogenesis and fatty acid ß oxidation pathways were enriched in EF compared to CON piglets. On the other hand, the significantly downregulated pathways in EF group were sirtuin signalling and immune response pathways (**Figure 5A**), such as CD28 signalling in T Helper cells, calcium induced T Lymphocyte Apoptosis, GP6 signalling, Th1 pathway and NFκB signaling among others. Three days after weaning (day+3), the colon transcriptome in EF piglets displayed upregulation of pathways such as Integrin, Leukocyte extravasation, p38 MAPK, Netrin, TREM1 signalling, associated with extra-cellular matrix (ECM), immune response and external (stimulus) stress response (**Supplementary figure 3B**), which could reflect the tissue's adaptive response to "weaning-associated stress".

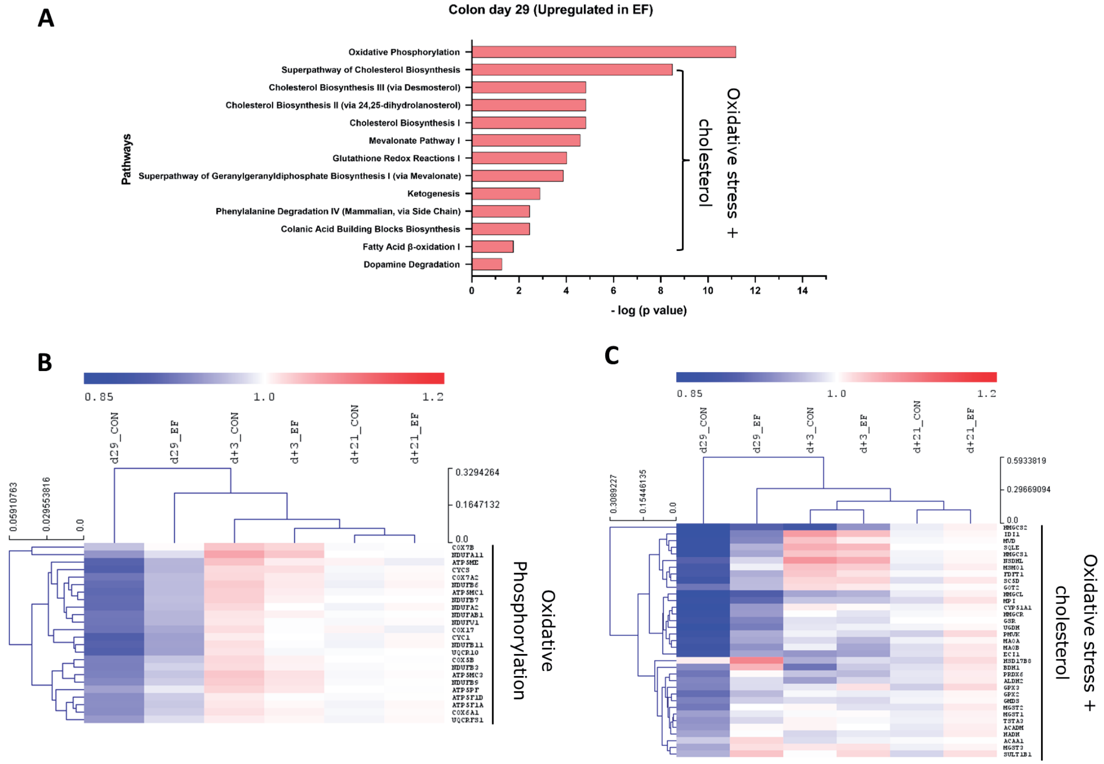

Figure 4: Pathway analysis (upregulated) in colon mucosa. (A) Canonical pathways upregulated in the early-fed (EF) group compared to the control (CON) group at day29. Identified in Ingenuity pathway analysis (IPA) having logP value ≥ 1.3 (enrichment score from Fisher's exact test) and an absolute Z score ≥ 2 (assessing the match of observed and predicted up/down regulation patterns). Hierarchical clustering of expression profiles over time for **(B)** Oxidative phosphorylation pathway genes **(C)** Oxidative stress + cholesterol related pathway genes including pathways including glutathione redox, ketogenesis, dopamine degradation. The normalised expression values (averaged per group per time-point) are scaled by the mean value of total day+21 expression (irrespective of treatment).

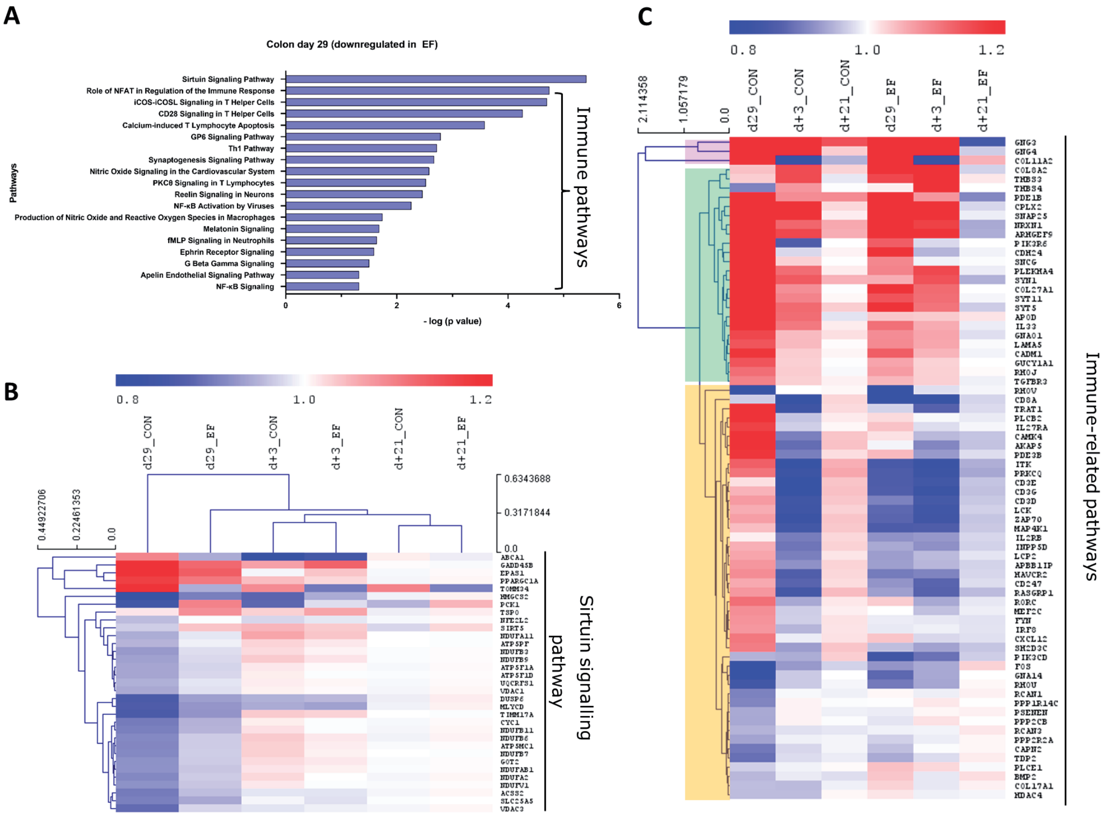

Figure 5: Pathway analysis (downregulated) in colon mucosa. (A) Canonical pathways downregulated in the early-fed (EF) group compared to the control (CON) group. Identified in Ingenuity pathway analysis (IPA) having logP value ≥ 1.3 (enrichment score from Fisher's exact test) and an absolute Z score ≥ 2 (assessing the match of observed and predicted up/down regulation patterns). **(B)** Hierarchical clustering of expression profiles over time for sirtuin signaling pathway genes. **(C)** Heatmap showing immune pathway genes (without hierarchical clustering). The normalised expression values (averaged per group per time-point) are scaled by the mean value of total day+21 expression (irrespective of treatment).

Notably, the persistence of pre-weaning altered pathways was not observed post-weaning in colon transcriptome of EF piglets. However, jejunum showed persistence in the upregulation of oxidative phosphorylation pathway at both day29 and day+3 (**Supplementary figure 3C**), although a modest numbers of genes were involved.

To investigate the evolution of gene expression in EF and CON groups over time, unsupervised hierarchical clustering was performed using the "point of convergence" (averaged day+21 expression values for each gene; irrespective of prior treatment) to scale and compare the transcriptome profiles over time. Time-resolved (colonic) gene expression patterns related to oxidative phosphorylation and oxidative stress-related pathways (including cholesterol pathway) displayed clear convergence of the gene expression towards day+21 (**Figure 4B, C**). In addition, the EF group (day29) was substantially closer to the post-weaning "convergent" time-point (day+21), compared to the CON group at the same time-point (**Figure 4B, C**), confirming an accelerated activation of these pathways due to early feeding. Sirtuin signalling associated genes displayed similar behaviour as we observed with the upregulated pathway genes, with similar expression as the post-weaning time-point (day+21) (**Figure 5B**), thus affirming the acceleration of the gene expression program due to early feeding. Notably, the hierarchical clustering of the DEG associated with immune system functions appeared predominantly age-driven (**Supplementary figure 4A**). However, this age-dependent gene expression conclusion was dictated by a subset of the immune system associated genes (purple and green clusters in **Figure 5C**). On the other hand, the other subset of immune system associated genes (yellow cluster in **Figure 5C**) did display the typical accelerated evolution of expression in EF piglets when analysed separately (**Supplementary figure 4B**), as previously seen in other pathways. Overall, we detect a mixed observation in immune-related genes where the upper panel of genes displays age-related programs, and the lower panel displays acceleration by the early feeding treatment.

The leading genes of day+3 in colon showed a similar acceleration pattern in the EF group as observed previously, with both day29 and day+3 clustering with the convergent day+21 transcriptome (**Supplementary figure 4C**). Of note, only one gene, GADD45B (part of sirtuin pathway; involved in cell cycle regulation) was found to be common on both day29 and day+3, although not significant at day29 and with an inverse fold change at day+3 (*P* = 0.01). Though in jejunum much fewer altered pathways were detected in EF as compared to the CON group, hierarchical clustering of those pathway identified genes at day29 and day+3 also support the accelerated (pre-weaning) maturation in the EF group (**Supplementary figure 4D, E**).

Additionally, protein-protein interaction networks were generated using the pathway associated leading gene in both day29 and day+3 (Cytoscape, Reactome FIviz plugin). From the colon transcriptome, a total of 180 pathway identified genes combining day29 (142 genes) and day+3 (38 genes) were used to construct the interaction network (**Figure 6**). In the network, the most prominent gene clusters were associated with oxidative phosphorylation, immune-related and sirtuin signalling pathways. Both oxidative phosphorylation and sirtuin signalling pathways are known to be active in mitochondria for redox metabolism, having common genes observed in the network such as ATP5F1A, ATP5F1D, ATP5MC1, ATP5PF (mitochondrial ATP synthase subunits), CYC1 (cytochrome bc1 complex subunit), NDUFA2, NDUFA11, NDUFB3, NDUFB6, NDUFB7, NDUFB9, NDUFB11, NDUFAB1, NDUFV1 (NADH:ubiquinone oxidoreductase subunits) and UQCRFS1, that encode key enzyme complexes in the electron transport chain reaction. Besides, sirtuin signalling is also involved in homeostasis and cellular adaptive response to external environmental stimuli, which can be observed in the altered transcription-regulating gene expression of PPARGC1A, NFE2L2 and EPAS1 (**Figure 6**), indicating a link between external stimuli and the regulation of cellular metabolism in the colon. Furthermore, PPARγ was identified as one of the potential upstream transcriptional regulators for the mucosal gene expression response to fibrous feed (Activation Z score = 3.4; Upstream Regulator Analysis, IPA). The other important large gene cluster in the network represents immune-related pathways, including tyrosine kinase family hub genes such as FYN, LCK, that are involved in cell growth and immune activation, chemokine receptors (CXCL12), as well as T cell development (proliferation, differentiation) and signal transduction (antigen recognition) genes like LCP2, ZAP70, ITK, TRAT1, CD3D, CD3E, CD3G, CD247, IL33 and IL2RB (**Figure 6**). Interestingly, this cluster of immune genes were previously observed to be deviating in the CON group at day+3, displaying an abrupt change of gene progression from day29 to day+21 (**Figure 5C**), which was not observed in the EF group.

In the functional network, we clearly observed the immune system to be more responsive in the EF piglets at day+3 potentially due to the newly emerging post-weaning situation (**Figure 6**). This is reflected by the activation of inflammation-related genes such as IL18, IL1RL1, TGFB1,TGFB2; leukocyte extravasation (i.e., leukocyte migration from peripheral blood into tissues site of inflammation) pathway identified genes including MMP12, MMP24, MMP25, as well as ECM (Integrin and Paxillin signalling; ACTG2, ACTN1, ACTN2, ITGA1, ITGA5, RHOD, RHOBTB1, VCL) genes that regulate cell migration and intercellular communication during inflammation/stress. MAPK11 can be one of the hub genes or central regulator of the above mentioned biological processes, as observed in the functional network. Notably, GADD45B gene (part of senescence and sirtuin pathway) was found at both day29 and day+3, with significant upregulation (fold change = 1.2; $P = 0.01$) after weaning (day+3), and is known to arrest cell growth and proliferation via blocking cell cycle proteins encoded by CDK1 and CCNB2, as seen in the network (**Figure 6**).

Even though jejunum mucosal transcriptomes only displayed a modest impact of early feeding (as described before), we investigated the functional interaction in the jejunum using a total of 43 pathway identified genes combining day29 (13 genes) and day+3 (32 genes) to build the interaction network (**Supplementary figure 5**). As anticipated, a substantially smaller functional network was observed, mostly revealing interactions between genes involved in oxidative phosphorylation and sirtuin signalling pathways, and some smaller networks associated with immune system functions. Notably, genes belonging to oxidative phosphorylation such as ATP5MC1, ATP5MC3, ATP5ME, COX17, COX7A2, NDUFA2 were found in both jejunum and colon at day29, whereas only one gene RHOD (Rho-related GTP-binding protein; involved in reorganization of the actin cytoskeleton and membrane transport) was found to be common between jejunum (part of ILK signalling) and colon (part of Integrin signalling) at day+3. Taken together, these findings indicate that EF piglets show an “alerted system” that displays an enhanced responsiveness to external stimuli of feed and microbiome development in colon and moderately in the jejunum, compared to the CON group.

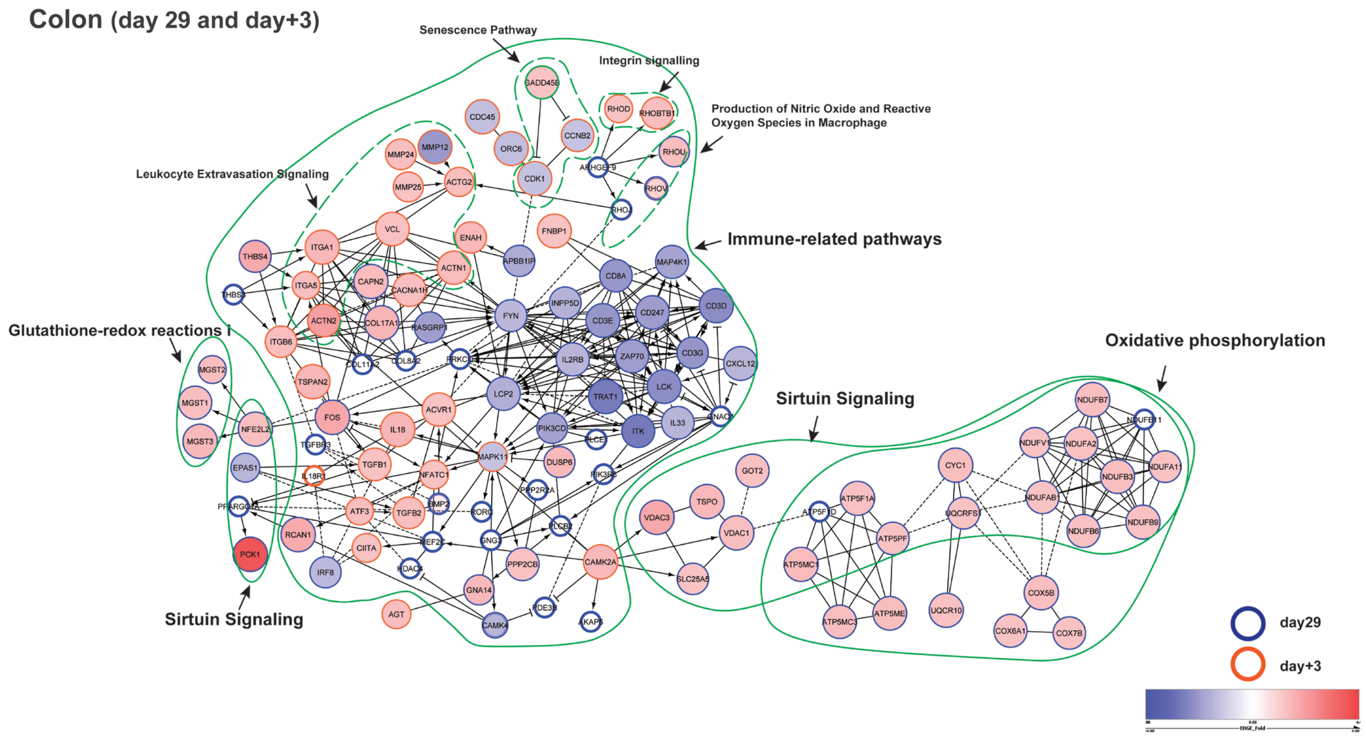

Figure 6: Functional network of pathway identified genes (colon) at day29 and day+3. Functional protein-protein interaction of up- and down-regulated genes in early-fed (EF) compared to control (CON) group. The circles or nodes represent genes and edges represent interactions between genes, as determined by Reactome. Arrows represent directed interactions, bar-headed arrows indicate inhibition reactions and dotted lines indicate predicted relationships. Pathway identified genes from time-points day29 (blue bordered) and day+3 (orange bordered) are shown in this figure. The upregulated (red nodes) and down-regulated (blue nodes) genes are represented by their fold change (EdgeR test) in a blue-red gradient scale, where the size of the node is proportional to their significance (*P* value; EdgeR test). The genes having *P* value > 0.1 are depicted as white nodes. The dotted green lines depict different pathways associated with those genes. Of note, the gene GADD45B (part of senescence and sirtuin pathway) was observed at both day29 (fold change =–1.3; *P* = 0.13) and day+3 (fold change = 1.2; *P* = 0.01).

Effect of early feeding on mucosal morphometry and proliferative cells at pre- and post-weaning period

In this study, we also investigated the intestinal mucosal changes (over time) in CON and EF piglets by evaluating their mucosal morphometry. Jejunum and colon samples (n = 8 per group per time-point per location) were assessed using H&E stained tissue images obtained at day29, day+3 and day+21. These analyses revealed clear age-related morphometric development of the jejunum mucosa, which was especially apparent in increasing crypt depth over time, while the villus length and width were not significantly different between the weaning day (day29) and three weeks post weaning (day+21). This observation is paralleled by a crypt depth dependent decrease in villus:crypt ratio over time (**Figure 7A**). Analogously, in colonic mucosa the crypt depth also increased over time, which was especially clear at day+21 (**Figure 7B)**. These differences between day29 and day+21 were observed in both groups of piglets (EF and CON).

Comparative analysis between the CON and EF group was performed for each morphometric measurement. Remarkably, at day+3 we observed significantly higher villus length in EF compared to CON piglets (**Figure 7C)**. In other words, EF (unlike CON) piglets, did not show a drastic change in villus length over time, indicating smoother weaning transition. This was also reflected in the significant difference between day29/+21 and day+3 within the CON group, suggesting an impact of weaning on jejunal mucosa in the CON group (**Figure 7A**). However, no other morphometric differences in crypt depth, ratio of villus:crypt, villus width (jejunum) and crypt depth (colon) were observed between the treatment groups (**Supplementary figure 6A, B, C**).

Next, we evaluated the potential alterations in epithelial proliferation (due to increasing crypt depth), and subsequently assessed the effect of early feeding. The number of epithelial proliferating cells were estimated in colon mucosa (n = 8 per group per time-point) by employing the previously standardised IHC quantification protocol (**Chapter 4**). Previous studies suggested that increased crypt depth is correlated with increased epithelial proliferation rates in the mucosa (Zhou et al., 2019), however, we did not observe a reflection of increasing crypt depth over time in the number of proliferating cells, which remained stable over time in both CON and EF (**Figure 7D**). Further, no difference was detected in the number of proliferating cells (ratio PCNA : Hoechst nuclear stain) between the CON and EF groups at any time-point (**Supplementary figure 6D**), which was further supported by the lack of (significant) changes in the PCNA gene expression level (**Supplementary figure 7**). This is contrasting to our previous study where increased number of proliferating cells were found in EF group at weaning (**Chapter 4**).

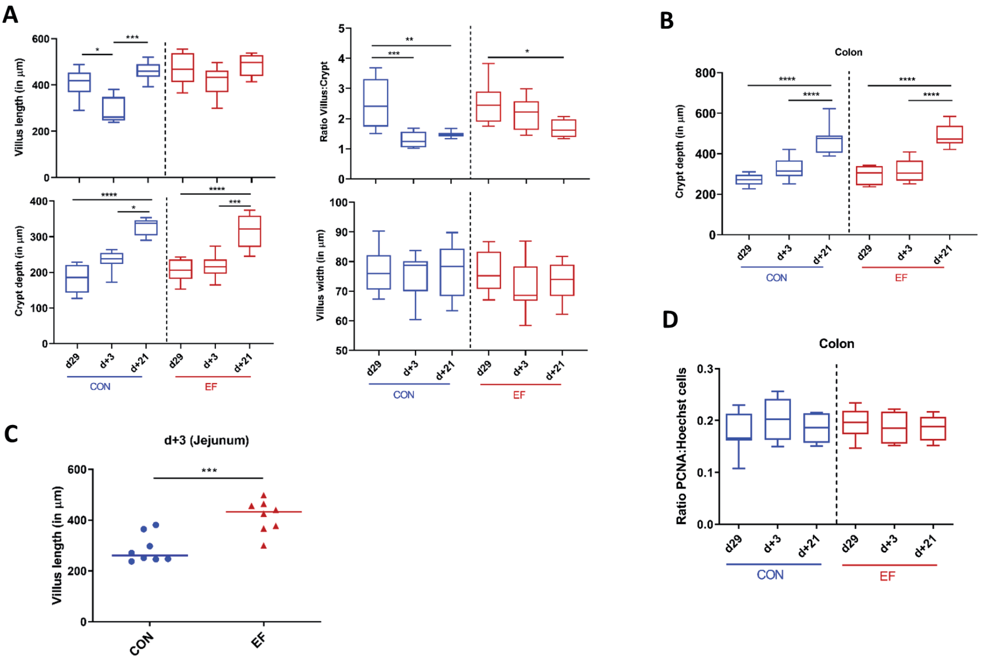

Figure 7: Intestinal morphometric analysis at day29, day+3 and day+21 time-points in jejunum and colon. **(A)** Measurements in jejunal tissue: Villus length, crypt depth, calculated villus:crypt (V:C) ratio and villus width. **(B)** Crypt depth measurement in colon tissue. **(C)** Comparison of villus length between early-fed (EF) and control (CON) group at day+3. **(D)** Colonic epithelial proliferating cells estimated by semi-quantitative image analysis by calculating the ratio of PCNA positive: Hoechst nuclei stain. Differences between groups were assessed by either Mann-Whitney U test or Kruskal Walis test (non-parametric).

Discussion

Dietary fibres are generally known to influence intestinal health, via unexplored biological mechanisms that have been proposed as a consequence of microbial fermentation into short chain fatty acids (SCFAs). In this study, we have confirmed the impact of early feeding (fibrous feed including dietary fibres like GOS, inulin, resistant starch) on colon microbiota, with accelerated microbiota maturation in suckling piglets, as also observed in a prior study employing a similar design (**Chapter 3**). The accelerated maturation in the early-fed (EF) piglets as compared with control (CON) piglets, was illustrated by the closer resemblance of pre-weaning microbiome with the post-weaning microbiota composition, with an expanded microbiota at day29 comprising of typical post-weaning microbial groups *like Prevotella, Subdoligranulum, Faecalibacterium, Roseburia* and *Megasphaera.* Moreover, we could additionally evaluate the persistence of EF-associated microbes post-wearing, and found persistence to some extent at three days post weaning (day+3) but not after three weeks (d+19). The similar microbiota composition in CON and EF piglets after weaning indicates the rapid microbiota adaptation due to diet and supports the previously observed “convergence” of microbiota post-weaning. Contrary to previous findings (**Chapter 4**), we observed a significant impact of early feeding on jejunal microbiota at day29, which did not persist post-weaning at day+3. This can be possibly explained by the lower pre-weaning feed intake in the previous study (**Chapter 4**) that may have been compromised by a diarrhoeic episode during the third week of age, thereby potentially weakening the EF impact. This is because we observed similar microbial signature in jejunum, with microbial groups like ***Subdoligranulum***, ***Coprococcus*** tending to correlate with the individual eating scores of the piglets (Chapter 4; data not shown), suggestive of the impact being driven by the amount of the feed consumed.

The primary aim of the current study was to investigate whether early feeding (pre-weaning provision of fibrous feed) can modulate the host mucosal transcriptome over time in colon and jejunum tissues. We show that early feeding accelerates not only the luminal microbiota (see above) but also the host transcriptome maturation in neonatal piglets, in addition to impacting the jejunal morphometry during the weaning transition. Intriguingly, the results demonstrate the coinciding time-frame of “accelerated maturation” as well as convergence after weaning both in the intestinal microbiota and transcriptome profile, indicating their potential interrelation. Maximum impact of early feeding was detected at weaning (day29), which was followed by convergence of transcriptome three weeks post-weaning (day+21). The “convergent” post-weaning time-point (day+21) was exploited to evaluate the maturation of the piglets at weaning and few days after weaning. It is to be noted that this study comprised of mixed effects of weaning-induced, age-related as well as treatment-associated transcriptome changes. Strikingly, the EF treatment effect seemed to overrule the aging/ weaning effect, as was clearly evident from the hierarchical clustering of transcriptome profiles, especially in colon and much modestly in jejunum. This is consistent with literature (Montagne et al., 2003; Chater et al., 2015; Capuano, 2017; Chen et al., 2019) as the microbial fermentation of fibres leading to SCFA production, predominantly occurs in the distal part of the gastrointestinal tract, thus a larger effect on colon mucosa due to fibre consumption is likely. However, separating the impact of the microbes from their metabolic products remains difficult. The key question is whether early feeding is causative for microbiome/transcriptome changes. We have previously established the association of eating and microbiome changes (**Chapter 3**), however the causative relation with mucosal transcriptome changes is difficult to determine in this study, although it seems likely that “feed in combination with microbiome” drives the predominant transcriptome changes (larger than age-effect).

SCFAs (particularly butyrate) form an important energy source for the intestinal epithelium and subsequently influences the epithelial cell homeostasis (Donohoe et al., 2011; Sivaprakasam et al., 2017; Allaire et al., 2018). Considering that SCFAs are fatty acids that are transported into colonocytes and eventually oxidised in mitochondria (Wojtczak and Schönfeld, 1993; Donohoe et al., 2011), it is reasonable that the pathways involved in cellular energy or mitochondrial metabolism, are altered in the EF group, which were enriched in SCFA producing microbes (***Prevotella, Subdoligranulum, Faecalibacterium, Roseburia*** and ***Megasphaera***)**.** This was further supported by the increased expression levels of SCFA transporters like SLC16A1, SLC26A3 (**Supplementary figure 7**) in the EF piglets at weaning, indicating that the CON piglets are less well-prepared to tackle the (post-weaning) changing circumstances (feed, microbiome, SCFA). At day29, EF piglets displayed an upregulation of genes associated with pathways including oxidative phosphorylation, cholesterol biosynthesis, glutathione redox reactions, ketogenesis and fatty acid ß oxidation (related to cellular metabolism and oxidative stress) and a downregulation of sirtuin signalling and immune-related pathways. This is consistent with previous studies which supplemented animal feed with non-digestible carbohydrates, and has shown to activate fatty acid oxidation in different animal models, including pigs (Haenen et al., 2013a), rats (Rodenburg et al., 2008). Moreover, a germ free versus conventionally raised mice study (Donohoe et al., 2011) has previously shown the impact of microbiota on energy homeostasis, specifically butyrate promoting oxidative metabolism in colonocytes by upregulating genes involved in fatty acid metabolism, glycerolipid metabolism, TCA cycle and mitochondrial oxidative phosphorylation (OXPHOS). The results presented here thus are in agreement with these studies, although our study highlights that these processes can be initiated during early-life by providing piglets with fibrous feed during lactation, whereby the transition to solid feed at weaning can be smoothened and does not require the mucosa to adjust abruptly to these novel feed components.

Our analysis identified PPARγ as an upstream transcriptional regulator that mediates the effects of the dietary fibres on gene expression, which is in accordance with a previous study (Lange et al., 2015) that evaluated different fibre sources in mice. Furthermore, EF piglets displayed an increased expression of oxidative stress pathways in colon mucosa. This is in line with previous knowledge that enterocytes contacted by enteric commensal bacteria (and/or their products) are known to stimulate transient oxidative stress generating physiological levels of reactive oxygen species (ROS) (Kumar et al., 2007; Jones et al., 2012). Microbial-elicited ROS may serve as second messengers to mediate cellular proliferation, motility and modulate innate immune signalling which is necessary for commensal-induced gut epithelial homeostasis (Marciano and Vajro, 2017; Dumitrescu et al., 2018). For instance, (probiotic) ***Lactobacillus*** strains have been reported as potent producers of ROS that acts as a signal-transducing molecule via the PPARγ pathway in intestinal epithelial cells (Voltan et al., 2008); PPARγ being a master regulator of energy metabolism and inducer of OXPHOS (Fan and Evans, 2015). The altered mucosal homeostasis is potentially regulated by sirtuin signalling, which is indicated by the activation of SIRT5 gene in the EF group, that is known to be exclusively located in mitochondria regulating energy metabolism and homeostasis (Yamamoto et al., 2007; Lee, 2019).

Weaning stress has been associated with altered gene expression of oxidative stress and immune pathways, regulated by MAPK signalling as a response to external stimuli (Yang et al., 2016; Xiong et al., 2019; Li et al., 2020). MAPK signalling are a class of mitogen-activated protein kinases that transduce signals from the cell membrane to the nucleus, leading to oxidative stress-induced differentiation, apoptosis, immune response. These response cascades were previously reported to be activated in pigs shortly after weaning (Hu et al., 2013; Luo et al., 2016; Li et al., 2020). Our study corroborates these results and identifies p38 MAPK activation at day+3 along with the increased expression of immune-related genes (IL18, TGFB1, TGFB2) in EF compared to CON piglets. TGF-beta signalling is known to play an important regulatory role in intestinal barrier restoration by stimulating epithelial cell migration, extra-cellular matrix, integrin production, and can be important for post-weaning adaptation in pigs (Xiao et al., 2014). Remarkably, EF piglets displayed an increased expression of TGFB1, TGFB2 as well as ECM (ACTG2, ACTN1, ACTN2, ITGA1, ITGA5, RHOD, RHOBTB1, VCL) genes, suggesting a relatively improved mucosa integrity. Improved barrier integrity in EF piglets was also substantiated in jejunum with the strong downregulation of MMP-9 gene (Matrix metalloproteinase-9; involved in re-modelling of ECM and wound repair), that is known to cause an increase in intestinal epithelial tight junction permeability (Al-Sadi et al., 2019). Taken together, the EF piglets seem to be more adequately coping with the weaning-stress induced mucosal challenges by pronounced activation of barrier repair reactions, which is a combination of immune activation, epithelial migration and “wound-repair” like processes. The lack of activation in the CON piglets might reflect failure of the intestine to respond adequately to the stimulus of feed and/or microbiota triggered by weaning.

In agreement with previous studies (Pluske et al., 1996; Vente-Spreeuwenberg et al., 2003; Montagne et al., 2007), temporal changes induced by weaning were observed in jejunal mucosal morphology, characterised by altered villus length and crypt depth. Strikingly, EF piglets displayed a significantly higher jejunal villus length three days post-weaning (day+3) compared to the CON piglets, which suggests a lower “weaning dip” or a smoother weaning transition in terms of mucosal morphology. Furthermore, lowered villus length could be indicative of the less appropriate activation of barrier and tissue remodelling in the CON group, possibly reflected in the altered expression levels of proliferation, tissue-remodelling associated genes (RHOC, RHOD, RHOE, RAC3) in jejunum. Unlike earlier work that focussed on jejunal morphology, this study also assessed the temporal changes in colon mucosa and detected an increasing colonic crypt depth over time, though this process did not appear to be affected by early feeding. Of note, it is remarkable to observe the impact of early feeding on both microbiota and host mucosa, in spite of the relatively low (fibrous) feed intake of (EF) piglets compared to the milk intake [approximately 28 grams/day/piglet from d21-30 in this study vs. 1 kg milk/day in (Quesnel et al., 2015)] that constitutes the main energy source of the piglet.

Performance parameters were also assessed in our study and a quite moderate smoothening of weaning transition was observed, reflected in the increased post-weaning feed intake (between day+1 and day+5), average daily gain (between day+1 and day+2) and consistently lowered coefficient of variation in body weight development (**Supplementary figure 8**), which was also seen previously (**Chapter 3**). However, we also observed a fluctuating average daily gain between the two treatment groups within 2 days post-weaning and no difference in relative body weight post-weaning, which makes the importance of early feeding in improving the post-weaning performance, unclear. It is although important to note that these piglets were healthy and unchallenged, and therefore a more stressful situation (e.g., nutritional or environmental challenge models such as low-hygiene, heat stress, fasting) can possibly detect differences in coping adaptability between the groups.

In conclusion, the results from the present study confirmed the acceleration in maturation of the microbiota and clearly demonstrated that this "accelerated maturation" is also reflected in the host mucosal transcriptome in early-fed piglets. The accelerated changes in the EF piglets were closely associated with upregulation of cellular energy metabolism and immune tolerance potentially induced by the colonised commensals. Remarkably, the EF piglets seem to be more responsive to the post-weaning situation (day+3), which may contribute to the smoothening of the weaning stress by supporting better maintenance of adequate immune responses, gut barrier integrity and intestinal morphology. Of note, our study addresses fundamental questions about how the microbiome regulates host metabolism, which might aid in the understanding of dietary fibre-microbiota-host mucosa interactions, in addition to exploring early feeding as an attractive strategy to prepare suckling piglets for weaning transition.

Acknowledgements

This study is part of research programme "Genetics, nutrition and health of agricultural animals" with project number 868.15.010, which is co-financed by the Netherlands Organization for Scientific Research, Cargill Animal Nutrition and Coppens Diervoeding. We would like to acknowledge the personnel and trainees of the animal research facilities in Wageningen (Carus, Wageningen University) for taking care of the animals and for their technical assistance, especially Arjan van Dolderen and Ries Verkerk. The authors are especially thankful to Anouschka Middelkoop for her help in scan sampling observation of the EF piglets to identify 'eaters'. The authors also want to thank Bjorge Laurenssen, Monique Ooms, Manon van Marwijk (Adaptation Physiology group, Wageningen University) who helped conducting the animal experiment. Special thanks to Pieter Roskam, Anja Taverne-Thiele, Soumya Kanti Kar, Bart van der Hee, Lara van Veen for assisting in sample collection and processing during the dissection procedure of the piglets. The authors are grateful to FrieslandCampina DOMO (Amersfoort, The Netherlands) for provision of Vivinal GOS.

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Supplemental information

Supplementary Table 1: Ingredients and calculated nutrient composition of the pre-weaning fibrous feed[1]

	Pre-weaning feed
Ingredients, %	
Wheat	21.9
Barley	15
Maize	15
Soy protein concentrate	7
Soybeans (heat treated)	5
Galacto-oligosaccharides[2]	**5**
Potato protein	4
Sugarbeet pulp	**4**
Oat hulls	**4**
Inulin[3]	**4**
Resistant starch[4]	**4**
Soybean oil	3
Blood meal (spray dried)	2
Dicalcium phosphate	1.7
Sucrose	1.5
Calcium carbonate	1.0
Sodium chloride	0.5
Premix[5]	0.5
Potassium bicarbonate	0.3
L-lysine hydrochloride	0.3
DL-methionine	0.2
L-threonine	0.04
L-tryptophan	0.04
Calculated nutrient composition, g/Kg	
Dry matter	891
Starch	290
NSP[6]	261
Crude protein	195
Crude fat	61
Crude fibre	44
Crude ash	57
Calcium	9.1
Phosphorus	6.1
Sodium	2.2
Standardized ileal digestible lysine	11.9
Standardized ileal digestible methionine	4.8
Standardized ileal digestible threonine	7.1
Standardized ileal digestible tryptophan	2.4
Net energy, MJ/kg	11.8

[1] Feed was mixed and pelleted (12mm diameter pellets) by Research Diet Services (Wijk bij Duurstede, The Netherlands). According to CVB (2007), nutrients are presented in g/kg dry matter, except for dry matter (g/kg) and net energy (MJ/kg).

[2] Source: Vivinal® GOS powder (Friesland Campina, Amersfoort, The Netherlands) containing 69% galacto-oligosaccharides.

[3] Source: Prebiofeed 95 inulin powder (Cosucra group, Belgium) containing 85% inulin.

[4] Source: AmyloGel® Native Starches (Cargill, Wayzata, USA) derived from high amylose maize with 75% amylose content.

[5] Vitamin and mineral premix (per kg of feed): vitamin A: 10000 IU, vitamin D3: 2000 IU, vitamin E: 40 mg, vitamin K: 1.5 mg, vitamin B1: 1 mg, vitamin B2: 4 mg, vitamin B6: 1.5 mg, vitamin B12: 0.02 mg, niacin: 30 mg, D-pantothenic acid: 15 mg, choline chloride: 150 mg, folate: 0.4 mg, biotin: 0.05 mg, iron: 100 mg, copper: 20 mg, manganese: 30 mg, zinc: 70 mg, iodine: 0.7 mg, selenium: 0.25 mg, anti-oxidant: 125 mg.

[6] Non-starch polysaccharide: Calculated as the difference between dry matter and the sum of starch, sugars, crude protein, crude fat and crude ash.

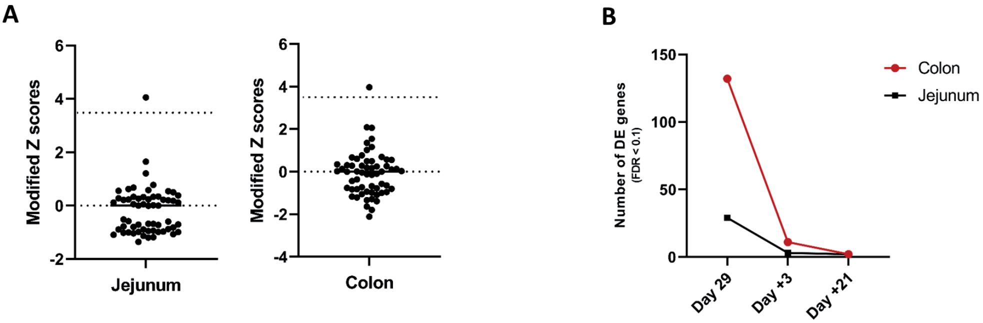
Supplementary Table 2: Ingredients and calculated nutrient composition of the weaner feed[1], that closely resembles the composition of standard commercial weaner diets

	Post-weaning feed
Ingredients, %	
Barley	37.9
Wheat	20.0
Corn	5.0
Soybean meal	4.0
Soybeans full fat toasted	5.0
Corn	5.0
Whey powder	5.0
Sugarbeet pulp	1.5
Provisoy™[2]	10.0
Soybean oil	2.5
Premix[3]	0.5
Limestone	1.05
Monocalcium phosphate	0.9
Sodium chloride	0.4
Sodium bicarbonate	0.1
L-lysine	0.52
DL-methionine	0.22
L-threonine	0.24
L-tryptophan	0.08
L-valine	0.1
Calculated nutrient composition, g/Kg	
Dry matter	886
Crude ash	54
Crude protein	175
Crude fat	57
Crude fibre	33
Carbohydrates	573
Starch	398
Sugar	68
Neutral detergent fiber (NDF)	109
Acid Detergent Fiber (ADF)	47
Calcium	6.7
Phosphorus, total	5.6
Magnesium	1.5
Potassium	8.3
Sodium	2.2
Chloride	5.0
Standardized ileal digestible lysine	11.0
Standardized ileal digestible methionine + cysteine	6.5
Standardized ileal digestible threonine	6.9
Standardized ileal digestible tryptophan	2.4
Standardized ileal digestible valine	7.7
Net energy (MJ/Kg)	10.6

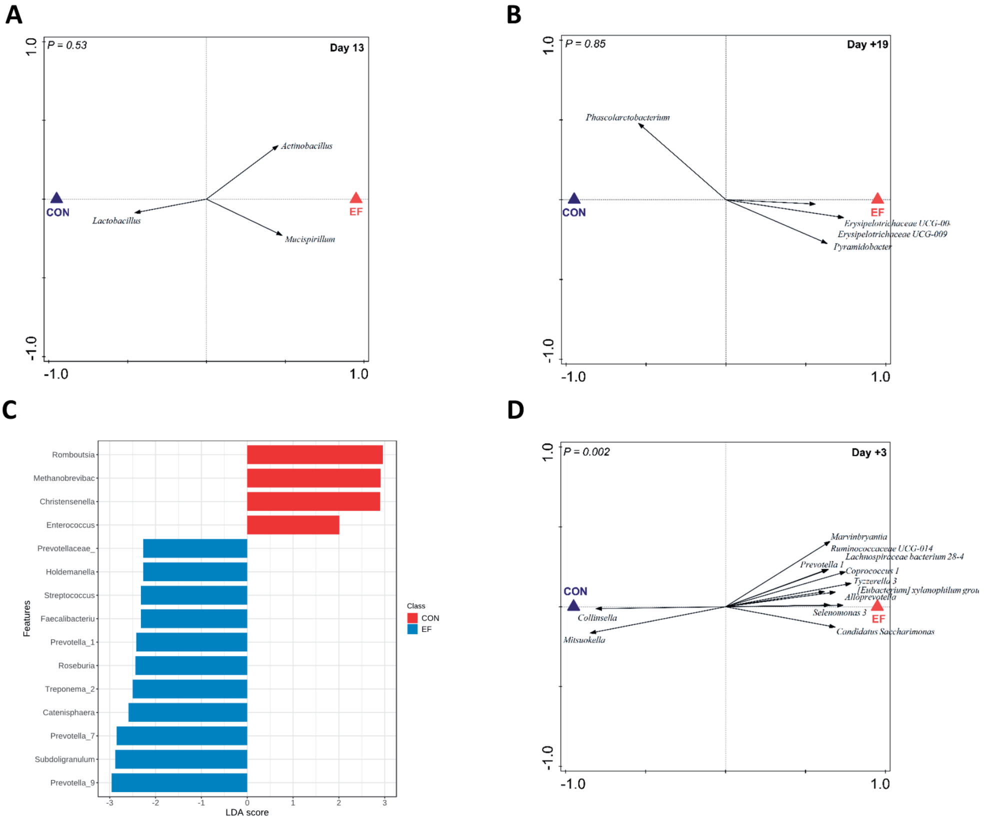
[1] Mixed and pelleted (3mm diameter) by Research Diet Services (Wijk bij Duurstede, The Netherlands).

[2] Provimi, Cargill, Rotterdam, The Netherlands.

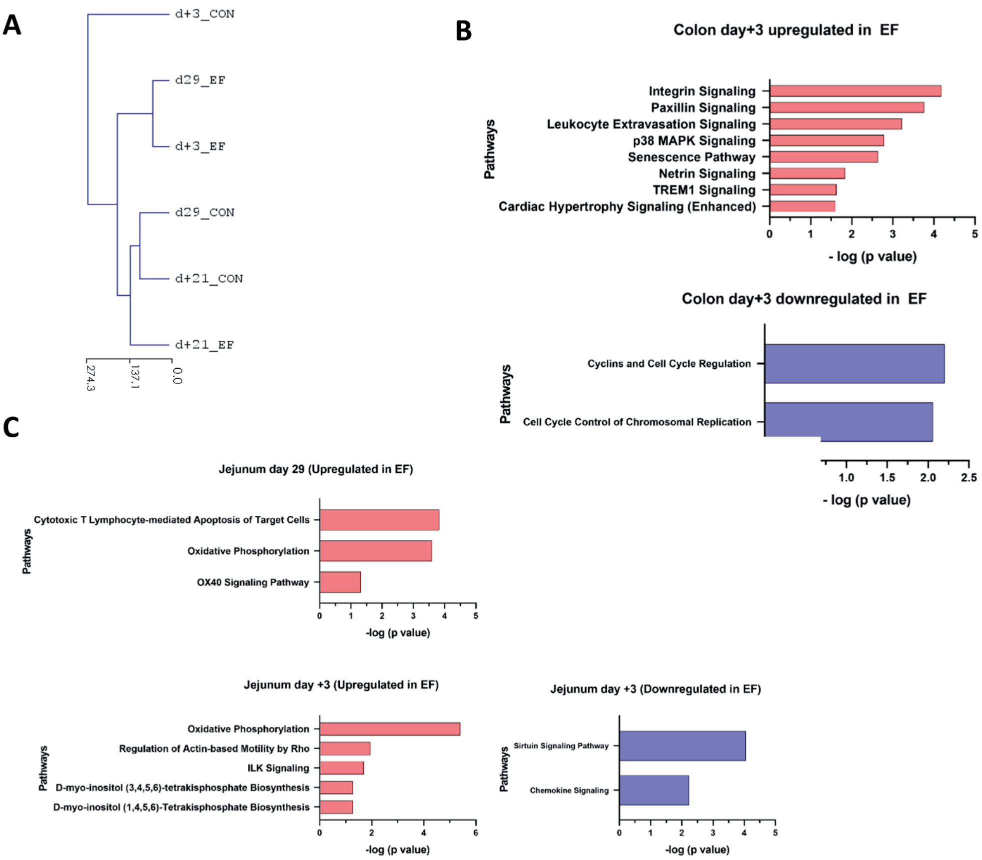
[3] Vitamin and mineral premix (per kg of feed): vitamin A: 10000 IU, vitamin D3: 2000 IU, vitamin E: 40 mg, vitamin K: 1.5 mg, vitamin B1: 1 mg, vitamin B2: 4 mg, vitamin B6: 1.5 mg, vitamin B12: 0.02 mg, niacin: 30 mg, D-pantothenic acid: 15 mg, choline chloride: 150 mg, folate: 0.4 mg, biotin: 0.05 mg, iron: 100 mg, copper: 20 mg, manganese: 30 mg, zinc: 70 mg, iodine: 0.7 mg, selenium: 0.25 mg, anti-oxidant: 125 mg.



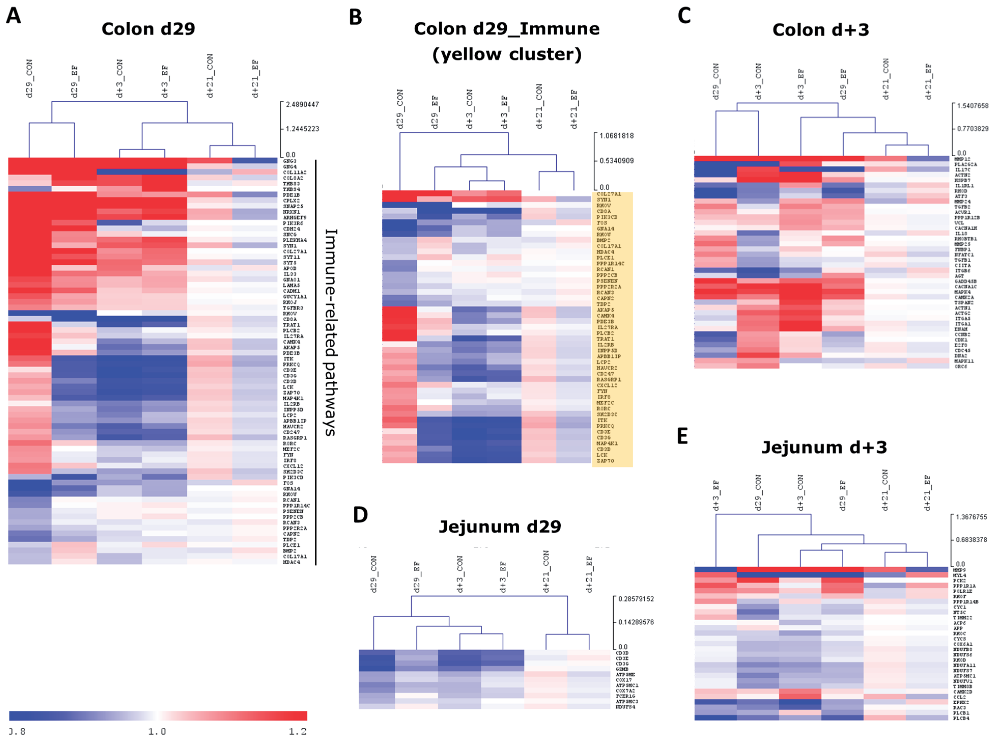
Supplementary figure 1: (A) Outliers were detected using modified Z scores of principal component loadings obtained from unsupervised principal component analysis in colon and jejunum samples. Samples exceeding absolute score of 3.5 are regarded as outliers. **(B)** Number of differentially expressed (DE) genes (EdgeR test: *FDR* < 0.1) at day29, day+3 and day+21 in colon and jejunal samples.



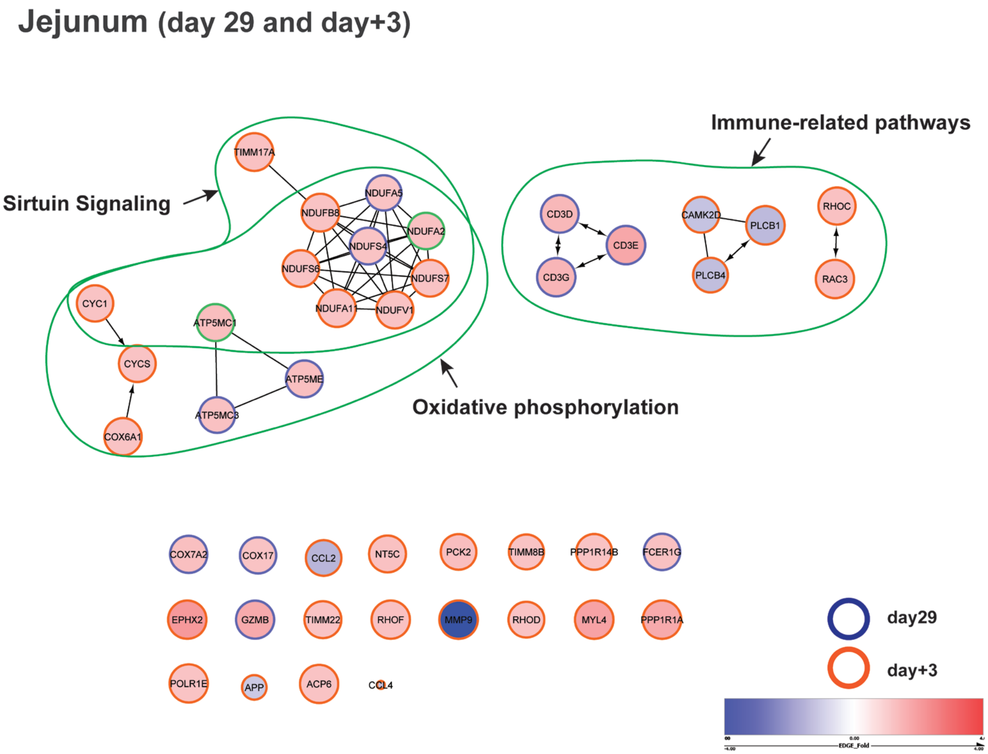
Supplementary figure 2: Microbiota composition (genus level) in early-fed (EF) and control (CON) group. **(A)** Redundancy analysis of EF and CON treatment groups at day 13 (swabs; explained variation = 0.00%; *P* = 0.53). **(B)** Redundancy analysis of treatment groups at day+19 (swabs; explained variation = 0.00%; *P* = 0.85). **(C)** LEfSe analysis (colon, d29) identified the differentially abundant microbial groups between EF and CON. **(D)** Redundancy analysis (colon, d+3) of treatment groups (explained variation = 9.8% ; *P* = 0.002).



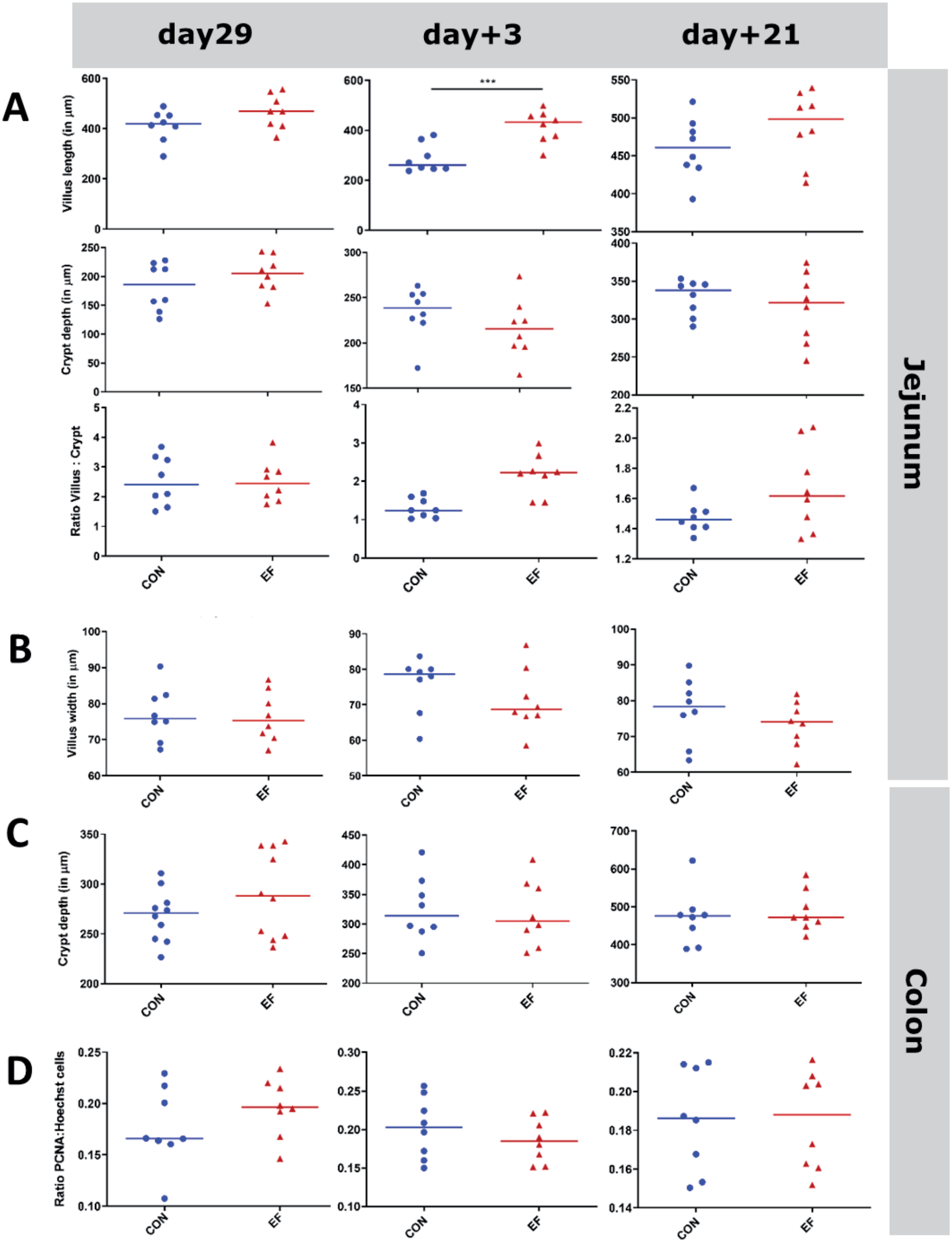
Supplementary figure 3: (A) Unsupervised hierarchical clustering (euclidean distance) of jejunal transcriptome using normalised (logCPM) expression values (averaged per group per time-point) and are scaled by the mean value of day+21 gene expression (irrespective of treatment). Pathway analysis in **(B)** Colon and **(C)** Jejunum. Canonical pathways up and downregulated in early-fed (EF) group compared to the control (CON) group are shown in red and purple bars respectively. Identified pathways have a logP value ≥ 1.3 (enrichment score from Fisher's exact test, Ingenuity pathway analysis) and an absolute Z score ≥ 2 (assessing the match of observed and predicted up/down regulation patterns).



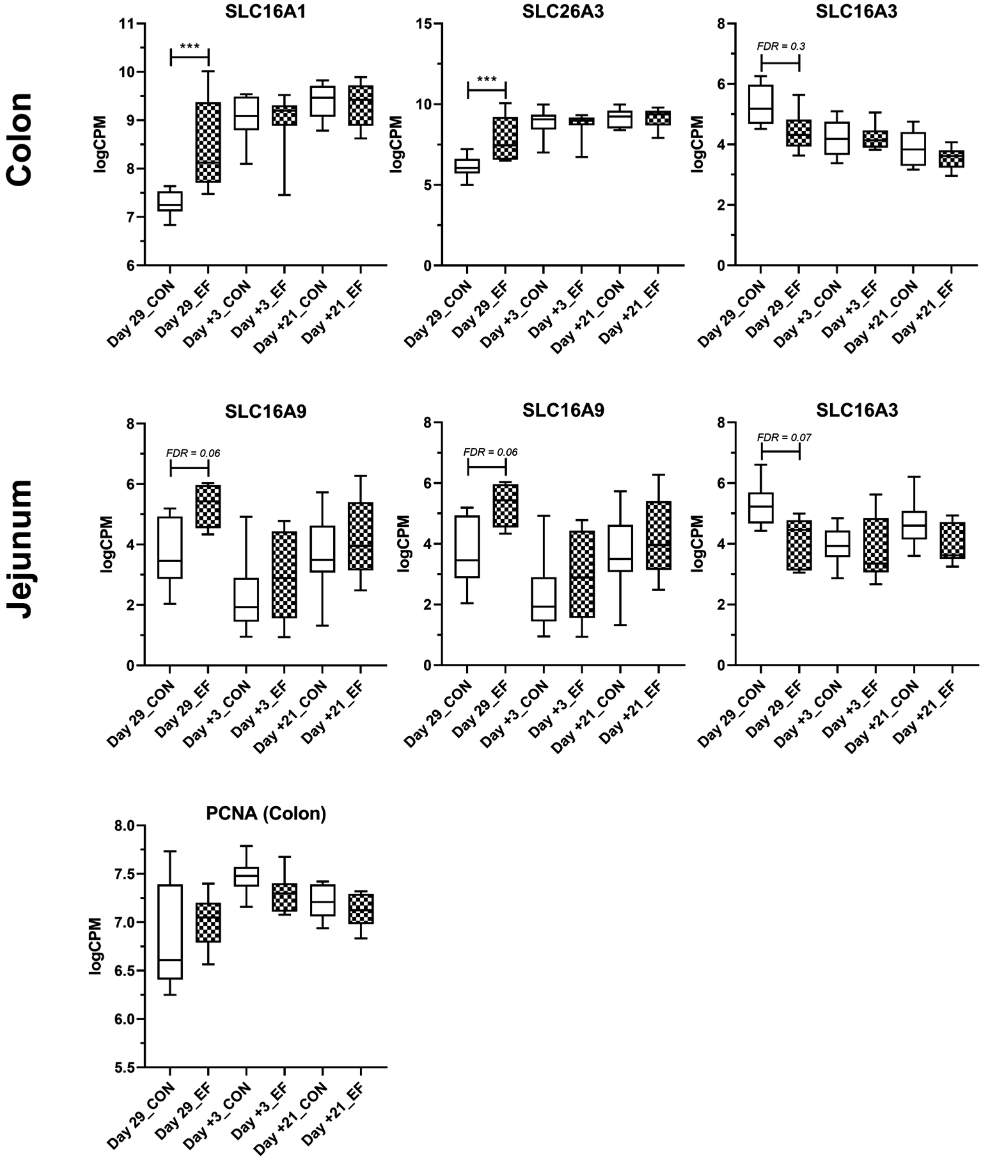
Supplementary figure 4: Hierarchical clustering of expression profiles over time for (**A**) immune pathway genes of colon at day29. (**B**) the third cluster of immune genes (yellow) observed in **Figure 5C** (**C**) colon day+3 (upregulated) (**D**) jejunum day29 (**E**) jejunum day+3. The normalised expression values (averaged per group per time-point) are scaled by the mean value of total day+21 expression (irrespective of treatment). EF = early-fed, CON = control.



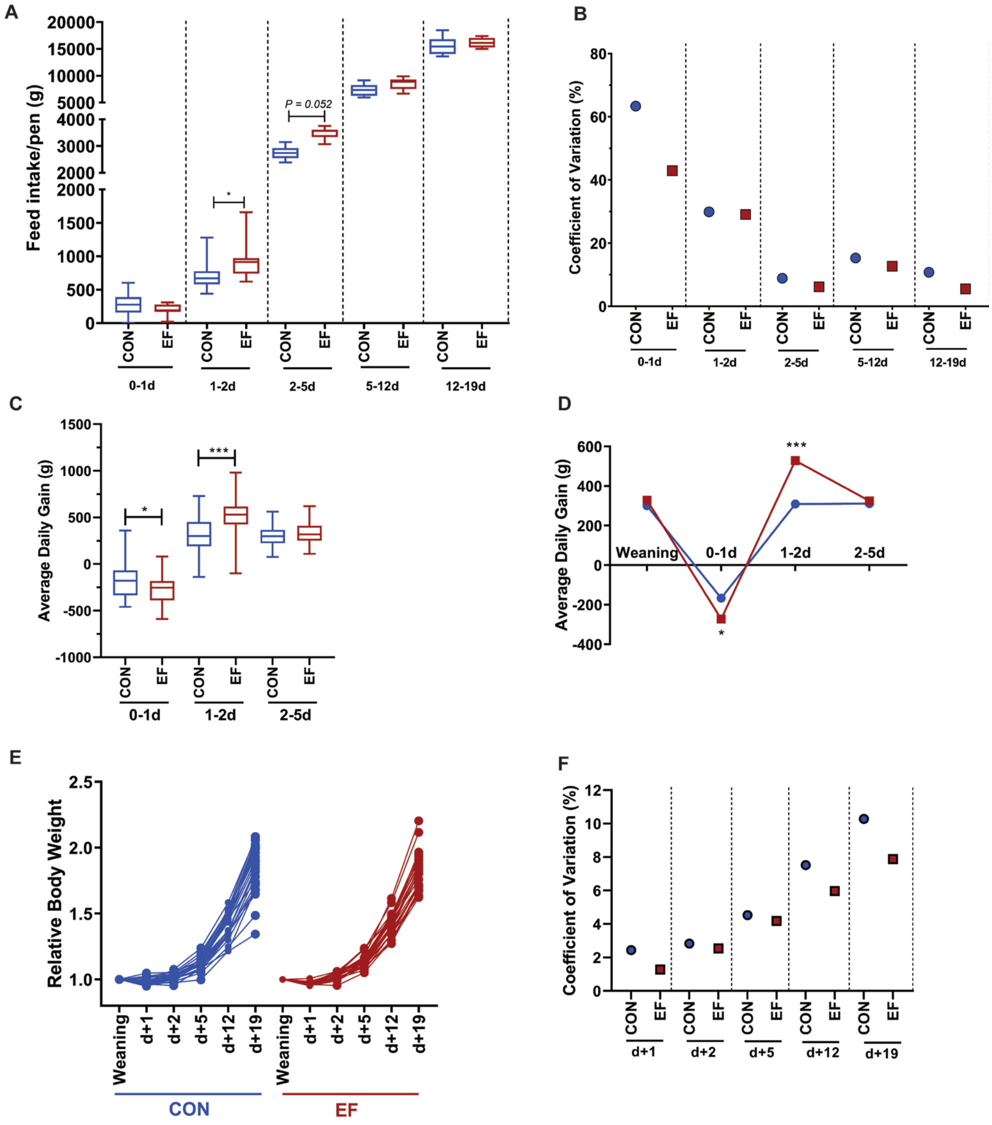
Supplementary figure 5: Functional network of pathway identified genes (jejunum) at day29 and day+3. Functional protein-protein interaction of up- and down-regulated genes in early-fed (EF) compared to control (CON) group. The circles or nodes represent genes and edges represent interactions between genes, as determined by Reactome. Arrows represent directed interactions, bar-headed arrows indicate inhibition reactions and dotted lines indicate predicted relationships. Pathway identified genes from time-points day29 (blue bordered), day+3 (orange bordered) and both (green bordered) are shown in this figure. The upregulated (red nodes) and down-regulated (blue nodes) genes are represented by their fold change (EdgeR test) in a blue-red gradient scale, where the size of the node is proportional to their significance (P value; EdgeR test). The dotted green lines depict different pathways associated with those genes. Two genes ATP5MC1 and NDUFA2 (part of oxidative phosphorylation pathway) were observed at both day29 and day+3 (fold change ~ 1.3; *P* < 0.05).



Supplementary figure 6: Jejunal and colonic morphometric measurements determined at the three sampling time-points indicated at the top (day29, day+3, and day+21) for individual piglets in the early-fed (EF; red) and control (CON; blue) groups. **Panel A:** Jejunal villus length (upper panels), crypt depth (middle panels) and villus:crypt ratio (lower panels). **Panel B:** jejunal villus width, **Panel C:** colonic crypt depth, and **Panel D**: colonic ratio of PCNA over Hoechst stained nuclei, indicative of relative epithelial proliferation activity. Differences between groups were assessed by t-test or Mann-Whitney U test (non-parametric).



Supplementary figure 7: Gene expression levels of SCFA transporters (belonging to solute carrier family) that were found significantly altered in early-fed (EF) as compared to the control (CON) piglets (EdgeR test, P < 0.05). In addition, gene expression levels of (colon) proliferating marker PCNA is shown.



Supplementary figure 8: (**A**) Pen-level post-weaning feed intake in control (CON; blue) and early-fed groups (EF; red) measured between 0-1 day, 1-2 day, 2-5 days, 5-12 days and 12-19 days post-weaning. All pens were provided with weaner diet after weaning (n = 12 pens per treatment group till day+2, after which n = 8 per treatment till day+19 due to sacrifice of piglets). (**B**) Coefficient of variation (%) which captures the dispersion of the feed intake data at each time-point per treatment. (**C**) Average daily gain (ADG, in grams; n = 24 per treatment group) in the early-post-weaning period. (**D**) Mean of the ADG shown during the first 5 days post-weaning, comparing with the pre-weaning (three days before weaning) ADG per treatment. (**E**) Relative body weight development (relative to weaning weight per individual piglet) between the groups (n = 48 per treatment) till 19 days post-weaning. (**F**) The coefficient of variation (CV%) capturing the variation in body weight in CON and EF groups. Statistical comparisons between the groups were assessed by student t test or Mann-Whitney U test in GraphPad Software 8.1.1.

6

Chapter 6

Exploring the association between microbiota and behaviour in suckling piglets

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Manuscript in preparation

Abstract

It is increasingly recognised that the microbes residing in the gastrointestinal tract can influence brain physiology and behaviour, via the microbiota-gut-brain axis. Here, we evaluated the association between the gut microbiota and behaviour in suckling piglets. 16S microbiota profiling information was obtained from two independent replicate experiments at two and four weeks of age. Piglets underwent a backtest to assess their personality or coping style at 2 weeks of age, and were subjected to a combined open field and novel object test at 3.5 weeks of age, recording anxiety-related and exploratory behaviour. The number of squeals vocalised during the open field test was associated with microbial groups such as ***Coprococcus 3*** and ***CAG-873***, whereas in the novel object test, explorative behaviour was significantly associated with microbial genera like ***Atopobium*** and ***Prevotella.*** Overall, this study explores the microbiota-behavioural relation by employing multivariate analysis and exemplifies the importance of individualised analyses when evaluating such relationships.

Keywords: anxiety, behaviour, exploration, microbiota, individual analysis, piglet, personality, backtest, open field, novel environment, coping style, fearfulness

Introduction

Increasing evidence indicates that the impact of gut microbiota extends beyond the gut, as it plays a crucial role in the bidirectional communication between the intestine and central nervous system (CNS), also known as the microbiota-gut-brain axis (Collins et al., 2012; De Palma et al., 2014; Sherwin et al., 2019). The mediators of this communication include short-chain fatty acids, neurotransmitters, immune system modulators, hormones, as well as the vagus nerve, which are all known to be affected by microbial metabolism (Bravo et al., 2011; Johnson and Foster, 2018; Valles-Colomer et al., 2019). During early-life the intestinal microbiota is dynamic and rapidly evolving, which coincides with important developmental processes in the brain. This overlapping timeline of early-life microbiota and brain development could provide a “window of opportunity” for influencing CNS development and function, via microbial modulation (Davidson et al., 2018). The microbiota-gut-brain axis consists of bidirectional communication, with the microbiota shown to play a role in neurodevelopment (from early-life to adulthood) and behaviour by influencing neural processes such as myelination, neurogenesis, neurotransmission and development of the hypothalamic-pituitary-adrenal axis (HPA) (Davidson et al., 2018; Sherwin et al., 2019). On the other hand, the brain regulates intestinal functions (e.g. motility, secretion and mucin production) as well as immune functions (e.g. modulation of cytokine production) (Collins et al., 2012) in the gastro-intestinal tract. However, the underlying mechanisms of the bidirectional communication between the gut and the brain are not fully understood.

Recent advancement of sequencing technology has allowed exploration of the microbiome and its neuroactive potential in the context of stress, anxiety and depression-related behaviour (Valles-Colomer et al., 2019). Animal models can play an important role in understanding the underlying mechanisms via which early-life experiences affect later life health. Mostly, gnotobiotic or germ free rodent models have been employed to get insight into the mechanism of the microbiota-gut-brain communication, using probiotics, antibiotics, drugs or faecal transplantation (Cryan and Dinan, 2012; Ezenwa et al., 2012). However, compared to humans, the CNS development is substantially different in rodent species as they have a less developed brain at birth (Dobbing and Sands, 1979), with maximum development occurring postnatally. On the other hand, pigs and humans exhibit striking similarity with respect to their physiology, brain development and gastrointestinal function (Conrad and Johnson, 2015; Nguyen et al., 2015), including an analogous “window of brain development” at birth as well as having a gyrencephalic brain (Dobbing and Sands, 1979). Apart from its value as a translational model for human development, disease and the underlying processes, the understanding of early-life development in pigs is also valuable in the context of veterinary and animal sciences. However, to the best our knowledge, there are no studies evaluating the relationship between microbial communities and behavioural characteristics in piglets using multivariate approaches, reflecting the putative communication with the gut and the brain.

It is well known that microbiota differs among individuals, and therefore needs to be explored as an individualised mechanism to explain differences in behaviour, which remains virtually unexplored (Davidson et al., 2018). The present study explores the association between the microbiota composition and behaviour of individual suckling piglets in challenging situations, (which could reflect their personality), aiming to take a first step towards better understanding of the biological relevance of microbiome variation in behaviour.

Materials and methods

Study design

The Animal Care and Use committee of Wageningen University & Research (Wageningen, The Netherlands) approved the protocol of the experiment (AVD104002016515). The protocol is in accordance with the Dutch law on animal experimentation, which complies with the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

Two independent (replicate) experiments were performed using 22 multiparous Topigs-20 sows housed and inseminated at research facility Carus (Wageningen University & Research, The Netherlands). The new-born piglets inhabited with the sow and littermates till weaning (4 weeks of age), and received ear tags for individual identification and an iron injection, standard to pig husbandry practice. Two days after birth, twelve litters were provided with fibrous diet (early-fed group or EF) in addition to sow's milk and the remaining ten litters suckled mother's milk only. Additional details about the treatment, housing and management have been described previously (**Chapter 3, 4**).

Personality test or backtest

At approximately 2 weeks of age, the piglets were subjected to a backtest as described previously (Bolhuis et al., 2003). Their response in this test, which is heritable (Velie et al., 2009; Zebunke et al., 2015; Iversen et al., 2017), reflects their preferred coping strategy, or coping style, which is considered a personality trait. Several studies, often studying the extreme pigs at either end of the population, have revealed links between the backtest response of piglets with neuroendocrine features, gene expression patterns and behavioural characteristics in later life, including behavioural flexibility (Bolhuis et al., 2004, 2005; Kanitz et al., 2019). Briefly, in the backtest, piglets are manually restrained by putting them on their back (supine position) for 60 seconds. The recorded parameters during the test were: (i) latency until the first struggling attempt (latency_resist); (ii) the total number of struggling attempts (frequency_resist); (iii) latency until the first vocalisation (latency_vocalise); (iv) the total number of vocalisations (frequency_vocalise).

Combined open field and novel object test (or novel environment test)

At 3.5 weeks of age, a subset of piglets (n = 47) was subjected to a 10 min combined open field test (OFT) and novel object test (NOT) in both the experiments (experiment1, n = 19; experiment2, n = 28 piglets). The selected piglets were balanced for pen, gender as well as average body weight of the litter at 21 days of age. Testing was carried out on two consecutive days and the order of piglets tested was balanced for gender and treatment. Fear-related behaviour and exploration were assessed using multiple behavioural observation scores obtained during the combined OFT and NOT, which have previously been described (Middelkoop et al.). The unfamiliar (or novel) environment, which was an arena of 3 x 3 m, with walls of 1.2 m and a concrete floor, was located in a test room at the end of the hallway, away from the home pen (visually and auditorily). The individual piglets were transported to the arena using a transport cart. Each piglet was placed in one corner of the test arena, in the same start position next to the wall. The pigs were given a 5-min period to explore the novel environment (OFT). After 5 minutes, a novel object (metal bucket) was slowly lowered from the ceiling into the centre of the arena until it touched the floor which, consequently, resulted in a noise. The piglets were given another 5 minutes to interact with the novel object (NOT). Behaviour and vocalisations (**Supplementary table 1**) were continuously scored live using Psion hand-held computers with the Pocket Observer 3.1 software package (Noldus Information Technology, Wageningen, The Netherlands). Two mutually exclusive behavioural classes were observed simultaneously, one recording the posture or locomotion of the piglet as states, and the other recording the (other) behavioural states displayed by the piglet whilst in a particular posture or locomoting.

Sampling, DNA extraction and 16S rRNA gene based amplicon sequencing

For microbiota analysis, rectal swab samples were collected at two and four weeks of age, by inserting a sterile cotton swab (Puritan Medical, Guilford, ME USA; Cat Number-25-3306-U) 20–30 mm into the rectum and rotating the swab against the bowel wall for a minute before placing it into a 5ml Eppendorf tube. The samples were kept on ice during transport to the laboratory and stored at −20°C until further processing.

As previously described in detail (**Chapter 3**), DNA extraction was performed by the repeated bead beating method (Yu and Morrison, 2004) using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Briefly, the DNA template was used to amplify the V3-V4 region of the bacterial 16S rRNA gene, purified and subsequently sequenced using (paired-end) Illumina MiSeq system at BaseClear BV (Leiden, The Netherlands). After quality filtering, the Illumina reads were imported into the CLC Genomics Workbench version 11.01, processed using the CLC Microbial Genomics Module version 2.5.1 (CLC bio, Arhus, Denmark) and the high quality sequences were finally clustered into operational taxonomic unit (OTUs) at 97% identity threshold using SILVA database v132 (Quast et al., 2013).

Correlation analysis

The backtest (at 2 weeks of age), reflecting a pig's coping style or personality, was analysed with microbiota information obtained from rectal swabs taken at the same timepoint (2 weeks of age), whereas the novelty test observations (at 3.5 weeks of age; related to anxiety, fear and exploration) were assessed with microbiota data collected at 4 weeks of age (**Figure 1**). To evaluate associations between microbiota composition (genus level) and behavioural variables, multivariate redundancy analysis was performed in CANOCO 5 software (Microcomputer Power, Ithaca, NY, USA) (Braak and Smilauer, 2012). Redundancy analysis (RDA) is a canonical version of principal component analysis where the principal components are constrained to be linear combinations of the explanatory variables. RDA is a type of constrained ordination that assesses how much of the variation in response variables (here, microbial taxa) can be explained by the variation in explanatory variables (here, behavioural observations), in addition to providing related microbial taxa with the explanatory variable. Partial redundancy analysis (pRDA) was employed in this study to analyse the associations between microbiota and behavioural variables, after experiment and treatment were removed (partialled out) from the ordination. Statistical significance was assessed by the Monte Carlo permutation procedure (MCPP) with 499 random permutations. The behavioural observations (**Supplementary table 1**) were categorised per test, i.e backtest, OFT, NOT, and analysed separately. Some of the behavioural observations were summed to obtain additional parameters, for example, frequency of squeals, grunt squeals and screams were summed as 'high pitched vocalisations'. Similarly, 'exploring behaviour' was obtained by summing nosing and rooting behaviour on the walls and floor of the test arena. Lying did not occur and sitting was very rare and displayed by only a number of piglets, and were not included in the analyses. Frequency of excretions was not included in the analyses, as it was found to be distant from the mean of coefficient of variation (CV%) (**Supplementary figure 1A**). We employed relatively permissive cut-off values in the RDA analyses to identify potential microbiota associations, where behavioural parameters having P value < 0.1 were considered significant and the associated microbial groups (genus level), having (i) a minimum response scores ≥ 0.35 and (ii) 0.1% relative abundance in at least 10% of samples, were considered relevant. The associations between individual behavioural scores and microbial groups (identified in pRDA) were subsequently tested by non-parametric Spearman correlation analysis ($P < 0.05$ considered significant) in GraphPad Software 8.1.1 (California, USA, www.graphpad.com).

In addition, Multivariate Analysis by Linear Models (MaAsLin) (Morgan et al., 2012) was employed in the galaxy platform (https://huttenhower.sph.harvard.edu/galaxy/) to further test the significant behavioural observations and their associated microbial taxa. MaAsLin is a multivariate statistical linear regression analysis that identifies associations between metadata and microbial community abundance. MaAsLin allows detection of the effect of one variable without the influence of other metadata in the study. Initially, the default MaAsLin parameters were applied (minimum 0.01% relative abundance present in 1% of samples, $P < 0.05$, FDR <

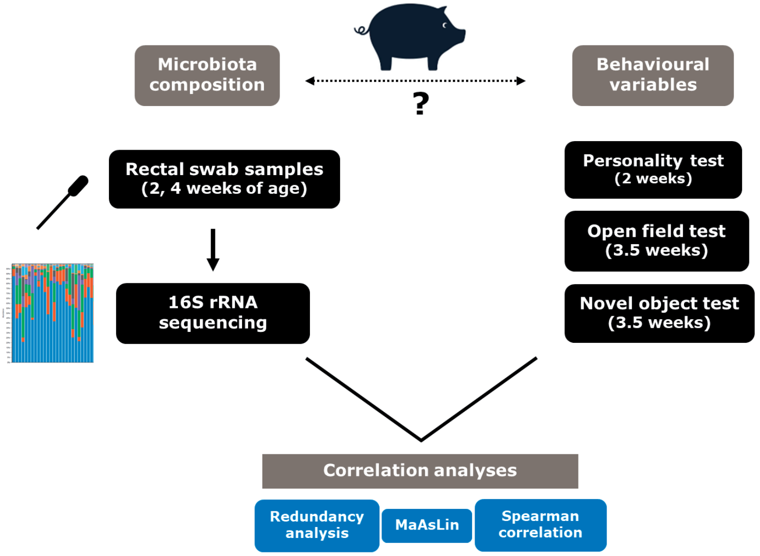

Figure 1: Schematic study design to assess association between the microbiota and behaviour. Microbiota and behavioural data were obtained from two independent experiments. Rectal swabs were collected at day 15 and day 29 pre-weaning for microbiota analysis. Personality (or back)test and Anxiety test (open field test, novel object test) were done at day 15 and day 25 respectively, in suckling piglets.

0.05), but to more accurately compare MaAsLin results with redundancy analyses, subsequent MaAsLin analyses employed taxa representing ≥ 0.1% of the total microbial composition prevalent in >10% of all samples.

Results

Group based analyses employing categorical variables such as treatment, gender and pen did not reveal any associations with the behavioural parameters. To understand individual differences in behavioural traits, we explored associations between microbiota composition and behavioural observations at an individual level. Correlation analyses were performed by combining data from two replicate experiments. The microbiota composition data were determined by llumina Miseq 16S rRNA gene sequencing of the V3-V4 region, generating 1,941,012 number of reads after quality filtering, with a mean sequencing depth of 20,128 ± 5905 reads per sample.

We analysed four backtest variables with the microbiota composition at two weeks of age by redundancy analysis (RDA), but did not find a significant correlation ($P = 0.66$; **Supplementary figure 1B**). To investigate fear-related as well as explorative behaviour in suckling piglets, a combined OFT and NOT was performed at 3.5 weeks of age. In the OFT, the frequency of squeals was significantly associated with the microbiota composition (**Figure 2A)**, displaying the strongest correlation in the RDA plot (longest arrow; **Supplementary figure 1C**). The frequency of squeals during the OFT was positively associated with the abundance of microbial genera such as *Coprococcus 3, CAG-873, Eubacterium coprostanoligenes, p-1088-a5 gut group* and ***Veillonella.*** Individual Spearman correlation analysis re-affirmed the RDA correlation observations, except for ***Veillonella*** whose association appeared to be insignificant (**Figure 2B**). Other behavioural variables in the OFT did not relate to the microbiota composition.

Similarly, NOT variables were assessed to evaluate whether an animal's reaction to a novel object was associated with its microbiota composition (**Supplementary figure 1D**). We found six observations linked to exploration behaviour, having significant associations with microbiota. Behavioural parameters such as nosing (walls + floor), exploring (nosing + rooting of walls and floor), walls (nosing + rooting walls), nosing floor, nosing walls as well as exploring the novel object were found significant ($P < 0.1$; **Figure 3A, B; Supplementary figure 2A, B, C, D, E**). However, no significant correlation was found between 'latency to touch the novel

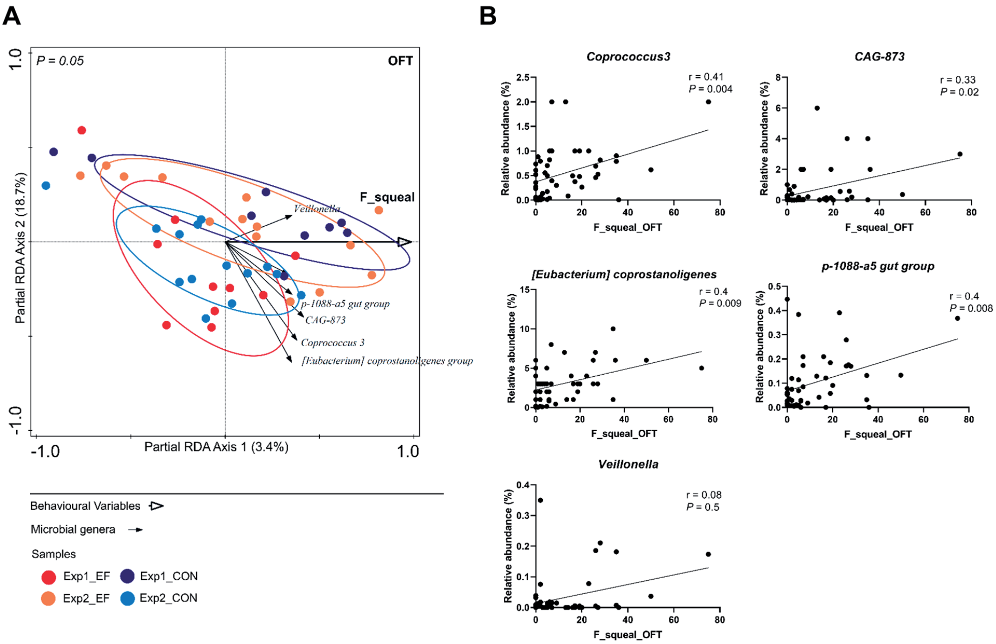

Figure 2: Assessing microbial associations of individual behavioural variables in open field test (OFT). (A) Partial redundancy analysis (pRDA) of the frequency of squeals (F_squeal_OFT), corrected for experiment and treatment. In the pRDA triplot, individual animals are indicated in coloured balls belonging to either one of the experiments and treatment group. Microbial groups having (i) RDA response score ≥ 0.35 as well as (ii) minimum 0.1% relative abundance in at least 10% of the samples, are visualised in the triplot. The perpendicular distance between microbes and environmental variable axes in the plot reflects their correlations. The smaller the distance, the stronger the correlation. (B) Spearman correlation of individual pRDA identified microbes with the frequency of squeals.

object’ and microbiota, that is related to the fear response of the pig (**Supplementary figure 2F)**. In most of the exploration-related variables, similar microbes were found associated at both genus and OTU level, supporting the inter-relatedness of the behavioural parameters used (data not shown). Notably, piglets spent much more time displaying nosing behaviour relative to rooting behaviour, indicating that the overall exploration behaviour is predominantly directed by the nosing behaviour (**Supplementary table 2**). Both nosing and total exploration behaviour negatively correlated with the abundance of *Prevotella 9* and *Prevotellaceae NK3B31 group* and positively with microbial groups like *Atopobium* and *UBA1819* (**Figure 3A, B**). Individual Spearman correlation analysis found moderate, yet significant correlations between the identified microbial groups and nosing/exploring behaviour (**Figure 3C, D**).

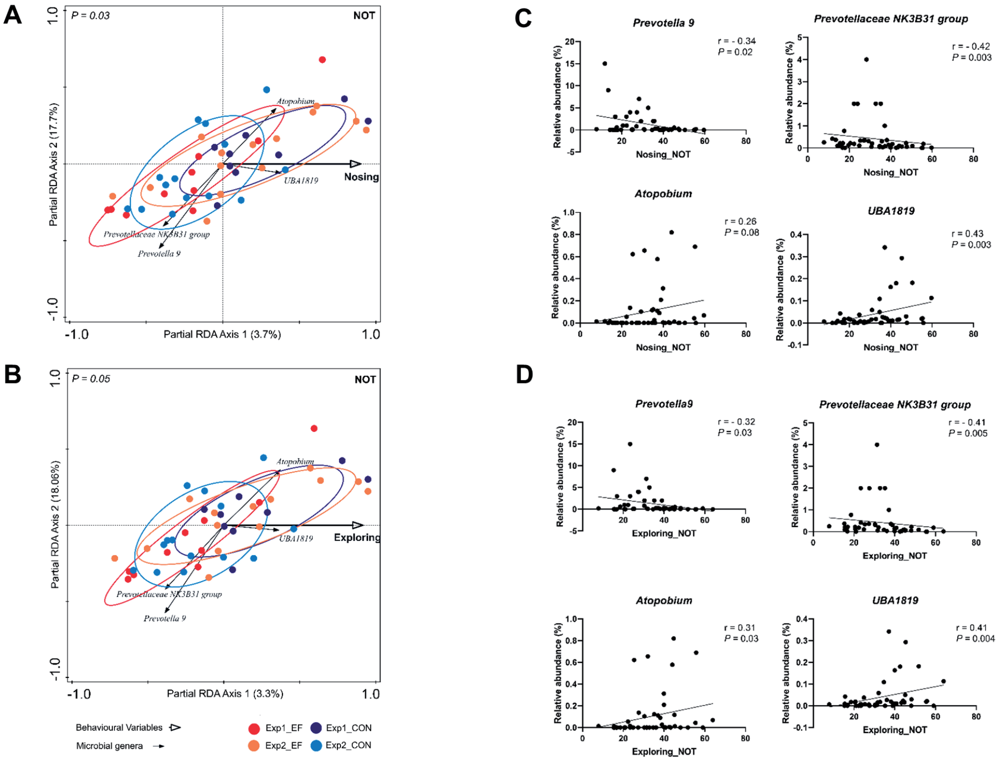

Figure 3: Assessing microbial associations of individual behavioural variables in novel object test (NOT). (A) Partial redundancy analysis (pRDA) of the nosing behaviour (% of time spent nosing) during the novel object test, corrected for experiment and treatment. (B) pRDA of the exploring behaviour (nosing + rooting: both walls and floor; % of time spent exploring), corrected for experiment and treatment. In the pRDA triplot, individual animals are indicated in coloured balls belonging to either one of the experiments and treatment group. Microbial groups having (i) RDA response score ≥ 0.35 as well as (ii) minimum 0.1% relative abundance in at least 10% of the samples, are visualised in the triplot. The perpendicular distance between microbes and environmental variable axes in the plot reflects their correlations. The smaller the distance, the stronger the correlation. (C) Spearman correlation of pRDA identified microbes with nosing behaviour. (D) Spearman correlation of pRDA identified microbes with exploring behaviour**.**

Notably, *Eubacterium coprostanoligenes* (5 OTUs), *UBA1819* (1 OTU) and *Atopobium* (1 OTU) were associated with respective behavioural observations at the OTU level. Further, most of these identified behavioural parameters were interrelated, indicated by similar (quartile) distribution of individual piglets (based on nosing behaviour) with other behavioural parameters (**Supplementary figure 1E**). Intriguingly, 'explore novel object' showed an opposite association with *Prevotellaceae NK3B31 group* compared to other exploration-related behaviour like 'nosing' and 'exploring' (**Supplementary figure 2**), which is also evident from their negative correlation (**Supplementary figure 3)** as well as the inverse distribution of individual piglets in 'explore novel object' behaviour when grouped by nosing behaviour (**Supplementary figure 1E**).

To compare results obtained by redundancy analyses, MaAsLin analysis was performed using both default as well as congruent settings. Using the MaAsLin default settings that are more stringent than those we employed in RDA analyses, we did not identify any significant microbial associations (Benjamini–Hochberg FDR corrected P value < 0.05). However, upon relaxing the correction method and employing microbiota cut-off settings analogous to the RDA analyses, the MaAsLin analyses revealed various microbial groups including several found by RDA, to be associated with behavioural parameters albeit with varying degree of strength (**Supplementary table 3**).

Discussion

The association between microbiota and behaviour is increasingly studied and accumulating evidence indicates that the gut microbiota can influence animal behaviour (Ezenwa et al., 2012; Johnson and Foster, 2018; Sherwin et al., 2019), although the underlying mechanisms largely remain to be deciphered. We previously investigated the influence of diet on gut microbiota and host mucosa development during early-life in pigs (**Chapter 3, 4** and **5**). In the present study we explored whether the intestinal microbiota composition in pigs is associated with host behavioural traits at an individual animal level (independent of dietary treatment), by employing multivariate correlation analyses. This study aimed to tentatively reveal associations between the intestinal microbiota composition and piglet behaviour in a test for coping style, (i.e. a personality trait), as well as anxiety- and exploration-related behavioural parameters in an open field-novel environment test. These associations between microbiota composition and behaviour of suckling piglets in challenging situations have, to the best of our knowledge, not been investigated before.

Previous studies stated that behavioural responses during the 'backtest' are indicative of individual coping responses to environmental stressors (Hessing et al., 1994; Zebunke et al., 2017), often classifying them into high and low-resisting animals based on the latencies and frequencies of struggling and vocalising (Bolhuis et al., 2003). High-resisting animals seem more likely to adopt an active coping style, whereas low-resisting animals that remain immobile and silent during the backtest adopt a passive, or reactive coping style (Hessing et al., 1994). In this study, the coping style of suckling piglets did not have any association with the corresponding microbiota composition at two weeks of age. A possible explanation for this might be that the microbiota colonisation in the first few weeks of life, is usually dynamic and apparently chaotic leading to relatively high individual variation, suggesting that an extended sample size would probably be required to assess microbial association with parameters obtained during the backtest. An alternative could be to perform the backtest at a later age, for instance just before weaning, when the microbiota has relatively stabilised, and investigate whether there is a relation between coping style and microbiota.

Prior studies indicate that vocalisations can reflect an animal's (pig) emotional and/or physiological state (Düpjan et al., 2008). Low-pitched vocalisations (grunts) might be used to maintain social contact, while high-pitched vocalisations, particularly squeals and screams, could relate to distress or anxiety, especially when the animal is in isolation (Fraser, 1975; Manteuffel et al., 2004; Düpjan et al., 2008). Suckling piglets were subjected to a combined OFT and NOT to evaluate their anxiety- and exploration-related responses in a novel environment. In the present study, we observed that the number of squeals uttered by piglets had a significant association with their microbiota. However, we found no significant microbiota correlation with the frequency of long grunts, which has been previously reported to be strongly associated ($r = 0.75$, $P < 0.05$) with the frequency of squeals in a similar test in adult pigs (Fraser, 1974). In the present study we only detect a minimal correlation between these two behaviour ($r = 0.3$, $P = 0.07$; **Supplementary figure 3**).

Exploratory behaviour in a challenging situation can reflect anxiety, with lower levels of exploration indicative of more fearfulness (Donald et al., 2011; Reimert et al., 2014). During the NOT in our study, the piglets displayed higher frequency of squeals, spent more time on standing alert and less time exploring the test arena (especially nosing floor), compared to the OFT (**Supplementary table 2**), that was not fully compensated by the time spent on 'exploration of novel object' during the NOT. This suggests that the piglets were more alert/ fearful in the NOT, possibly indicating 'exploration during the NOT' as a better reflection of (lack of) fear/anxiety compared to OFT. This is in line with a previous study (Ursinus et al., 2013) that found associations among platelet serotonin, brain serotonin and behaviour during the NOT phase, but not during the novel environment (or OFT) phase. Another study (Andersen et al., 2000) demonstrated the effect of an anxiolytic drug on pig behaviour, only in an elevated plus maze but not in a novel environment test. This potentially suggests that exposure to a novel environment alone (OFT) was not particularly fear-provoking for pigs, as their level of fearfulness is more evident in a relatively challenging situation (e.g., NOT), which is also coherent with our findings with more associations observed in the NOT.

In our study, *Coprococcus* showed a moderate positive correlation with the frequency of squeals in the OFT, however, this is contrary to previous mice/human studies that showed an inverse correlation with stress (Bailey et al., 2011) or depression (Valles-Colomer et al., 2019). In addition, we found microbial groups like ***Atopobium*** and ***Prevotella/***Prevotellaceae to be positively and negatively associated with exploration behaviour, respectively. This is consistent with a previous mice study on maternally separation which reported that 'anxious' mice have a reduced ***Atopobium*** abundance (De Palma et al., 2015). Moreover, our results are also in agreement with existing literature reporting positive associations between ***Prevotella*** and depression/ anxiety in rodents or humans (Cussotto et al., 2019; Simpson et al., 2020), which would be in line with 'less exploring' behaviour in the present study. Although these parallel findings are intriguing, it is important to note that comparisons of microbiota-behavioural associations across different animal species are challenging, as we might be potentially compare apples and oranges. This is because both behavioural observational scores as well as the coinciding development of the central nervous systems and intestinal microbiota during early-life can be potentially very different across species. In the previous study (Valles-Colomer et al., 2019), apart from an inverse relation with depression, the ***Coprococcus*** relative abundance was proposed to coincide with abundance of genes associated with dopamine biosynthesis in the microbiota. The paper further assembled a catalogue or framework called gut brain modules (GBMs), that represent 'neuroactive potential' of microbes annotated for function, pathway, structure and potential to cross the intestinal epithelium and the blood–brain barrier. The microbial groups identified in the present study, such as ***Coprococcus, Eubacterium*** and ***Prevotella*** are described in the GBMs having microbial neuroactive potential, which is noteworthy.

Interestingly, 'exploring novel object' revealed an opposite trend of association with the intestinal microbiota, possibly reflecting the ambiguity of 'general exploration' behaviour and 'novel-object-oriented' behaviour and their connection to an animal's level of fear. As mentioned above, squeal ('high-pitch' vocalisation) and exploratory behaviour directed at the environment have previously been positively and negatively linked with anxiety in piglets respectively. However in the present study, we did not observe a significant negative correlation between the frequency of squeals/ latency to explore NO (fearfulness) and nosing (exploratory) behaviour (**Supplementary figure 3**). In other words, we did not identify an 'anxious' group of piglets displaying both high squeal behaviour (OFT) and low exploration behaviour (NOT). A possible explanation for this apparent discrepancy can be because pigs are opportunistic omnivores and relatively neophilic (unlike rodents), with 'novelty' not only perceived as a potential threat but also as an opportunity. Besides, pigs have a natural instinct or 'behavioural need' to explore, which is extremely limited in their barren, stimulus-poor housing (not enriched). Thus, during their exposure to novel environment (e.g., OFT), social isolation might be the most stressful part and the test could well be experienced as an outlet for exploring the environment. Hence, their behaviour can be reflecting a mixture of fear(fulness), motivation to explore ("curiosity"), and motivation to join their pen mates and mom (for warmth and food).

Another point to recognise is our lack of understanding of the complexity of the (animal) behavioural phenotype. For instance, although the open-field test has been widely used to assess pig emotion, there are insufficient evidence to justify its validity as a test for fearful or anxious behaviour in pigs (Forkman et al., 2007). Apart from the lack of consensus in assessing and interpreting individual behavioural parameters, it is even more complex to interpret the overall open field behaviour in pigs (Donald et al., 2011). For example, ‘freeze’ behaviour (or standing alert) in a novel environment, has been differently interpreted by previous studies, where it has been considered to reflect either a ‘fear response’ (Clouard et al., 2015) or as a ‘state of arousal’ (Backus et al., 2017) in which the animal orients itself to toward the stimulus to investigate, and likely reflects both. Furthermore, suckling piglets are hardly tested, so we do not know whether their open field behaviour reflects the same as weaned/adult pigs as reported in other studies. Therefore, the ambiguities in behavioural interpretations has to be taken into account when inferring the overall biological consequence of correlations between microbiota and a single observed behavioural parameter. Nevertheless, the porcine microbiota-behavioural associations observed in this study deserve further evaluations, to establish their biological relevance and underlying mechanism(s).

Taken together, this study provides insights into the association of early-life behaviour and microbiota in suckling piglets. It also demonstrates the importance of individual analysis when evaluating behavioural and microbiota associations, both of which are individual-specific traits. Notably, most behavioural tests have been developed and standardised for post-weaning or growing pigs, which are more mature as compared to the suckling (still developing) piglets employed in our study. For future studies in young piglets, the behavioural tests might need to be adjusted and verified accordingly to ensure that they adequately reflect anxiety and/or explorative behaviour. Although there is a lack of similar studies reported in the literature which disables the comparative analysis of the results obtained in this study, the approaches used in this study may inspire the design of new experiments and strategies to evaluate the role of the microbiota-gut-brain axis in behavioural development of young animals.

Acknowledgements

This study is part of research programme ‘Genetics, nutrition and health of agricultural animals’ with project number 868.15.010, which is co-financed by the Netherlands Organization for Scientific Research, Cargill Animal Nutrition and Coppens Diervoeding. We would like to acknowledge the personnel and trainees of the animal research facilities in Wageningen (Carus) for taking care of the animals and for their technical assistance. The authors are grateful to Bjorge Laurenssen, Monique Ooms, Manon van Marwijk (Adaptation Physiology group) as well as the students who helped conducting the animal experiment and the behavioural tests.

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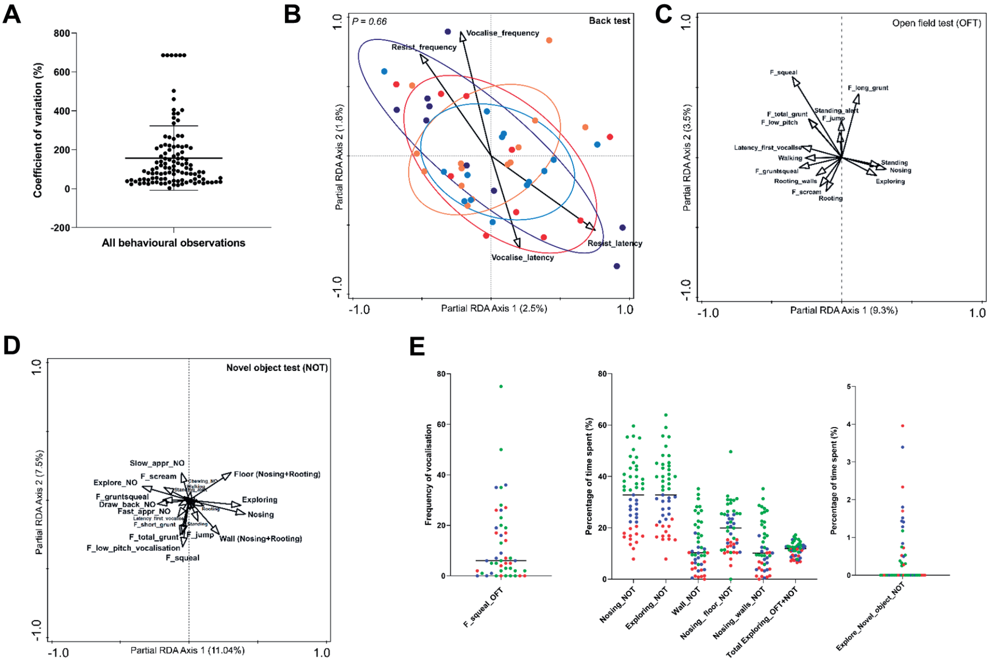
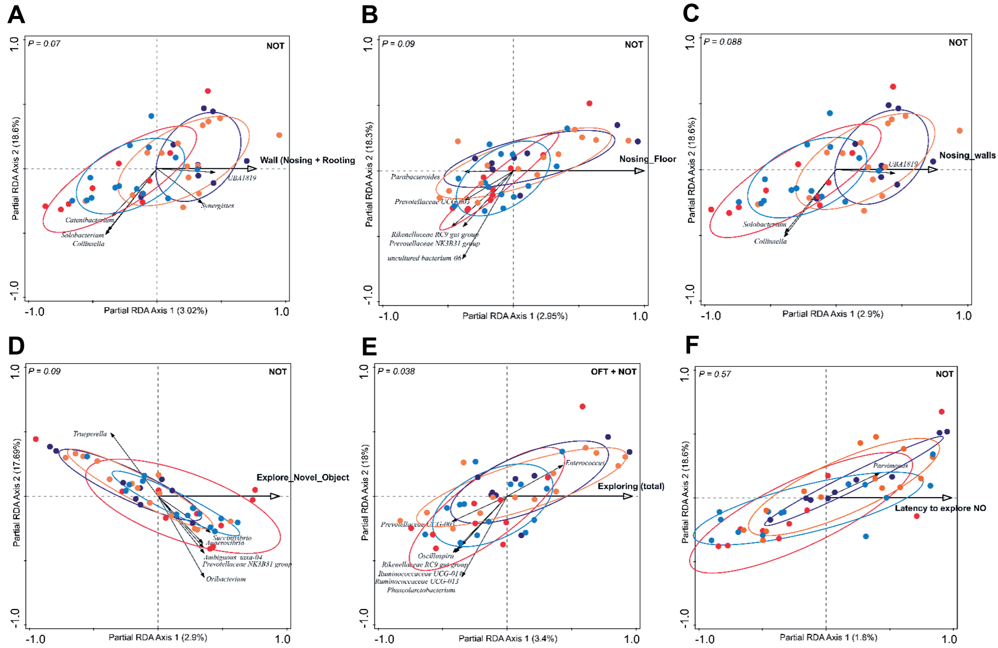
Supplemental information

Supplementary table 1: Ethogram of behavioural states and vocalisations scored in the combined open field and novel object test. Behavioural states were scored in two mutually exclusive classes: "Locomotion and postures" and "Behaviour". The behaviours mentioned under "novel object test only" were added to the "Behaviour" class for that phase of the test.

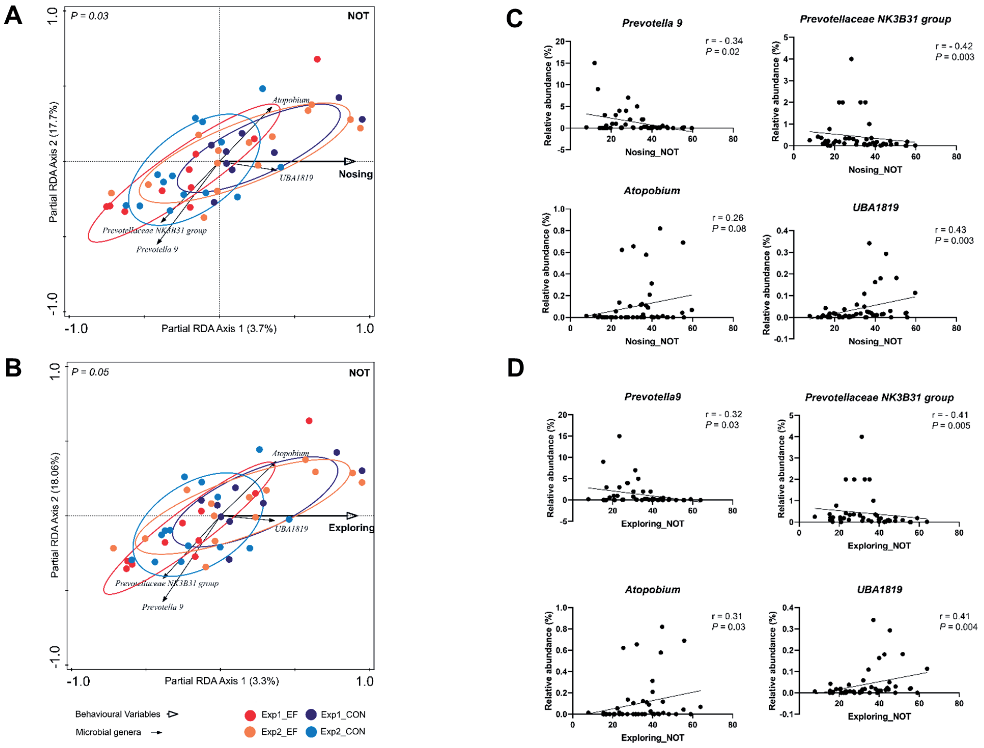
Behaviour	**Definition**
Open field test/ Novel object test	
Locomotion and postures	
Walking	All four legs move or the piglet turns around at the same spot without moving all four legs
Standing	Standing with four paws on the floor without moving them, excluding standing alert
Standing alert	Standing motionless with ears upright and head fixed (up or down)
Sitting or lying	Sitting or lying on the floor without performing any other described behaviour
Behaviour	
Exploring floor	
Nosing floor	Sniffing, touching (with snout) or licking the floor. Rooting disc is either in contact or very close to the floor
Rooting floor	Rooting the floor with the rooting disc that exerts some force
Exploring walls	
Nosing walls	Sniffing, touching (with snout) or licking the walls of arena. Rooting disc is either in contact or very close to the surface
Rooting walls	Rooting the walls of arena with the rooting disc that exerts some force
Low-pitched vocalisations (events)	
Short grunt	A low tone of less than half a second (one note)
Long grunt	A low tone of more than half a second (one note)
High-pitched vocalisations (events)	
Squeal	A high tone (different notes)
Grunt-squeal	A low tone that transforms into a high tone
Scream	A high, long and loud tone, often as long as an expiration
Novel object test only	
Draw back	Drawing back from the novel object (NO; bucket) by walking away from the object or turning around at the same spot. At first the head is directed to the object, during the draw back the head can be either directed to the object or turned away from it
Approaching novel object	
Slow approach novel object	Approaching the novel object slowly (step by step) within 1m distance
Fast approach novel object	Approaching the novel object quickly (easy walking or running) within 1m distance
Exploring novel object	
Touch novel object	Sniffing, touching (with snout), rooting or licking the novel object. Rooting disc is in contact with the object
Explore novel object	Sniffing the novel object without touching it. Rooting disc is not in contact with the object
Chew novel object	Touching novel object with open mouth and making biting movements
Latency to explore novel object	Latency until the time the piglet either touched or explored (sniffing without touching) the novel object



Supplementary figure 1: (A) Coefficient of variation (CV%) of all the behavioural variables observed in the backtest, combined open field test (OFT) and novel object test (NOT). (B) Partial redundancy analysis (pRDA) of the personality or backtest showing individual piglets belonging to exp1_EF (red), exp1_CON (dark blue), exp2_EF (orange) and exp2_CON (light blue) groups. (C) pRDA of all the variables in OFT. (D) pRDA of all the variables in NOT. The arrow length represents the strength of the correlation between the environmental variables and the microbes. The longer the arrow length, the stronger the correlation. (E) Scatter plot of the individual behavioural variables showing the spread of the data. Based on the 'nosing behaviour' quartiles, the individual piglets were divided into three groups: High (green; maximum to median), moderate (blue; median to 25th percentile) and low (red; 25% percentile-minimum) nosing behaviour. The other behavioural parameters were visualised using this classification.



Supplementary figure 2: Partial redundancy analysis of the behavioural variables (corrected for experiment and treatment), with a P < 0.1. (A) Wall (nosing + rooting behaviour on the walls) in novel object test (NOT). (B) Nosing_floor (nosing behaviour in the floor only) in NOT. (C) Nosing_walls (nosing_behaviour on the walls only) in NOT. (D) Exploring_novel_object (exploring the bucket) in NOT. (E) Exploring_total (total exploring behaviour: nosing + rooting in both floor and walls) in combined OFT and NOT. (F) Latency to explore NO (novel object) in NOT.



Supplementary figure 3: Spearman correlation analysis between different behavioural parameters: vocalisations (“squeal” and “long grunt” in OFT); vocalisation and exploring (“squeal” in OFT and “nosing” in NOT); exploration directed towards novel object (“explore NO”) and general exploration (“nosing” and “exploring” during NOT); latency to explore NO and general exploration (“nosing”); latency to explore NO and explore NO.

Supplementary table 2: Behavioural responses observed in the combined open field test (OFT) and novel object test (NOT) at 3.5 weeks of age in suckling piglets (n = 47), expressed as Mean ± SEM. The variables with a prefix “F” denotes frequency of that event and “P” denotes the percentage of time (%) spent on that behavioural state.

Open field test (OFT)		**Novel Object test (NOT)**	
Variable	**Mean±SEM**	**Variable**	**Mean±SEM**
F_shortgrunt	113 ± 9.2	F_shortgrunt	121 ± 8.8
F_longgrunt	55 ± 4.3	F_longgrunt	48 ± 3.9
F_bark	0.1 ± 0.1	F_bark	0.7 ± 0.4
F_lowpitch	168 ± 8.7	F_lowpitch	170 ± 8.3
F_squeal	12 ± 2.2	F_squeal	20 ± 3.1
F_gruntsqueal	31 ± 6.0	F_grunt_squeal	22 ± 4.7
F_scream	2.3 ± 1.3	F_scream	2 ± 0.7
F_highpitch	45 ± 7	F_highpitch	44 ± 5.7
F_jump	2.2 ± 0.45	F_jump	3 ± 0.6
P_walking	48 ± 1.9	P_walking	35 ± 1.8
P_standing	50 ± 1.7	P_standing	61 ± 1.8
P_standing_alert	1.9 ± 0.4	P_standing_alert	3 ± 0.5
P_nosing_floor	42 ± 2.2	P_nosing_ floor	19.5 ± 1.3
P_rooting_floor	1.7 ± 0.5	P_rooting_floor	1.2 ± 0.3
P_nosing_walls	14 ± 0.9	P_nosing_walls	13 ± 1.4
P_rooting_walls	0.39 ± 0.1	P_rooting_walls	0.2 ± 0.1
Nosing	56 ± 2.2	Nosing	32 ± 1.9
Rooting	2.1 ± 0.6	Rooting	1.4 ± 0.4
Floor	44 ± 2.4	Floor	21 ± 1.4
Wall	14 ± 0.9	Wall	13 ± 1.4
Exploring	58 ± 2.4	Exploring	34 ± 1.9
		P_draw_back_NO	0.3 ± 0.1
		P_slow_approach_NO	0.6 ± 0.23
		P_fast_approach_NO	1.0 ± 0.1
		P_touch_NO	13 ± 1.5
		P_explore_NO	0.5 ± 0.13
		P_chewing_NO	1.2 ± 0.39
		Latency_explore_NO	18 ± 2.1

Supplementary table 3: Correlation of behavioural variables with microbial taxa having 0.1% relative abundance in at least 10% of samples, using MaAsLin Analysis in Galaxy (Huttenhower lab). The variables listed here have been identified in both MaAsLin ($P < 0.05$) and redundancy (RDA) analysis ($P < 0.1$), along with the corresponding regression coefficient, number of observations, number of non-zero observations, P-value, and Q-value (FDR-adjusted P-value using Benjamini–Hochberg) determined by MaAsLin analysis. The microbial taxa marked in deep green (response score ≥ 0.35), light green (response score ≥ 0.1) represent taxa that are identified in both the analysis. The light red coloured taxa indicate associations identified in RDA in opposite direction.

Variable	Microbial genera	Coefficient	N	N.not.0	P-value	Q-value
F_squeal_OFT	Bacteria|Firmicutes|Clostridia|Clostridiales|Ruminococcaceae|Anaerotruncus	0.0003	47	9	0.012	1.00
F_squeal_OFT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|Oribacterium	0.0017	47	41	0.013	1.00
F_squeal_OFT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|Rikenellaceae|Alistipes	0.0009	47	32	0.020	1.00
F_squeal_OFT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|Muribaculaceae|CAG-873	0.0041	47	41	0.020	1.00
F_squeal_OFT	Bacteria|Proteobacteria|Gammaproteobacteria|Pasteurellales|Pasteurellaceae|Actinobacillus	0.0010	47	35	0.024	1.00
F_squeal_OFT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|uncultured|uncultured bacterium	0.0008	47	31	0.028	1.00
F_squeal_OFT	Bacteria|Synergistetes|Synergistia|Synergistales|Synergistaceae|Pyramidobacter	0.0009	47	28	0.040	1.00
F_squeal_OFT	Bacteria|Cyanobacteria|Melainabacteria|Gastranaerophilales|Clostridium sp. K4410. MGS-306|Clostridium sp. K4410.MGS-306	0.0004	47	11	0.043	1.00
Nosing_NOT	Bacteria|Proteobacteria|Deltaproteobacteria|Desulfovibrionales|Desulfovibrionaceae|uncultured	-0.0015	47	20	0.003	1.00
Nosing_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Family XI|W5053	0.0019	47	27	0.005	1.00
Nosing_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|Syntrophococcus	-0.0064	47	7	0.007	1.00
Nosing_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|[Eubacterium] hallii group	-0.0141	47	43	0.024	1.00
Nosing_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Family XIII|Mogibacterium	-0.0081	47	41	0.032	1.00
Nosing_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|[Eubacterium] eligens group	-0.0015	47	10	0.032	1.00
Nosing_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Family XIII|Family XIII UCG-001	-0.0006	47	39	0.039	1.00
P_explore_bucket_NOT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|Prevotellaceae|Prevotellaceae UCG-001	0.0172	47	27	0.000	0.87
P_explore_bucket_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|[Eubacterium] eligens group	0.0079	47	10	0.002	1.00
P_explore_bucket_NOT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|Prevotellaceae|Prevotella 9	0.0526	47	45	0.003	1.00
P_explore_bucket_NOT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|Prevotellaceae|Prevotellaceae NK3B31 group	0.0269	47	45	0.014	1.00
P_explore_bucket_NOT	Bacteria|Cyanobacteria|Melainabacteria|Gastranaerophilales| Ambiguous_taxa| Ambiguous_taxa	0.0051	47	23	0.015	1.00

Continue

Continued

Variable	**Microbial genera**	**Coefficient**	**N**	**N.not.0**	**P-value**	**Q-value**
P_explore_bucket_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|Coprococcus 2	0.0096	47	11	0.042	1.00
P_explore_bucket_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|Coprococcus 1	0.0178	47	39	0.046	1.00
P_explore_bucket_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Ruminococcaceae|CAG-352	0.0060	47	10	0.047	1.00
P_explore_bucket_NOT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|Prevotellaceae|Alloprevotella	0.0171	47	47	0.048	1.00
P_nosing_ floor_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Family XIII|Mogibacterium	0.0092	47	41	0.023	1.00
P_nosing_ floor_NOT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|uncultured bacterium|uncultured bacterium	0.0019	47	7	0.036	1.00
P_nosing_ floor_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|[Eubacterium] hallii group	0.0144	47	43	0.036	1.00
P_nosing_walls_NOT	Bacteria|Firmicutes|Erysipelotrichia|Erysipelotrichales|Erysipelotrichaceae|Erysipelotrichaceae UCG-007	-0.0045	47	27	0.030	1.00
P_nosing_walls_NOT	Bacteria|Elusimicrobia|Elusimicrobia|Elusimicrobiales|Elusimicrobiaceae|Elusimicrobium	0.0010	47	27	0.033	1.00
P_nosing_walls_NOT	Bacteria|Proteobacteria|Deltaproteobacteria|Desulfovibrionales|Desulfovibrionaceae|uncultured	0.0007	47	20	0.035	1.00
P_nosing_walls_NOT	Bacteria|Fusobacteria|Fusobacteriia|Fusobacteriales|Fusobacteriaceae|Fusobacterium	-0.0029	47	42	0.035	1.00
P_nosing_walls_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Family XI|W5053	-0.0012	47	27	0.037	1.00
P_nosing_walls_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Defluviitaleaceae|Defluviitaleaceae UCG-011	-0.0006	47	39	0.037	1.00
P_nosing_walls_NOT	Bacteria|Actinobacteria|Actinobacteria|Corynebacteriales|Corynebacteriaceae|Corynebacterium 1	0.0025	47	44	0.039	1.00
P_nosing_walls_NOT	Bacteria|Synergistetes|Synergistia|Synergistales|Synergistaceae|Cloacibacillus	0.0005	47	30	0.039	1.00
P_nosing_walls_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Ruminococcaceae|CAG-352	0.0004	47	10	0.040	1.00
P_nosing_walls_NOT	Bacteria|Bacteroidetes|Bacteroidia|Bacteroidales|Muribaculaceae|Ambiguous_taxa	0.0019	47	34	0.044	1.00
Wall_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|[Eubacterium] fissicatena group	0.0050	47	41	0.049	1.00
Exploring_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|[Eubacterium] eligens group	0.0016	47	10	0.019	1.00
Exploring_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|Syntrophococcus	0.0045	47	7	0.030	1.00
Exploring_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|Marvinbryantia	-0.0041	47	46	0.039	1.00
Exploring_NOT	Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|[Eubacterium] hallii group	0.0133	47	43	0.046	1.00

7

Chapter 7

General Discussion

Background

Weaning stress leading to microbial dysbiosis is a major concern in pig commercial farming, with a pressing need to evaluate feeding strategies to prepare pigs for the weaning transition (Gresse et al., 2017; Guevarra et al., 2019). Commercial pig production systems involves early and abrupt weaning, which contrasts with the gradual transition from mother's milk to solid feed in nature. Due to such abrupt weaning, a piglet is challenged with multiple stressors (including environmental-, nutritional- and psychological-) which is usually associated with changes in gut microbiota and a high incidence of diarrhoea. An optimally functioning gastro-intestinal tract is important for the overall host metabolism and physiology, with gut microbiota playing a crucial role (Pluske et al., 2018). Modulating gut microbiota by dietary fibres has been previously proposed to be beneficial to the gastro-intestinal health of the host (Montagne et al., 2003; Jha et al., 2019), especially during 'early-life' development (Pohl et al., 2015; Everaert et al., 2017). However, there is a scarcity of information on the impact of dietary fibres on intestinal microbiota and mucosal development in young piglets, utilising the early-life 'window of opportunity' (**Chapter 1**). Therefore, this thesis aimed to evaluate early-life nutritional strategies to prepare the pig's intestine for weaning stress, focussing on assessing the (molecular) consequences of the early feeding intervention (during lactation phase) in piglets.

The principal aims of this thesis were: (i) To assess the optimal sampling type for (longitudinal) microbiota analysis in young piglets (**Chapter 2**), (ii) To investigate the age-related gut microbiota colonisation and the impact of fibrous feed on the gut microbiota colonisation as well as intestinal physiology in neonatal piglets (**Chapter 3, 4**), (iii) To investigate the host mucosa (transcriptome) response due to pre-weaning fibrous feed (**Chapter 5)**, (iv) To explore associations between the gut microbiota and behaviour in suckling piglets (**Chapter 6)**. Three animal experiments were conducted to address the above-mentioned aims, starting immediately after the birth of the piglets, and following the piglets until a few weeks post-weaning (**Figure 1**). The first five days after weaning is usually considered to be the period where piglets experience maximum weaning stress, whereas the adaptive phase (day 5 to 15 post-weaning) corresponds to an adaptation of the gut to the weaning diet (Montagne et al., 2007). Keeping this in mind, the study design was prepared (**Figure 1**), with neonatal piglets being offered a customised fibrous feed (including dietary fibres such as galacto-oligosaccharides (GOS), inulin, resistant starch) till weaning (around 4 weeks of age). Post-weaning effects were investigated at two stages: an early post-weaning time-point (generally a few days after weaning) and a later post-weaning time-point (around 2-3 weeks after weaning).

This chapter is divided into three sections: (**A**) Evaluating early-life feeding as an alternative nutrition strategy in suckling piglets, where the major findings of the thesis will be discussed and aligned with existent literature; (**B**) Technical considerations, where methodological

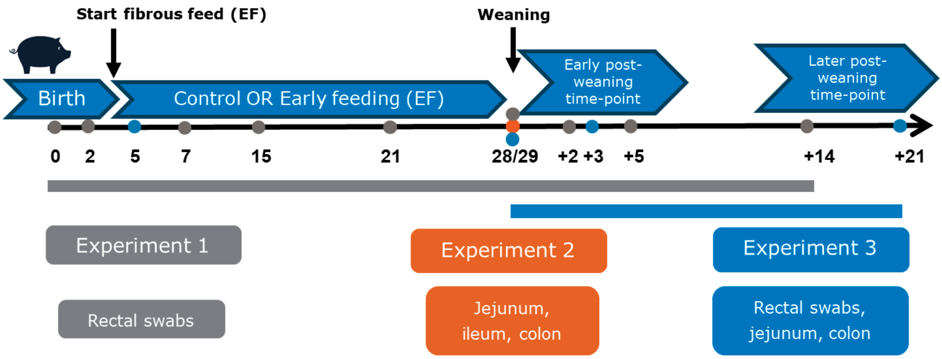


Figure 1: Schematic overview of the experiments performed in this PhD thesis (**Chapter 3, 4, 5, 6**) along with the sampling time-line and sample-type/location. The pilot experiment (to evaluate rectal swabs as an alternative to faecal sampling) described in **Chapter 2** had a different set up and is not illustrated in this figure.

limitations will be discussed along with proposed alternatives; (**C**) Future perspectives, where the implications of our findings as well as considerations for future research will be addressed.

(A) Evaluating early–life feeding as a nutrition strategy in suckling piglets

Stimulating early feeding in piglets

The work presented in this thesis was part of an integrated project titled "*Stimulating early foraging in piglets to accelerate their development and improve their performance around weaning*", that allowed the investigation of the connections among "stimulating early feeding in piglets", their "behavioural development" and the "molecular effects" (microbiota and host mucosa development) due to the early-life feeding.

Before investigating the impact of early feeding, it is essential to stimulate solid feed intake in suckling piglets. In our studies, we opted for the "natural" method of pre-weaning feed provision (*ad libitum* feed consumption), "mimicking" natural conditions where piglets can show their foraging behaviour. In modern pig farming, creep feeding is an optional method to provide complementary feed during lactation, where suckling piglets are given access to solid feed to familiarise them to independent feeding. This may ease the weaning transition as well as stimulate post-weaning feed intake (Bruininx et al., 2002) in piglets. However, "traditional" creep feed is usually highly palatable, easily digestible and mainly based on milk proteins (Okai et al., 1976), which is distant from more fibrous solid feed exposure in both natural and commercial post-weaning conditions. In this thesis, the pre-weaning diet was customised to stimulate the intestine of a suckling piglet, having a fibrous feed composition containing dietary fibres, such as galacto-oligosaccharides (GOS), inulin and resistant starch with a varying degree of solubility and fermentability.

To stimulate early feeding, we provided the fibrous feed two days after birth in a long feed trough (100 x 24 x 8 cm; having approximately 8 feeding spaces) in experiment 1 and 2 (EF; **Chapter 3, 4**). Observational (video analysis) data from both the experiments indicated that eating started mostly during the second week (between 9-11 days of age), increasing over time and displayed maximum eating in the last week before weaning (**Figure 2**).

However, the increasing eating score was not consistent for all piglets, and a high individual variation was observed between and within litters. Against expectations, the eating score did not display a consistent development over time (i.e., steady increase or reaching a plateau), but appeared to display considerable daily variation up to the weaning timepoint (**Figure 2**), illustrating the unpredictability of solid feed intake in suckling piglets.

Our observations are in agreement with previous studies on creep feeding (Pajor et al., 1991; Bruininx et al., 2002, 2004; Heo et al., 2018), that reported a large individual variation in feed intake ranging from "no eating" to "high" feed intake within the creep feeding group. Therefore there is a need to find strategies for stimulating early feed intake in suckling piglets, i.e., increase the number of piglets consuming the feed and enhance the feed consumption. This habituation to ingesting and digesting (some) feed during lactation may lead to "more robust" pigs around weaning for which the dietary transition at weaning is less challenging.

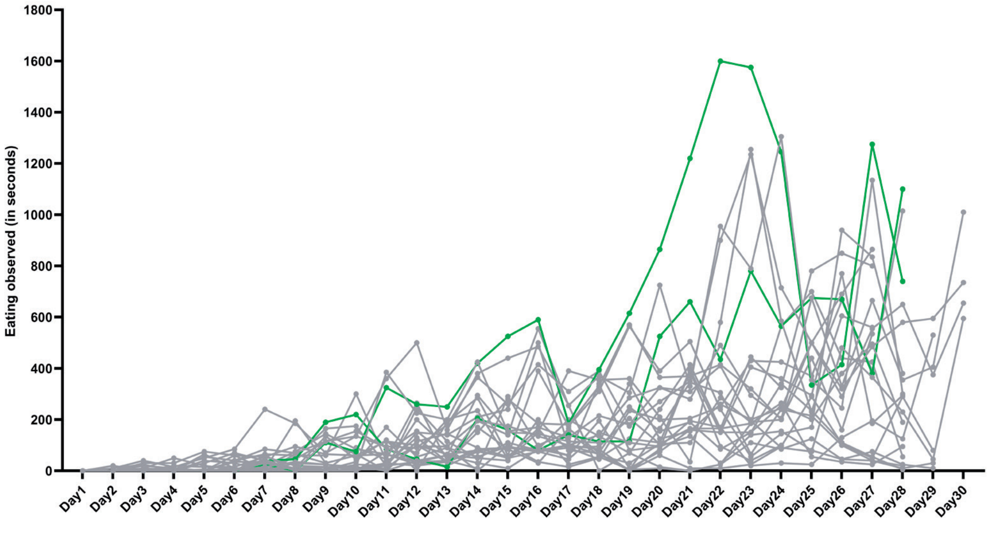


Figure 2: Daily eating behaviour in suckling piglets over 4 weeks (data from experiment 1 and 2). Two piglets having the highest eating score (video observation) in the respective experiments are shown in green lines.

In the (integrated) project, different strategies have been evaluated to stimulate foraging behaviour in suckling piglets such as an ‘enriched pen’ with foraging materials, a ‘foraging-stimulating’ play-feeder, dietary diversity as well as feed presentation in sand (Middelkoop, 2020 PhD Thesis). The most effective and feasible strategy was chosen to stimulate EF piglets in experiment 3 (**Chapter 5**) which was a combination of (i) the ‘foraging-stimulating’ play-feeder and (ii) learning from the sow (**Figure 3**). The play-feeder strategy (Middelkoop et al., 2019a) stimulated the time spent exploring the feeder and led to a higher number of piglets within a litter to visit the feeder (video observations), compared to litters provided with a conventional feeder. Furthermore, ‘learning from the sow’ was included since a previous study (Oostindjer et al., 2011) has shown that ‘learning to eat’ is more effective when piglets can participate with the mother as well as observe her while she is eating. Applying diversity to the feed, which has been shown to be effective in stimulating feed intake in suckling piglets (Middelkoop et al., 2018, 2019b), may lead to confounding effects on the intestinal microbiota and was therefore not included in experiment 3.

Contrary to our expectations, inclusion of these feed-intake stimulating strategies in experiment 3 (**Chapter 5**) did not seem to improve eating in EF piglets, as these had pre-weaning feed intake (at pen level) values comparable with those in the previous experiments in this thesis (**Figure 3**). This is in agreement with previous observations (Middelkoop et al., 2019a) that

Figure 3: Experiment 3 included strategies like providing a ‘play-feeder’ and allowing ‘learning from the sow’ to stimulate eating behaviour in suckling piglets. An extra ‘foraging area’ was built in front of the sow, with two feeders (eight spaces) attached with ‘play materials’ like canvas clothes, braided natural cotton ropes and PVC spiral tubes. The feeders were placed in front of the sow for ‘learning from sow’ strategy, where sows were allowed to partially enter the ‘foraging area’ and eat with the piglets for 10 mins two times a day till weaning.
In the panels on the right, the average feed intake per pen (in grams) are shown for all three experiments conducted. The black dots are individual pens followed over different time-points. At day 30, the average feed intake was 3.96 Kg/pen in experiment 3 whereas it was 4.19 Kg/pen for experiment 1 and 2 (combined).

reported a minimal effect of the play-feeder on pre-weaning feeding (i.e., creep feed intake per pen) although it was successful in eliciting and sustaining exploratory behaviour directed at the feed(er) in suckling piglets. Furthermore, effects of the 'learning from the sow' strategy, on sow and piglet behaviour during the procedure varied (R. Choudhury, personal observation). This strategy involved allowing the sow (with her snout; two 10-min sessions per day) access to a 'foraging arena' that was normally only accessible to her piglets, during which the piglet feed was presented on the floor and in the feeders (**Figure 3**). In four of the seven EF pens, sow and piglets ate together during these 10-min sessions, in line with our expectations. However, that was not the case for all pens, for example, some of the sows were resistant to consume the piglet feed and were waiting for their own feed, which was provided after each session. In another pen, the piglets did not enter the 'foraging arena' when the sow was eating, and started suckling the standing sow for milk. The above-mentioned behaviours were quite consistently observed for those particular pens on all days. All these individual (or pen specific) behavioural variations can thus render such feeding strategies (in suckling piglets) difficult to be uniform in their effect. On one hand, the lack of uniformity poses a challenge to form a homogenous, well-eating group in piglets. On the other hand, a natural gradient of eating behaviour is formed, allowing us to evaluate the consequences of these individual variations in piglets (**Chapter 4**).

Dynamic microbiota colonisation in early–life followed by subsequent homogenisation

Prior to evaluating the effect of early feeding on gut microbiota, we aimed to assess the microbial colonisation or microbiota development over time (regardless of treatment groups), i,e., from birth to 7 weeks of age (**Chapter 3, 5**). As anticipated, we observed distinct pre- and post-weaning associated microbes (irrespective of treatment) with a gradual increase in microbial diversity and "microbiome age" (as determined in redundancy analysis of age) over time (**Figure 4**), eventually reaching a plateau. After 2-3 weeks post-weaning, a relatively stable microbial composition was reached predominantly induced by post-weaning (feeding) conditions. Early colonisers (generally facultative anaerobes that can survive in relatively higher levels of oxygen) such as *Escherichia-Shigella, Enterococcus* and *Streptococcus* first create an anaerobic environment in the neonate gut, allowing the colonisation and/or proliferation of strict anaerobes like *Romboutsia, Intestimonas, Methanobrevibacter, Christensellaceae group R-7* during 3-4 weeks of age (**Chapter 3**). After weaning, a drastic shift in microbiota composition occurred, dominated by fibre-degrading bacteria such as *Prevotella, Faecalibacterium, Subdoligranulum, Roseburia, Megasphaera*, which is likely due to the dietary shift from sow's milk to plant-based solid diet.

The sole dependence on solid feed (after commercial weaning around 4 weeks of age) is a key event that affects the intestinal microbiota as well as the host physiology of the developing piglet (Heo et al., 2013). Our results are consistent with previous studies (Davenport, 2016; Rothschild et al., 2018; Scepanovic et al., 2019) that collectively suggest diet as one of the

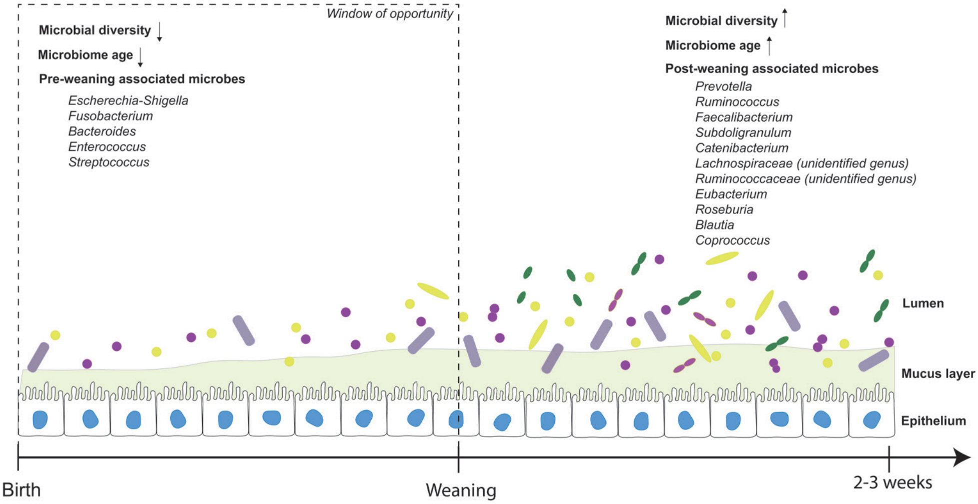


Figure 4: Age-associated intestinal microbiota dynamics at pre- and post-weaning.

prominent drivers of microbiome composition and diversity in various mammals. Specifically in pigs, a previous study concluded that the combination of age, introduction of solid food, and weaning as the main driving forces for the succession and establishment of the microbiota (Bian et al., 2016). They reported that although the impact of breed or host genetics (internal factor) was evident in the early suckling period (< 2 weeks of age), it was overruled by the introduction of solid food/weaning (external factor) in the later ages. After weaning, piglets also undergo (small intestinal) morphological changes at the mucosa level, having a lower villus length as well as villus:crypt ratio (**Chapter 5**), which is in line with previous literature (Bruininx et al., 2004; Al Masri et al., 2015). The post-weaning morphological alterations could also be caused by the reduced nutrient intake (or transient starvation) in the first 48 hours after weaning, independent of the dietary change from mainly sow's milk to (solid) feed.

While the intestinal microbiota during early-life is known to be dynamic and variable among individuals, initial bacterial settlers like ***Escherichia-Shigella, Fusobacterium*** showed similar colonisation patterns in both the experiments conducted in this thesis (**Figure 5**; **Chapter 3, 5**). Experiment 1 and 3 of this thesis enable the assessment of microbiome development over time in piglets. Remarkably, the colonising microbes at various ages were mostly comparable between the two studies, though there were a few inconsistencies. For example, in experiment 1, ***Prevotella*** was the dominant post-weaning microbe with relative abundance increasing from 2% at weaning (day28) to 15% at day14 post-weaning (day+14), whereas in experiment 3 ***Lactobacillus*** was detected as the dominant genus increasing from 6% at weaning (day29) to 35% at day+19 (**Figure 5**). The main differences between the two experiments were the lack of repeated sampling (from the same piglets) in experiment 3 and the later post-weaning

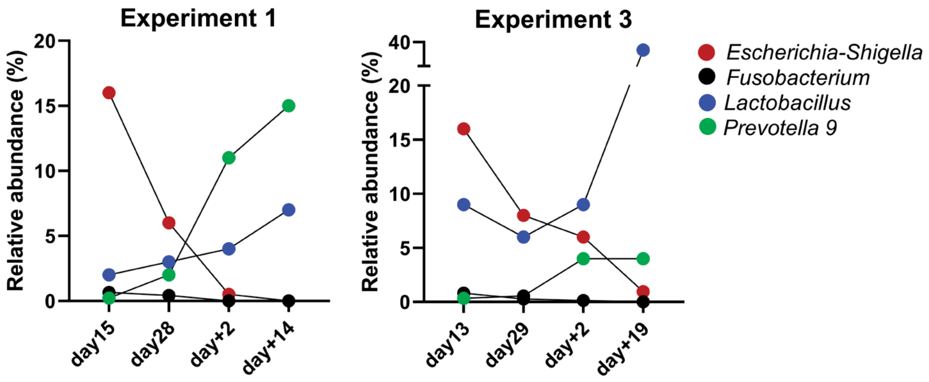


Figure 5: Pre- and post-weaning associated microbes in experiment 1 and 3 showing their average relative abundance at each time-point.

sampling point (day+14 vs. day+19), however that seems unlikely to cause these variations. Both *Prevotella* and *Lactobacillus* have been reported to be prevalent in weaned piglets in several studies (**Chapter 1**). Members of the *Prevotella* genus are known to metabolise plant-derived non-starch polysaccharides to produce short-chain fatty acids (SCFAs), via degrading polysaccharides in plant cell wall with enzymes (such as β-glucanase, mannase, and xylanase) (Flint and Bayer, 2008). In addition, some *Lactobacillus* species are well-equipped to metabolise carbohydrates like oligosaccharides and starch (Gänzle and Follador, 2012), that are abundant in the post-weaning diet providing favourable conditions for members of this genus. Notably, the relative abundance of the *Lactobacillus* genus has been previously shown to be variable among pig breeds (Bian et al., 2016), but that has no relevance in our studies as the experiments were conducted with the same genetic line. The establishment of a "dominant species" can potentially be just a chance or timing event rather than a consistent predicted outcome, with microbes having a fermentative capacity getting a benefit to compete against others immediately after weaning, setting a stage for a relatively stable adult microbiome.

Early feeding leads to accelerated maturation in intestinal microbiota as well as host mucosa

The primary aim of this thesis was to evaluate the impact of early feeding (of fibrous feed) on the intestinal microbiota and host transcriptome in suckling piglets, compared to the control (CON) piglets that did not receive any pre-weaning feed and suckled mother's milk exclusively (**Chapter 3, 4 and 5**). Early-fed (EF) piglets displayed an 'accelerated maturation' in their microbiota colonisation (pre-weaning; **Figure 6**), which was illustrated by a higher (alpha) diversity, emergence of typical post-weaning associated microbes (e.g., *Prevotella, Roseburia, Faecalibacterium, Ruminococcus)* (**Figure 6**) as well as rapid decline of pre-weaning microbial genera (e.g., *Fusobacterium, Eschechichia-Shigella, Intestimonas, Bacteroides*). The effect of early feeding started to be evident from 15 days of age till weaning, which coincided with increased eating scores in video observation. Importantly, eating behaviour was quantitatively correlated with the degree of microbiota acceleration at individual animal level. This illustrates the relation between amount of feed consumed and the microbiota adaptations, thus supporting that these changes are caused by feed intake (i.e., more eating = more microbiome change; **Chapter 3**). Interestingly, our findings are analogous with a previous study (Vo et al., 2017) which reported 'accelerated microbiota maturation' as a result of exposure to agricultural soil (for unrestricted rooting behaviour) from 4 days of age till weaning (day21). Rooting is a natural behaviour in pigs, where they use their snout as a tool for foraging i.e., explore their surroundings and find food (Studnitz et al., 2007). Similar to EF piglets, an increased abundance of microbial groups such as ***Prevotella, Blautia, Faecalibacterium, Coprococcus*** were detected in soil-exposed piglets, which can be possibly explained by the presence of plant material (mainly fibres) available in the soil, hence making it analogous to our (fibrous) intervention. However, the soil consumption by the piglets (during rooting) would be possibly minimal (soil intake not reported in the study), which is reflected by the considerably smaller effect size of the microbiota changes due to soil exposure compared to our study.

Early feeding exhibited maximum impact on colon, while being much more moderate on small intestinal locations (**Chapter 4, 5**), which is in line with previous literature reporting the effect of dietary fibres on the distal regions of the intestine. Short chain fatty acids (SCFAs), such as acetate, propionate and butyrate, are important metabolites in maintaining intestinal homeostasis, produced by bacteria that feed on dietary fibres (predominantly in the distal part of the intestine). SCFAs, particularly butyric acid, can modulate the expression of genes involved in gut motility, host defence and inflammatory responses, contributing to adequate establishment and maintenance of the intestinal barrier (Hamer et al., 2008; Leonel and Alvarez-Leite, 2012; Den Besten et al., 2013; van der Beek et al., 2017). The EF-associated microbiota were typical SCFA-producers, which was also reflected by the increased amount of total SCFAs, as well as specific SCFA butyrate and acetate, and the lower luminal pH in EF relative to CON piglets at weaning (**Chapter 4**). It can be anticipated that there will be an age-related increase in the SCFA concentration levels post-weaning (Ayuso et al., 2020), although the persistence of the impact of early feeding on SCFA levels was not evaluated in our studies. However, it should be noted that microbial SCFAs production in the intestinal lumen and their absorption by the colonic epithelium is a highly dynamic process and the measurements (***in vivo***) merely display a snapshot view. Therefore this provides an inadequate representation of the actual SCFA-flux that is subject to substantial fluctuation, depending on feeding timeframe and history, as well as mucosal adaptation of absorptive capacity (Von Engelhardt et al., 1989; Verbeke et al., 2015). For a better understanding of the SCFA kinetics, future studies might apply stable isotope-labelled nutrients in combination with metabolic modelling (meta-transcriptomics or meta-proteomics) to assess metabolic changes in the microbiota and the host (de Graaf and Venema, 2007; Verbeke et al., 2015).

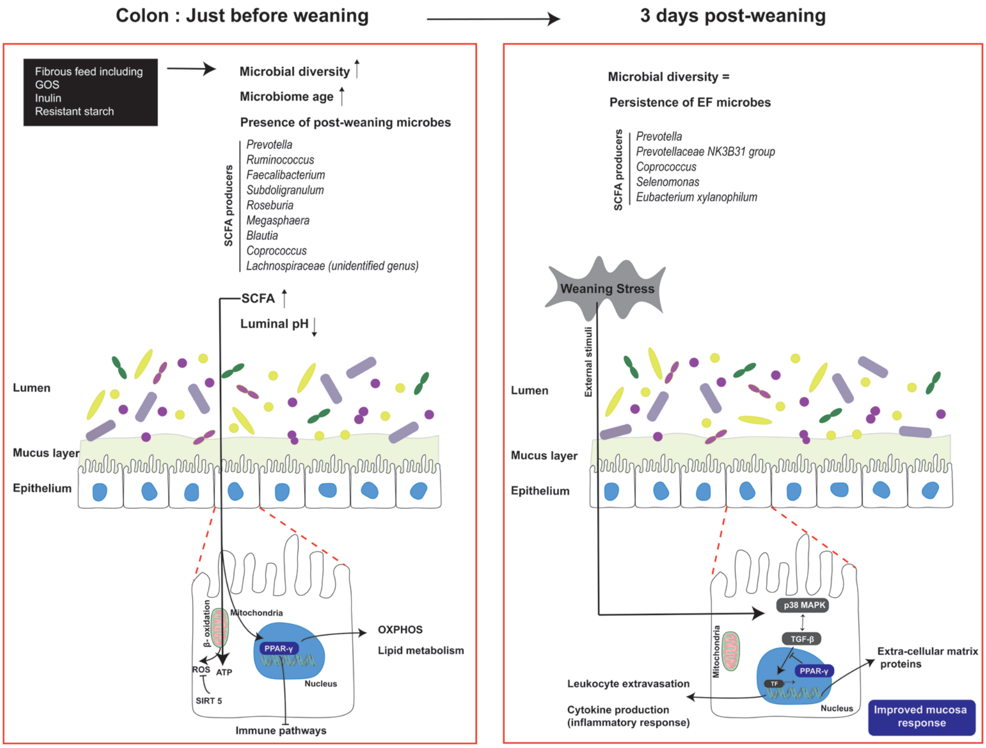


Figure 6: Graphical representation of the molecular changes due to early feeding (fibrous feed) at weaning (day 28/29) and at an early post-weaning time-point (day+3) in the colon mucosa. The early-life dynamics reached stability 2-3 weeks post-weaning, where both the treatment groups converged in their microbial composition and transcriptome. The intestinal microbiota in early-fed (EF) piglets showed an accelerated maturation with a higher microbial diversity, microbiome age and presence of post-weaning microbes (typically SCFA producers) just before weaning, compared to the control (CON) piglets. At weaning, cellular energy metabolism (mitochondrial oxidative phosphorylation or OXPHOS) and immune response were altered in EF piglets, which is modulated by the key transcription factor PPAR-γ. Colonic epithelial cells consume SCFAs (particularly butyrate) through ß-oxidation in mitochondria, producing energy (ATP) and generating reactive oxygen species (ROS). The cellular homeostasis is maintained by sirtuin proteins like SIRT5 which balances the altered cellular ROS levels. Day+3: Weaning stress activates the p38 mitogen-activated protein kinase (p38 MAPK) signalling as a response to external stimuli or stress, along with TGF-ß (transforming growth factor-beta) pathway, that regulates the post-weaning adaptation process. Increased gene expression of cytokines (IL18, TGFB1, TGFB2), leukocyte migration, extracellular matrix proteins indicates an improved mucosal response in EF piglets compared to the CON group. ↑ significant increase; ↓ significant decrease; = no difference.

The host transcriptome displayed a similar trend of progression as was seen for the microbiota maturation during the same timeline, with maximum impact of early feeding just before weaning and reaching relative stability 3 weeks post-weaning (**Chapter 5**). Moreover, the effect of early feeding was more pronounced in the colon transcriptome, analogous to the more extensive microbiome changes in the same intestinal region. Just before weaning (day29), the colon transcriptome of EF piglets showed significantly altered pathways involved in cellular energy metabolism (mitochondrial oxidative phosphorylation or OXPHOS), lipid metabolism and immunity, including the upstream activation of PPAR-gamma (**Figure 6**). The upregulated (cellular) energy metabolism is expected due to the increased SCFAs production (particularly butyrate) in EF piglets, which are utilised by the colonic epithelial cells as an energy source, and subsequently metabolised in mitochondria to produce ATP (Wojtczak and Schönfeld, 1993; Haenen et al., 2013a). Import of SCFA by colonic epithelia is mostly carrier-mediated with proton coupled transporters mediating SCFA influx from lumen and SCFA efflux into blood (Sivaprakasam et al., 2017; Venegas et al., 2019). This was reflected in the increased expression of solute carriers such as SLC16A1 (monocarboxylate transporter) and SLC26A3 (chloride anion exchanger) in the EF group, mediating the SCFA transport across the cellular membranes. However, expression of other transporters such as the sodium coupled transporter SLC5A, known to be regulated by colonic microbes (Sivaprakasam et al., 2017) did not seem to be altered in EF piglets (**Figure 7**). Through binding to G protein-coupled receptors such as GPR41 (FFAR3), GPR43 (FFAR2) and GPR119 or by inhibiting histone deacetylase (HDAC), SCFAs can influence regulatory pathways that impact intestinal mucosal homeostasis, immunity and barrier integrity (Mingming Sun, Wei Wu, Zhanju Liu, 2017; Fellows and Varga-Weisz, 2020). Although a tendency of repressed HDAC7 expression was found in EF compared to CON piglets (**Figure 7**), GPR41, GPR43 and GPR119 were not differentially expressed and actually had a very low expression level (counts per million ≤ 7) in most of the samples irrespective of the treatment. This might be explained by the cell-lineage-specific and time-associated variations

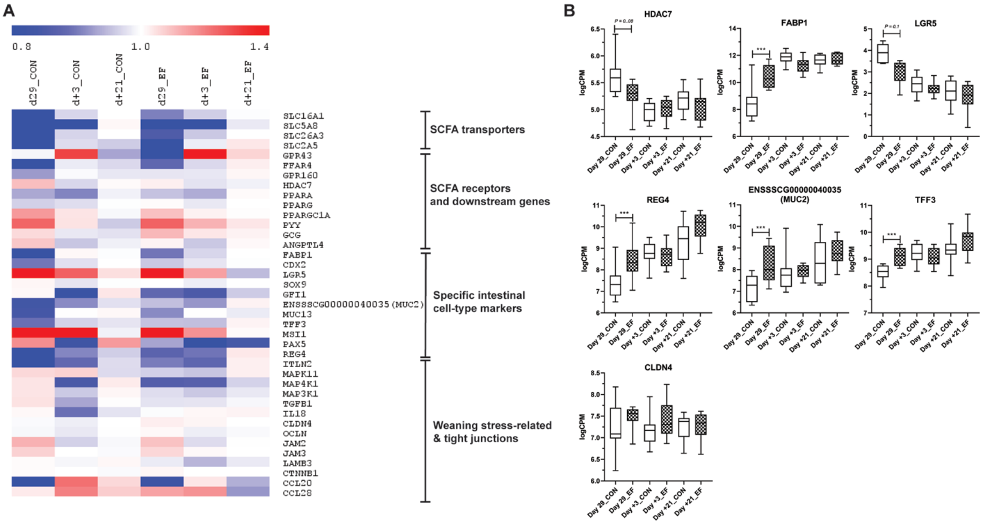


Figure 7: (**A**) Heatmap displaying colonic gene expression of SCFA transporters, SCFA sensing receptors, specific intestinal cell-type markers and tight junction proteins (literature identified) in CON and EF piglets. The expression values (averaged per group per time-point) are scaled by the mean value of total day+21 expression (irrespective of treatment). (**B**) A few genes shown in box plots along with their *P* value (differential expression analysis, EdgeR test) (***: $P < 0.001$).

in the expression levels of GPR41 and GPR43, which was found lowest in the colon tissue of 4 weeks old pigs (Li et al., 2014). Furthermore, this is consistent with previous pig studies (Haenen et al., 2013b; Venegas et al., 2019) that found no fibre-induced changes in the gene expression of these SCFA-sensing receptors, indicative of potential species-related differences in the regulation of the receptor itself (may not be required in pigs) to elicit the downstream signalling cascades. To further evaluate the sensing receptors in the porcine colon, ***in vitro*** experiments can be designed using porcine organoids (or colonoids derived from 4-week old pigs for instance) combined with RNA-sequencing to understand their response to SCFAs exposure. Taken together, it appears that SCFA transporters (especially SLC16A1) in the colon mucosa are regulated by the microbe-derived metabolites in EF piglets, while additional studies would be necessary to assess the effective SCFA-sensing GPRs in pigs.

Our results corroborate with findings of previous fibre intervention studies (Haenen, 2013; Lange et al., 2015) which proposed PPAR-gamma as a central regulator of (colonic) transcriptional responses to dietary fibres, modulating lipid metabolism and immune pathways. Peroxisome proliferator-activated receptors (PPARs) are a group of ligand-activated transcription factors that regulate various metabolic processes such as fatty acid metabolism (Fan and Evans, 2015) and are also involved in regulating microbiota-mediated intestinal homeostasis and immunity (Su et al., 2007; Oh et al., 2019; Cai et al., 2020). Although PPAR-gamma was activated in EF piglets at weaning, the (fibre) fermentation associated genes reported in rodent studies (Zhou et al., 2006, 2008), such as glucagon (GCG) and peptide YY (PYY) were not differentially altered in EF piglets (**Figure 7**), which may be indicative of species-specific regulatory differences and supports the need for further mechanistic exploration in ***in vitro*** cell models using porcine cells.

In **chapter 5**, we demonstrated the "accelerated maturation" in the intestinal mucosal transcriptome at weaning, due to early feeding which was closer to the "convergent" post-weaning transcriptome obtained three weeks post-weaning. At weaning (around four weeks of age), the gastro-intestinal tract of a piglet is immature and thus early-life intervention can induce transcriptomic changes in the mucosal tissue. The intestinal epithelium is one of the most rapidly renewing tissues, where the epithelial cells are continuously renewed approximately every 3–5 days, leading to a reformed monocellular lining of the intestine. The epithelial integrity and rapid turnover are supported by intestinal stem cells (ISCs) located at the base of intestinal crypts that generate the progenitors of all intestinal epithelial cell-lineages, including absorptive cells (enterocytes and colonocytes), secretory cells (mucus-secreting goblet cells, hormone-secreting enteroendocrine cells), and antimicrobial peptide-secreting Paneth cells. Previous studies have demonstrated the influence of microbiota in determining specific cell-types in the intestinal epithelium via modulating signalling pathways like Notch and Wnt/b-catenin signalling (Peck et al., 2017; Lee et al., 2018; Troll et al., 2018; Xing et al., 2020). For example, it has been previously (Lee et al., 2018) shown that microbiota-derived lactate can impact stem cell-mediated intestinal development, having an increased expansion of intestinal stem cells, Paneth cells, and goblet cells in mice fed with ***Bifidobacterium*** and ***Lactobacillus*** spp. The impact of early feeding (i.e., accelerated microbiota) on the specific intestinal cell-type markers (literature-known) was thus evaluated and the maturation of the EF piglets was further apparent from the modulation of certain markers, such as FABP1 (fatty acid-binding protein 1; associated with differentiated enterocytes) (Gregorieff, 2005; Rodriguez Sawicki Luciana et al., 2017), CDX2 (caudal type homeobox 2; associated with enterocyte differentiation and proliferation) (Coskun et al., 2011), LGR5 (leucine rich repeat containing G protein-coupled receptor 5; stem cell marker) (Barker et al., 2007; Fernandez Vallone et al., 2020), TFF3 (trefoil factor 3; expressed within goblet cells) (Scholven et al., 2009; Aihara et al., 2017) as well as REG4 (regenerating family member 4; deep crypt secretory cells functioning as the colon equivalent of Paneth cells) (Sasaki et al., 2016) (**Figure 7A, B**). Furthermore, an increased expression of a common goblet cell marker MUC2 gene (ENSSSCG00000040035) was found in EF piglets at weaning. Initially, the gene was not detected in our RNA-seq dataset, however a recent study has shown Ensemble ID ENSSSCG00000040035 corresponding to the MUC2 gene (NCBI gene tracks of NC_010444.4; Chromosome 2) in porcine organoids (van der Hee et al., 2020). Nevertheless, the differential expression between the treatment groups did not hold true for other known Paneth and goblet cell markers such as, SOX 9 (SRY-Box transcription factor 9; involved in Paneth and goblet cell differentiation) (Bastide et al., 2007), GFI1 (growth factor independent 1 transcription repressor) (Shroyer et al., 2005; Chen et al., 2018), MSI1 (musashi RNA binding protein 1; suppresses Paneth cell differentiation) (Murayama et al., 2009), which were not differently expressed in EF compared to CON piglets. Differential expression of marker genes for specific cell-types (mentioned above) would imply that the numbers of those cells are different between the treatment groups, and requires further evaluation for example, using cell-lineage specific staining in intestinal tissue samples. This may in turn inspire molecular hypotheses and further studies can strengthen our understanding of the mechanism of how the microbes modulate the host function and how the host regulates this homeostatic dialogue.

During the weaning stress period, EF piglets had a better mucosal response (day+3) reflected in the activation of p38 MAPK, TGF-beta signalling and increased cytokine production (IL-8, TGFB1, IFNGR1), as well as increased expression (responsiveness) of genes ACTG2, ACTN1, ACTN2, ITGA1, ITGA5, MMP(s), RHOD, RHOBTB1, VCL that are associated with intestinal barrier function, which suggests that EF piglets maintain an improved mucosal barrier during weaning-stress (**Figure 6**) compared to the CON piglets. Although TGF-beta stimulated actin cytoskeleton in EF piglets necessary for maintaining barrier integrity during external stress, the gene expression level of tight junction (TJ) proteins (e.g., Claudin, Occludin, JAM3, LAMB3) was not altered (**Figure 7**). However, the gene expression does not necessarily reflect the TJ protein levels or their post-translational modifications and/or functional as well as barrier supporting interactions in the actual tight junction complex (Karczewski et al., 2010; Lechuga and Ivanov, 2021).

Taken together, we demonstrate that early feeding in young piglets modulates their microbiota development as well as intestinal maturation, making the piglet better prepared (more mucosal responsiveness) for the weaning transition. It is remarkable that we observed these effects in spite of the fact that the (fibrous) feed intake in suckling piglets (including good eaters) was very low as compared to the milk intake that constitutes the main nutrient and energy source of the young piglet. This thesis further underlines the importance of the early-life “window of opportunity” to accelerate the intestinal development towards a relatively matured state, thereby better preparing the piglets for the post-weaning adaptation. It remains unclear whether the effects of early feeding can persist long-term, as the experiments conducted in this thesis do not go beyond 3 weeks post-weaning where the differences between the two treatment groups seem to have vanished. However, this does not exclude the possibility that there are “early-life programming” effects (not evident in the transcriptome profile of the intestinal mucosa within this timeframe), that may be present in different body-parts of the piglets (e.g., liver, brain or even intestine), and can be apparent only in challenging situations (e.g., responsiveness or coping capacity of the piglets). Hence, it can be useful to study the long-term consequences of early feeding on metabolic, immune and behavioural flexibility of pigs, especially when employing stressful conditions (e.g., low-hygiene, environmental and social stress like heat stress, fasting etc.).

(B) Technical considerations

Sampling method: Mucosa-adhered versus Lumen microbes

Prior to investigating the impact of early-life feeding on microbiota, we initially performed a study to assess the optimal sampling method for (longitudinal) microbiota analysis in young piglets (**Chapter 2**). Although faecal sampling is a commonly used sample type for microbiota analysis, it is not feasible in all situations, especially considering the difficulties in obtaining fresh faeces from neonatal piglets (< 2 weeks of age; **Chapter 2**). Rectal swabs were therefore evaluated, as an alternative to faecal samples, to study the porcine microbiome development in early-life. Previously, we concluded that regardless of the sample type (swab, faeces), the biological interpretation with respect to age-related microbiota colonisation was found to be mostly comparable. Consistent with previous observation (**Chapter 2**), in later studies (**Chapter 4, 5**) we also found that rectal swabs tend to capture additional mucosa-adhered microbial groups (when evaluating treatment differences) that potentially confounded the variability of the data (explained variation: 5.3% in colon vs. 2.4% in swabs; day29, Experiment 2). To further investigate the microbiota differences in the sample types and refine our former conclusions, we analysed colon (representing luminal) and rectal swabs (representing both luminal and mucosa-adhered) samples collected from the same animals at the same time-point (n =28 per sample type; day29, Experiment 2). Global comparison by principal component analysis showed clustering based on sample types predominantly, except for a few paired sample types that were close to each other (PCA; **Figure 8A**). This is most probably related to the variable degree of adhered populations in swab samples due to the spontaneous defecation by some piglets during swab collection, which also explains the higher variability observed in the swab samples (reflected by the distance between swab and colon sample). Microbial signatures associated with each sample type were assessed using redundancy analysis (RDA; **Figure 8B**). This enabled the identification of rectal-swab associated microbes such as ***Escherichia-Shigella, Enterococcus, Actinomyces, Trueperella, Peptostreptococcus, Anaerococcus, Peptoniphilus*** as well as microbes that are more abundant in the colon such as ***Oscillibacter, Prevotellaceae UCG-004, Prevotellaceae UCG-003 and Rikenellaceae RC9 gut group.*** This is in line with our previous study (**Chapter 2**) that highlighted some of these typical mucosa-adhered microbes to be enriched in swabs. However, it is to be noted that except *Escherichia-Shigella* (**Figure 8C**)*,* most of these mucosa-adhered microbial genera have very low relative abundance.

To evaluate whether the EF intervention impacted the mucosa-adhered microbial community, we make two selections of the adhered microbes: (1) unique genera found in swabs (strictly reflecting the swab sample type) and (2) genera strongly enriched in the swab (not strict but still strongly associated with the swab), and perform the redundancy analysis to address the question. Comparative analysis of the identified genera in rectal swabs and colon samples detected 19 microbial groups unique to rectal swabs, that capture only 2.24% of total microbial population (**Figure 8D**). Importantly, the shared bacteria between the two sample types, encompassed about 96–98% of the total microbial population, thereby indicating the relatedness of the swab and colon microbiota. We re-evaluated the data in the context of the "acceleration effect", by separately analysing the mucosa-adhered and luminal population in the rectal swabs. Post-hoc segregation of the rectal swab data was performed, followed by re-normalisation of the relative abundance and redundancy analysis of the treatment variable. The acceleration effect in EF piglets was recaptured in the luminal population of the rectal swabs, but not by the mucosa-adhered microbes (unique to rectal swabs**; Figure 8D**). However, it is to be noted that microbial groups such as ***Escherichia-Shigella, Enterococcus, Actinomyces*** (enriched but not exclusive to rectal swabs), were not included in the previous analysis with 19 mucosa-adhered microbial groups. Subsequently, the second option of assessing the mucosa-adhered microbes was employed by selecting the microbes strongly enriched (not unique) in the swab samples (top 20; identified in **Figure 8B**). With this set, we observed a near significant tendency of EF intervention impacting the mucosa-adhered microbial community ($P = 0.056$; **Figure 8E**), with an altered abundance of microbial groups like ***Clostridium sensu stricto 1, Peptoniphilus, Escherichia-Shigella, Pasteurella***. Intriguingly, the microbial group ***Escherichia-Shigella***, which is often associated with post-weaning diarrhoea, appeared to be enriched in CON group, potentially suggesting the prevention of their enrichment due to early feeding. Overall, in this thesis we have employed both rectal swabs (**Chapter 3, 5**) and colon (**Chapter 4, 5**) samples to investigate the impact of early feeding on microbiota development, and the biological inference of "accelerated maturation" was detected in both sample types.

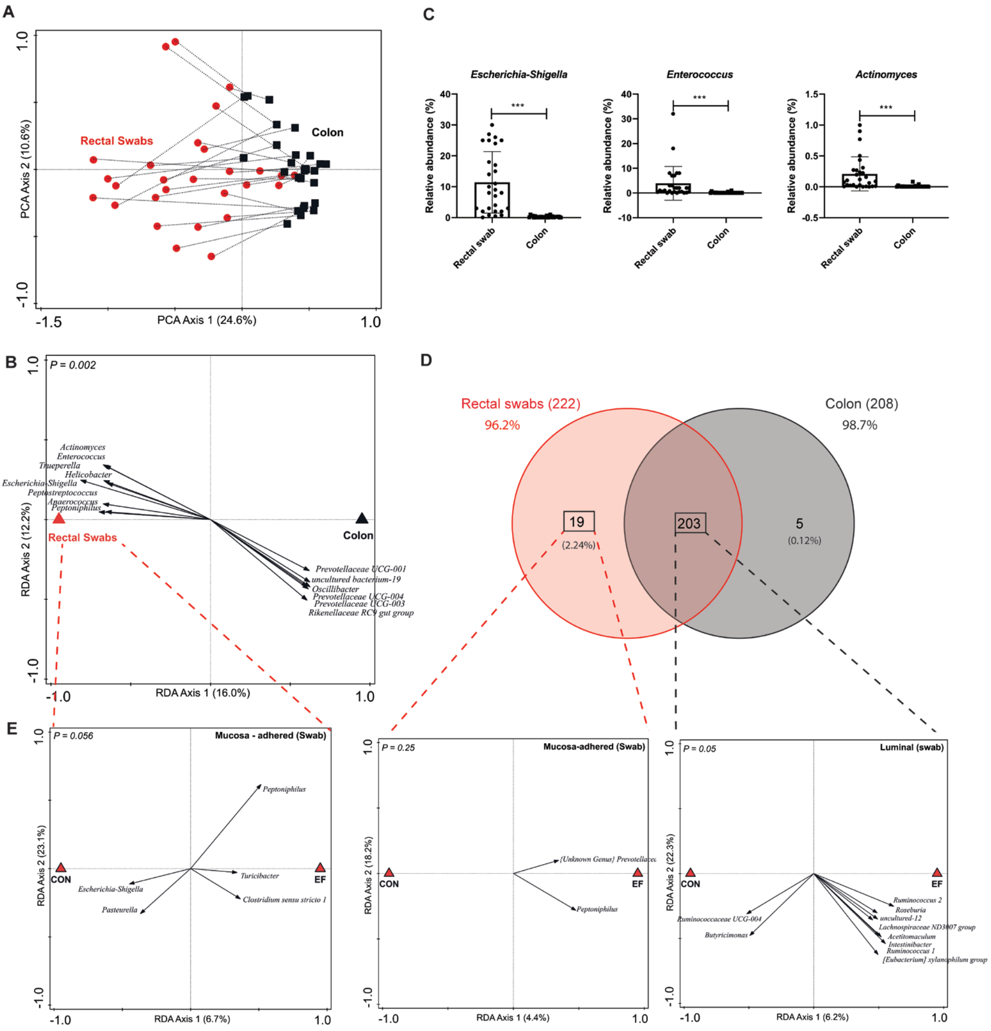


Figure 8: Evaluating microbiota composition (genus level) in rectal swabs and colon content, collected from the same animals at weaning (experiment 2). (**A**) Principal Component Analysis (PCA) of rectal swab (red) and colon (black) microbiota, with samples from individual piglets joined by dotted lines. (**B**) Redundancy analysis (RDA) of rectal swab and colon samples ($P = 0.002$), displaying discriminating microbial groups (response score > 0.60). (**C**) Relative abundance of representative microbes enriched in the rectal swab samples compared to the colon samples (Mann Whitney t test; ***: $P < 0.0001$). (**D**) Venn diagram representing unique and shared microbial genera between rectal swab and colon samples, having <0.01% relative abundance in at least one of the sample types (relevant microbes). The percentages (outside the diagram) indicate the relative abundance captured by the shared genera, i.e., 203 microbes capture 96.2% and 98.7% of total microbiome in rectal swabs and colon samples respectively. The mucosa-adhered (or swab-specific) and luminal population of the rectal swab samples were separately analysed (filtered out and re-normalised) by RDA to evaluate the acceleration effect in early-fed (EF) piglets. Additionally, (**E**) the treatment effect was evaluated in the mucosa-adhered population (top 20; identified in RDA of sample types; figure 7B).

For future studies in neonatal piglets, we need to keep in mind the variability brought by the presence of mucosa-adhered populations in rectal swab samples (possibly due to spontaneous defecation during sample collection). Nevertheless, the rectal swab samples provided the opportunity to evaluate the impact of EF intervention on the mucosa-adhered populations. A data filtering method (as depicted above) can be applied to segregate the luminal and mucosa-adhered microbial populations in the rectal swab samples and assess the biological response (of an intervention) separately.

Assessing feed intake of individual piglets

Feed consumption in the suckling period as well as post-weaning can vary greatly among individual piglets (Bruininx et al., 2004). Moreover, microbiota composition and diversity can differ substantially due to various environmental factors such as diet, age, housing, litter and stress. Assessing the consequences of early-life feed-intake requires correction for these potentially confounding factors, although diet is an overruling factor over the above mentioned factors. It is therefore essential to determine the individual feed intake of piglets in order to evaluate the microbiota and mucosal consequences of the nutritional intervention. This was also evident from our previous observation that the eating time (reflecting feed intake) quantitatively associated with the microbiome adaptation (**Chapter 3**). In this project (Middelkoop, 2020), individual feed intake of piglets was determined either by continuous video recordings (12 hours every day for 4 weeks; **Chapter 3, 4**) or by 1 minute instantaneous scan sampling (**Chapter 5**), identifying the (good, moderate, bad) eaters within the EF litters. The video scores provided an "estimate" for the amount of eating per piglet and were quantitatively related to the observed microbiota compositional changes during the pre-weaning period (**Chapter 3, 4**). However, assessing individual feeding of piglets via observations can be quite tedious and time-consuming, in addition to observer dependent subjectivity that includes for instance the problem of reliably distinguishing eating behaviour from chewing or playing behaviour.

An alternative method would be using "dietary markers" that are routinely applied in nutritional studies to assess digestibility of nutrients and its kinetics in animals. The total feed intake can be calculated from the total faecal marker-output, provided that the relative faecal recovery of the marker (preferably completely) is known. Dietary markers can be broadly divided into two types: (a) ***external markers*** that are additionally added in the feed, for example chromium oxide (Cr_2O_3), titanium dioxide (TiO_2) and other indigestible markers (Kim et al., 2010; Jacobs et al., 2017), (b) ***endogenous or internal markers*** such as acid (HCl)-insoluble ash (Moughan et al., 1990), lignin (Jagger et al., 1992) and n-alkane (Sandberg et al., 2000) that are naturally present (and/or can be deliberately increased) in the feed ingredients. In pigs, chromium oxide has been the most commonly used faecal marker for digestibility studies, however it has mutagenic and carcinogenic properties (Mamyrbaev et al., 2015) and can influence microbiome composition and function (Wu et al., 2017). Although metal markers are generally assumed as "inert", a clear effect of titanium dioxide (at physiological doses) on microbiota and intestinal homeostasis was found (Pinget et al., 2019), with increased abundance of ***Parabacteroides***, ***Lactobacillus*** and ***Allobaculum*** in mice. This highlights the importance of thoroughly evaluating these dietary markers prior to employing them in microbiome-oriented studies. A previous study in weanling pigs (Tian et al., 2017) showed that faecal samples retained the non-starch polysaccharides (NSP) of the fibrous diet, with constituent monosaccharides (glucose, xylose, arabinose, uronic acid and galactose) correlating with produced acetate (SCFA) as well as microbiota. Keeping that in mind, we tried to estimate the individual feed intake of piglets by evaluating the monosaccharide composition of their faeces by acid hydrolysis (i.e., conversion of manure fibre into fermentable reducing sugars) (Wen et al., 2007). We hypothesised that faecal samples of the piglets that consumed more fibrous feed (good eaters) would display increased amounts of neutral plant-polysaccharide derived sugars than those of the EF piglets that consumed a lesser amount (bad eaters) and CON piglets (which had no exposure to fibrous feed). Surprisingly, against our expectations, similar amounts of all constituent neutral mono-sugars detected were found in the faeces of good- and bad-eating EF as well as CON piglets (**Figure 9**). This is puzzling because the milk-suckling CON piglets had no exposure to fibrous feed pre-weaning, and their faeces were therefore not expected to contain monosaccharides derived from typical plant-polysaccharides like arabinose and xylose, which are abundant in the cereal grains (plant cell wall). A possible explanation may be found in the observed chewing on the sows faeces (coprophagy) (Aviles-Rosa et al., 2019) by the CON piglets whereby they may

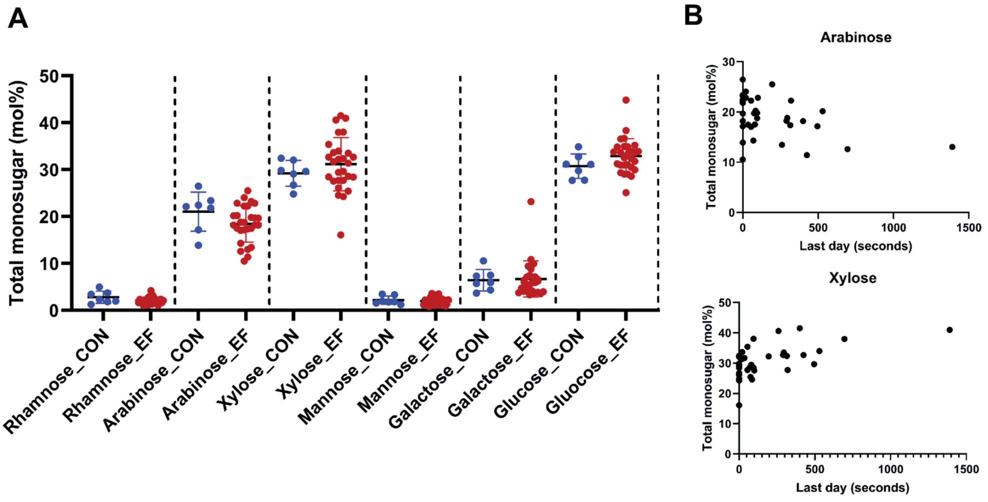


Figure 9: Constituent monosaccharide composition (in mol%) of the faecal samples obtained from EF (red dots) and CON (blue dots) piglets in the pre-weaning period (Experiment 1) by acid hydrolysis to cleave the glycosidic bonds, thereby releasing mono-sugars, which were measured in gas chromatography. Following previous protocol (Tian, 2016), freeze dried faecal samples were pre-hydrolysed (72% w/w H_2SO_4 at 30 °C for 1 h), followed by hydrolysis (1M H_2SO_4 at 100 °C for 3 h), and derivatisation of monosaccharides to their alditol acetates with inositol as an internal standard (Englyst and Cummings, 1984). On the right panel: Pearson correlation plots between mono-sugars (arabinose and xylose) and the eating behaviour in the last day before sampling (video scores in seconds).

be exposed to plant-derived sugars, but the amount of sow faecal matter ingested would be minimal (mostly rooting behaviour; (Middelkoop, 2020)) compared to the feed consumed by EF piglets and would (if substantial) have been also reflected in their microbiota composition. In future studies, closer inspection is needed to eliminate or employ this method based on monosaccharide composition of faeces for determining the individual feed intake of piglets.

Quantitative immunohistochemistry to evaluate intestinal cells in lamina propria

Automated image analysis provides an objective, quantitative, and reproducible method of measurement for evaluating specific cell populations of interest. In **Chapter 4** and **5**, we employed a semi-quantitative immunohistochemistry (IHC) method to quantify proliferating cells in the intestinal mucosa. The sections were double stained with proliferating cell nuclear antigen (PCNA stain; proliferating cells) and Hoechst (nuclei), which were subsequently identified and enumerated using Cell Profiler and FCS Express software. As both were nuclear stains, it was possible to normalise the PCNA positive cells by the number of nuclei per image (PCNA: Hoechst ratio), as this number can considerably vary between images/sections. This method enabled us to estimate the relative level of epithelial proliferation in mucosal tissue sections in a consistent and relatively high-throughput manner. However, these analyses revealed that we were not able to detect age-related increase in proliferating cells corresponding to the increasing crypt depth, that contrasts with rodent studies (Zhou et al., 2019). In the post-natal period of piglets, the immune cells infiltrate the lamina propria from 2-4 weeks of age (Everaert et al., 2017), for instance CD4+ T helper cells start appearing around three weeks of age (Bianchi et al., 1992). We thus wanted to assess whether early feeding impacted the number of T cells (CD4+) and mucosal dendritic cells (CD16+) in the lamina propria. Using previous protocols (Inman et al., 2010), the immune cell populations were visualised in the jejunal cryo-sections from 4-week old piglets (**Figure 10**). However we could not establish a standardised reproducible procedure to quantify these cells. Analogous to our previous method (in quantifying proliferating cells; **Chapter 4**), we needed to have a reference stain (or ‘primary object’ in Cell Profiler) that would (a) provide the total number of cells in an image for normalisation purposes and (b) help in detecting the target immune cells by co-localised staining (reference for guided detection). To achieve this, we tried co-staining immune cells with Wheat germ agglutinin (stains lectin in the cell membrane) as well as Phalloidin (stains F-actin surrounding the cell), however there were inconsistencies in staining and their visual overlap (co-localisation) with the target cells, rendering the chosen approach unsuitable for quantitative evaluation. Furthermore, we observed that the CD16 marker was also staining granulocytes in the mucosa, which is probably because these low affinity Fc receptors are also expressed at moderate levels in granulocytes and tissue macrophages (Naeim et al., 2018). Finally, the density of stained cells, in particular when targeting the CD4+ populations, provided a challenge for the discrimination of individual cells, which is necessary to obtain accurate relative cell-counts in a tissue.

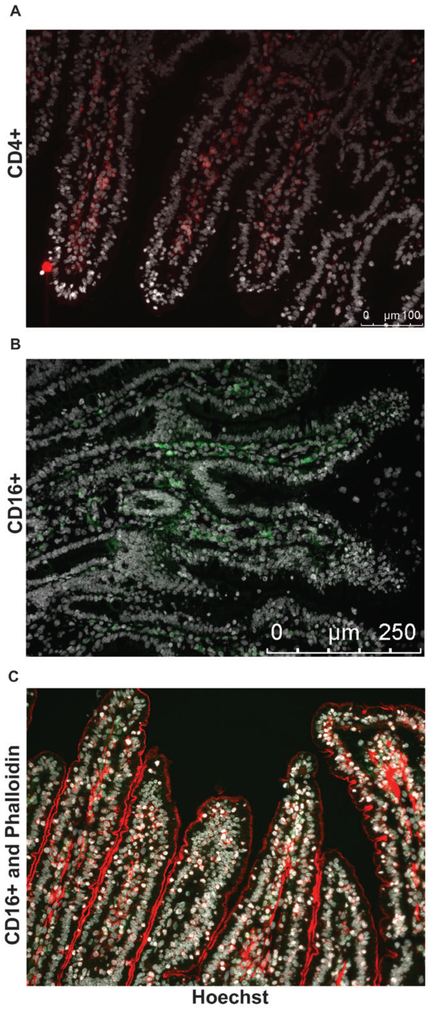


Figure 10: Representative (overlay) images of Hoechst+ cells (grey) along with (**A**) CD4+(red) (**B**) CD16+(green) (**C**) CD16+(green) and Phalloidin(red) in jejunal cryo-sections of 4-week-old piglets.

In conclusion, further work will be needed to optimise and evaluate the feasibility of this type of discriminative co-staining method, which may require the use of multiple stains including membrane stains (to better define the periphery or boundary of individual cells) to asses accurate co-localisation with immune cell markers on individual cells. Such novel protocols would also require substantial adjustments to the image analysis pipeline to allow the correct, semi-automated and objective evaluation of the relative abundance of targeted cell populations.

Several intrinsic technical aspects of IHC make this methodology quite challenging for quantitative analyses. One of these is the fact that staining intensities can be affected by multiple factors, making it crucial to standardise each step carefully, from tissue processing, sectioning, primary and secondary antibody selection, dilutions, blocking (non-specific staining), autoflorescence (e.g. eosinophils in intestinal crypts) to bleaching, filter settings for image acquisition (gain, intensity) as well as correct cell recognition (by automated software). Needless to say, it is not easy to standardise all these factors and only a few laboratories have succeeded to effectively employ IHC for the quantitative assessment of cell-populations in mucosal tissues. For instance, automated counting of specific cells was previously done (Inman et al., 2003, 2005, 2007) by pixel-based counting of digitised images (represented as proportion of total pixels), although some part of manual input was needed for determining threshold levels (for each colour or filter). Immunohistochemistry staining can be helpful for visualising and evaluating specific cells and their interaction with a few other cell types, however to further elucidate the complex mucosal interactions, IHC staining combined with transcriptomics and/or matrix assisted laser desorption ionisation (MALDI) imaging (Bailey et al., 2019) can provide targeted information and validate our observations at both mRNA and protein level.

(C) Future perspectives

Need to address the technical issues the field is facing

In recent years, the intestinal microbiota has emerged as an important target for evaluating and modulating health, with alterations in microbial composition associated with imbalance of homeostasis or disease (Vandeputte et al., 2017). Besides, the extensive application of next-generation sequencing technologies has revolutionised microbiota research via high-throughput profiling of the genetic contents of bacterial communities. Over the past decade, powerful computational pipelines and numerous protocols have been established to enable efficient analysis of large complex datasets, ranging from raw data processing, annotation to downstream statistical analysis and functional interpretation. However, deciding on the optimal method remains challenging as there seems to be a lack of consensus with respect to the different steps (or methods) employed during sample collection, processing as well as *in silico* pipeline analysis and statistics, which can lead to confusion and an inability to compare results across studies. Variations in microbiota composition can be merely caused by the technical choice of the sample collection procedure, DNA extraction protocol, 16S primers, sequencing platform (MiSeq, HiSeq,), data analysis (amplicon sequence variant or ASV vs operational taxonomic unit or OTU), reference database (Greengenes, SILVA, RDP Classifier) and downstream analysis steps (cut-off for taxa inclusion, transformation method and statistics). The initial steps of sample prepping and processing (for sequencing) especially limit the possibilities for comparisons across studies, while the aspect of data processing can be compensated by downloading the original data and re-running the analysis, although seldom done. Therefore, the field encounters a pressing need for the development of more standardised working protocols accepted in the microbiome field. However, the constant changes in the technological possibilities and rapid development of “improved” procedures

(Bailey et al., 2019), hampers the building of consensus, suggesting that the field needs to further mature to reach a stage where standardisation becomes more realistic. We can probably draw inspiration from the field of bacterial genomics, which evolved from "non-standardised mode" two decades back to a more unanimous and uniform approach in current years. Until we reach the "decided and accepted" golden standard, it would be advisable to form informed agreements starting within a group or facility.

Apart from the technical aspects, part of the variation in microbiota composition may be explained by different host characteristics and environmental factors including age, gender, breed, litter and housing. Multivariate analysis should be employed to evaluate the environmental variables in the study and their consequences on the intestinal microbiome, prior to focussing on the treatment variable (**Chapter 2, 3**). We observed a strong age effect, a moderate litter effect (which was confounded with pen in our studies) and no significant gender effect on microbiota composition, which was consistently observed in all experiments conducted in this thesis.

Moving from group level analyses to individual-based analyses

Animal individuality is challenging to explain because individual differences are regulated by multiple selective forces that can lead to unique combinations of characteristics (Goursot et al., 2019). Feed intake, microbiota and behavioural (personality) characteristics are all individual-based traits, and have not been explored at all beyond human studies (Davidson et al., 2018). This is not to say that group level analyses are not valid or can be replaced, as they are important for addressing our research questions initially. However, to further explore interactions (e.g., diet-microbiota-behaviour) and better understand the mechanism of action, individual level evaluation could be vital, even this does bring in more noise in the data (**Chapter 4, 6**). This is also illustrated by the varying degree of microbial abundance, in piglets belonging to the same treatment group at the same time-point (for example, EF piglets at day29; **Figure 11**). Despite all that noise [especially in this type of study (outbred lines) where within-treatment individual variation is already high], we do detect significant developmental signatures that are affected by EF intervention, indicating the strength of the 'detected signal'. Another important aspect to consider for the individual microbiota analysis is longitudinal sampling, which employs repeated measures over a period of time and can provide crucial individual microbiota information. Previously (**Chapter 3**), we have observed homogenisation of the microbiome within two weeks post-weaning as well as presence of post-weaning microbes in EF piglets (acceleration; following 20 piglets for 8 time-points). However, we did not consistently observe that at an individual level in **Chapter 5**, because we could not follow the same animals over time as they had to be sacrificed to obtain intestinal tissue or luminal content. Separate sets of (dissected) piglets were evaluated per time-point, that were colonised by partially different (**Figure 11**; red and green boxes), and partially the same groups of (SCFA producing) "accelerated microbes". Some of these microbes that are abundant at day29 (e.g., red box) do not seem to persist at

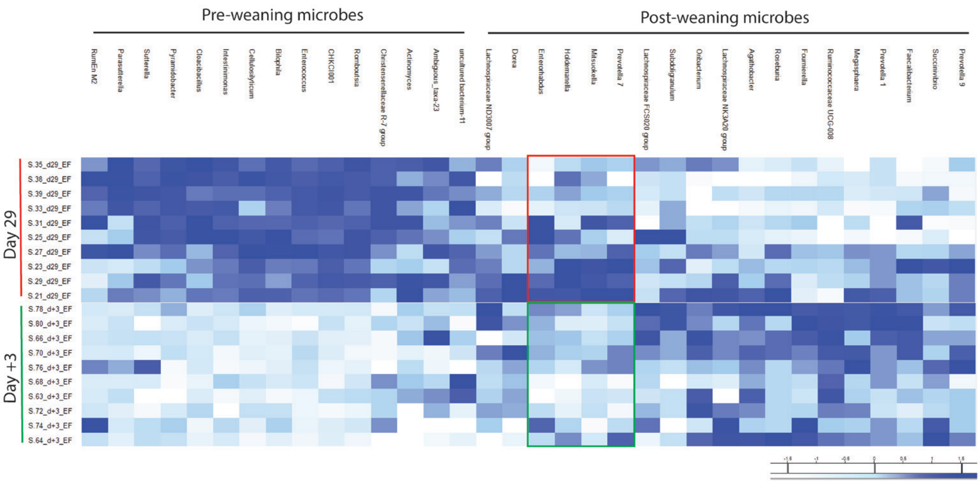


Figure 11: Heatmap displaying the relative abundance of pre- and post-weaning microbes in EF and CON piglets (Experiment 3) at an individual level. Ten EF piglets were sacrificed at each time-point: day29, and day+3, hence breaking the timeline of following individual animals. Some of the EF-associated microbes that are abundant at day29 (red box) do not persist at day+3 (green box), which is potentially because we are visualising microbiota information from different animals.

day+3 (green box), which is potentially because we are visualising microbiota information from different animals, thereby underlining the importance of longitudinal sampling.

Another approach of interest for repeated tissue sampling from the same animal could be endoscopy (biopsies) or cannulation method (post-valve T-caecum or PVTC) (Kik et al., 1988; Köhler, 1992; Jacobson et al., 2001). The PVTC method (implanting a cannula 5-15cm anterior to the ileo-caecal valve) has been standardised in growing pigs for collecting mid-gut samples (ileal digesta or proximal colon). However, both of these methods have their shortcomings and can be invasive, for example, for endoscopy, sedation needs to be applied that can potentially act as a confounding variable as well as welfare of the animal. Yet, these methods could be optimised and further modified to be suitable for suckling piglets, keeping in mind the welfare state of the young animal.

Pig as a model to study diet–microbiota–behaviour interaction

In **Chapter 6**, we explored the association between the microbiota and behaviour of suckling piglets irrespective of the treatment and found some intriguing correlations. Obviously, further in-depth analysis and dedicated study designs will be necessary to understand the biological relevance of these associations, as well as the role of 'microbiota-gut-brain axis' in behavioural development of young piglets. Pre-weaning personality (backtest), exploratory and anxiety-related behavioural parameters were assessed in our study. For pig farming, studying the associations between microbiota and damaging behaviour (tail and ear biting) towards other pen-mates, which is a major problem, would be of particular relevance. Interestingly, feather pecking behaviour in chickens (that is analogous to pig tail biting in aetiology) seems to be related to their microbiota composition, where early-life microbiota transplantation had an effect on behavioural and physiological characteristics related to feather pecking (Eijk, 2019).

Apart from their resemblance with respect to diet, physiology, and gastrointestinal function, the post-natal 'window of brain development' of pigs coincides with human brain development (**Chapter 1**). Moreover, pigs are outbred animals that partially mimic the genetic diversity as well as behavioural variation in human population, compared to the inbred rodent models. As a consequence, one can expect a higher intrinsic noise in these (pig) studies, requiring a substantial signal to detect the effect. Certainly, the noise or variability will hamper the detection of (behavioural) effects by group based analyses, leading to inconclusive results. Alternatively, pigs can be utilised as a developmental model to understand the fundamental aspects of gut-brain interaction (gut-brain axis) as well as spatial learning and memory (Gieling et al., 2011). This can be done by high resolution measurements in larger groups of individual animals (without intervention) merely tracking individual relationships in complex multivariate data (using advanced data-mining and ***in silico*** applications in pattern recognition, data modelling and machine learning), that may give directions for future (more targeted) research. For example, the designing of personalised glycaemic response algorithm (enabling accurate postprandial glucose response prediction in humans) (Zeevi et al., 2015) can serve as an inspiration for the field of animal science to tackle the inter-individual variability. Omics-based technologies such as proteomics or metabolomics of brain samples (targeting specific brain locations like frontal cortex, hippocampus, amygdala etc.) could be integrated with microbiota and transcriptomics, for a holistic approach to understand the microbiota-gut-brain interaction. Furthermore, recent advances in porcine brain imaging (functional magnetic resonance imaging or fMRI) could also be implemented in such studies to evaluate this relation non-invasively (Sauleau et al., 2009; Conrad and Johnson, 2015). This highlights the need to develop data acquisition tools (e.g., microsensors, individual recognition, continuous and real-time parameter recording systems) for individual animal information necessary to investigate these associations.

Implications in animal husbandry

Creep feeding i.e., provision of solid feed before weaning is increasingly studied and considered as an option to improve piglet adaptation after weaning. In this thesis, we have employed a customised (fibrous) feed composition for suckling piglets which is distant from the traditional, milk-based creep feed composition. It is striking that a relatively lower amount of feed ingested before weaning which is negligible in terms of nutrient and energy, can influence the microbiota as well as the host molecular processes. Furthermore, in our experiments, the piglets seem to cope well with the pre-weaning fibrous diet, that is often considered to be unsuitable for young piglets. Since we do not have a "traditional creep feed" treatment group in the experiment, it will be difficult and speculative to make comparisons with our study. The anticipation will be that the non-fibrous, highly digestible traditional creep feed will probably have substantially lower impact on microbiota (and host processes) compared to the customised feed provided in our study. This is because diet is one of the major determinant of the microbiome (also observed in this thesis), which also potentially suggests that the impact of early feeding in piglets is a combination of nutrition (fibrous composition) and microbiota that is difficult to separate (i.e., two sides of the same coin).

Combining high-resolution molecular analyses (metagenomics, transcriptomics) and animal performance parameters can be difficult, as they require very different numbers of animals to reach the statistical power of analysis. To assess pig performance during the weaning transition, parameters such as average body weight gain, daily feed intake and diarrhoea scores are routinely measured, although it is unclear whether the currently used parameters can reliably indicate the health and performance of the animal in the long-term (up to slaughter age). Besides, it is important to acknowledge the resilience and adaptive capacity of individual animals, which can very adequately cope with stressful situations, leading to equally efficient performance per animal in the long run. In future follow up studies, the long-term effect of the early feeding thus needs to be examined, which will show whether such “accelerated maturation” at 4 weeks of age provides any advantages to the animal in the long run or not. To assess the long-term effect of early-life nutrition on resilience or coping capacity of the animals, challenge models (nutritional or environmental) can be utilised, which can potentially be relevant for human studies as well.

The current pig farming system consists of unnatural settings with a barren environment (lack of enrichment), too many animals on a small area, a short suckling period, abrupt weaning transition, lack of possibilities to learn from the mother and often procedures with negative implications for welfare (ear notching, teeth clipping or grinding and tail docking). Taking inspiration from nature, the future farming systems can be designed with a view to support the ‘natural’ development of the pigs, such as, creating more natural habitats (closer to nature) and/or extending lactation period, rearing family groups of multiple sows with their offspring (loose-housed) in addition to foraging possibilities with the mother. The feeding strategy explored in this thesis i.e., providing piglets with a stimulus-rich (fibrous feed) environment in the lactation period, mimics natural conditions and can be easily applied to existing farming systems. Adopting a more sustainable animal production system and accepting substantially higher price of animal protein products (milk, eggs, meat) as well as lower meat production (and consumption) than at present, is something to ponder upon. Of course, to achieve that, a revolutionary change is needed in the mindset of the society at large. This may sound idealistic in the present society that is mostly dictated by the minimal production costs needed to sustain the low margin achieved on the products, but the long-term solutions probably ask for a more fundamental change in our attitude towards animal husbandry, meat consumption, and the maintenance of a liveable planet.

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Summary

Early-life bacterial colonisation can be of particular importance to the overall growth and health of an animal, especially influencing intestinal and immune system development with long-term implications. This is especially relevant in pig production where post-weaning enteric infection is one of the major concerns related to the gut health of pigs, and is associated with economic losses and welfare problems. Commercial pig production systems involves early and abrupt weaning, which contrasts with the gradual transition from mother's milk to solid feed in nature. Due to such abrupt weaning, a piglet is challenged with multiple stressors (including environmental-, nutritional- and psychological-) which is usually associated with changes in gut microbiota and a high incidence of diarrhoea. Modulating intestinal microbiota to reduce weaning-associated problems in pigs, is getting increasing scientific and commercial interest, as microbial dysbiosis (or imbalance) has been identified as a leading cause of post-weaning intestinal infections. The work described in this thesis focussed on the evaluation of early-life feeding (pre-weaning provision of fibrous feed) on the intestinal microbiota and mucosa development in neonatal piglets, thereby preparing piglets for weaning transition i.e., consuming plant-based solid feed after weaning.

In **Chapter 2**, we first focussed on assessing a reliable sampling method to study microbiota colonisation over time, especially suitable for neonatal piglets. We showed that rectal swabs are a suitable alternative sample type to study the porcine microbiome development in early-life, when faecal sampling is challenging. Yet, the results were further refined in **Chapter 7** (combining later experiments), where we found that rectal swab samples bring a certain degree of variability in the presence of mucosa-adhered population.

In **Chapter 3,** we performed a longitudinal study (using rectal swabs) to evaluate the impact of early feeding (fibrous feed) on the microbiota colonisation at pre- and post-weaning time-points. The results revealed that the early-fed (EF) piglets had an accelerated maturation of the microbiota, compared to the control (CON) piglets that consumed milk exclusively. Accelerated maturation at pre-weaning time-points were characterised by the simultaneous emergence of typical post-weaning-associated microbial groups (such as ***Prevotella, Roseburia, Faecalibacterium, Ruminococcus, Megasphaera, Subdoligranulum***) and a more rapid decline of typical early-life/pre-weaning microbial genera (e.g., ***Fusobacterium, Finegoldia, Bacteroides, Eschechichia-Shigella***). Moreover, we found a quantitative association between eating behaviour of EF piglets (video scores) and their microbiota signature, indicating that the piglets who spent more time at the feeding trough had a higher abundance of 'accelerated' microbial groups. Subsequently, in **Chapter 4**, we characterised the early feeding effect on the local intestinal microbiota (using luminal content) and intestinal (macroscopic and microscopic) physiology. Although the feed intake was relatively low in this experiment, it provided us the opportunity

to evaluate the relation between the individualised quantification of eating behaviour (with piglets classified into good, moderate and bad eaters) and the piglet-specific microbiota composition and intestinal measurements. At weaning, we found that early feeding altered the colonic microbiota composition, increased microbial fermentation products (SCFA) in the colon and modulated intestinal development i.e., increased weights and lengths of several intestinal tract segments, as well as a decreased villus-crypt ratio in jejunal mucosa and an increased abundance of proliferative cells in colon mucosa.

In **Chapter 5**, we investigated the mucosa transcriptome response to ingesting pre-weaning fibrous feed in suckling piglets. In this study, we aimed to explore the molecular effect of early feeding (pre-weaning fibrous feed) and also its impact during weaning transition. Maximum impact of early feeding was detected at weaning (day29; compared to other time-points), which was followed by convergence of the transcriptome three weeks post-weaning (day+21) in EF and CON piglets. We showed that early feeding not only accelerates the gut microbiota (re-confirmed in this study), but also the host transcriptome maturation at weaning. The results revealed that in the EF group, oxidative phosphorylation, cholesterol biosynthesis and oxidative stress-related pathways were significantly enriched, whereas sirtuin signalling and immune response pathways were downregulated in the colon mucosa at day29. Remarkably, three days after weaning, the EF piglets displayed a stronger mucosal responsiveness compared to the CON piglets, reflected in the increased expression of genes (transcriptomics) related to immune activation, epithelial migration and “wound-repair” like processes necessary to maintain gut barrier integrity (during weaning transition) as well as jejunal morphometry.

Chapter 6 explored fundamental aspects of host-microbe interactions, by evaluating the associations between the intestinal microbiota and behaviour of suckling piglets. We aimed to tentatively reveal associations between the intestinal microbiota composition and piglet behaviour in a test for coping style, (i.e. a personality trait), as well as anxiety- and exploration-related behaviour in a novel environment test. These associations between microbiota composition and behaviour of suckling piglets in challenging situations have, to the best of our knowledge, not been investigated before. A multivariate approach was used employing redundancy analysis (RDA), individual spearman correlation and Multivariate Analysis by Linear Models (MaAsLin). A number of microbial groups such as ***Coprococcus, CAG-873, Atopobium and Prevotella*** were identified to be associated with anxiety- and/or exploration-related behaviour, although these results are premature and need further validation for their biological relevance. However, this study provides insights into the association of early-life behaviour and microbiota in suckling piglets and can be an inspiration towards future studies that are more dedicatedly designed to understand the microbiota-gut-brain axis.

Finally, **Chapter 7** summarises and discusses the main findings of the research and describes possible directions for future research. Overall, the findings of this thesis indicate that EF piglets

show an “alerted system” with an enhanced responsiveness to external stimuli of feed and microbiome development, compared to the CON group, thus exemplifying the potential of early-life programming to modulate intestinal development in piglets. Furthermore, the results of this thesis indicate that early feeding of fibrous feed has considerable potential to better prepare young piglets for the weaning transition.

Acknowledgements

“ বিপদে মোরে রক্ষা করো এ নহে মোর প্রার্থনা--
বিপদে আমি না যেন করি ভয়।
দুঃখতাপে ব্যথিত চিতে নাইবা দিলে সান্ত্বনা,
দুঃখে যেন করিতে পারি জয়॥
সহায় মোর না যদি জুটে নিজের বল না যেন টুটে,
সংসারেতে ঘটিলে ক্ষতি, লভিলে শুধু বঞ্চনা
নিজের মনে না যেন মানি ক্ষয়। ”

The above song written by Nobel Laureate Rabindranath Tagore has been an inspiration for me from my childhood and I want to thank Tagore for his insightful writings and understanding of life that especially provides hope and positivity in times of trial.

It is almost surreal to look back on my journey so far. And when I do, I feel deeply grateful to everyone who has been a part, as this would not have been possible without their support and kindness. I am thankful to so many people who have contributed directly or indirectly to the completion of this thesis, all of whom I might not be able to mention in this section.

I would like to immensely thank my promoter and supervisor, **Michiel Kleerebezem**, for providing this wonderful opportunity to pursue my PhD and believing in my ability that allowed me to grow in the last four years. Michiel, I am truly inspired by not only your knowledge, scientific aptitude, critical thinking, but also your mentoring skills that is balanced with right amount of support, guidance, encouragement and challenge. You can easily win “the best supervisor in the world” title :D. I am glad that we will continue to collaborate in the coming years for the post-doc project. My co-promoter, **Liesbeth Bolhuis**, introduced me to the world of animal behaviour and I gained considerable insight in this field, thanks to her. Liesbeth, I have found positivity and persistent support in you whenever I needed it, for which I am truly grateful. The fun-filled brain-storming Friday morning sessions with you, Michiel and Anouschka will be dearly missed.

I would like to thank all the project members of the “early foraging team”, **Bas Kemp**, **Walter Gerrits**, **Hendrik de Vor**, **Evelien Alderliesten, Maarten Hollemans, Delphine Melchior, Moniek Van Den Bosch** and **Rosalie van Emous**, for their constant support and commitment towards reaching the objectives of this project. **Bas**, I will always remember how you saved me by arranging the shipping of resistant starch from the United States, which was especially challenging during the holiday period. Thank you, **Walter**, for the crucial inputs on the feed and also providing critical comments on improving the manuscripts. Sincere thanks to the industrial partners (**Coppens Diervoeding and Cargill**) for your active participation and feedback on this project. I really appreciate **Henry van der Brand** for his pertinent inputs and suggestions as an advisor.

A special thanks to my fellow PhD candidate in the project, **Anouschka Middelkoop**, with whom I have planned and performed multiple experiments together. I admire your dedication and I have definitely learnt how to behave in a pig stable from you! :D Wishing you great success in your future endeavours.

Deepest appreciation to all the technicians (**Anja Taverne-Thiele, Fleur Bartels, Monique Ooms**, **Bjorge Laurenssen, Manon van Marwijk**), for providing their relentless help in my PhD, especially during the animal experiments. **Anja**, thank you for your help during the dissections and for sharing your expertise on microscopy. **Fleur**, although I have worked with you only for a brief period, I am so thankful to you for training me at the Cargill farm (pilot study) "how to catch a running young piglet while saving yourself from the angry mother". It was a life-saving lesson literally! **Monique**, thank you for being an approachable, kind person whom I could rely on. **Bjorge**, you are a creative person who can always come up with ideas to work on. **Manon**, I worked with you much later in my PhD, and found you very helpful and diligent. Profound thanks to all the animal caretakers at Carus especially **Arjan van Dolderen** and **Ries Verkerk** for taking care of the animals so well. **Arjan** and **Ries**, in spite of my novice Dutch language skills, you both communicated with me and made my experience at Carus a fun one! Thank you for that! Special thanks to **Pieter Roskam**, **Sandra Vreman** and **Soumya K. Kar** for their help with intestine/brain dissections.

I would like to extend my sincere gratitude to **Prof Mick Bailey** (University of Bristol, UK) for having me at his lab for 5 weeks. I felt really welcome and it was great fun learning from you, although I must admit that it can be quite intimidating by your inexhaustible knowledge. Thanks for being such a wonderful host, showing me around your beautiful houseboat and the Christmas Market tour at Bath. Hope to collaborate with you someday in future. **Emily Porter**, thank you for guiding me around the lab and for being so friendly and supportive. I also want to thank **Prof. Henk Schols** for providing the lab facilities at Food Chemistry and **Christiane Rösch** for all the help during the work. Even though we did not reach conclusive results in the mono-sugar (faecal) analysis, it was a good learning experience for me. I am also thankful to **Klaas Frankena** for helping with statistical queries.

During the four years of my PhD, I enjoyed supervising five students in their bachelors/MSc thesis: **Xantia van den Brink**, **Tim Dekker**, **Jaqueline Gama de Souza**, **Lara van Veen** and **Yuner Gu**. I am thankful to each one of you for showing great enthusiasm and motivation for the project, and contributing to this thesis. I also feel proud to see you all doing so well in your respective careers.

“*Home-away-from-home*”, **Host-Microbe Interactomics** group has been such a place for me. **Jerry**, thank you for your suggestions and comments during my presentations, which I always found very helpful. Also, I thoroughly enjoyed being a part of the “cheese team” during the 2016 lab outing at Mill. I loved the competitive-yet-fun element that you brought in and we even won the contest! **Peter**, I am sincerely grateful for all your inputs and perspective on LefSe and STEM analysis, which were very useful. **Jos**, I first met you at NIZO for your advice on Canoco, and I am glad that now you are a part of HMI, and that I do not need to cycle ‘all the way’ (read: poor biking skills) to Ede for your advice. Your readiness to discuss any problem and your helpful attitude is much appreciated.

I am thankful to all my wonderful present and former colleagues for making this PhD journey special, many of whom I can call my friends. **Loes**, **Nico**, **Anja**, **Ellen**, **Marcela**, **Edo**, **Jori**, **Bart**, **Nuning**, **Jonna**, **Hugo**, **Simon**, **Berdien**, **Isabela**, **Linda**, **Tim**, **Rogier**, **Soumya, Nirupama**, **Stefan**, **Juri**, **Nadya**, **Bruno**, **Marjolein**, **Agnieszka**, **Kim**, **Femke**, **Laura**, **Saartje**, **Sylvia**, **Joyce**, **Simen**, **Blanca**, **Jeroen**, **Arabela**, **Aisha**, **Alex**, **Avis**, **Tiantong**, **Zhan**, **Kristiina**, and **Stéphane**. Thank you for all the interesting conversations, lab-outings, international dinners and lunch/coffee breaks. **Loes**, you are simply the best! You are kind, positive, elegant and ever-ready with solutions to all our problems! Thank you for that and for hosting *gezellige* Sinterklaas celebrations. **Nico** and **Anja**, you are a power couple whom I admire for your unfading commitment and professionalism. Both of you are my dear friends, and you know I love you guys. **Ellen**, thank you for all your help, and for managing numerous purchase orders over the years. Special thanks to my dear (ex) office-mates: **Marcela, Jori, Bart, Isabela**, for our hilarious discussions which covered quite a range of subjects. It would not have been the same fun without you guys. **Marcela** (my paranymph), you have been there for me from the beginning and were my “go-to person” for any kind of help/advice. Over time, we developed a bond and I am glad you are my friend. I sincerely admire your contagious positivity and happy vibe irrespective of the demanding situations. **Jori**, I am glad that we finished our theses around the same time as we could discuss (read: complain) about our comparable situations. My best wishes for your defence! **Bart**, thank you for your inputs on RNA-seq data analysis, numerous illustration-related queries and help during dissection. I still struggle to differentiate between your jokes and sarcasm, oh wait are they the same?! :D. **Edo**, thank you for being my data analysis-buddy! Discussing (and mostly complaining) with you all my difficulties, made them somehow resolvable :D. I cannot thank you enough and will certainly miss that! **Simon**, thank you for your philosophical depth and insights, I really enjoyed those conversations with you. **Nuning**, you are one of the sweetest person I know. Thank you for being you. Hope to visit you in Indonesia some day! **Berdien**, thank you for guiding me with random forest analysis in R and Perseus software. I cherish our frank conversations too! **Linda**, I am able to write my acknowledgements because of you! ;) Thank you!

My heartfelt gratitude to all my (former) colleagues at **Food and Biobased Research** (WFBR) institute, especially **Jurriaan Mes** and **Harry Wichers**. I will be forever grateful to you for your constant support during my 'PhD hunt'. Thank you **Alexia Lépine**, for being an excellent guide (and friend) and for providing the opportunity to work in your project as a volunteer. I am also thankful to **Mari Smits**, **Maria Forlenza** (also my PhD advisor), **Geert Wiegertjes and Leo Kruijt** for their suggestions during that period which was helpful. I am grateful to **all my teachers** and **supervisors** over the years, who guided me in following my interest and passion.

I consider myself fortunate to have met several wonderful people coming from different parts of the world, which has substantially broadened and enriched my experience. In the last five years, valuable friendships have been developed that are close to my heart. **Mari** and **Thilly**, thank you for inspiring us to start hiking (wandelroutes) and for your warmth and support. **Olaf** and **Anouk**, thank you for your friendship. I miss our dinners, fun trips and I hope to see you both at the other side of the world in Australia. My Zodiac friends, **Eva**, **Elsa**, **Uros**, thank you for all the fun we had together, I miss Eva's hearty laugh, Elsa's contagious energy and Uros's Tiramisu. I hope to see you all someday. **Hugo** and **Mirelle**, thank you for being such awesome people, and I cherish our friendship. **Nirupama** and **Rajaram**, you have been an inspiration for us, thank you for all the help and support, especially during my surgery and corona times. In the last phase of writing my PhD, our activities together (hiking trips, dinners, video gaming etc.) were the much-needed breaks that I always looked forward to. Thank you for that and also for introducing Overcooked2 :D. I am super grateful to the Hoevestein family members: **Balaji, Sindhu, Neeraj, Arathi, Rini, Liana, Janardhan, Yasmeen, Katerina, Abhiroop, Tanya, Vani, Athul, Nitin, Kamesh, Ananth, Aditya, Sascha, Tom,** and **Suraj**, for all the special or random dinners, board games and dancing. All of you made me feel at home here at Wageningen. **Balaji**, thank you for our interesting discussions and debates, I always enjoy them. **Sindhu**, thank you for hosting us at Göttingen during Christmas last year, and I hope to meet you at Boston soon! **Rini** and **Liana** (my paranymph), I met you both separately through common friends, and did not expect that we would become such close friends. I treasure our friendship dearly, and thank you both for being there in the challenging moments of my life. "Some friendships are timeless", especially the ones I share with my childhood friends has been time-tested: **Madhureema, Madhuswana, Aparajita, Sreyashi** and **Simontini**, thank you for being there. **Madhureema Sen**, my bestie, you have been involved in all crucial phases of my life. Thank you for being my unpaid therapist :D. Your daily motivational reminders in the last phase of PhD definitely helped me sail through.

"Family is not an important thing. It's everything." I am very lucky to have a strong support system upon whom I can constantly rely. **Maa** (Mausumi Choudhury), thank you for being the best mother in the whole wide world, I can vouch for that ! Your love and unbreakable trust in me provides a strength that I am otherwise unaware of. Thank you for being 'my mom'. **Papa** (Tridev Choudhury), in a world where patriarchal norms have been sub-consciously standardised, I am truly grateful for having a progressive man like you as my father. I love you as my father, but more than that I respect you as an individual that could retain his child-like enthusiasm at seeking knowledge constantly. I hope to keep that spirit alive when I reach your age. **Bhai** (Apratim Choudhury), my baby brother, I am too emotional talking about you. Thank you for coming into my life and making me the person I would have never explored without you. You have been the shoulder to cry on in the last months of finishing my thesis, I certainly couldn't have done it without you. **Dida**, I wish you were here with us today, as I could have shared how proud I feel to be your grand-daughter. You were a feminist (or equalist might be a better term) in the true sense of the word, who pursued her dreams and aspirations at a time when society had different expectations from a woman. Thank you for instilling your spirit in me. I am grateful to **Misti mashi**, **Kutti mashi** and **Dadu** for their love and support throughout. A special thanks to my in-laws (**Maamoni**, **Baba**, **Dada**) and all the relatives who have been very kind and encouraging always.

Soumya, I do not know where to begin. I have known you far too long and I undoubtedly cherish our friendship that has evolved over the years. I am forever grateful to you for believing in me at a time when I myself forgot to do so. Needless to say, you have a humongous contribution in whatever (little) I have achieved in my life. Thank you for that and everything else.

Raka Choudhury.

About the author

Raka Choudhury was born in Diphu, Assam, India on 8th October, 1988. She was fascinated by the world of biology from a young age and eventually pursued her passion for the subject after finishing high school studies. She started Bachelor of Science (BSc) in Botany, Zoology and Chemistry at GC College, Assam University (Silchar, India) in 2008, during which she discovered her interest in the microscopic world and chose to do a BSc thesis studying endophytic fungi in young tea plants (*Camellia sinensis*). She graduated with a *Gold medal* by topping in the BSc program (Hons) in 2011. Subsequently she joined MSc in Molecular Biology and Biotechnology at Tezpur University (Tezpur, India) with a scholarship from the Department of Biotechnology (DBT), Govt. of India. Her MSc thesis was focussed on venom biochemistry that characterised "three finger toxin" protein in the venom of *Naja siamensis* (Indo-Chinese Spitting Cobra). After completing her masters with distinction in 2013, she cleared multiple state- and national-level eligibility tests in Life Sciences which led to working as a junior research fellow at the Dept. of Veterinary Microbiology & Virology, Faculty College of Veterinary Science, Assam Agricultural University (Guwahati, India). In 2015, she moved to the Netherlands and got an opportunity to work as a volunteer/trainee at Food and Bio-based Research (WFBR) Institute, Wageningen University and Research (WUR; Wageningen, The Netherlands). The research project aimed at elucidating the immune effects of pre-, pro-and synbiotics on intestinal (human) cell lines *in vitro*.

From January 2016, Raka joined as a PhD candidate at the Host-Microbe Interactomics group, WUR. During her PhD, she focussed on understanding the impact of early-life (nutritional) intervention on the intestinal microbiota and host mucosal development; the findings of the research are described in this thesis. In 2017, on receiving the WIAS Travel Grant, she visited the Infection and Immunity Laboratory (group of Prof. Mick Bailey), Bristol University (Langford, UK) for academic training focussing on quantitative immuno-histochemistry analysis. As of March 2020, Raka started working as a Post-doctoral Researcher at Host-Microbe Interactomics group (WUR), on a research project funded by the Nestlé Research Centre (Lausanne, Switzerland), that aims to evaluate strain-level relatedness of oral and small intestinal microbiomes in healthy and celiac disease individuals. During her career, she would like to continue understanding the role of microbiota in animal/human health and disease.

List of Publications

Das, S., **Choudhury, R**., Sultana, A., Balamurugan, V., Chakravarty, I., Devi, M., Bora, M., Sharma, K. Effect of peste des petits ruminants virus (PPRV) infection on the host immune response in naturally infected goats. Indian J. Anim. Sci. Vol 87, No 3. (2017).

Middelkoop, A., **Choudhury, R**., Gerrits, W. J. J., Kemp, B., Kleerebezem, M., and Bolhuis, J. E. Dietary diversity affects feeding behaviour of suckling piglets. Appl. Anim. Behav. Sci. 205, 151–158. (2018).

Choudhury, R., Middelkoop, A., Bolhuis, J. E., and Kleerebezem, M. Legitimate and reliable determination of the age-related intestinal microbiome in young piglets; rectal swabs and fecal samples provide comparable insights. Front. Microbiol. 10. (2019).

Middelkoop, A., **Choudhury, R.**, Gerrits, W. J. J., Kemp, B., Kleerebezem, M., and Bolhuis, J. E. Effects of Creep Feed Provision on Behavior and Performance of Piglets Around Weaning. Front. Vet. Sci. 7:520035 (2020).

Choudhury, R., Middelkoop, A., Gerrits, W. J. J., Kemp, B., Bolhuis, J. E., and Kleerebezem, M. Early-life feeding accelerates gut microbiome maturation in piglets (bioRxiv 2020.09.30.320275). *Submitted for publication.*

Choudhury, R., Middelkoop, A., De Souza, J.G., van Veen, L. A., Gerrits, W. J. J., Kemp, B., Bolhuis, J. E., and Kleerebezem, M. Impact of early-life feeding on local intestinal microbiota and digestive system development in piglets. *Submitted for publication.*

Choudhury, R., Gu, Y., Bolhuis, J. E., and Kleerebezem, M. Early feeding leads to molecular maturation of the gut mucosa in suckling piglets. *To be submitted.*

Choudhury, R., Middelkoop, A., Bolhuis, J. E., and Kleerebezem, M. Exploring the association between microbiota and behaviour in suckling piglets. *To be submitted.*

Overview of Completed Training Activities

Completed in fulfilment of the requirements of the Wageningen Institute of Animal Sciences (WIAS)

The Basic Package (3 ECTS)

WIAS Introduction Day	2016
Course on philosophy of science and/or ethics	2019
Course on essential skills	2017

Disciplinary Competences (18 ECTS)

Writing a WIAS literature survey	2016
Laboratory Animal Science (LAS)	2016
Multivariate Statistics (VLAG)	2016
Species Specific Course (Pigs), Utrecht University	2017
The Intestinal Microbiome and Diet in Human and Animal Health (4th edition; VLAG)	2017
Introduction to R (VLAG)	2017
Big data in the life sciences (VLAG)	2017
Visit Infection and Immunity Lab, Bristol University (WIAS Travel Grant)	2017
Analysis of microarray and RNA Seq expression data using R/BioC and web tools	2018

Professional Competences (13 ECTS)

WGS PhD Workshop Carousel	2016
Convener in 3rd PhD Symposium, Wageningen, WPC	2016
Systematic approaches to reviewing literature	2016
Information Literacy including Endnote Introduction	2016
Project and Time Management (PTM)	2018
Teaching and supervising Thesis students	2018
Organising WIAS Science Day	2018
Scientific Writing	2018
Scientific Artwork–Vector graphics and images	2018
The Final Touch: Writing the General Introduction and Discussion (WIAS)	2019

Presentation Skills (4 ECTS)

Federation of European Microbiological Societies (FEMS) 2017, Valencia, Spain (**Poster**)	2017
14th International Symposium on Digestive Physiology of Pigs (DPP), Brisbane, Australia (**Oral**)	2018
WIAS Science Day, Wageningen, The Netherlands (**Oral**)	2019
Host-Microbe Genetics Meeting, Leiden, The Netherlands (**Oral**)	2019
21st Annual Gut Day, Amsterdam, The Netherlands (**Poster**; best poster prize)	2019

Teaching competences (6 ECTS)

Supervised 2 bachelor and 3 master students 2016–2020

Total: 44 ECTS (one ECTS credit equals a study load of 28 hours)

Colophon

The research described in this thesis was financially supported by the Netherlands Organisation for Scientific Research (NWO), Cargill Animal Nutrition and Coppens Diervoeding and was part of research programme ‘Genetics, nutrition and health of agricultural animals’ with project number 868.15.010. Financial support from the Host-Microbe Interactomics Group (Wageningen University and Research) for printing this thesis is gratefully acknowledged.

Concept Cover: Raka Choudhury, Apratim Choudhury, Soumya K. Kar
Cover Illustration: Swarnavo Datta || www.swarnavod.com
Lay-out and Printing: ProefschriftMaken || www.proefschriftmaken.nl


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