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Piglet Gut Microbiota Responses to Exposure to Outdoor Simulated Environment and Formula Feeding

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Abstract

Several lines of evidence suggest that early-life gut microbiota composition is a crucial determinant of the susceptibility to diseases later in life. Understanding the evolution of early-life gut microbiota and critical factors that can modify this microbial community will espouse the prevention or reversion of health risk originating from dysbiosis of the gut microbiota in the early stage. In this research, we hypothesized that alterations in the environment (simulation of rural conditions by soil exposure) and feeding mode (breastmilk versus formula milk) will have significant impacts on the establishment of the gut microbiota in early life. At first, in literature review discipline, we present a holistic view on the current knowledge of gut microbiota and environmental modulations. Then, through empirical studies, we investigate the impacts of soil exposure and different feeding modes on gut microbiota composition. We applied 16s rRNA sequencing to examine the composition of gut microbiota based on fecal samples in two separate studies: 1) 4-day-postpartum piglets were exposed to agricultural soil until weaning day (day 21) and then grew under the same conventional condition with control treatments; 2) 4-day-old piglets were either mother reared and breastfed or artificial reared and formula fed, then grew in the same conditions at weaning. The results revealed that agricultural soil exposure and feeding mode could significantly modify the gut microbiota composition in early stages although this trend was not maintained until the later phases of life. The alterations in the early gut microbiota, albeit in a short period of time, caused by those extrinsic environmental modulators may still be sufficient to ameliorate or exacerbate health risk of the host.

Keywords: Early-life, gut microbiota, feeding mode, agricultural soil.

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Sincerely,

Nguyen Vo

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Introduction

The adequate establishment of infant gut microbiota is pivotal to regulate the maturation of other systems in the host including but not limited to immune system and metabolic functions. A deficit in beneficial members of gut microbiota in early life may have detrimental effects on health status of the host in later phases. Remarkably, there is a critical time-frame to avert microbial dysbiosis or induce a healthier gut microbiota. Environmental modulators in early stages play a crucial role in the evolution of gut microbiota composition which, in turn, significantly affects the health risk prevalence. Understanding the evolution of gut microbiota and intrinsic as well as extrinsic factors that modulate this evolution will shed light on potential prevention of diseases such as asthma, allergy, and obesity.

While the knowledge from human studies are germane to the delineation of the mechanism of how early intervention of gut microbiota composition leads to shifting in disease risk, there remain limitations in using human model regarding ethical regulations and hard-to-achieve controlled conditions. The usage of the animal model allows researchers to obtain a more controlled experiment and apply other factors which are difficult or even impossible to achieve in the human model. In this study, we leverage the swine model with a high degree of similarity to the human in term of physiology and immune responses to investigate the effects of environmental modulators including agricultural soil exposure and feeding modes on the gut microbiota composition in young pigs. The first chapter will present current knowledge on the evolution of the gut microbiota and environmental modulators. Then, in the next two chapter, we will report our empirical studies on the effects of rural simulation and feeding modes on gut microbiota composition.

1. Current knowledge on the gut microbiota evolution in early-life and environmental modulation

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Abstract

Early-life gut microbiota composition is increasingly seen as critical determinants of future gut microbiota and associated health and disease. Exposure to intrinsic and extrinsic factors in this critical timeframe of early stage –potentially even before birth- has a pivotal role in the establishment of the first group of microbial taxa in the digestive tract and the large intestine in particular. It is important to understand the dynamics of gut microbiota and the impacts of the newly established microbial community on health risk. In this review, we first present current knowledge of factors affecting gut microbiota dynamics and how the alterations of early-life gut microbiota can affect disease risk with a focus on human studies. Then, studies in mice and swine models are taken into account to present possible mechanisms for factors defining gut microbiota development and the protective effects of a healthy gut microbiota on disease prevalence. Prenatal conditions, delivery modes, antibiotics usage in infancy, environmental factors and feeding modes are crucial factors that affect the evolution of gut microbiota. In addition, alterations in early-life gut microbiota are directly linked to disease prevalence such as atopic, asthma, obesity. Animal models provide supportive evidence corroborating the role of early intestinal microbial community in immune responses and metabolic functions which directly exacerbate or ameliorate health risks in the host.

Key words: Early-life, gut microbiota, environmental factors, human health, animal models

1. Introduction

With the advent of next-generation sequencing, our ability to gain insights into the gut microbiota composition and understand more about its pivotal role in maintaining the health status of the host has been multiplied. Remarkably, there is increasing evidence that early-life gut microbiota assembly significantly influences the make-up of the host gut microbiota on medium and long-term. In this context, it is hypothesized that external and internal factors, even before birth, are strong determinants of gut microbiota and associated health conditions later in life. While the significance of this research problem is warranted, there are obvious hurdles, in particular that longitudinal human studies are extremely difficult to justify and conduct. However, it is likely that dynamics and mechanisms in early-life gut microbiota development could be relatively well extrapolated from studying animal models.

In this review, we provide knowledge on early colonization of the human gut microbiota as well as factors that significantly affect this microbial community and how the alterations of early gut microbiota could impact disease risk based on research with a human model. In addition, we also look into mice and swine models in order to offer some mechanisms that can expand on the effects of alteration of early gut microbiota on disease prevalence.

2. Early-life human gut microbiota

Since human gut microbiota is increasingly associated with health and disease, understanding the forces driving its development becomes crucial. The early-life establishment of microbial communities clearly represents the first, and possibly the most important stage, as colonization by foreign taxa becomes increasingly unlikely later in life. Therefore, it appears important to define what constitutes a healthy early-life gut microbiota, and how external factors may modulate it detrimentally or beneficially. In this section, we will present the knowledge from

human studies on the development of the gut microbiota since birth, the significance of this microbial community dynamics in bolstering health status, and extrinsic factors that critically affects the composition of infant gut microbiota.

2.1 Early colonization of the gut microbiota

According to macroscale ecosystem theory, the order of initial species colonizing the community will determine the structure of that community. For instance, types of animal that enter a newly formed island will define the composition of organisms living on that island. Interestingly, gut microbiota of babies/infants could be pictured as an "island" in which the first microbial community colonizing will strongly affect the composition and function of the future intestinal microbial community (Turnbaugh et al., 2007). Thus, to maintain a healthy gut microbiota, early exposure is a critical point to take into consideration. Infants gut microbiota composition may be shaped since prenatal period, at birth and postnatal growth (environmental exposure and feeding mode).

The establishment of symbiont relationships between human host and microbial community may start even before birth (Blaser & Dominguez-Bello, 2016). It is hypothesized that a healthy maternal microbiota plays a key role in promoting fetal development, averting premature birth and other potential health risks (Charbonneau et al., 2016). This hypothesis is supported by numerous studies on epidemiology, placenta microbiota and prenatal related gut microbiota (Tamburini et al., 2016, Stensballe et al. 2013, Metsala et al., 2013, Lahtinen et al., 2008, Aagaard et al., 2014). Other evidence for the prenatal effects on neonates' microbiota laid on the scientific proof of an unsterile environment in the uterus which used to be believed to be sterile by discoveries of microbes' presences in amniotic fluid, placenta, fetal membrane, umbilical cord blood and meconium (Tamburini et al., 2016, Perez-Munoz et al., 2017). By 16s rRNA

sequencing and whole-genome shotgun metagenomic, a study on placental specimens of 320 pregnant subjects described a unique microbiome for the placenta in the uterus including Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes and Fusobacteria phyla in which Proteobacteria is the most dominant phylum. Interestingly, when compared with oral, stool, skin, nasal vaginal microbiome, the placental microbiome cluster was more closely related to the cluster of oral microbiota (Aagaard et al., 2014). The fetus may obtain the very first colonization from maternal microbiota through the bloodstream which is enabled by association with dendritic cells. Dendritic cells could transport the bacteria from the gut lumen to other locations through the bloodstream (Jimenez et al., 2008).

Another evidence for the translocation of mother microbiome to a fetus is the increase of health risk in babies whose maternal microbiomes are disrupted by the use of antibiotics. Maternal antibiotics usage correlated with the development of allergy and asthma in infants (Metsala et al., 2013, Stensballe et al., 2013, Zhao et al., 2015, Mueller et al., 2015). For instance, an epidemiological study in a relatively large Finland cohort including 6690 pairs of asthma diagnosed children and their matched control (without asthma) revealed that maternal antibiotics usage was associated with asthma risk in which the use of Cephalosporins resulted in the strongest correlation (Metsala, 2015). Moreover, a distinct pattern of meconium microbiota of preterm infants compared to their fecal samples collected within the first week of life also suggested the transfer of maternal microbes to the fetus took place as early as in pregnancy period (Romano-Keeler and Weitkamp, 2015). Together, these results indicate a strong link between prenatal phase and the formation of neonatal microbiota. However, more direct evidence is necessary to claim that mothers transferred and/or influenced microbial communities to their child during pregnancy.

Right after being born, neonates experienced a profound exposure to the outer microbial community, which is mostly derived from maternal microbiota. Delivery mode is crucial in the formation of neonatal microbiota in term of initial microbial members that colonize infants' intestinal microbial community. Various studies have confirmed the different pattern of gut microbiota among infants with different delivery mode (Rutayisire et al., 2016, Mueller et al., 2016, Dominguez-Bello et al., 2016, Stewart et al., 2017). A 16s rRNA sequencing based-study of Venezuelan cohort revealed that newborn babies' microbiota was not disparate among skin, oral mucosa and nasopharyngeal aspirate, yet the initial microbiota composition was essentially different between C-section and vaginally delivered babies (Dominguez-Bello et al., 2010), which was consistent with a recent study on US cohort and China infants (Chu et al., 2017, Kuang et al., 2016). Specifically, unweighted Unifrac distant matrix reflected a cluster of vaginally delivered babies with mother's vaginal microbiota while C-section ones clustered with the skin microbiota. In term of taxonomy, babies vaginally born were dominant by vaginal microbiota such as *Lactobacillus, Prevotella, Atopobium* and *Sneathia* spp. while skin dominant taxa like *Staphylococcus* spp., prevailed in C-section neonates (Dominguez-Bello et al., 2010). With a focus on gut microbiota, Backhed et al., (2015) applied the metagenomic approach to longitudinally examine the dynamics of the gut microbial community of neonates. The results also confirmed different delivery modes greatly influenced the gut microbiota structure of neonates with C-sectioned babies enriched with microbes belonging to oral and skin microbiota and vaginally delivered babies are significantly dominant by *Bacteroides, Bifidobacterium, Parabacteroides* and *Escherichia/Shigella*. Recently, other studies on US cohort infants' microbiota returned consistent results which showed the dearth of Bacteroidetes/*Bacteroides* population in C-section infants (Bokulich et al., 2016 and Madan et al., 2016). The very first

contact of neonates to the surrounding environment either through passing birth canal or C-section may shape microbiota composition in the direction to be similar to vaginal/gut mother's microbiota or skin mother's microbiota, respectively.

Aberrations in the inoculation of the first microbial member to the intestinal tract have been suggested to be linked with predispositions to various diseases due to the development of dysbiosis gut microbiota. One of the typical factors which could interfere with the normal development of neonates' gut microbiota is antibiotics usage in infancy (Kemppainen et al., 2017). Antibiotics usage in childhood was shown to perturb gut microbiota composition by decreasing bacterial diversity, altering phylogenetic composition as well as microbiota metabolisms (Bokulich et al., 2016, Yassour et al., 2016, Cho et al., 2012, Schulfer and Blaser, 2015). Data from previous studies indicated that antibiotic treatment in early childhood might lead to a higher susceptibility to various disease including asthma, allergy, and obesity (Russell et al., 2012, Alm et al., 2014, Love et al., 2016, Azad et al., 2014, Ajslev et al. 2011, Thomas et al., 2006, Deshmukh et al., 2014). The usage of antibiotics in infants does not only decrease specific normal taxa including *Clostridiales* and *Ruminococcus* in the first year of life but also delay the maturation of gut microbiota later in life (Bokulich et al., 2017).

Besides mode of delivery and antibiotic usage, other factors such as feeding modes and living environment also greatly contribute to the formation of a healthy gut microbiota (Tamburini et al., 2016, Munyaka et al., 2014). These two factors will be discussed further later in this review.

2.2 Early gut microbiota and health

2.2.1 Immune system

Infant human gut microbiota plays a pivotal role in regulating the immune system and disturbances of the intestinal microbial community can result in diseases caused by immunological malfunctions such as allergy, asthma, autoimmunity, and inflammatory bowel disease (IBD) (Round & Mazmanian, 2009, Reynolds & Finlay, 2017). Moreover, numerous studies highlighted that early microbial composition determined the risk of disease (Arrieta et al., 2015, Wegienka et al., 2015, Bunyavanich et al., 2016 & Suzuki et al., 2007). Atopic and asthma, -related microbial community had lower diversity and depletion of certain groups of bacteria, namely *Coprococcus eutactus* (butyrate-producing bacteria), *Lachnospira, Veilonella, Faecalibacterium* and *Rothia* (Nylund et al., 2015, Abrahamsson et al., 2014, Arrieta et al., 2015).

The mechanism of how microbiota facilitates the proper function of the immune system in early life is not fully delineated. However, it is intuitive that commensal bacteria participate in the education of immature immune system (Wesemann & Nagler, 2016). The underrepresentation of certain groups of commensal microbes in infants could disturb the immune system leading to disease phenotype like food allergy (Ling et al., 2014, Azad et al., 2014, Chen et al., 2015). For example, the group with the highest susceptibility to asthma from a US birth cohort had a unique gut microbiota with enrichment of fungi (*Candida* and *Rohodotorula*) and depletion of *Bifidobacterium, Akkermansia* and *Faecalibacterium*) with pro-inflammatory fecal metabolites. To test the effects of this distinct metabolome on the immune system, the authors incubated peripheral T-cell in sterile fecal water containing metabolites from the gut microbiota of the

highest risk of asthma group. The results showed a remarkable increase of IL-4 producing CD4+ and decrease of CD4+CD25+Foxp3+ cells. This pattern of CD4+ T-cell dysfunction supported for the establishment of atopic asthma (Fujimura et al., 2017).

The structure of the intestinal microbial community in early time is critical in the establishment of the immune system which affects the risk of getting diseases. It is rational for us to focus on a promising method which can harness the capacity of beneficial microbiota to prevent immune-relating disease risk in the future by modifications of the microbiota in early time.

2.2.2 Metabolic functions

Gut microbiota is strongly associated with obesity and metabolic dysfunction (Tilg and Adolph, 2015, Tremaroli & Backhed, 2012). A meta-analysis study incorporated recent 10 original articles using sequencing approach to study gut microbiota and obesity provided a significant, albeit small, correlation between microbial diversity and obesity risk. In addition, the study failed to establish a microbiota signature for predicting obesity risk based on microbiota composition. The authors suggested the reason for this might be because there were high variations among individuals' gut microbiota composition which lead to a distinct signature of obesity for each person (Sze and Schloss, 2016). Notably, in the meta-analysis study above only included study on adult objects. It is possible that the effects of microbiota composition that lead to obesity status may be significantly expressed in early time rather than in adult phase. Epidemiologic studies revealed gut microbiota composition in infancy correlated with weight gain in later life stages (Koleva et al., 2015).

2.3 Early gut microbiota and environmental factors

Environmental exposure in early life may shape the gut microbiota via horizontal transmission of environmental microbes. Exposure to a more diverse microbial community in an early time of life may provide beneficial outcomes in children's health (Tasnim et al., 2017). Early exposure to a wide spectrum of microorganisms could benefit the host in multiple aspects. Firstly, by exposing to various types of organism, the human body created a memory of molecular structure which can be quickly retrieved for prompt reactions against harmful organisms. Second, exposure to microbial peptidoglycans and lipopolysaccharide (LPS) allows proper induction of innate immune system. Last but not least, a biodiversity environment maintains the tolerance by constructing a regulatory system where Treg cells play a key role. This system averts abnormal immune responses to innocuous agents which decrease the risk of allergy, autoimmune diseases and IBD (Rook et al., 2014).

2.3.1 Living environment

Recently, Blaser (2017) proposed the theory of disappearing microbiota and the increased risk of chronic diseases such as obesity, asthma, hay fever, IBD, diabetes, and autism which first occurred in developed countries. In the theory, he underscored the significance of early life gut microbiota establishment and claimed that the disappearance of specific human microbiota community through modern life practice inevitably resulted in abnormalities in shaping human physiology. There are three main causes for the absence of crucial microbial taxa. First, the shift to C-section delivery mode and the usage of antibiotics during pregnancy in industrialized countries impede the vertical transmission of important maternal microbiota taxa to the child.

Second, hygienic practice in modern life, especially clean water, decreases the opportunity for horizontal transmission of commensal bacteria among people. Third, antibiotics usage in infancy and formula feeding hindered the colonization of beneficial microbiota which has been inherited through generations (Blaser, 2017). A microbial "rescue" in early life by confronting these three mentioned causes as well as providing a more diverse microbial exposure for infants may help restore the disappearing microbiota that is likely to the origin of disease incidences in modern life. Early-exposure to furry pets modified the gut microbiota of 3-4 months old by increasing the abundance of *Ruminococcus* and *Oscillospira* which decrease in childhood atopy and obesity (Tun et al., 2017). Farm exposure plays a role in epigenetic regulation of asthma and allergy genes such as T-cell differentiation and IgE regulation by affecting DNA methylation (Michel et al., 2013). The protective effects of biodiversity exposure in early life on immune-relating diseases were proven by multiple epidemiological studies (Jackson et al., 2017). Data of atopy and living environment from 1044 children and adolescents (0.5-20 years old) in Finland and Estonia was integrated to elucidate the association of atopy risk and green areas around the home. The results reported a significant relationship between atopic sensitization and the cover of the forest and agricultural land. Atopy risk decreased with the increase of green covered areas. Interestingly, analysis of children cohort who had relocated at the early time (1-2 years old) indicated that it was the environmental exposure at birth that predicted atopy later in life rather than the current exposure (Ruokolainen et al., 2015). Of our interest, this evidently supports the significance of early exposure to a high diversity environment for protection from diseases like allergy.

2.3.2 Parasites/Helminth infections

Besides rural factors, parasitic infections in less hygienic life practice also play a role in shaping microbiota in the direction of protecting the host from immune-relating disease risks (Ramanan et al., 2016). A study on two communities: Pygmy hunter-gatherer and Bantu farming in the rainforest of Southwest Cameroon revealed the crucial role of gut protozoa, *Entamoeba*, in defining gut microbiota composition with accuracy up to 79% when predicting a subject's gut microbiota based on the colonization of these common protozoa. *Entamoeba* infected individual's gut microbiota had higher alpha diversity comparing to Entamoeba free ones. Remarkably, the presence of *Entamoeba* was directly associated with taxonomic signatures such as enrichment of *Clostridiales Ruminococcaeae* and decrease of *Prevetella copri* and *Fusobacteria*. This taxa pattern was reported for protective effects on autoimmune disorders (Morton et al., 2015). The effects of helminth on microbiota composition may differ across species. For instance, different types of helminth colonization may either augmented or decrease the diversity of gut microbiota (Lee et al., 2014, Cattadori et al., 2016). Nonetheless, it is likely that helminth infections play a role in shaping the gut microbiota in early life and affect the disease susceptibility of the host. Helminth infection ameliorated the severity of allergic airway inflammation in mice by reducing infiltrating eosinophils and activating IL-10- producing Treg cells. Notably, gut microbial community presence was critical for the efficacy of helminth in protecting the host from allergic responses. Vice versa, helminth infection shifted intestinal microbiota composition into a higher production of short-chain fatty acid (SCFA) (Zaiss et al., 2015).

2.4 Gut microbiota and feeding mode

Numerous studies emphasized the pivotal role of breast milk in bolstering a normal and healthy growth of infants (Davis et al., 2016). Besides nutritious values, human milk is composed of a myriad pool of bioactive and immunological factors including secretory IgA (sIgA), lactoferrin, lysozyme, α-lactalbumin, complex lipid and free oligosaccharides and glycoconjugates (Ballard & Morrow, 2013). The constellation of these molecules critically supports infants in multiple aspects, namely immunological maturation and colonization of beneficial commensal gut microbes like *Bifidobacterium* spp. Remarkably, when comparing with mature breast milk, the composition of colostrum (peripartum breast milk) is enriched with molecules like sIgA and free oligosaccharides compared with mature milk. sIgA and free oligosaccharides assist neonates in establishing immunoglobulin production and reducing pathogenic colonization, respectively (Gordon et al., 2012, Zivkovic et al., 2010, Coppa et al., 2004). The differences in composition between colostrum and mature milk (Drago et al., 2017) suggest that breastfeeding in the first few days after birth is crucial in shaping healthy infants' gut microbiota composition, as well as immune system.

Different feeding modes may lead to divergent patterns of gut microbiota composition (Guaraldi and Salvatori, 2012). A study comparing exclusively breastfed (BF) infants and exclusively formula-fed (FF) infants at three months old revealed significantly distinct gut microbiota structures between the two groups. Both BF infants and FF infants gut microbiota were dominated by Actinobacteria at phylum level and *Bifidobacterium* at genus level. However, PCoA plotting Unweighted UniFrac distance of the intestinal microbiota of BF infants and FF infants reflected two distinct clusters which indicated each group had their own distinct gut microbiota structure. At phylum level, Bacteroidetes was significantly higher in BF infants while

FF infants were enriched with Firmicutes which was consistent with other studies (Wang et al., 2015 & Praveen et al., 2015). At genus level, BF infants had an increase of *Bacteroides* and decrease *of Clostridium XVIII, Lachnospiracea incertae sedis, Streptococcus, Blautia, Clostridium XI, Clostridium sensu stricto, Eubacterium, Erysipelotrichaceae incertae sedis* and *Haemophilus* compared with FF infants. Interestingly, human milk oligosaccharides (HMOs) composition could be used to predict certain genera from the gut microbial community (Wang et al., 2015). In addition, in another study, infant twins who were separated and had different feeding mode also showed differences in infant gut microbiome (Yatsunenko et al., 2012). This strongly underscores the importance of early life feeding mode in shaping gut microbiota structure.

Feeding mode in early life may also affect immune system via altering gut microbiota. Intestinal metagenome and transcriptome of BF and FF babies espoused this notion when revealing different patterns of gene expression and microbial diversity between two groups. Microbiota activities in early age were dominated by expressions of immune system-related genes in both groups. While BF infants had a lower diversity gut microbiota, they could co-express more genes compared with FF infants. Bioactive compounds in breast milk may possibly explain for a denser network of co-expressed gene since the presence of those compounds could activate multiple pathways (Praveen et al., 2015). Bioactive compounds in breast milk like HMOs can also serve as a prebiotic that induces the growth of beneficial microbial community such as *Bifidobacterium* spp. with a vital role in competing with pathogenic organisms, maintaining epithelial barrier function and activating immunological and inflammatory responses (Mueller et al., 2015, Wang et al., 2015, Donovan & Comstock, 2016).

3. Animal models in studying early life gut microbiota

Experiments on animal models allow scientists to investigate mechanisms beyond the limitations of the human model. Research on animal models facilitates our understanding of the impacts of early life gut microbiota modifications on the health status of the host. In this section, we will review studies on two popular models including murine and swine with the intention to elaborate some mechanisms that are associated with early life gut microbiota.

3.1 Mice model

The advantages of mice model not only include the low cost, high reproductive rate, and easy handling but also a high level of human shared genes (up to 99%) as well as the availability of various inbred/outbred strains, mutants and disease models (Kostic et al., 2013). Although there are human features that mouse model cannot replicate such as differences gastrointestinal tract structure and living behaviors (coprophagia), they are still useful in offering general knowledge of the mammalian gut microbiota (Nguyen et al., 2015) and insights into possible mechanisms that can translate to human model with further evidences (Kostic et al., 2013).

Early establishment of gut microbiota is significant for the proper function of immune system and metabolic function which can be proven using germ-free model (Hrncir et al., 2008, Sudo et al., 1997). For instance, germ-free mice without commensal microbes in the intestine witnessed the accumulation of invariant natural killer T (iNKT) cells in lamina propria and lung, which significantly exacerbated the severity of oxazolone-induced-colitis and ovalbumin-driven-allergic asthma models compared with induced diseases model of specific pathogen-free mice (Olszak et al., 2012). Disturbance of early gut microbiota also increases the susceptibility to

disease. Antibiotics treated neonatal mice, when challenged with *E. Coli* and *K. pneumoniae*, are extremely more susceptible to infection than control mice. Specifically, mice with antibiotic exposure survived only for only 8 to 9 hours while the control mice could survive more than 72 hours. The study discovered that gut microbiota alterations cause neutrophils to plummet in antibiotics treated mice which proposed the role of this microbial community in the regulation of postnatal granulocytosis through Interleukin 17A (IL-17A) (Deshmukh et al., 2014).

Mice models also support the delineation of the mechanism of how gut microbiota can protect the host from immune relating disease (Tlaskalova-Hogenova et al., 2011). Commensal bacteria protected mice from food allergy through activations of both innate and adaptive immune system. Clostridia colonization reinforced the production of innate IL-22 by RORϒt+ ILCs and T cells. IL-22 is important in enhancing epithelial barrier upon introduction of food allergen through induction goblet cells to secrete mucus. In addition to the innate immune system, commensal bacteria also affected adaptive immune system by expanding intestinal Treg production and promoting class switching to IgA which is critical in food allergen tolerance and epithelial barrier, respectively (Stefka et al., 2014). Another study using mice as a model underscored the role of Clostridium colonization in 2-week-old neonatal SPF mice in promoting colonic Treg population. Clostridium-treated mice, when challenged with dextran sodium sulfate (DSS) to trigger colitis, showed the ability to avert disease symptoms such as weight loss, rectal bleeding and ameliorate edema and hemorrhage comparing to control mice. Clostridium enrichment in early time also bolstered up the immune system in exposure to allergy inducing factor, Ovalbumin (OVA) by lowering IgE, IL-4 and increasing IL-10 production. The results confirmed that early exposure of Clostridium can positively alter systematic immunological responses in mice (Atarashi et al., 2011).

Mice model allows us to closely investigate the effects of nutrition in early life on the gut microbiota composition which is strongly associated with disease risk (Smith et al., 2013, Houghteling & Walker, 2015, Turnbaugh et al., 2006, Canani et al., 2011). In a study, neonatal mice were fed milk containing or excluding milk oligosaccharides (sialyllactose) to examine the impacts of this milk component on the intestinal gut microbiota. The results revealed that despite insignificant differences in mucosal leukocyte populations development, treatment with or without sialyl(α-2,3)lactose in the milk had different gut microbiota composition pattern. Remarkably, adult mice with supplementation of sialyllactose were more protective from induced colitis by dextran sulfate sodium (DSS) compared with other treatments. To confirm it was the gut microbiota composition that influences colitis risk, the authors colonized germ-free mice with the gut microbiota from above treatments and then challenge with DSS-induce colitis. The results from germ-free mice experiment were consistent with the previous experiment which confirmed the role of milk oligosaccharides in preventing DSS-induced colitis (Fuhrer et al., 2010). Studies in animals with highly controlled environment may provide a more descriptive mechanism of how biodiversity modifies the gut microbiota and in turn, affects the health. An experimental study in mice with exposure to soil, house dust, and decaying plants reported consistent results in the influence of a low-hygiene environmental on gut microbiota composition. Soil, house dust and decaying plants exposed mice showed a higher diversity of gut microbiota which correlated to suppression of serum IgE level when treated with 2-4-dinitrofluoreobenzene (DNFB) (Zhou et al., 2016). Disturbances or restorations of gut microbiota were only effective in neonatal mice, not adult mice. Neonatal germ-free mice with restored gut microbiota via inoculation of healthy microbiota did not experience invariant natural killer T cells accumulation and thus, was protected from associated pathological symptoms. The

colonization did not result in measurable protective effects in adult mice (Olszak et al., 2012). In a different context, yet similar effects, antibiotic could only increase the risk of allergic asthma in neonates, not adult mice (Russell et al., 2012).

Experimental studies with mice confirmed the strong impacts of disruption of intestinal microbiota on adiposity (Cox et al., 2014, Cho et al., 2012). Cox et al., (2014) treated mice with low-dose-penicillin (LDP) and longitudinally examined intestinal microbiota, body composition, adiposity and ileal gene expression. Exposing mice to penicillin at low dose resulted in microbe-induced obesity (MIO) phenotype with abnormally high adiposity. The mechanism for this can be because LDP mice possessed a shortage of specific groups of bacterial taxa including *Lactobacillus, Allobaculum, Rikenellaceae* and *Candidatus Arthromitus* (SFB) which played a role in controlling weight gain in mice. Interestingly, analysis of ileal gene expressions of LDP microbiota recipients reveals that even with the restoration of the gut microbiota later in life, the perturbation of this microbial community had sustainable effects in metabolic processes. The wiping of protective microbial groups caused by the usage of antibiotic in the early stage of life pivotally afflicted the metabolism of the host in the direction of increasing obesity risk (Cox et al., 2014). Timing of modifications for gut microbiota therefore appears to be critical for life-long effects on health.

3.2 Swine model

Pig is an eminent model for early gut microbiota influences in both immune responses and nutritional metabolisms. First, piglets shared a high degree of the genome, protein sequences as well as immune responses which are >80% similar to that in human while it is only <10% in

mice. Second, the anatomy and physiology of the gastrointestinal of pigs are resembled human in which they are both colon fermenters (Wang & Donovan, 2015). Those features make swine model a more relevant model when studying the interaction of early gut microbiota and its impacts on health risks.

A metagenomic research based on pig fecal samples confirmed the similarities between the gut microbiota of piglets and human gut microbiota in diversity pattern and the two most abundant phyla, namely Firmicutes and Bacteroidetes (Lamendella et al., 2011) which was consistent with other studies (Kim et al., 2011, Niu et al., 2015). Nonetheless, when taking into consideration of the relative abundance, the swine gut microbiota was different from human gut microbiota at both phyla and genus level. Pig gut microbiota had a lower level of Actinobacteria and enrichment of Spirochetes. In addition, at genus level, Prevotella was enriched in pigs while Bacteroides was more abundant in human gut microbiota. Intriguingly, human metagenomes were 70% similar to that of swine model (Lamendella et al., 2011). The swine gut microbiota experienced different stages of gut microbiota development over time. The gut microbiota composition can reach a stable stage at the age of 4 weeks (Unno et al., 2015). The intestinal microbial community is significantly affected by dietary with distinct functional microbiome before and after weaning (Frese et al., 2017). Although gut microbiota composition of piglets and human are not entirely similar, we still recognize some similar trend in the evolution of both models. For instance, there is a critical time window for both human and pig to modify the gut microbiota which in turn affects the growth and health risk of the host (Tamburini et al., 2016, Lalles et al., 2007, Thompson et al., 2008). Antibiotic usage at 3 weeks old did not alter the development of gut microbiota in pigs or weight gain compared with the control. However, the

use of antibiotic at 10 days old pigs significantly increased the growth performance (Unno et al., 2015).

In piglets, differences in the environment during the first days of life were sufficient to exert significant alterations in the gut microbiota and urinary metabolisms at 35 days old in farm piglets. Specifically, two different batches of piglets from the same sows were isolated from their mother after one day and housed in a strictly controlled environment. This means that variations in the environmental exposure could only occur on their first day of life, yet 16s rRNA sequencing of fecal samples from two batches still showed significant differences in term of gut microbiota composition. Not only gut microbiota but also metabolic process was affected in the same manner which led the authors to suggest the promise of 'metabolic rescue' for infants who had a high risk of getting diseases due to anomalous initial gut microbiota colonization (Merrifield et al., 2016). This corroborates the notion that environmental exposure right after birth is sufficient for a sustained effect on gut microbiota composition which defines health status of the host. More studies are necessary for delineating of the mechanism of high-microbial-diversity environment beneficial impacts on gut microbiota. The knowledge from that will shed light on the development of methods involving interfering early establishment of gut microbiota to protect children from disease risks.

Isolation from mothers and formula feeding increased intestinal dendritic cells (DC). Meanwhile, farm pigs (mother rearing and breastfeeding) showed a higher level of IL-4 production. The discrepancies in the immune response of two treatments were strongly associated with the gut microbiota composition. Evidently, four outliner pigs at 2-5 days old's microbiota which clustered with the microbiota of 12-28-day-old pigs possessed the intestinal DCs number essentially more consistent with that of pigs at 12-28 days old than their matching age piglets

(Inman et al., 2010). A caveat to this study is that they were not able to provide a whole detailed picture of the gut microbiota composition due to the limitation of the method (Denaturing gradient gel electrophoresis) they applied for microbiota analysis. Yet, the study still could confirm a strong link of environment plus feeding mode and immune system through alterations of gut microbiota. Neonatal gnotobiotic pig model is a promising animal model to study the immune responses which occurs under the modifications of the gut microbiota (Wen et al., 2014).

4. Conclusion

The evolution of gut microbiota may start as early as the prenatal phase. Then, delivery mode and external factors such as feeding mode and environmental exposure exert profound impacts on shaping the intestinal microbiota. The alteration of gut microbiota in early life determines health risk of the host later in life. This represents a potential to treat high-risk infants via modifying their gut microbial community. Rural/farm exposure in early age was reported to have beneficial effects on decreasing immune-relating disease prevalence. It is promising to consider exposure the children to a high biodiversity environment or even create a simulation for that environment to prevent potential disease and increase health status. By using animal models, we can gain insights into the mechanism of early life gut microbiota, and their role in host health and disease.

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2. Early exposure to agricultural soil accelerates the maturation of the early-life pig gut microbiota

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Abstract

Reduced microbial exposure in early childhood is postulated to be associated with subsequent immune deficiencies and associated health conditions. This corollary to the "hygiene hypothesis" has grown of popularity in the medical field, but can only be really tested with animal models. Based on the previous observation that access to outdoor environment improves piglets' growth performance, we simulated early microbial exposure by providing pigs with topsoil during the lactation phase. Specifically, pigs from 20 litters were assigned to either control treatments (C) or soil treatments (S): pigs exposed to topsoil from day 4 postpartum to the end of lactation. At weaning, five unisex littermates of 10 sows from each treatment were penned together and grew in the same conditions. Fecal samples were collected at on d 13 (Lactation: L), 21 (Weaning: WT), 35 (Maintenance, MNT), 56 (End of Nursery: EONT) and 96 (End of Growth: EGT) for 16s rRNA amplicon high-throughput sequencing. Overall, common trends of gut microbiota maturation, associated with diet switch from maternal milk to a plant-based diet, were observed. *Bacteroides, Clostridium XIVa* and *Enterobacteriaceae* were most abundant during lactation, while *Prevotella*, *Megasphaera*, and *Blautia* became abundant after weaning. Remarkably, exposure to soil resulted in a faster maturation of the piglets' gut microbiota at weaning, while a completely distinct phase was observed at day 35 for control piglets. Soil-exposed piglets tended to harbor a more diverse gut microbiota at weaning and day35, however, the more significant changes were at those time points in terms of composition. *Prevotella* and a wide range of Firmicutes members were significantly enriched in soil-exposed piglets from the lactation to the end of nursery phase. It can be hypothesized that those taxa were either directly transmitted from the soil or stimulated by the presence of plant material in the soil. Those changes were accompanied by depletion of several potentially harmful taxa, as well as improved growth

performance between weaning and the end of nursery phase. Our findings suggest that early exposure to soil strongly influences the maturation of the early-life piglets, probably allows for a better adaptation to the plant-based diet and possibly improves overall health

Key words: Gut microbiota, Hygiene Hypothesis, Early-life, Agricultural soil

1. Introduction

As the importance of the gut microbiota in health and disease was rediscovered (Lozupone et al., 2012; Gordon, 2012; Kau et al., 2011), the hygiene hypothesis has become increasingly popular (Blaser et al., 2008; Azad et al., 2013; Abrahamsson et al., 2014). Importantly, the human microbiota, especially the gut microbiota, has been increasingly reported as the probable main parameter, leading to suggestions to change the hypothesis name to "microbial exposure" or "microbial deprivation" (Mulder et al., 2009). Since one of the corollaries of the hypothesis is the observation that modern hygiene and westernization appear to drive the recent rise in so-called western diseases, there have been several recent studies focused on determining the "primitive" human microbiome. There is now substantive evidence that populations that are still living ancestral way of life harbor higher gut microbial diversity with the presence of distinguishing genera/species(Gomez et al., 2016; Morton et al., 2015; Obregon-Tito et al., 2015; O'Keefe et al., 2015; Ou et al., 2013; Rampelli et al., 2015; Schnorr et al., 2014). However, it should be noted that the majority of studies only include one time-point sampling, and rarely children (Grzeskowiak et al., 2012; Kemppainen et al., 2015; Kuang et al., 2016; Yatsunenko et al., 2012), thus the dynamics of the "natural" early-life human gut microbiota remain elusive.

Early-life human gut microbiome has been studied quite extensively in relation to mode of delivery (Frese & Mills, 2015; Rutayisire et al., 2016) and nutrition (breastfeeding vs formula)(Wang et al., 2015; Piacentini et al., 2010) and to a lesser extent to antibiotic and pre-probiotic exposure (Gibson et al., 2015; Vangay et al., 2015). These variables and sometimes the gut microbiome have been associated with disease risk (Abrahamsson et al., 2012; Abrahamsson et al., 2013; Abrahamsson et al., 2014; Alm et al., 2014; Anderson & Jackson, 2016; Jakobsson

et al., 2014). Since it is clearly not possible to set up studies with controlled environmental (hygiene and cleanliness) variables with infants or children, any data available relies on self-reported parameters, often broad in scope. For instance, there have been reports that exposure to environments with higher microbial diversity has a negative relationship with atopic sensitizations and asthma prevalence (Ruokolainen et al., 2015; Feng et al., 2016). It is commonly postulated that disruption of "normal" microbiota leads to allergic and other auto-immune diseases through impaired immunological development (Chu & Mazmanian, 2013; Dzidic et al., 2016; Gill & Finlay, 2011; Lee & Mazmanian, 2010; Furusawa et al., 2013; Stefka et al., 2014).

As it is commonly the case in medical-oriented studies, the vast majority of studies attempted to link gut microbiota and immune status rely on rodent models (mice in the overwhelming majority). While this choice of model is supported by its advantages for short-term well controlled studies, it is well known that any findings cannot be directly translated to conclusions relevant to human biology and health. An emerging animal model is the swine that is characterized by closer similarity to human in terms of size, digestive physiology and metabolic processes (Heinritz et al., 2013) and specifically intestinal microbiota composition (Zhang et al., 2013). Indeed, there are several reports of promising simulation of early-life human gut microbiota, as well as gut microbiota/immune system interaction using swine models. In addition to be a medical model, gut microbiota dynamics in early-life piglets has recently been reported to potentially affect subsequent health and more generally growth performance, a crucial outcome in the context of animal production.

In the present study, we simulated the outdoor rearing-like environment by exposing piglets to topsoil during lactation (since day 4 to day 21 postpartum). Our objective was to

investigate the potential impact of early exposure to environmental soil microbes on the gut microbiota composition through time and between treatments and overall growth performance.

2. Method

2.1 Experimental design-exposure of top soil to pigs

Piglets (PIC-29 x 380) from 20 litters (litter size>10) were assigned to be either managed conventionally in farrowing crates (C) or daily exposed to topsoil (S) from 4 days postpartum (d 0) to the end of lactation (d 21). Approximately 1kg of topsoil from Tontitown, AR (Sod Store, INC.) was placed and maintained throughout the 21 days into flat containers in S pigs farrowing crates for unrestricted rooting.

At weaning, unisex littermates were penned together in groups of 10 (total of 10 pens and 50 pigs per treatment), and were all subjected to identical growing conditions (no more soil exposure) until marketing. Pigs were fed common antibiotic-free corn-SBM-DDGS nutrient-adequate diets, and individual pigs body weight (BW), was measured at birth and weaning (d 21), together with feed disappearance at each phases change (d 25, 38, 56, 77, 100, 123 and 142), and again at the end of trial (d 163) to determine the growth performance. Fresh grab fecal samples for gut microbiota sequencing were collected on d 13 (Lactation: L), 21 (Weaning: WT), 35 (Mid Nursery, MNT), 56 (End of Nursery: EONT) and 96 (End of Growth: EGT).

Ten samples from each pre-selected animal were taken for each treatment at mentioned time and were processed for microbiota analyses except for L phase. In L, we collected 8 samples for C treatments and 12 samples for S treatments.

2.2 16s rRNA sequencing for gut microbiota composition

Fecal samples received from the Animal Science Department (University of Arkansas, AR, USA) were stored at -70 Celsius for subsequent processing steps.

We extracted DNA using the QIAamp® Fast DNA Stool Mini kit (Qiagen) following the manufacturer's instructions with addition of a bead-beating step, as commonly advised (Zoetendal et al., 2006). Specifically, we weighted around 180mg-220mg fecal samples into autoclaved tubes containing 100 mg of each 0.1 mm and 0.5 mm diameter Zirconia-silicate beads (BioSpec Products) and the InhibitEX Buffer provided in the kit. The tubes were then subjected to bead-beating at 5.5 m/s for 60 seconds in a Fastprep®-24 bead beater. To obtain more concentrated DNA, we added 50µl of Buffer ATE to dilute in the last step instead of 200µl as stated in the manufacturer's instruction. DNA quality and quantity were checked by gel electrophoresis and Nanodrop and/or Qbit (ThermoFisher) measurement.

For library preparation, we followed the dual-index amplicon sequencing approach developed by Kozich et al (2013) with slight modifications. Briefly, DNA extracts were used as template for PCR using the dual index primers targeting the V4 region of the 16S rRNA gene sequence in 96 well plates according to the scheme. PCR conditions were as follows: initial denaturation (2 min at 95°C) 25 amplification cycles (95°C for 30s, 55°C for 30s, 72°C for 1 min) and final elongation (72°C for 5 min). A random row was submitted to gel electrophoresis to confirm successful amplification, and all amplicons were subjected to purification and normalization using SequalPrep™ Normalization Plate Kit (ThermoFisher). Amplicons were pooled, and the pool subjected to library quality control: quantitative PCR by NEBNext® Library Quant Kit for Illumina® (New England Biolabs) and TapeStation Bioanalyzer (Aligent) following manufacturer recommendations. Because resulting libraries were found to have

unexpected additional bands, gel extraction of the correct band was performed with the QIAquick Gel Extraction Kit (Qiagen) and resulting libraries submitted again to quality control. Based on previous sequencing runs on our MiSeq, libraries were diluted to 6pM and pooled with PhiX (internal sequencing standard; 5%) and sequenced on an Illumina MiSeq using the Illumina MiSeq® Reagent Kit v2 (500 cycle). The runs were monitored with Sequence Analysis Viewer with particular emphasis on appropriate cluster density (700-800k/mm^2) and quality scores (final >Q30 score of >70%). When satisfactory sequencing was obtained, preliminary sequence analysis was performed on BaseSpace (illumina) with the 16S Metagenomics application. Resulting Fastq files were readily demultiplexed and assigned to samples and downloaded for bioinformatics analyses.

Sequences were processed following the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP). Briefly, sequences were screened and aligned to the Silva database for 16S RNA gene sequences. Subsequently, OTUs were picked and assigned to taxonomic groups. Resulting OTUs and taxonomic tables were exported to Excel sheets for basic analyses and JMP and PAST software for univariate (T-test, ANOVA) and multivariate (ANOSIM, PcoA, NMDS) statistical analyses. We used subsampled data to calculate observed OTUs, Chao, Shannon and Inverse Simpson index via Mothur (Schloss et al., 2009) and compared results by non-parametric Wilcoxon test using JMP software.

3. Results

From 102 samples, a total of 5194218 raw sequences were obtained of which 2995037 high-quality reads were used for the analysis. Samples yielding less than 2807 high-quality reads were discarded, the remaining samples analyzed had an average of 29950 reads. All reads were analyzed together in the Mothur pipeline, leading to a total number of 4544 OTUs identified, belonging to 16 phyla.

3.1 Overall early-life piglets gut microbiota composition and dynamics

In all piglets, Firmicutes and Bateroidetes were always the two dominant phyla, with the remainder of the bacterial community mainly comprised of Proteobacteria and Synergistes and to a lower extent Spirochaetes (Figure 2.1). Somewhat unexpectedly for such a mammalian gut microbiota, Actinobacteria and Verrumicrobia numbers were extremely low. During the suckling phase (day 13 to day 21), Proteobacteria and Synergistes were found in higher numbers (5% each) and declined to marginal levels after weaning. Spirochaetes became abundant around weaning (5% at days 21 and 35).

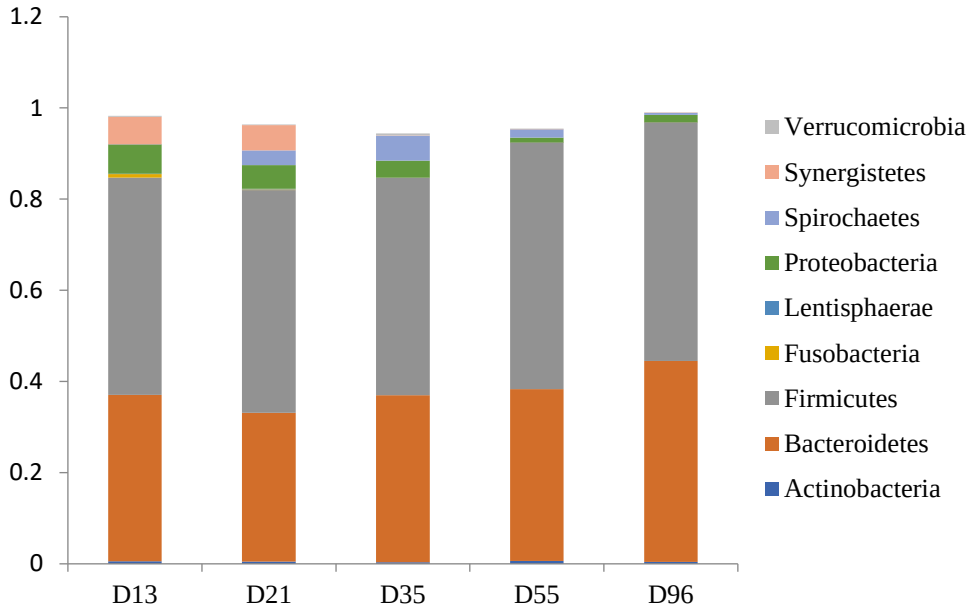

Figure 2.1: Early-life piglet gut microbiota dynamics. Averaged relative abundance at the phylum level of all pigs at each time point.

Firmicutes were dominated by a few genera during the suckling phase (including day 35): Unclassified Ruminococcaceae, Lachnospiraceae and Acidaminococcaceae and *Clostridium* XIVa members. The weaning phase was characterized by a significant decrease of some of those dominant Firmicutes (Particularly *Clostridium* XIVa members, and Ruminococcaceae on day 96), This decline was accompanied by a large diversification and increase of several genera (*Megasphaera*, *Streptococcus*, unclassified Veillonellaceae, *Blautia*, *Faecalibacterium*, *Dialister*…). Somewhat unexpectedly, Lactobacillus decreased at day 21 (from 5.8 to 2%) but bounced back during weaning (Figure 2.2). Significant changes were observed especially distinguishing the suckling (day 13 and 21) from the weaning phase (days 35, 55 and 96). Among the Bacteroidetes, *Bacteroides* switched from most abundant to minor, while *Prevotella* followed an inverse trajectory from low to major. Day 35 was interestingly marked by a peak in other Bacteroidetes, Bacteroidales and Porphyromonodaceae (20% combined). Proteobacteria

were dominated by Enterobacteriaceae and Desulfovibrio during the lactation phase, with only Enterobacteriaceae staying at high numbers at day 35. The end of growing phase was characterized by the presence of unclassified proteobacteria and Gammaproteobacteria, albeit in very low abundance (Figure 2.2). *Cloacibacillus* were relatively abundant during the lactation phase, while *Treponema* appeared at weaning, with numbers fading after day35.

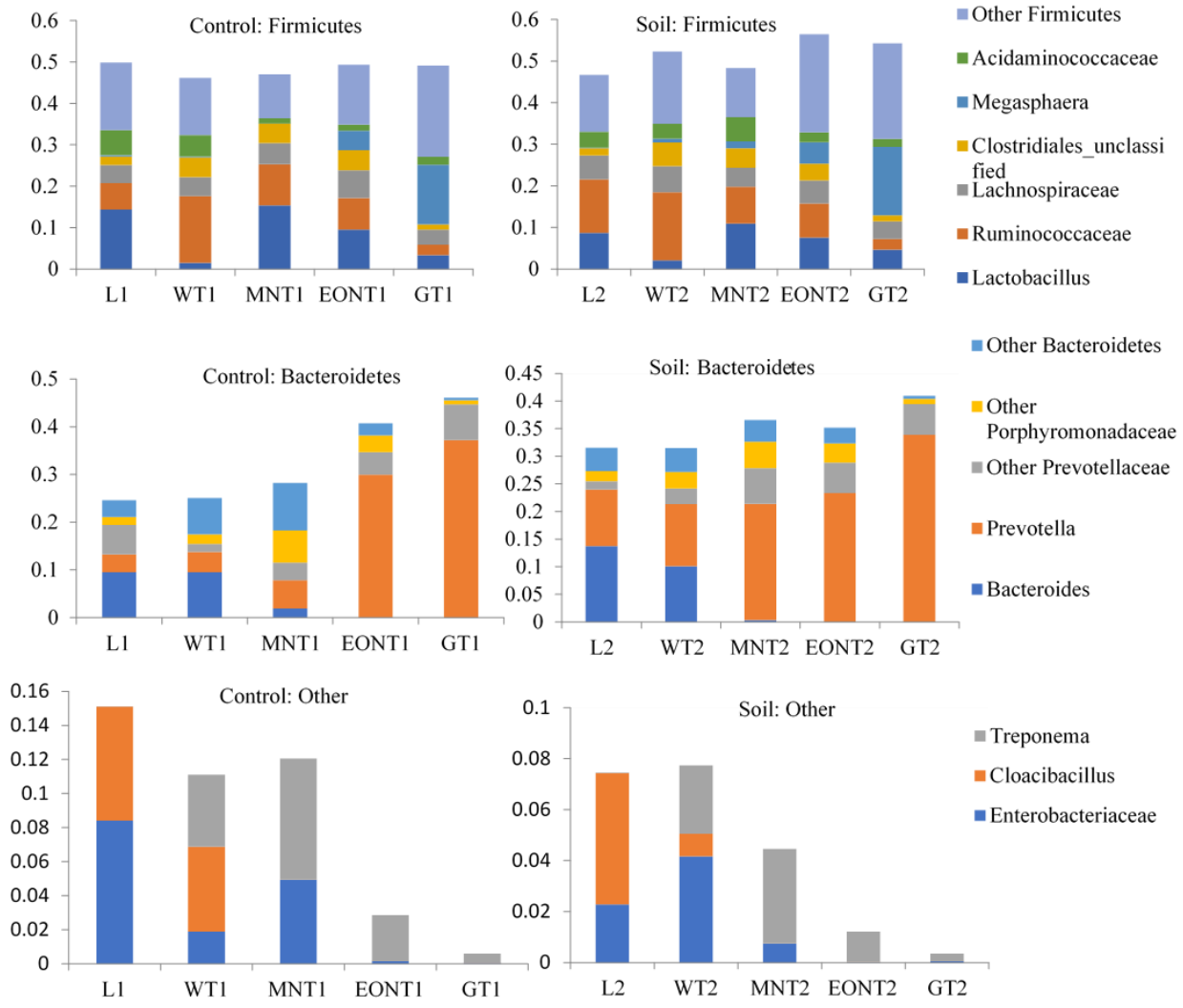

Figure 2.2: Early-life piglet gut microbiota dynamics. Relative abundance of taxa representing at least 5% of the total community for at least one of the different stages: d13 (Lactation: L), 21 (Weaning: WT), 35 (Mid-nursery, MNT), 56 (End of Nursery: EONT) and 96 (End of Growth: EGT).

3.2 Impact of soil exposure on gut microbiota structure and dynamics

During the lactation phase, soil exposure had no significant impact on gut microbiota diversity. However, a significant (p=0.028) difference in the number of taxa (203 in C, 258 in S) and in Chao index (290 in C, 373 in S), was observed at weaning (but not reflected in other diversity indices; Table 2.1). At the end of nursery, all diversity indices were significantly higher in S pigs except observed OTUs and Chao index (Table 2.1). Diversity indices became entirely similar at day 96.

Table 2.1: Mean of observed OTUs and diversity indices including Inverse Simpson, Chao and Shannon index of two treatments in different time points.

Time	Group	OTUs	Invsimpson	Chao	Shannon
Lactation (D13)	Control	177	13.7	256	3.44
	Soil	175	15.3	254	3.45
Weaning (D21)	Control	203*	20.8	290*	3.5
	Soil	258*	25.6	373*	4
Mid Nursery (D35)	Control	361	24	520	4.18
	Soil	333	23	477	4.09
End nursery (D56)	Control	321	22*	448	4*
	Soil	328	38*	382	4.4*
End growth (D96)	Control	265	13.9	406	3.58
	Soil	262	14.7	401	3.6

Note: T1: Control treatment (C), T2: Soil treatment (S). L: lactation (13 days), W: weaning (21 days), MN: mid nursery (35 days), EON: end of nursery (56 days), G: Growth phase (96 days). Observed OTUs and diversity indices were compared between treatments at specific time point using non-parametric Wilcoxon test (performed in Jmp 11 Software. (*) indicates significant difference ($P<0.05$)

At the phylum level, Bacteroidetes were significantly higher and Proteobacteria significantly lower at day 13 and 35 in S pigs. C pigs had significantly higher Synergistes numbers at weaning (day 21). At day 55 and 96, the composition was similar with a trend for lower Bacteroidetes and higher Firmicutes in S pigs (Figure 2.3.).

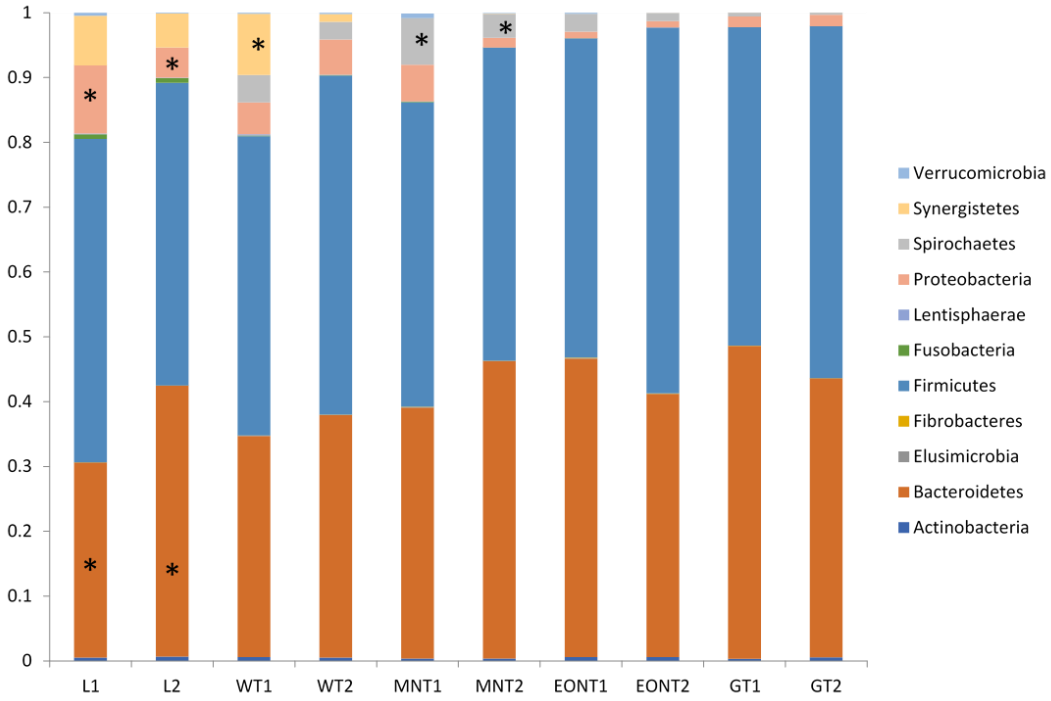

Figure 2.3: Differences in gut microbiota composition at phylum level between two treatments. Control (1) and soil (2) exposed pigs at d13 (Lactation: L), 21 (Weaning: WT), 35 (Mid-nursery, MNT), 56 (End of Nursery: EONT) and 96 (End of Growth: EGT). (*) indicates significant differences ($p < 0.05$)

Multivariate analyses at the genus level indicated significant differences in the gut microbiota trajectories in developing piglets depending on soil exposure (Figure 2.4). S and C pigs microbiota during the lactation phase formed two separate but overlapping clusters. The gut microbiota was separated in significantly ($p<0.001$, ANOSIM) distinct clusters at the Mid Nursery timepoint (Day 35). Subsequently, the gut microbiota was found to converge to form an end of nursery and an end of growth cluster, regardless of initial treatment. Remarkably, the S

cluster at day 35 was distinct from every other cluster, while the C cluster showed overlap with the end of nursery cluster.

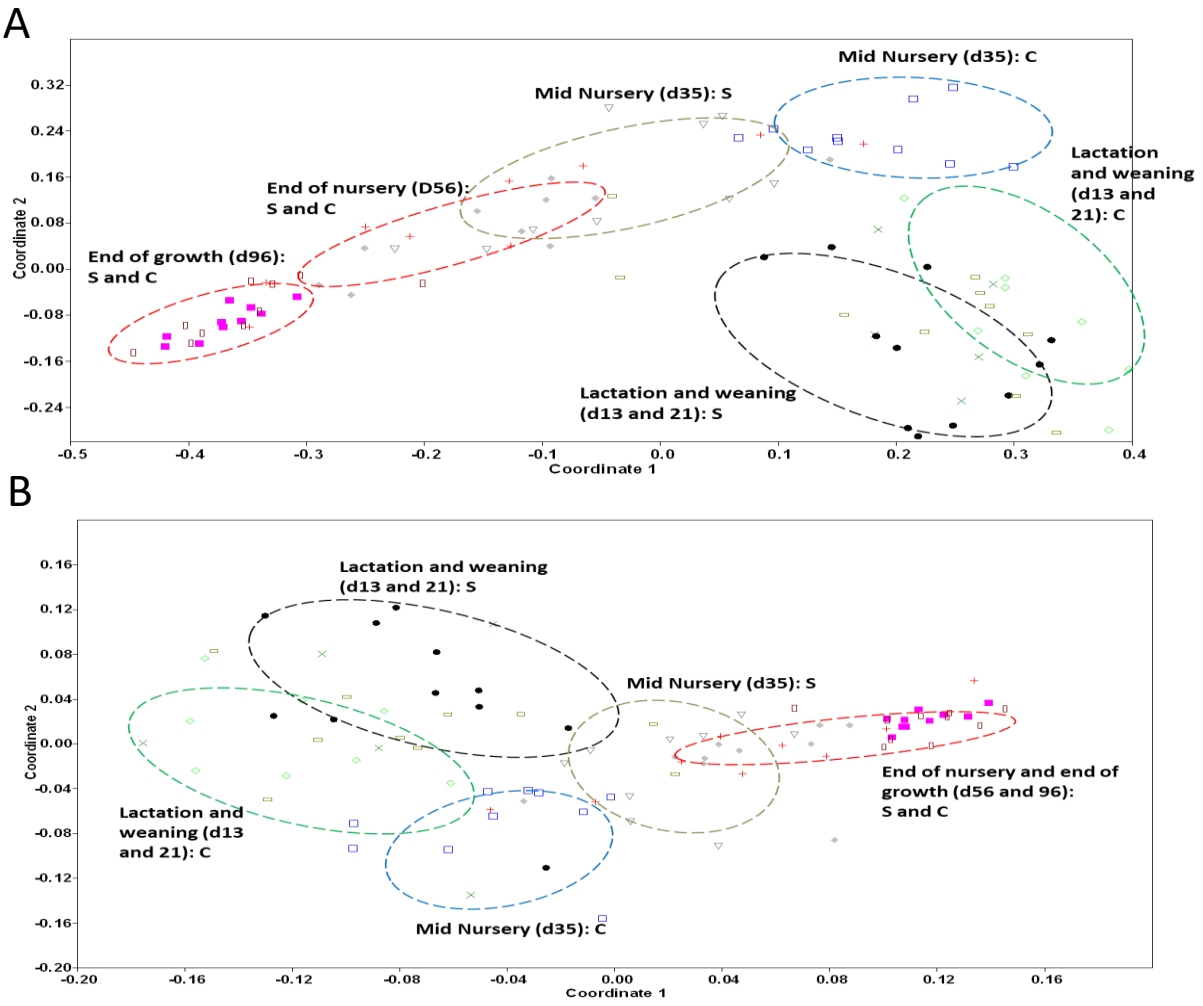

Figure 2.4: Gut microbiota dynamics in control (C) and soil exposed (S) piglets. (A) PcoA and (B) non-Metric Multidimensional scaling (NMDS) based on Bray-Curtis similarity (outlining circles indicating the different phases were drawn on the ordination plots to facilitate interpretation). Control pigs: d13: green diamonds, d21: green crosses, d35: blue squares, d56: gray diamonds and day 96: purple filled squares. Soil exposed pigs: d13: black filled circles, d21: gray rectangles, d35: gray inverted triangles, d56red crosses and day 96: red open squares.

Significant differences were observed at the genus level, especially at weaning and day 35 and 55 (19, 9 and 11 abundant genera significantly different). During lactation (day 13), S pigs were characterized by significantly higher numbers of *Prevotella* and *Butyricimonas* (Bacteroidetes) as well as Ruminococcaceae. At weaning, C pigs had higher numbers of non-*Prevotella* Bacteroidetes (*Butyricimonas*, *Alistipes* and *Odoribacter*), members of the Clostridium XIVa cluster and Proteobacteria (*Campylobacter*, *Desulfovibrio*) and Synergistes (*Cloacibacillus* and *Pyramidobacter*) (Figure 5.). At weaning, S pigs had higher numbers of *Prevotella* and several firmicutes genera (*Clostridium* and *Clostridium* clusters XI and XIVb, *Faecalibacterium*, *Coprococcus*, *Blautia*, *Oscillibacter*, *Butyricioccoccus*, Peptostreptococcacceae and *Veillonella*) (Figure 2.5).

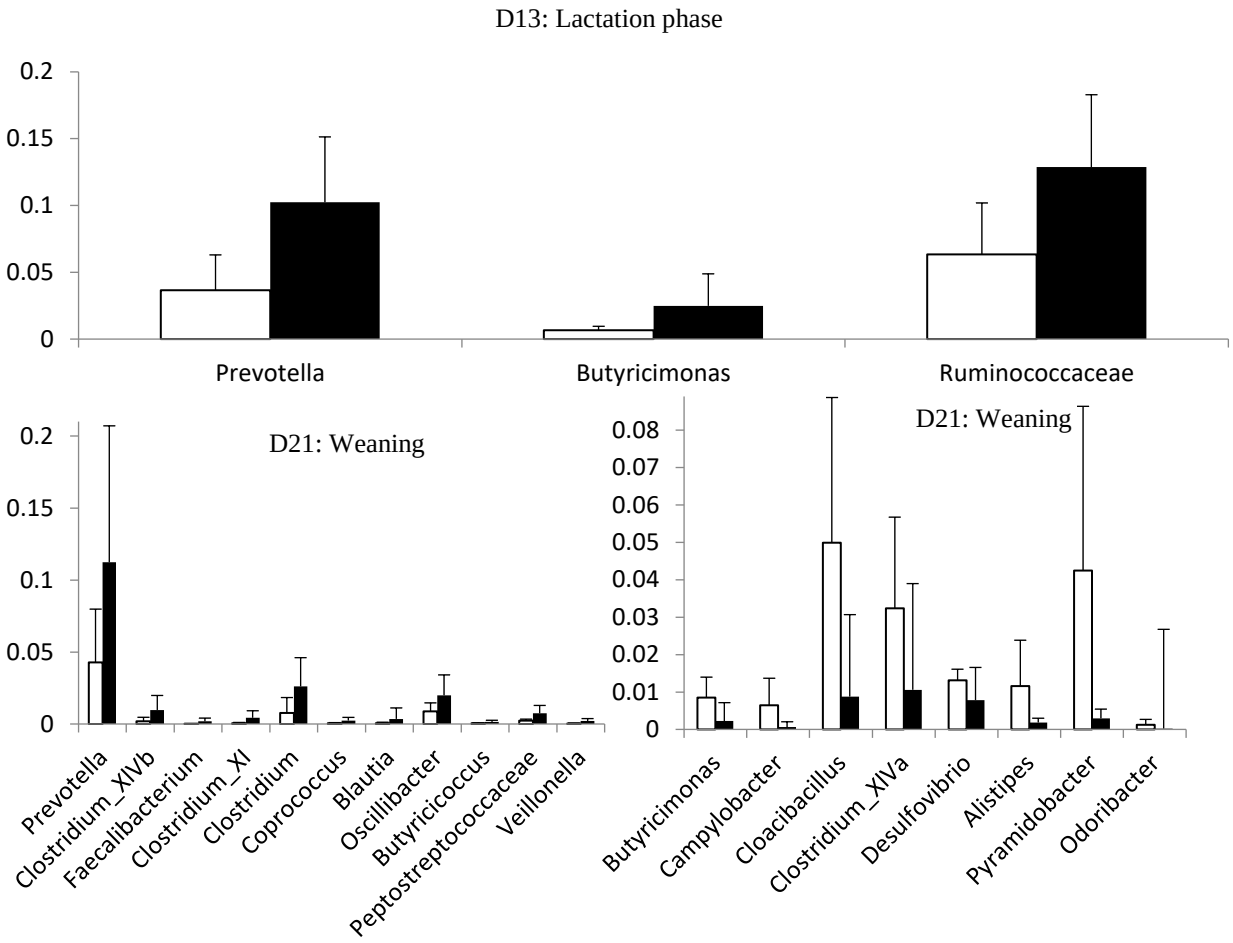

Figure 2.5: Significantly different of gut microbiota at genus level between two treatments in early time. Average relative abundances for genera significantly ($P < 0.05$) enriched in one group (control or soil); Lactation (d13) and weaning (d21) timepoints White: Control treatment; Black: Soil treatment.

At day 35, C pigs had higher *Bacteroides* (and other Bacteroidetes), *Clostridium* XIVb and Enterobacteriaceae; while S pigs had higher *Prevotella*, *Faecalibacterium*, *Megasphaera*, Veillonellaceae and Acidaminococacceae (Figure 6.). At day 55, C pigs had higher *Bacteroides* and *Succinivibrio*. At day 55, S pigs had higher *Clostridium* and *Clostridium* cluster XI, *Coprococcus*, *Campylobacter*, *Streptococcus*, *Mitsuokella*, *Ruminococcus*, *Dialister* and *Sarcina*. At the end of the growth period (day 96), S pigs had higher *Faecalibacterium*. (Figure 2.6)

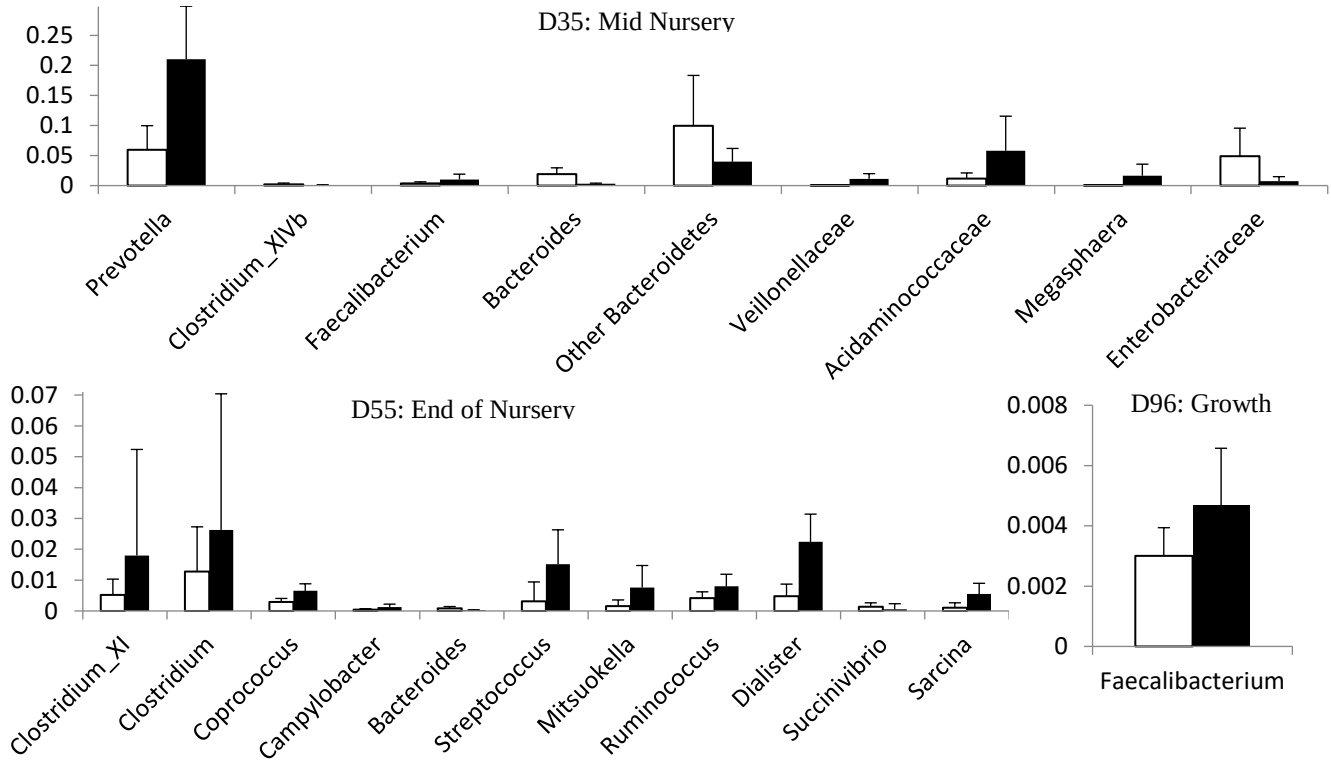

Figure 2.6: Significantly different of gut microbiota at genus level between two treatments in later phases. Average relative abundances for genera significantly ($P < 0.05$) enriched in one group (control or soil). Post-lactation time points: d35 (Mid-Nursery, MNT), d56 (End of Nursery: EONT) and d96 (End of Growth: EGT). White: Control treatment; Black: Soil treatment.

4. Discussion

In this study, we observed that exposure to soil increased the piglet gut microbiota diversity and accelerated its maturation after weaning. It can be logically postulated that the agricultural soil contained both plant-derived compounds (carbohydrates, fibers) and soil-borne microorganisms that were transferred in the piglet colon and allowed for an increased ability to switch from the milk-based to the plant-based diet.

While differences between treatments were indeed observed, there were general trends in the gut microbiota dynamics that were clearly not affected by treatments. As reported previously (Frese

et al., 2015; Mach et al., 2015), *Bacteroides* were the predominant Bacteroidetes during the suckling phase, replaced with *Prevotella* after weaning. However, the gut microbiota dynamics we observed differed significantly from both studies. We found *Lactobacillus* to be relatively stable, with a slight increase in the last days, in agreement with Frese et al., (2015), and since the other studies may suffer from lower read counts per samples, the hypothesis that plant-based diets stimulate *Lactobacillus* activity appears to be reinforced. We observed higher levels of Ruminococcaceae during the suckling phase in accordance with Mach et al., (2015) but in contrast with Frese et al., (2015). Those differences suggest that factors other than diet play a role in the establishment of the early-life swine gut microbiota, which would require more systematic testing.

In human, microbial diversity in early time of life may be critical in predicting allergic risk. An analysis of infant fecal samples at 1 week, 1 month and 12 months revealed that children with low gut microbiota diversity in the first month, had increased asthma incidence at 7 years of age (Abrahamsson et al., 2014). Antibiotic use, which interferes with the microbial diversity and stability of infant gut microbiota (Yassour et al., 2016), was also linked to the higher risk of asthma and allergic disease at school age (Alm et al., 2014; Ong et al., 2014). In fact, antibiotics were proven to associate with only allergic asthma (Ong et al., 2014) which once again confirmed the potential role of microbial diversity in educating the immune system at early stage. In the present study, at W (21 days), S pigs microbiota possessed higher diversity comparing to C pigs in term of richness when they have significantly higher observed species as well as Chao index. After 56 days (EON), S pigs showed a higher diversity in term of evenness when they have statistically higher value of Shannon and Inverse Simpson index. Although these trends were not maintained at all time and dissimilate after 96 days, it was evident that exposing

pigs to soil had potential positive effects to modify the gut microbiota in term of increasing richness and evenness. This suggests a role of microbial exposure in increasing the diversity at early age which may in turn help reduce the risk of allergic diseases.

There are numerous reports indicating that housing with outdoor access increases pig feed intake and growth performance (Wenner et al., 2013; Lebret, 2008; Dourmad et al., 2009; Lebret et al., 2011). Indeed, it was reported that the piglets exposed to soil in this study had significantly higher body weight (BW; 2.21 kg more, $p<0.05$) at the end of-nursery despite having lower BW at weaning (6.87 vs. 7.40 kg, $P < 0.05$). Pigs exposed to soil tended to have higher BW at end of growth (4.6 kg), resulting in significantly higher average daily gain throughout the nursery period (0.42 vs. 0.35 kg/d, $P < 0.01$) (Tsai et al., 2016). These trends correlate very well with the trends observed in the gut microbiota diversity and composition over the lactation and nursery periods. These combined observations suggest that piglets GI tract colonization by environmental microbes is slightly detrimental during the lactation phase, but becomes highly beneficial when switching to the plant-based diet after weaning, even as piglets are no longer exposed to soil. Different mechanisms can be hypothesized to be involved in the apparent role for microbiota maturation in piglets' growth performance.

The gut microbiota of lactating piglets exposed to soil were rapidly enriched in Bacteroidetes (Prevotella mainly) and Ruminococcaceae. These microbial groups are characterized by known presence in soils and ability to degrade complex polysaccharides and fibers (Thomas et al., 2011). These properties are probably the reason why they were enriched in the soil exposed piglets; however, it is not clear why other taxa with similar properties were not enriched as well. It is possible that the specificity of plant material available in the soil dictated

this trend (Thomas et al., 2011). Many taxa distinguished the two groups at the end of the lactation period, but the most remarkable difference was again the presence of twice more *Prevotella* in the soil exposed piglets. Pigs from S group were also enriched in different short-chain fatty acid (SCFA) producing clostridia: Acetate (*Blautia*), Butyrate (*Faecalibacterium*, *Butyriciococcus*, *Oscillibacter*) or both (*Coprococcus*, *Clostrium* spp and cluster XI and XIVb) and propionate + acetate (*Veillonella*). In contrast, only other *Clostridium* cluster XIVa and *Butyricimonas* (both butyrate producers) were depleted. The increased abundance presumably limited the amount of Proteobacteria and Synergistes, allowing for potentially more beneficial fermentation processes. Somewhat similar trends were observed in the days after weaning (day 35 and 55), with S pigs having higher levels of several SCFA-producing firmicutes (*Megasphaera* in addition at day 35) and *Prevotella* at day 35.

Prevotella is well known as the dominant genus in gut microbiota of mammals and humans consuming a diet rich in plant polysaccharides and fiber (O'Keefe et al., 2015; Ou et al., 2013; Schnorr et al., 2014; Gomez et al., 2016), indicating that Prevotella is a primary degrader, realeasing propionate and plant-derived compounds for further microbial fermentation. A few studies have reported Prevotella as significantly enriched in obese patients (Zhang et al., 2009; Hu et al., 2015), and it is thus possible that increased Prevotella numbers is one factor involved in increased weight gain in pigs exposed to soil. Indeed, it has been reported that post-weaning piglets belonging to the *Prevotella*/*Mitsuokella* enteroype, as the soil exposed piglets in this study, had significantly higher ADG and final BW (Ramayo-Caldas et al., 2016).

There is extensive evidence that microbial-derived SCFA, in particular butyrate, have beneficial effect to the host, both by increasing energy harvest from dietary compounds and serving as energy source for colonocytes. Here, we observed that soil exposure resulted in

significant increases of known butyrate producers over the lactation and post weaning periods. Among these butyrate-producers, *Faecalibacterium* is of particular interest, as it is known for its anti-inflammatory properties in human. More generally, butyrate was shown to play a key role in the induction of Tregs in vitro and in vivo (Furusawa et al., 2013). Butyrate produced by commensal gut bacteria was known for its inflammatory effects as well as immunology tolerance (Xu et al., 2016). In addition to produce butyrate, members of the Clostridia class have been shown to play an important role in maintaining gut homeostasis by regulating immunological tolerance and preventing harmful organisms to outgrow in the gut (Lopetuso et al., 2013). More specifically, direct evidence showed that *Clostridium* species educate the immune system by inducing the differentiation of CD4+ regular T cells (Tregs) Supplementing *Clostridium* orally in early life in mice also helped decrease colitis and immunoglobulin E (IgE) response in adult mice which suggests an allergy-protective effect for this bacterial genus (Atarashi et al., 2011). The fact that S pigs maintained significantly higher numbers of butyrate-producing clostridia from W to EON supports our hypothesis on the benefit of soil exposure by improved immune function and/or energy harvest.

Besides butyrate producers, S pigs also had significantly higher number of other SCFAs producing bacteria and genera generally associated with beneficial properties such as *Blautia, Veillonella* and different Clostridia members. Especially at weaning and day 35, these trends were associated with depletion of potential pathogens (*Campylobacter*, Enterobacteriaceae) and potentially detrimental taxa such as the mucin-degrading *Cloacibacillus* (Looft et al., 2013) and hydrogen sulfide producer *Desulfovibrio* (Carbonero et al., 2012). All considered, it can be hypothesized that exposure to soil reduced the risk of mild dysbiosis in the piglet microbiota, thus transiently favoring health and growth.

5. Conclusion

Our results confirm the intuitive hypothesis that short-term exposure to soil-borne microbes could mimic outdoor rearing and that it drives strong compositional differences in the gut microbiota of piglets during lactation and several weeks after weaning. Specifically, soil exposure promotes the abundance of *Prevotella* and several SCFA producing taxa, while restricting detrimental taxa. Moreover, we show that gut microbiota maturation towards the optimum state for plant-based diet consumption is accelerated for piglets exposed to soil. It is likely that gut microbiota modulation by soil exposure correlates with the transient improvement in piglets' growth. Many factors can potentially explain this trend: improved energy harvest potential (*Prevotella* enterotype), increased production of SCFA and modulation of the immune system. These data also suggest that similar gut microbiota modulation may be relevant to human children, in line with the Hygiene Hypothesis.

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3. Divergent gut microbiota composition in early stage under influences of different feeding mode

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Abstract

Feeding mode plays a pivotal role in the establishment of the symbiont relationship between gut microbiota and the host which is critical in defining the prevalence of health risk later in life. In the current study, we leveraged the piglets model which facilitated more controlled experimental conditions, yet possessed a handful of resemble characteristics with human model, to investigate the differences of gut microbiota composition with different feeding modes. Specifically, from day 4 postpartum to weaning (day 21), littermate piglets blocked by body weight were assigned into either breastmilk treatment (M treatment) with mother rearing and breastmilk feeding or formula treatment (F treatment) with artificial rearing and formula feeding. Fecal samples were collected at day 21, day 64 and day 78 for V4 16s rRNA sequencing using Illumina Miseq system. We analyzed the obtained sequencing data using Mothur workflow, PAST3, and Jmp11 Software to examine the gut microbiota composition. Regardless of feeding modes, the two most dominant phyla in the gut microbiota were Firmicutes and Bacteroidetes and the gut microbiota was more diverse with time factor. *Prevotella* significantly increased over time indicating a more mature gut microbiota while Proteobacteria belonging genus essentially decreased post-weaning. F and M piglets' gut microbiota diverged at weaning (day 21) with an enrichment of Firmicutes in F piglets and a more abundance of Bacteroidetes in M piglets. The gut microbiota in F treatment had higher diversity compared with M treatment. Dominant genera in F piglets gut microbiota were *Clostridium sensu stricto* (0.07), *Streptococcus* (0.05), *Mogibacterium* (0.05), *Blautia* (0.09), and *Leuconostoc* (0.12) while they are *Bacteroides* (0.12), unclassified Enterobacteria (0.07) and *Desulfovibrio* (0.006) in the case of M piglets. However, this trend quickly faded during time leading to a relatively resemble gut microbiota composition in day 64

and day 78. Our results suggest that different feeding mode is sufficient to modify the early gut microbiota composition which may have great impacts on immune system maturation in infants.

Key words: Breastmilk, gut microbiota, feeding mode, piglets, early life

1. Introduction

Gut microbiota plays an important role in maintaining the health status of the host (Kahrstrom et al., 2016). There is a critical window for modifications of initial gut microbiota to either ameliorate or exacerbate the prevalence of health risk (Arrieta et al., 2015, Wegienka et al., 2015, Bunyavanich et al., 2016 & Suzuki et al., 2007). The symbiont relationship of gut microbiota and human host may initiate as early as the prenatal period (Charbonneau et al., 2016) then this intestinal microbiota experiences other evolution at birth and post-natal period (Dominguez-Bello et al., 2010; Chu et al., 2017). Among important extrinsic factors such as environmental exposure, early feeding mode in infants is imperative in shaping the gut microbiota which in turn, affects the health of infants (Guaraldi & Salvatori, 2012). Besides lactose, breast milk also contains a myriad of components such as defensins, lactoferrin, short-chain fatty acids that actively support the healthy development of infants.

Especially, breast milk supports the establishment of a well-functioned gut microbiota. A study on 107 healthy mothers and their infants underscored the role of breast milk in establishing the gut microbiota when reporting that in the first 30 days of life, breastfed infants acquired 75% of their gut microbiota composition from the breast milk and only 10.3% from areolar skin (Pannaraj et al., 2017). Among the first anaerobes that dominantly colonized the infants' gut microbiota within the first and second week of life are *Bifidobacterium* spp. (Jost et al., 2012) which are enriched in human breast milk (Solis et al., 2010). A study on bifidobacterial supplementation impacts on the gut microbiota composition of infants with early onset of atopic eczema revealed that supplementation of bifidobacterial helped decreased the numbers of *Escherichia Coli* and *Bacteroides*. These two microbial members were directly proportional to serum total IgE. The result suggested that bifidobacterial might alleviate allergic inflammation

by promoting a healthy gut microbiota (Kirjavainen P.V. et al., 2002). In addition, the breast milk stimulated intestinal microbial community helps harmonize the balance of Th1/Th2 and activates T-regulatory cells. These immunological responses are critical for oral tolerance and immune homeostasis which prevents the host from immune-mediated and allergic disease (Walker & Iyengar, 2015).

It is critical to understand how differences in feeding mode can lead to different gut microbiota structure in a more controlled condition which is difficult to obtain in human studies. For instance, a research studied on piglets' gut microbiota could set up the conditions in which two treatments were only differences in the first day of life. This allowed them to study the significance of early life exposure on gut microbiota, albeit with short time exposure (Merrifield et al., 2016). By using animal models, a more insightful view of immune responses differences from breastfed and formula-fed neonatal mice could be achieved. A study reported that SigA from breastmilk could enhance epithelial barrier function in neonatal mice. (Rogier et al., 2013). In addition, another study on pig model indicated breastfed neonates showed a higher level of Interleukin 4 (IL-4) production and these differences were strongly associated with gut microbiota composition (Inman et al., 2010).

In the current study, we aim to investigate the gut microbiota composition of piglets that assigned to either mother rearing and breastfeeding or isolated nursery rearing and formula feeding. We hypothesize that the gut microbiota of two treatments will significantly different under the effects of different feeding mode.

2. Materials and methods

2.1. Experimental design- milk and formula feeding

The animal study was conducted at Animal Science facility under the Institutional Animal Care and Use Committee (IACUC) approved protocol #13060. Body weight (BW) blocked- piglets (PIC-29x380) from the same litters were assigned either to Milk treatment (M) or Formula (F) treatments. There were total 80 piglets from 20 litters involved in this experiment. Specifically, when the pigs were 4 days old, we selected 4 unisex pigs with close-to-average BW from each litter, then either left them be with their mother and had full access to breastmilk (M treatment) or isolated them into artificial rearing system and fed them with formula milk (F treatment). At weaning (day 21), pigs blocked by gender and feeding mode treatment were randomly allotted to nursery pens (4 pigs/pen) with the same conventional growing condition for both treatments.

Fecal samples were collected at weaning (day 21) and post-weaning including day 64 and day 78. During experiment time, we collected a total of 165 samples including 40 samples for M treatment and 38 sample for F treatment in day 21, 36 samples for M treatment and 33 samples for F treatments in day 64 and 10 samples per treatment in day 78. Fecal samples were collected at Animal Science Department (University of Arkansas, AR, US) and transferred in a cooler with ice to the Molecular laboratory in Biomass Research Center (Arkansas, US). Then, the samples were stored in the freezer at -80oC before performing DNA extraction and 16s rRNA sequencing.

2.2 Gut microbiota 16s rRNA sequencing

We extracted DNA using the QIAamp® Fast DNA Stool Mini kit (Qiagen) following the manufacturer's instructions with the addition of a bead-beating step, as commonly advised (Zoetendal et al., 2006). Specifically, we weighted around 180mg-220mg fecal samples into autoclaved tubes containing 100 mg of each 0.1 mm and 0.5 mm diameter Zirconia-silicate beads (BioSpec Products) and the InhibitEX Buffer provided in the kit. The tubes were then subjected to bead-beating at 5.5 m/s for 60 seconds in a Fastprep®-24 bead beater. To obtain more concentrated DNA, we added 50µl of Buffer ATE for elution in the last step instead of 200µl as stated in the manufacturer's instruction. DNA quality and quantity were checked by gel electrophoresis and Nanodrop and/or Qbit (ThermoFisher) measurement.

For library preparation, we followed the dual-index amplicon sequencing approach developed by Kozich et al (2013) with slight modifications. Briefly, DNA extracts were used as templates for PCR using the dual index primers targeting the V4 region of the 16S rRNA gene sequence in 96 well plates according to the scheme. PCR conditions were as follows: initial denaturation (2 min at 95°C) 25 amplification cycles (95°C for 30s, 55°C for 30s, 72°C for 1 min) and final elongation (72°C for 5 min). A random row was submitted to gel electrophoresis to confirm successful amplification, and all amplicons were subjected to purification and normalization using SequalPrep™ Normalization Plate Kit (ThermoFisher). Amplicons were pooled, and the pool subjected to library quality control: quantitative PCR by NEBNext® Library Quant Kit for Illumina® (New England Biolabs) and TapeStation Bioanalyzer (Aligent) following manufacturer recommendations. Because resulting libraries were found to have unexpected additional bands, gel extraction of the correct band was performed with the

QIAquick Gel Extraction Kit (Qiagen) and resulting libraries submitted again for quality control. Based on the previous sequencing runs on our MiSeq, libraries were diluted to 6pM and pooled with PhiX (internal sequencing standard; 5%) and sequenced on an Illumina MiSeq using the Illumina MiSeq® Reagent Kit v2 (500 cycles). The runs were monitored with Sequence Analysis Viewer with particular emphasis on appropriate cluster density (700-800k/mm2) and quality scores (final >Q30 score of >70%). When satisfactory sequencing was obtained, preliminary sequence analysis was performed on BaseSpace (Illumina) with the 16S Metagenomics application. Resulting Fastq files were readily demultiplexed and assigned to samples and downloaded for bioinformatics analyses.

2.3 Bioinformatics and Statistical analyses

Sequences were processed following the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP). Briefly, sequences were screened and aligned to the Silva database for 16S RNA gene sequences. Subsequently, OTUs were picked and assigned to taxonomic groups based on Ribosomal Database Project (RDP) database. Resulting OTUs and taxonomic tables were exported to Excel sheets for basic analyses and JMP and PAST software for univariate (non-parametric Wilcoxon test) and multivariate (ANOSIM, NMDS plot) statistical analyses. We used subsampled data to calculate observed OTUs, Chao, Shannon and Inverse Simpson index via Mothur (Schloss et al., 2009) and compared results by non-parametric Wilcoxon test using JMP software.

3. Results

In this study, we sequenced the V4 region of 16s rRNA genes from DNA extracted from piglets' fecal samples. Our objective is to compare the gut microbiota composition and evolution between breastfed pigs (isolated from the mothers) and formula-fed pigs (mother rearing) through three time-points: day 21 (weaning), day 64, and day 78.

From 165 samples, we obtained a total of 7169525 raw sequences of which 4207000 high-quality sequences (aligned with Silva Bacteria Database) were used for further analysis. Three samples with lower reads were eliminated from the dataset which left remaining samples with an average of 25576 (standard error = 9013) reads per sample. When clustering those sequences, we obtained 5554 OTUs which could be classified into 261 bacterial groups at genus level and belonged to 19 phyla with reference to Ribosomal Database Project (RDP) database.

3.1 Overall gut microbiota dynamics in different feeding mode and environment

Regardless of feeding modes, the two most dominant phyla in the gut microbiota were Firmicutes and Bacteroidetes (Figure 3.1). Other remarkable phyla, albeit in lower abundance, were Actinobacteria, Proteobacteria, Synergistetes, and Spirochaetes. At phylum level, in both treatment, after weaning time (day 21), Actinobacteria, Proteobacteria, and Synergister relative abundance significantly decreased while there was an increase in Bacteroidetes and Spirochaetes. Notably, Firmicutes abundance in formula-fed piglets statistically decreased, yet it was maintained the same in breastfed treatment.

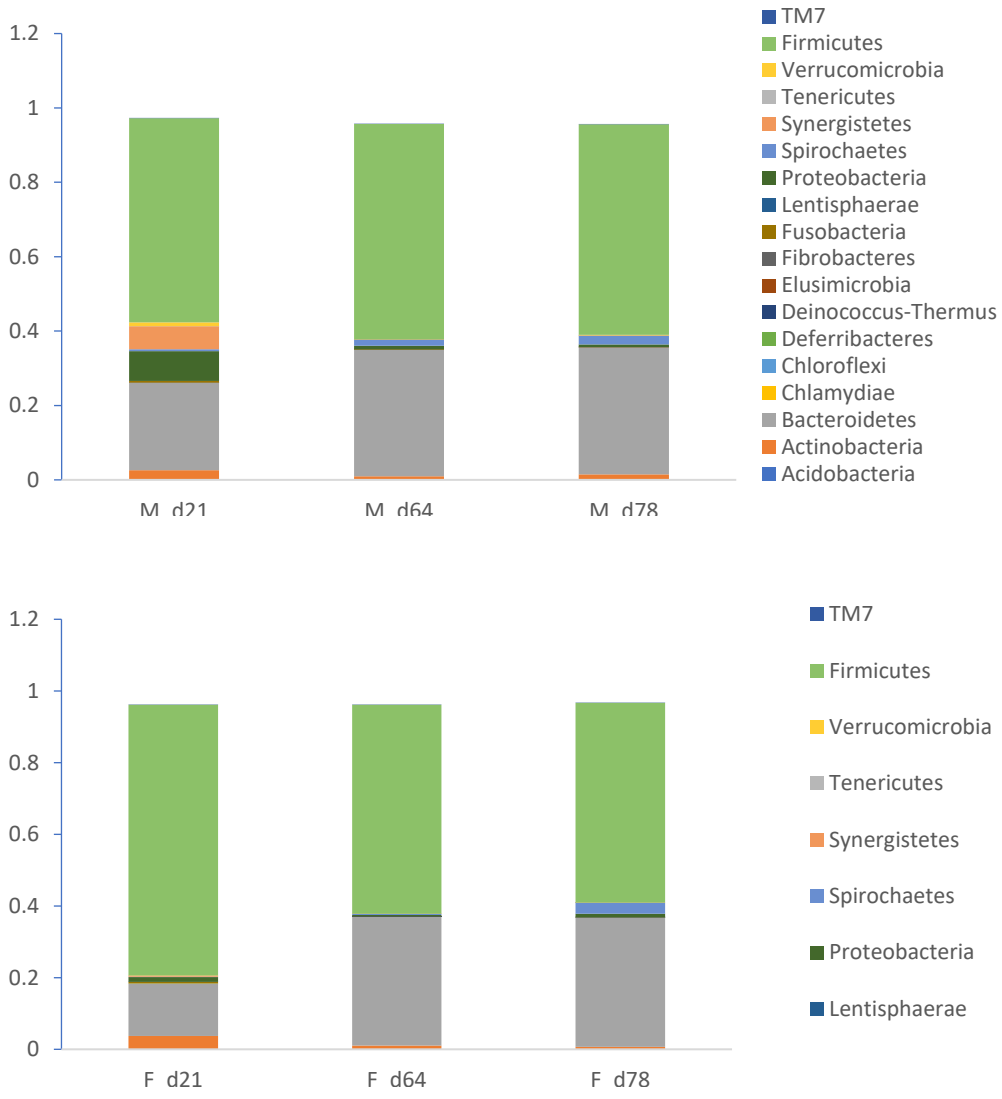

Figure 3.1: All phyla evolution within each treatment. M: Milk treatment, F: Formula treatment.

In term of richness, diversity of the gut microbiota significantly increased through time (based on observed OTUs and Chao 1 index). However, when taking into account of both evenness and richness (based on Shannon and Inverse Simpson index), we witnessed different patterns of the gut microbiota diversity evolution between mother-rearing piglets and isolated piglets. Specifically, in M treatment, Inversed Simpson index as well as Shannon index significantly

accrued in day 64 and day 78 compared with day 21 while F treatment only showed significantly increased in Shannon index, not in Inversed Simpson index. (Table 3.1)

Table 3.1. Comparison of diversity indices among time points. We used non-parametric Wilcoxon test to compare each pair (time point). Different letters indicate significantly different. Two treatments were tested independently. Invsimpson: Inversed Simpson; OTUs: observed OTUs. Richness measurement: OTUs and Chao 1 indices; Evenness and Richness measurement: Invsimpson and Shannon

Treatment	Time	OTUs	Invsimpson	Chao 1	Shannon
Milk	Day 21	235 a	14 a	323 a	3.3 a
	Day 64	441 b	23 b	583 b	4.1 b
	Day 78	511 c	27 b	691 c	4.3 b
Formula	Day 21	275 a	23a	359 a	3.7 a
	Day 64	440 b	24a	571 b	4.1 b
	Day 78	530 c	25a	714 c	4.2 b

To investigate the development of the gut microbiota per treatments, we calculated Bray-curtis distance based on genus level, then plotted using non-Metric Multidimensional Scaling (NMDS) to identify clusters of gut microbiota composition through time (Figure 3.2). Interestingly, the intestinal microbial community of breastfed piglets created two main clusters in which reflected gut microbiota at weaning (day 21) was significantly (ANOSIM tested, $p<0.05$) disparate from that after weaning (day 64 and day 78). On the other hand, formula-fed piglets gut microbiota composition clustered into three distinct areas on the plot which confirmed that the evolution of the gut microbiota was significantly different among 3 collected time points. The driving forces for the evolution (based on SIMPER test of Bray-curtis distance) of the gut microbiota after weaning (day 21) to day 64 appeared to be more evenly distributed in M pigs compared with F

pigs. Specifically, the first two contributors, *Prevotella* (17.7%) and *Lactobacillus* (10.85%), accounted for up to 28.55 % dissimilarity after weaning till 64 days old in F piglets while in M treatments, the major contributions were more dispersed with *Prevotella* (13.9%), *Ruminococcace* (6.9%) and *Lactobacillus* (6.9%). Only did F treatments express a significant difference between day 64 and day 78 with *Clostridium sensu stricto* (16.8%) and *Prevotella* (13.23%), *Lactobacillus* (11.52) as major factors affecting the evolution of the microbial composition.

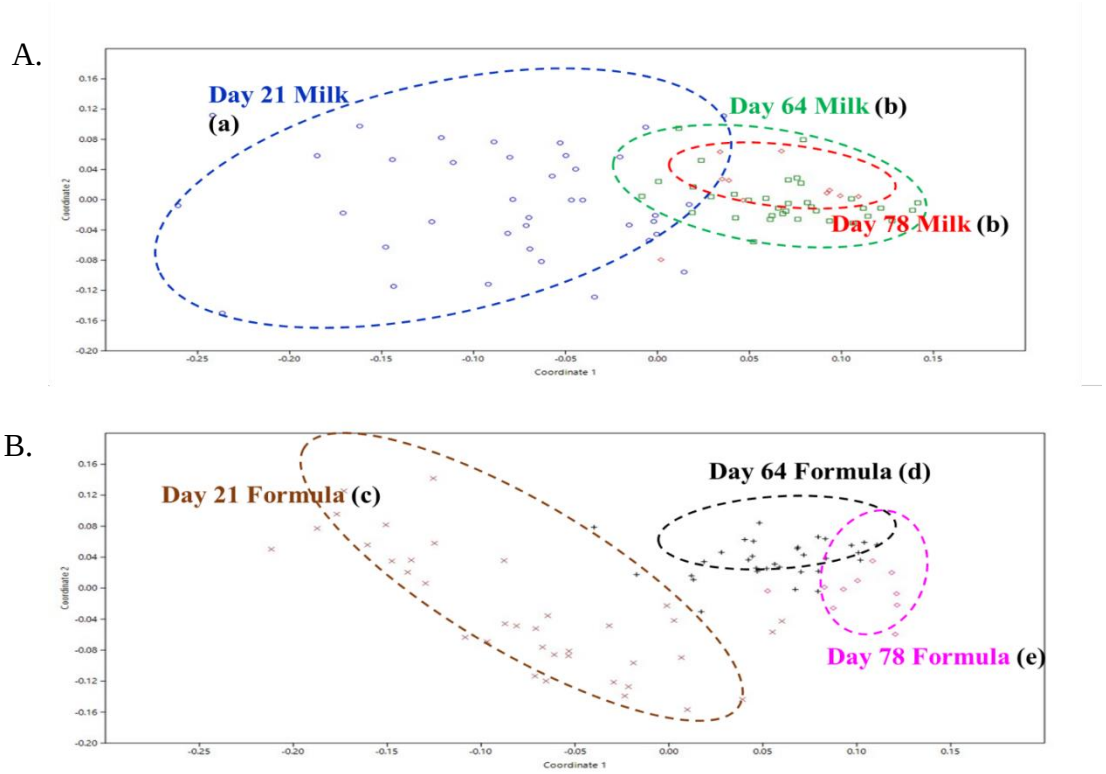

Figure 3.2: Evolution trend of the gut microbiota in pigs at day 21, day 64 and day 78. Non-Metric Multidimensional scaling (NMDS) plot based on Bray-Curtis distances calculated from genus-based relative abundance (outlining circle represented clusters of each phase for facilitating interpretation). A, Breastfed piglets. Blue round: Day 21 (weaning time); Green square: Day 64; Red diamond: Day 78. B, Formula-fed piglets. Brown cross: Day 21 (weaning time); Black plus: Day 64; Pink diamond: Day 78. Statistical differences among time points in each treatment were tested by One-way ANOSIM, corrected p-value via PAST3. We only tested separated time points within each treatment, not between treatments. Different letters indicate significant differences.

At genus level, the gut microbiota of both F and M piglets showed a relatively similar trend of evolution (Figure 3.3). Right after weaning (day 21), there were a lower level of *Prevotella*, unclassified Prevotellaceae and *Megashaera* and an enrichment of *Bacteroides*, unclassified *Ruminococcaceae*, *Cloacibacillus* and unclassified Enterobacteriaceae compared with pigs at 64 days old. Gut microbiota of piglets at the age of 78 days witnessed a substantial increase of other genera including *Clostridium XI* and *Clostridium sensu stricto* while still maintained abundance of enriched genus since day 64 (*Prevotella*, unclassified Prevotellaceae and *Megashaera)*. In addition, *Lactobacillus* was less enriched in day 78 compared with remaining time points. Remarkably, in F pigs, we witnessed a significant decrease of unclassified *Lachnospiraeae* in day 64 and a recovery, albeit lower than day 21, in day 78 which was not the case in M pigs with no significant differences through time.

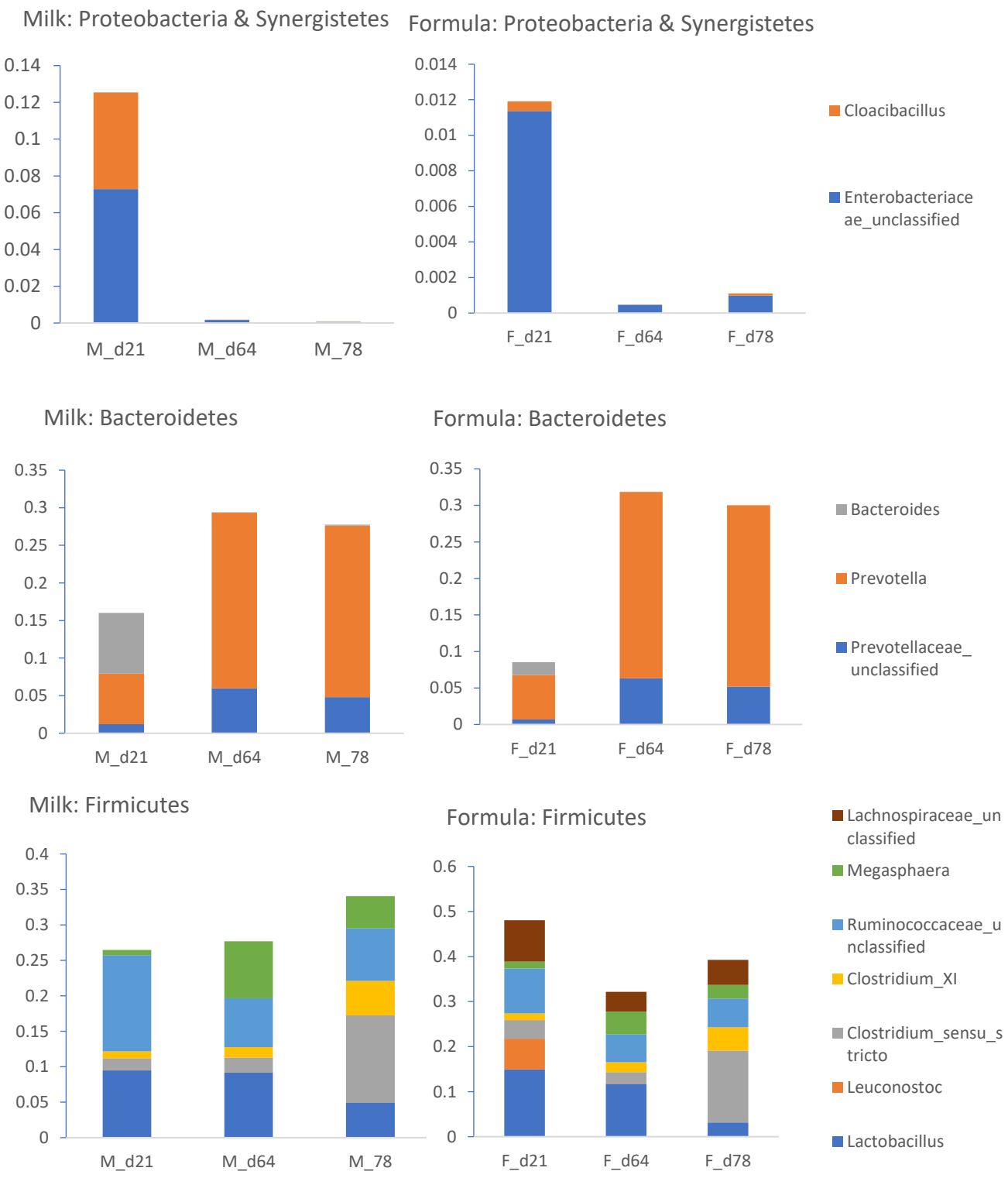

Figure 3.3. Evolution of the gut microbiota at genus level of the two most abundant phyla, Bacteroidetes and Firmicutes. Genus represented had relative abundance larger than 0.05 and significantly different at least at 1 time point during day 21 to day 78. Significant differences were tested using non-parametric Wilcoxon compared each pair (data not shown).

3.2 Feeding mode impacts the early gut microbiota composition

At weaning (day 21), F pigs gut microbiota composition was more diverse compared with M pigs, but this difference quickly disappeared at day 64 and day 78. All of the means of observed OTUs and diversity indices (Chao, Inverse Simpson, and Shannon) were higher in F treatment. On day 78, only Chao index which measured the richness was higher in F treatment. (Table 3.2)

Table 3.2: Mean of observed OTUs and diversity indices including Chao, Inversed Simpson and Shannon of two treatments in Day 21, Day 64 and Day 78.

Time	Treatments	OTUs	Chao	Invsimpson	Shannon
Day 21	Milk	235*	323*	15*	3.4*
	Formula	275	359	23	3.8
Day 64	Milk	441	583	23	4.1
	Formula	440	571	24	4.1
Day 78	Milk	511	691*	27	4.3
	Formula	530	714	25	4.3

(*) indicate significant differences between two treatments
Observed OTUs and diversity indices were calculated using Mothur program
Statistical differences were tested by non-parametric Wilcoxon test using Jmp11 software

At phylum level, the gut microbiota composition was significantly different between F and M piglets at weaning (day 21). Nonetheless, this trend was not maintained later in life (day 64 and day 78) (Figure 4.). The ratio of Firmicute: Bacteroidetes was essentially higher in piglets fed with formula. Notably, piglets that were reared with mother and fed with breast milk were dominant by Synergistetes and Proteobacteria while Actinobacteria enrichment occurred in F piglets. On day 64, Proteobacteria remained to be more abundant in M treatments even though

other phyla were similar in both treatments. At the end of the experiment, day 78, the intestinal microbial composition was entirely the same for F and M piglets (Figure 4.).

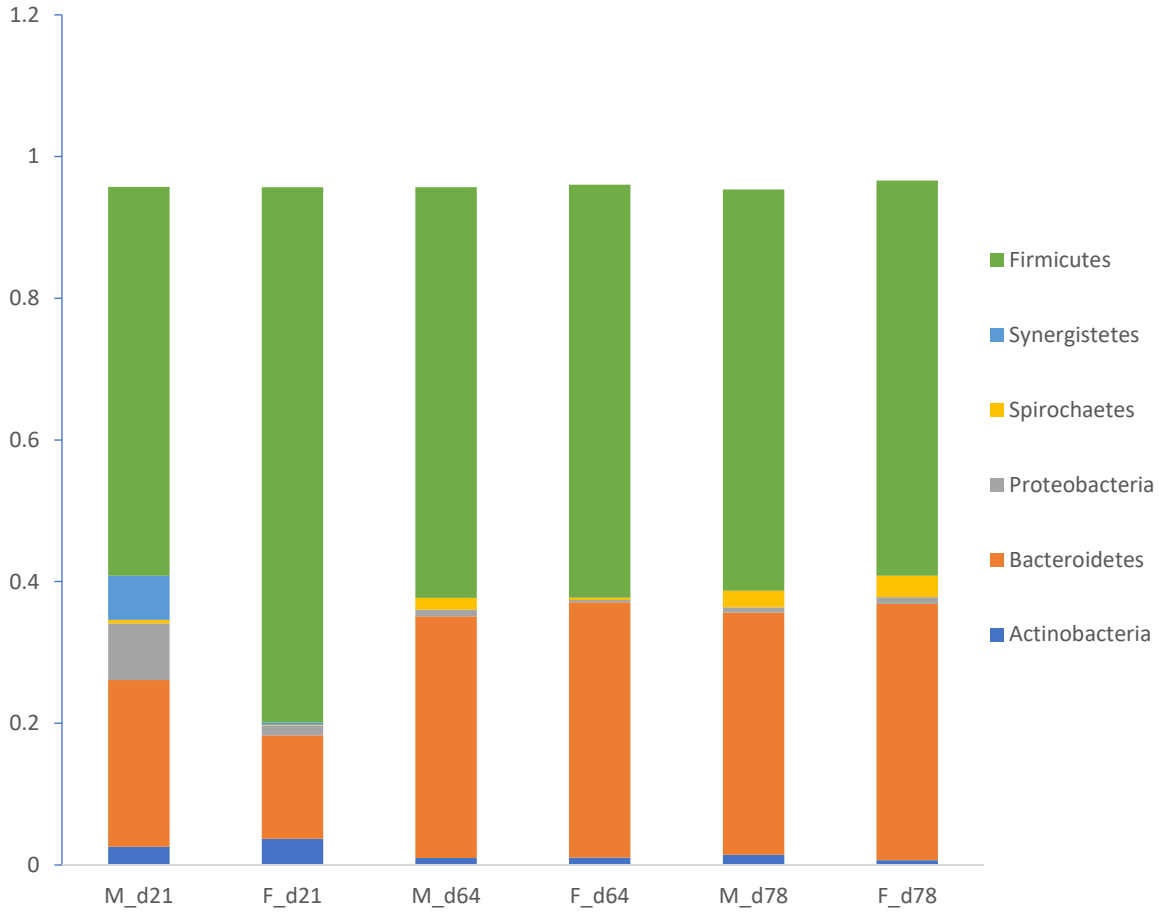

Figure 3.4. Gut microbiota dynamics in two treatments. Representative phyla which were most abundance in the gut microbiota of two treatments. Significantly different phyla: day 21, all phyla; day 64: Proteobacteria, Spirochaetes (non-metric Wilcoxon test performed by Jmp11 software). M: Milk; F: Fomular; d: Day.

Based on Bray-curtis dissimilarity at genus level of gut microbiota and statistically tested by one-way ANOSIM, there were two overlapped but distinct cluster on day 21 for two treatments ($P<0.05$). However, at day 64 and day 78, F and M piglets' microbiota composition clusters were not significant anymore (Figure 5.). SIMPER test showed that distinct clusters at weaning (day

21) were contributed by Lactobacillus (10.86%), Ruminococcaece (6.76%), and Prevotella (6.13%).

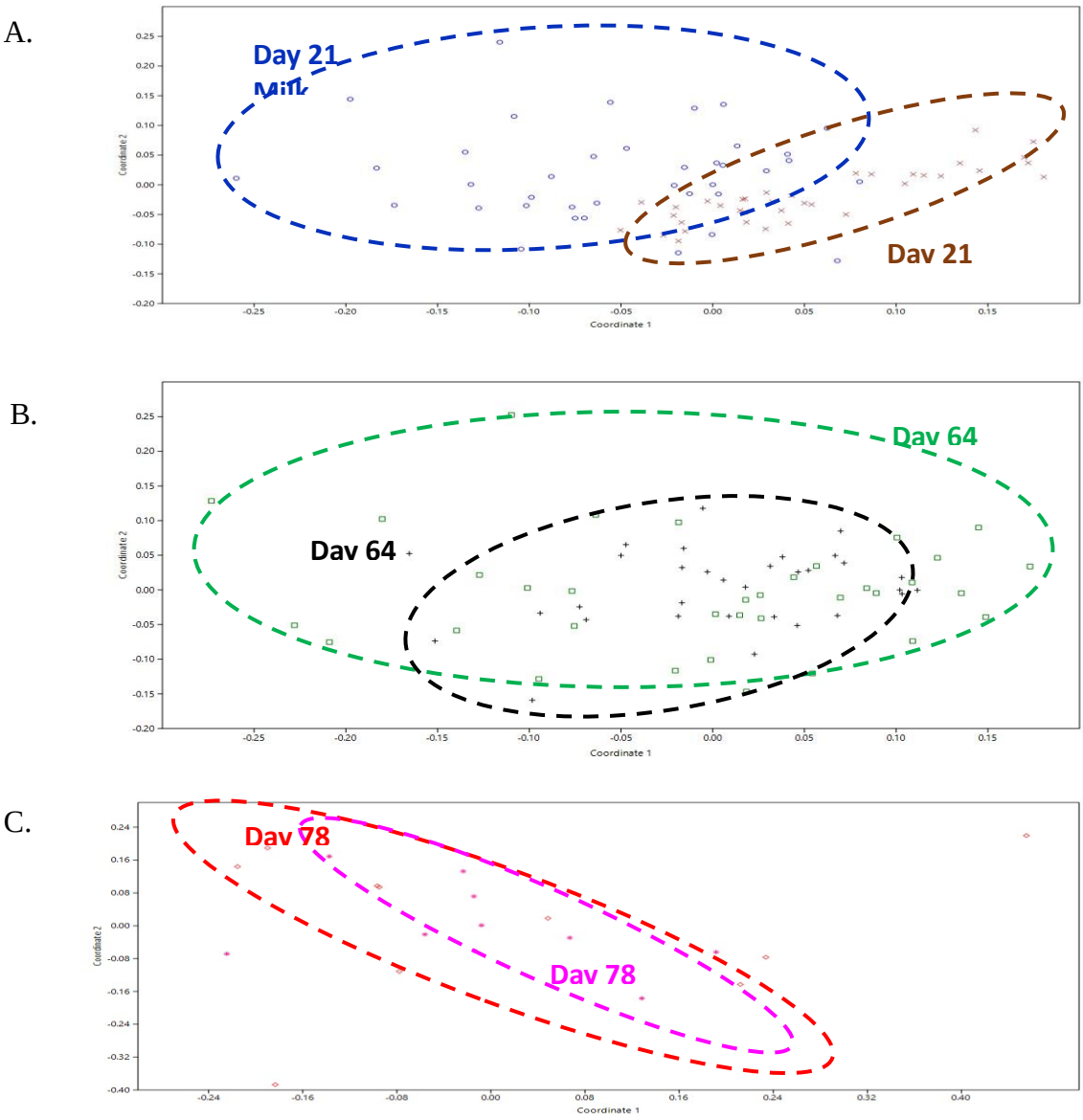

Figure 3.5: Comparison of gut microbiota composition between formula-fed and breastfed piglets. Non-Metric Multidimensional Scaling (NMDS) based on Bray-Curtis distance (Outlining circles indicate different treatments to facilitate interpretation). Significant differences were test by one-way ANOSIM. Blue round: Day 21 (weaning time); Green square: Day 64; Red diamond: Day 78.B, Brown cross: Day 21 (weaning time); Black plus: Day 64; Pink diamond: Day 78.

As reflected in the differences at phylum level mentioned earlier, significant differences in relative abundance at genus level between two treatments mostly occurred at the age of 21 days (Figure3.6 & Figure 3.7). Genera belonging to Firmicutes phylum were enriched in F treatment except for *Clostridium XIa, Succiniclasticum,* and *Eubacterium*. Relative abundance of *Clostridium sensu stricto* (0.07), *Streptococcus* (0.05) and *Mogibacterium* (0.05) were twice as abundant in F piglets as that in M piglets, namely 0.07 and 0.03, respectively. Moreover, *Blautia* and *Leuconostoc* of which relative abundance accounted for 0.09 and 0.12 of the gut microbiota were substantially dominant in F treatments while they were almost absent in M treatments (Figure 3.6). In contrast to this, genera in Bacteroidetes and Proteobacteria phyla were augmented in piglets fed by breastmilk compared with formula-fed pigs. For instance, *Bacteroides,* unclassified Enterobacteria, and *Desulfovibrio* were extremely higher in M treatments, namely 0.12, 0.07 and 0.006, respectively compared with that in F treatments, namely 0.03 and 0.002, respectively. In other phyla including Actinobacteria and *Synergistetes, Collinsella* was the dominant genus in F pigs while *Cloacibacillus* and *Pyramidobacter* had high relative abundance in M pigs.

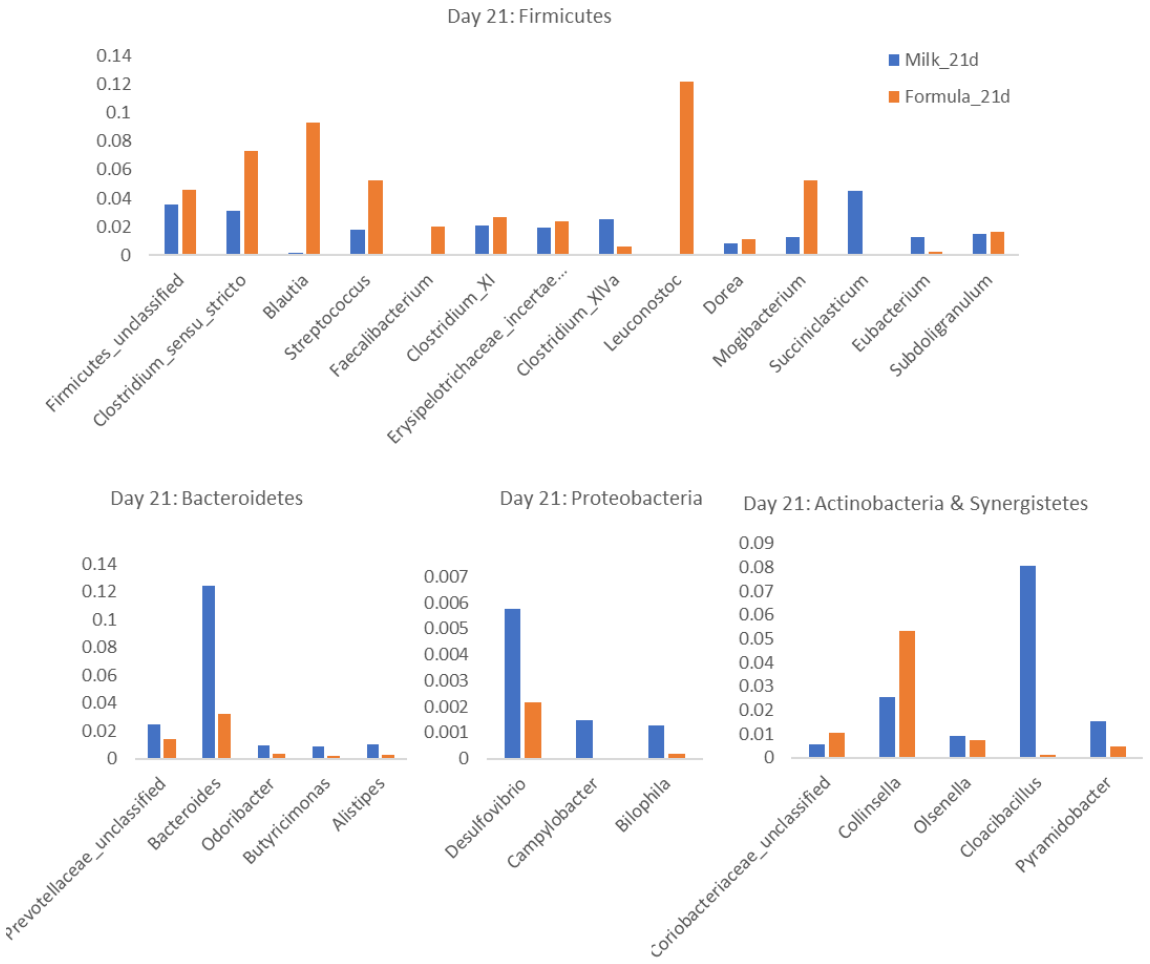

Figure 3.6: Genera with significantly different relative abundance (P-value <0.05) between two treatments at day 21. We compared two treatments using non-parametric Wilcoxon test.

The unique pattern of gut microbiota between two treatments faded through time. After weaning (day 64 and day 78), dominant genera that had significant differences in relative abundance essentially decrease leading to 11 genera in day 64 and only 2 genera in day 78 (Figure 3.7) *Streptococcus* and *Clostridium XI* dominance in F treatment to M treatment was still maintained from day 21 to day 64 but disappeared in day 78. Until day 64, unclassified Proteobacteria was enriched in M treatments. On day 78, only two genera were significantly different between two treatments, namely *Olsenella* and *Turicibacter*.

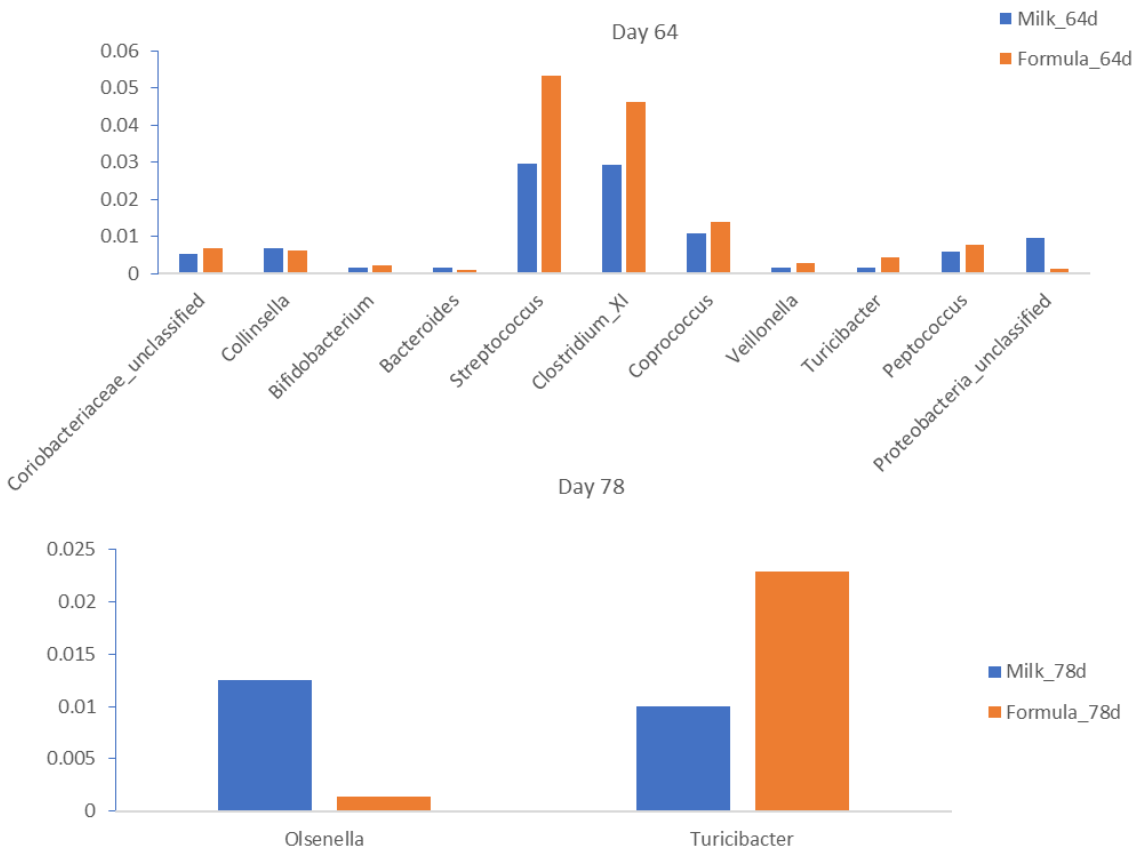

Figure 3.7: Genera with significant different relative abundance between two treatments at day 64 and day 78. We compared two treatments using non-parametric Wilcoxon test.

4. Discussion

In this study, we investigated the impacts of different feeding modes and environmental exposure in pre-weaning (4 to 21 days old) on the gut microbiota composition in piglets using 16s rRNA sequencing approach. By longitudinally sampling, we were able to examine not only the gut microbiota composition between two treatments but also the evolutional trend of the gut microbiota through time within each treatment.

In general, despite the significant differences in gut microbiota composition at day 21, both F and M piglets were concordant in the trend of gut microbiota development. For instance, the

diversity of all the piglets increased with age in which Firmicutes and Bacteroidetes to be the two most abundant phyla in the gut microbiota in consistenc with various studies with either pigs or human model (Yatsunenko et al., 2012; Backhed et al., 2015; Hill et al., 2017; Avershina et al., 2013; Niu et al., 2014). Moreover, the signature of early gut microbiota right at weaning (day 21) was the enrichment of Proteobacteria and Synergistetes at phylum level and unclassified-*Ruminococcaceae* at genus level in agreement with studies using pigs as a model for longitudinal investigate of dynamic distribution of the gut microbiota (Niu et al., 2014; Slifierz et al., 2015). The resemblance in the evolution trend of gut microbiota during time between two treatment suggests that feeding formula may not significantly disrupt overall development of the gut microbiota, yet impact the health of the host by modifying the composition of gut microbiota in early time.

In addition, we also witnessed an extremely higher relative abundance of *Prevotella* post-weaning (day 64 and day 78) compared with day 21 (from 0.06 in day 21 to 0.23 in day 78) which is consistent with other studies (Hu et al., 2016). This result corroborates that the transition of diet after weaning has a strong impact in modifying the gut microbiota since *Prevotella* was reported to link with a plant-rich diet which was introduced to the piglets later in life (Ley, 2016). Along with the increase of *Prevotella* was the decrease of Proteobacteria belonging genera such as unclassified Enterobacteriace following the growth of pigs. This can be explained by the competition of other short-chain fatty acid (SCFA) producing genera such as *Clostridium XI, Clostridium sensu stricto* and *Megasphaera* (Kaakoush et al., 2016; Slifierz et al., 2015). Especially, the enrichment of *Megasphaera* may exert beneficial effects in supporting host health since this genus had extensive functions in vitamin production, amino acid and SCFA (Slifierz et al., 2015).

Breastfeeding was reported to increase infant's infection resistance, lower risk of obesity and allergic disease (Tamburini et al., 2016). This may be done by shaping the gut microbiota in the direction that is beneficial for infants' health. When comparing breastfed infants and formula-fed infant at three months old, a study reported a significant different gut microbiota structure (Wang et al., 2015). In the current study, we leveraged swine models with anatomy and immune system more resemble to human compared with mice model (Hvistendahl, 2012) and designed a more controlled experimental set-up to test the impacts of different feeding mode and environmental exposure on gut microbiota composition postulated that feeding mode would modify the gut microbiota. Piglets from two treatments only experienced discrepancies in feeding mode (breast milk or formula) and growing condition (mother-rearing or isolated in nursery facility) from day 4 to day 21 of age. Then, they were grown in the same condition. The seventeen-day period was sufficient to alter the gut microbiota establishment in neonatal piglets. At weaning, the gut microbiota composition was significantly different between two treatments with F piglets having a higher diversity of microbial community compared with M piglets. A study in infants with closely similar set-up also reported that the gut microbiota in F treatment was more diverse than that in M treatment (Praveen et al., 2015).

The differences in the gut microbiota composition between two treatments were not maintained in a later phase of life (day 64 and day 78) which indicates the similar rearing environment and diet in the later life can shape a more similar gut microbiota regardless of initial treatment. However, gut microbiota composition in early time resulted from different feeding mode may be sufficient to create different responses in the immune system which affects the health status of the host. Previous studies confirmed the diet in early life affected intestinal microbial community

defining the immune response and the growth of the host (Yeruva et al., 2016; Davis et al., 2016; Rogier et al., 2014).

5. Conclusions

Feeding mode are imperative factors in shaping the initial gut microbiota composition. We observed a distinct patterns of gut microbiota only in early life of the piglets (day 21) with F treatment had higher diversity compared with M treatment. More studies on the immune response as well as functional microbiota during time with disease challenging should be carried out to delineate the mechanism of protective effects of breastmilk induced gut microbiota on disease risk.

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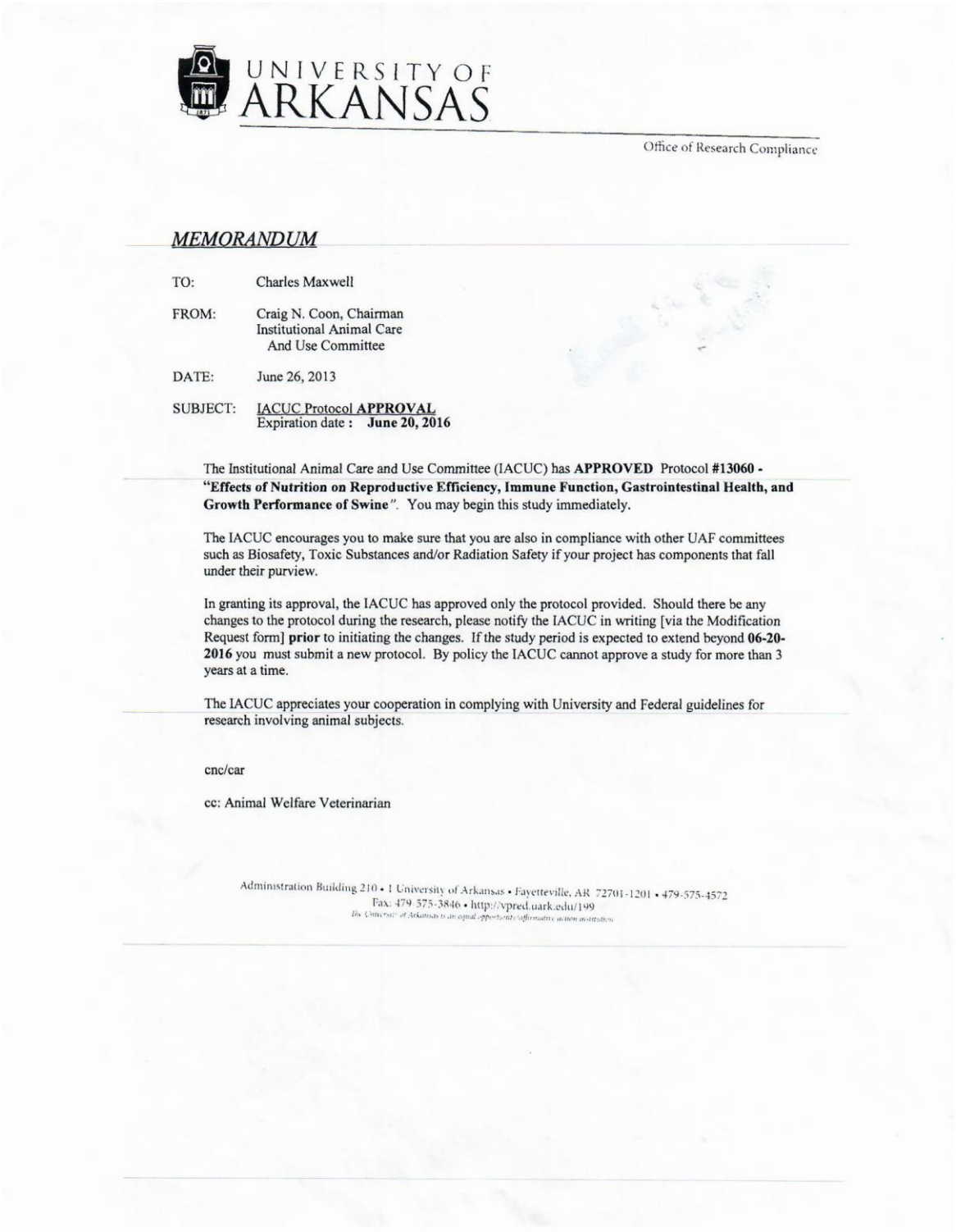
Conclusion

The establishment of the gut microbiota may initiate even before birth and proceed under the profound influences of delivery mode and external factors such as feeding mode. Our results indicated that feeding mode and short-term rural/farm simulation by soil exposure in early life could alter the gut microbiota composition which is in line with our hypothesis, even though this trend faded through time. Soil exposure enhanced the diversity of the gut microbiota and accelerate the maturation of the microbial community. On the other hand, despite the same trend in evolution, the gut microbiota composition of piglets with different feeding modes was still significantly different at weaning. More studies on the immune response as well as functional microbiota during time with disease challenging should be carried out to delineate the mechanism of protective effects of early-life environmental factors induced gut microbiota on disease risk.

Appendix

Animal Care and Use Committee (IACUC) Approval



UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Charles Maxwell

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: June 26, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **June 20, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13060 - "Effects of Nutrition on Reproductive Efficiency, Immune Function, Gastrointestinal Health, and Growth Performance of Swine"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **06-20-2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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