PROBIOTICS AND BOTANICAL SUPPLEMENTATIONS ON GUT MICROBIOTA OF WEANED PIGS UNDER DIARRHEAL STRESS

By

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ABSTRACT

Probiotics and botanical extracts are investigated as potential feed additives for weaned pigs to strengthen the intestinal function or overall immunity by improving nutrient digestibility and immunity in weaned pigs. Under proper manipulation, gut microbiota can additionally offer great physiological and immunological benefits to growing pigs, however, gut microbiota yet tends to be overlooked when assessing the viability of potential feed additives to weaned pigs under diarrheal stress. Therefore, three experiments were conducted to determine the impacts of various feed additives (*Bacillus* (*B.*) *subtilis*, *B. amyloliquefaciens*, and botanical blends) on gut microbiota of newly weaned pigs using a disease challenge model. The first study investigated modifications in fecal and intestinal microbiota of weaned pigs challenged with enterotoxigenic *Escherichia coli* (ETEC) and compared the gut microbiota when supplemented with *B. subtilis* DSM 25841 or carbadox, an antibiotic growth promoter. ETEC infection decreased ($P < 0.05$) microbial richness in feces on d 7 post-inoculation (PI) compared with d 0 before ETEC infection. Relative abundance of Firmicutes was decreased ($P < 0.05$) while Proteobacteria and *Enterobacteriaceae* were increased ($P < 0.05$) in fecal samples of pigs challenged with ETEC on d 7 PI than on d 0. Supplementation of carbadox reduced ($P < 0.05$) the relative abundance of *Lactobacillaceae* in feces on d 0 and 21 PI. *Lactobacillus* was more ($P < 0.05$) abundant on d 7 PI and *Lactobacillaceae* was greater ($P < 0.05$) in relative abundance on d 21 PI in feces from pigs supplemented with *B. subtilis* than with carbadox. Among intestinal segments, *Enterobacteriaceae* was more ($P < 0.05$) abundant in ileum than in jejunum and colon of weaned pigs on d 21 PI. Pigs supplemented with *B. subtilis* increased ($P < 0.05$) the relative abundance of gram-positive bacteria than pigs supplemented with carbadox, including *Lactobacillaceae* and *Bifidobacteriaceae* in ileal digesta on d 21 PI. Overall, the present study indicated that *B. subtilis*

supplementation modulated the fecal and intestinal microbiota differently from carbadox. The modulation of gut microbiota might contribute to the reduced diarrhea and enhanced intestinal health of ETEC infected pigs. The second study investigated the supplementation of *B. amyloliquefaciens* on growth performance, diarrhea, systemic immunity, and intestinal microbiota of weaned pigs infected with ETEC and compared the efficacy of *B. amyloliquefaciens* vs. carbadox on weaned pigs. ETEC infection reduced ($P < 0.05$) average daily gain (ADG) from d 0 and 21 PI, gain:feed from d 14 and 21 PI, increased ($P < 0.05$) frequency of diarrhea during the entire experiment and white blood cell and lymphocyte counts on d 7 and 21 PI when positive control was compared with negative control. *B. amyloliquefaciens* or carbadox supplementation did not affect diarrheal frequency throughout the experiment. However, carbadox supplementation enhanced ($P < 0.05$) ADG from d 0 to 21 PI than positive control. Pigs fed with carbadox had greater ($P < 0.05$) monocyte percentage in serum samples on d 7 and 21 PI but lower ($P < 0.05$) neutrophil percentage on d 21 PI than pigs fed with *B. amyloliquefaciens* under ETEC challenge. Pigs supplemented with *B. amyloliquefaciens* had greater ($P < 0.05$) Shannon index in ileal digesta than pigs fed with carbadox on d 21 PI. Positive control pigs had a greater ($P < 0.05$) abundance of Bacteroidota and Proteobacteria and lower ($P < 0.05$) Firmicutes in ileal digesta than negative control on d 21 PI. Carbadox addition increased ($P < 0.05$) the relative abundances of Firmicutes and *Clostridiaceae* but reduced ($P < 0.05$) *Bifidobacterium* in ileal digesta compared with *B. amyloliquefaciens* on d 21 PI. The results of the present study indicated that supplementation of *B. amyloliquefaciens* tended to increase ADG and had limited effects on diarrhea of ETEC infected pigs. However, pigs fed with *B. amyloliquefaciens* exhibit milder systemic inflammation than control. *B. amyloliquefaciens* differently modified intestinal microbiota of weaned pigs,

compared with carbadox. The third study characterized the impacts of two types of botanical blends on gut microbiota composition and serum and ileal mucosa metabolites in weaned pigs challenged with ETEC. Botanical blends (BB1 and BB2) were comprised of 0.3% capsicum oleoresin and 12% garlic oil extracted from different sources. Throughout the experiment, the relative abundance of *Lachnoclostridium* was decreased ($P < 0.05$) in feces as the age of pigs was increased from d -7 to d 21 PI. ETEC infection reduced ($P < 0.05$) the relative abundance of fecal *Faecalibacterium* and *Prevotella* on d 5 and 21 PI when positive control was compared with negative control. On d 21 PI, the relative abundance of Firmicutes and *Lactobacillaceae* were lower ($P < 0.05$) in feces of pigs fed with 100 ppm BB1 than pigs in positive control. Pigs supplemented with 100 ppm BB2 had a greater ($P < 0.05$) relative abundance of *Lachnospiraceae* in feces than pigs fed with 100 ppm BB1 on d 21 PI. Pigs in positive control had the highest ($P < 0.05$) Shannon and Chao1 indices in ileal digesta on d 5 PI. Pigs supplemented with 100 ppm BB1 had greater ($P < 0.05$) abundances of *Enterobacteriaceae* and *Escherichia-Shigella* in ileal digesta than pigs in positive control on d 5 PI. The relative abundance of *Prevotellaceae* was greater ($P < 0.05$) in cecum of pigs supplemented with 100 ppm BB1 than positive control pigs on d 5 PI. The metabolomics analysis revealed that ETEC infection downregulated pinitol, malic acid, and methionine on d 4 PI and upregulated methionine and guanosine on d 21 PI when positive control was compared with negative control. Supplementation of 100 ppm BB2 upregulated serum pinitol on d 5 PI and serum cholesterol and aminomalonic acids on d 21 PI compared with positive control. In ileal mucosa, asparagine was downregulated by 50 ppm BB2 in comparison to positive control. The present study indicated that supplementation of botanical blends modulated ileal microbiota and serum metabolomics profiles in weaned pigs under ETEC challenge. Overall, feed additives, including probiotics and

botanical blends modified gut microbiota of weaned pigs challenged with ETEC to different extent, which may potentially contribute to reduced diarrhea and enhanced intestinal integrity and health of newly weaned pigs when these feed additives were supplemented into the diet.

KEY WORDS: Botanical blends, Diarrhea, Enterotoxigenic *Escherichia coli*, Gut microbiota, Probiotics, Weaned pigs

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CHAPTER 1

LITERATURE REVIEW

A dynamic and diverse population of microbes are found extensively in the gut of pigs. As the relationship between humans and their gut microbiota is increasingly recognized as crucial to human health, understanding the importance of gut microbiota in livestock animals such as pigs cannot be overlooked. A healthy gut microbiota is especially crucial in early developmental phases in pig production: pre-weaning and post-weaning stages. Newly weaned pigs encounter many stress factors, which induce post-weaning diarrhea due to enterotoxigenic *Escherichia coli* infection. Post-weaning diarrhea results in a decrease in feed intake, induced growth retardation, and disrupted gut microbiota balance in piglets. Pigs undergoing post-weaning diarrhea can eventually lead to death, negatively impacting the profit of the swine industry. By further understanding the host-microbe interaction and pathogenic behavior of enterotoxigenic *E. coli*, dietary manipulation of pig gut microbes may improve pig performance by enhancing disease resistance and pig growth.

1.1. Gut microbiota in pigs

The gastrointestinal tract, the gut, is crucial for nutrient metabolism and immune regulation in pigs. However, microbes including bacteria, archaea, and eukarya, are present inside the gut and have been overlooked to understand the full functioning of the gut and the benefits for the host (Quigley, 2013). As various microbes assemble within the gut, they are collectively referred to as the gut microbiota (Sokol, 2019). Gut microbiota has been used interchangeably with the term “gut microbiome”; however, studies today define microbiota and microbiome separately. The definition of gut microbiome is more broad, taking into account the gut microbiota and the environment where the microbes reside, which includes their genomic

material and metabolites (Berg et al., 2020). Gut microbiota is also used interchangeably with the term gut microflora. However, microflora refers to plants and not microbes, which is why future studies are suggested not to use microflora as a term in microbe-related studies (Marchesi and Ravel, 2015).

1.1.1. Measuring gut microbiota

In human studies, gut microbiota was observed to be strongly associated with several diseases, including inflammatory bowel disease and diabetes (Khan et al., 2019; W.-Z. Li et al., 2020). Pigs have been commonly used as human models for biomedical research on gastrointestinal illnesses and microbial associated diseases (Rose et al., 2022). However, limited studies have investigated the gut microbiota of pigs raised for pork production, hence the universal standard for a healthy gut microbiome has not been fully understood. As they are becoming more affordable and accessible, culture-independent methods are common practices to quantify and identify bacterial communities in the gut of swine and other livestock species. Genetic material (DNA, mRNA) can be extracted from microbes present in gut content samples and sequenced using various platforms to analyze interactions between pigs and their gut microbes (National Academies of Sciences, 2018).

The sequencing of the 16S ribosomal RNA (16S rRNA) gene is one of the most practiced methods to identify the microbial composition in the gut of pigs. Samples can be sourced from different sites in the intestine for bacterial DNA extraction. Extracted DNA samples are amplified using universal PCR primers to target hypervariable regions of the 16S rRNA gene, as they are ubiquitous among bacteria and can provide enough genetic material to identify and quantify bacteria within samples based on the DNA sequence within a hypervariable region. The 16S rRNA genes contain nine hypervariable regions with V4 region commonly observed to

characterize human gut microbiota with approximately 254 base pairs (Liu et al., 2020). Barcode sequences for each sample can be attached to the universal PCR primers to allow amplified DNA samples to be pooled within one lane for sequencing (Pjevac et al., 2021). This greatly cuts the time and cost for microbial community analysis in an animal experiment. Bioinformatics tools and pipelines are accessible at no cost to pre-process sequenced reads and classify the taxonomy of the resulting bacterial sequences.

The use of 16S rRNA amplicon sequencing has been greatly applied to characterize the gut microbiota in pigs. Longitudinal studies were also performed to address how dietary shifts influence the microbial composition of the fecal microbiota of pigs throughout their life stages (Frese et al., 2015, Slifierz et al., 2015). The microbial community along the length of the intestinal tract within pigs including the small and large intestines has also been characterized (Crespo-Piazuelo et al., 2018, Holman et al., 2017). With many more studies than these using 16S rRNA sequencing in pigs, it is feasible to characterize and quantify the relative gut microbial populations across projects. Sufficient collection of gut microbiota results being publicly available allows future studies to provide meta-analyses and execute machine learning. Meta-analyses in human gut microbiota have been performed globally, observing similar patterns in shifts in microbiota when humans were in a disease state and/or difference ages (Lozupone et al., 2013). Core gut microbiota, or commonly observed bacteria, was also characterized in swine with meta-analysis of 16S rRNA sequence data, which allows more research targeting specific bacteria to investigate their significance on the gut and the host (Holman et al., 2017). In Maltecca et al. (2019), machine learning was performed on multiple 16S rRNA sequencing datasets and showed that microbiome composition can be used as a predictor of swine growth and carcass traits such as fatness. Although limited studies have performed machine learning on

pigs in regards to gut microbiota, Morota et al. (2018) predicted that machine learning would be applied more in animal agriculture, merging previous studies into one large dataset to find more significance while removing data bias.

Shortcomings are also observed when using 16S rRNA amplicon sequencing to characterize the microbiota in any sample, including swine. Only amplifying a short region of the 16S rRNA gene limits microbial identification down to the genus level at best (Mignard and Flandrois, 2006). A common alternative to 16S rRNA sequencing is shotgun sequencing, which examines all genomic DNA in a sample and allows the identification of more taxa at the species level. However, shotgun sequencing is also more costly because the whole genome needs to be sequenced per sample and fewer samples can be pooled per lane for sequencing (Durazzi et al., 2021). Shotgun metagenomics can also provide functional profiling of a microbial community (Silva et al., 2016). However, bioinformatics tools can be utilized in 16S rRNA sequenced data to generate predicted data for functional profiling of a microbial community when running shotgun metagenomics is not possible (Douglas et al., 2020; Wemheuer et al., 2020).

1.1.2. Host-microbe interaction in swine gut

Newly born piglets are sterile before birth but gut microbial colonization is first observed within 24 hours after birth (Kenworthy and Crabb, 1963). Similar to human infants who have intestinal microbiota reflecting their mother's vaginal microbiota, piglets are likely to have acquired microbes through vaginal delivery (Dominguez-Bello et al., 2010; Perez-Muñoz et al., 2017). However, fecal microbiota of piglets shift away from their initial microbiota one day after birth, signifying that multiple environmental factors can greatly alter their gut microbiota (Kubasova et al., 2017; Lührmann et al., 2021). Throughout the pre-weaning and post-weaning stages of pigs, Firmicutes and Bacteroidetes are observed as the two primarily abundant phyla in

the fecal microbiota of piglets, accounting for more than 90% of the microbial population in the gut (Holman et al., 2017). But for these gut microbes to provide a beneficial role to pig health, symbiotic relationship between microbes and the pig (host) becomes more crucial after pigs are weaned from their sows because weaned pigs can no longer depend on sow milk for further nutritional needs and intestinal maturation.

1.1.2.1. Importance of intestinal structure to gut microbes

Microbes can enhance pig health only if the pig provides a favorable environment for the microbes to survive. Gut microbes are present at the highest density in the colon followed by the lower segment of the small intestine (O'Hara and Shanahan, 2006). The proximal section of the small intestine is more acidic from gastric acid secretions from the stomach and bile acid secreted into the small intestine which reduces the survivability rate of microbes. The small intestine also has a high level of oxygen, enabling only facultative anaerobes to survive (Donaldson et al., 2016). The colon, or large intestine, has been shown to have a much lower level of oxygen and maintains pH at approximately 7, which is preferable for facultative anaerobes and obligative anaerobes to reside (Evans et al., 1988; Zheng et al., 2015). Oxygen level in the large intestine is also reduced due to the demand for oxygen by facultative bacteria and enterocytes for nutrient metabolism (Ward et al., 2014; Donaldson et al., 2016).

The unique structure of the small and large intestines and large surface area makes the gut the most predominate site for microbes to colonize within the host. In the intestines, the luminal layer is observed to be greatly colonized by microbes for its availability of digesta for fermentation. The three mucosal layers (epithelium, lamina propria, and muscularis mucosae) and the muscularis externa play a major role in digesting and absorbing nutrients but also interplay with the gut microbes to accommodate intestinal homeostasis.

The epithelial layer or the epithelium is located on the surface of the intestinal villi. Villi are comprised of specialized epithelial cells, including enterocytes that are absorptive cells making up at least 80% of the epithelial cells and secretory cells such as goblet cells and Paneth cells (Snoeck et al., 2005). Tight junctions connect adjacent epithelial cells, preventing microbes from crossing the epithelium and controlling diffusion and electrolytes translocation (Itoh and Bissell, 2003). The intestinal epithelial cells are shielded by a mucus layer, which also help repel luminal microbes from directly interacting with the intestinal epithelial cells. The single layer of mucus has minimal microbial growth and contains mixtures of antibacterial mediators secreted by Paneth cells and digestive enzymes secreted by the epithelial cells (Schoenborn et al., 2019; Herath et al., 2020). Unlike the small intestine, the large intestine has two mucus layers, in which microbes colonize in the outer mucus layer but not the inner mucus layer that is close to the epithelial cells (Herath et al., 2020). Goblet cells are responsible for mucus secretion and the fast turnover rate of mucin in the inner mucus layer minimizes microbial colonization on the villi (Johansson, 2012). However, mucus-degrading bacteria can break down mucin to monosaccharides, supplying substrates to bacteria within the vicinity for further microbial metabolism (Herath et al., 2020). Resident microbes ferment undigested feedstuff and produce short-chain fatty acids (SCFA), which can increase luminal acidity (Rowland et al., 2018). Enterocytes located in the large intestine can effectively absorb SCFA and secrete bicarbonate to reduce luminal acidity, maintaining intestinal health and the balance of gut microbes (Akiba et al., 2001; Holzer, 2015). Thus, intestinal epithelial cells are crucial to flush out these byproducts before killing these microbes. Major SCFAs (acetate, butyrate, and propionate) are also metabolized via beta oxidation as fatty acids and further catabolized into energy by enterocytes to maintain their functionality (Cummings et al., 2004). Between the villi, the crypt is comprised

of transit-amplifying cells, progenitor cells, that can differentiate into enterocytes or secretory cells on the villi. The constant cell development from the crypt and the cell renewal of the villi will ensure normal intestinal functionality, as well as, the balance of intestinal microbes (Nigro and Sansonetti, 2015).

The lamina propria (LP) is a layer of connective tissue underneath the intestinal epithelium and above the muscularis mucosae (Okumura and Takeda, 2017). It extends inside the villi and contains complex capillary networks and a lacteal per villus for nutrient absorption (Shroyer and Kocoshis, 2011). The LP is also rich in immune cells and nerve endings (Boudry et al., 2004). In particular, B and T immune cells reside underneath the epithelium to allow immediate immune responses (Shi et al., 2017). Peyer's patches are lymphoid structures that also house immune cells, however, Peyer's patches are present in the small intestine and not the large intestine for mucosal immunity (McDermott and Huffnagle, 2014). Macrophages also emerge from the LP and are responsible for eliminating apoptotic epithelial cells (Bain and Schridde, 2018). Local immune cells are responsible for eradicating intestinal pathogens, however, hyporesponsiveness or increased anti-inflammatory functions of the host immune system can be observed under the presence of normal gut microbiota to prevent themselves from being eradicated. For example, macrophages are responsible for promoting systemic inflammation and secreting toxic cytokines and chemokines for stimulating further immune response (Rezania, 2018). However, an *in vitro* study observed macrophages that emerge from the LP did not respond to Toll-like receptor ligands on various microbes to produce pro-inflammatory cytokines and macrophages also did not promote inflammatory response by down-regulating adapter proteins that would facilitate NF-κB signaling (Smythies et al., 2010). This prevents the mucosal immunity from LP from constantly inducing an immune response to luminal bacteria and food

antigens. A normal microbiota also regulates inflammatory responses by controlling chemokine receptor CX_3CR1-expressing intestinal mononuclear phagocytes, which restrain pro-inflammatory helper T cells and promote anti-inflammatory regulatory T cells (Kim et al., 2018). Although the mechanism is not well understood, host immune cells are selectively eliminating commensal microbes, presuming to maintain intestinal homeostasis and prevent microbial overgrowth in the intestines. In Morikawa et al. (2016), phagocytes emerging from the LP and Peyer's patches engulf *Lactobacillus murinus* more frequently than *Lactobacillus taiwanensis* despite both microbes being indigenous residents in the small intestine of mice. Although complex, adaptation of the host intestinal immune system to the gut microbiome contributes to the stabilization of host mucosal health.

The muscularis externa is located outside of muscularis mucosae with submucosa between the two muscular layers. The muscular layers consist of various smooth muscle cells, playing an important role in muscle contraction. Muscularis mucosae contracts the smooth muscles that surround intestinal tissue and assists in the secretion of epithelial cells, such as goblet cells, for mucin secretion (Percy et al., 2003). The muscularis externa is responsible for gut mobility by inducing peristalsis using inner circular and outer longitudinal layers of smooth muscles (Patel and Thavamani, 2022). Gut motility is notably known to guide bolus through the gastrointestinal tract, but gut motility is also crucial for regulating the gut microbiota. It allows the host to introduce undigested feedstuffs to the microbes and to excrete microbial byproducts that were not utilized by the host. Peristalsis induces propulsive forces to minimize microbial overgrowth in the small intestine by flushing out bacteria (Gunnarsdottir et al., 2003; Mirbagheri and Fu, 2017). Bacterial overgrowth in the small intestine is undesirable as it can hinder host nutrient absorption and increase the risk of bacterial pathogenicity (Dukowicz et al., 2007).

However, recirculation flow is generated near the muscular contraction from peristalsis, allowing some microbes to remain colonized in the intestines (Cremer et al., 2016).

1.1.2.2. Dietary impacts on gut microbiota

Diet is a major factor that can reshape the gut microbiota of pigs from birth. Milk constituents and perhaps microbes introduced during the ingestion of sow milk (i.e., teats and environment) could contribute to the gut microbiota of pre-weaning pigs, and a shift in microbiota was observed throughout the pre-weaning phase of the piglets (Frese et al., 2015; Chen et al., 2018; Zhang et al., 2018). Longitudinal studies of fecal microbiota in pigs observed great shift between pre-weaning and post-weaning stages, likely due to the major dietary changes from liquid sow milk to a solid plant-based diet during the weaning process (Frese et al., 2015; Slifierz et al., 2015). For example, the relative abundance of *Bacteroidaceae* and *Clostridiaceae* were reduced in the gut microbiota of pigs, but the relative abundance of *Prevotellaceae* and *Ruminococcaceae* was increased after weaning (Guevarra et al., 2019).

Although research about sow milk on the gut microbiota of pre-weaning pigs is limited, the impacts of maternal milk composition , especially milk oligosaccharides, on gut microbiota in infants have been examined in numerous human studies. Milk oligosaccharides are indigestible and complex carbohydrates that constitute the third most abundant nutrient in human milk and human and sow milk have shown similarity in the composition of oligosaccharides (Lönnerdal and Hernell, 2016; Salcedo et al., 2016). Milk oligosaccharides cannot be digested by the host but can be utilized by gut bacteria, in particular *Bifidobacterium* and *Bacteroides* (Marcobal et al., 2010). The presence of milk oligosaccharides can increase the abundance of *Bifidobacterium* in the piglet gut, thus, facilitating the digestibility of other milk carbohydrates and the release of SCFA (Taft et al., 2018; Lawson et al., 2020). Lysozyme, an antimicrobial

enzyme found in milk, is another example of a milk constituent that could shift the gut microbiota of young pigs. In Maga et al. (2012), dietary supplementation of six-week old pigs with lysozyme-rich milk was shown to reduce Firmicutes abundance, while increasing the abundance of Bacteroidetes, *Bifidobacteriaceae* and *Lactobacillaceae.*

Upon withdrawal of sow milk, post-weaning pigs are provided with dry and plant-based diets, mostly based on corn and soybean meal. Gut microbes in the lumen attempt to adapt to the fluctuating nutrient availability. Gut microbes are challenged by the complex carbohydrates that host enzymes cannot digest (Turnbaugh et al., 2010). *Bifidobacteria*, again, has been shown to not only efficiently metabolize milk glycans but also break down plant glycans using their microbial enzymes such as carbohydrate active enzymes and carbohydrate esterases (Kelly et al., 2021). According to a metagenomics study on fecal samples from pigs, starch was mainly fermented by Firmicutes and Bacteroidetes, but fructans were fermented by microbial enzymes secreted by Bacteroidetes and *Lactobacillus* (Wang et al., 2019b). Protein also contributes to host-microbe metabolism. Protein sources for microbes are present endogenously and exogenously in the gut and some proteins are fermented in the small intestine (Dai et al., 2010). Undigested dietary protein and internally secreted mucins are fermented by microbes in the distal large intestine after carbohydrates are depleted in the proximal large intestine (Pieper et al., 2016). Protein is an energy source to bacteria, including Bacteroidetes, *Clostridium, Bifidobacterium,* and *Enterococcus*. These microbes can produce branched-chain fatty acids when fermenting branched-chain amino acids (Pugin et al., 2017; Diether and Willing, 2019). Branched-chain fatty acids are generally known indicators of microbial protein fermentation and aid in increasing villous height in the duodenum and increasing levels of immunoglobulins in the jejunum and ileum of weaned pigs, imposing benefits by fortifying immune health (Ren et al.,

2015; Pieper et al., 2016). Fat is another important feed ingredient to fulfill the energy requirement in pigs. However, limited studies have observed the peripheral effects of dietary fat on the gut microbiota in pigs. A high fat diet has been shown to reduce microbial diversity and decrease SCFA availability according to an *in-vitro* study (Agans et al., 2018). The exact mechanism is not clear, but the altered secretion of bile acid by a high fat diet might be the potential reason for indirectly impacting gut microbiota (Yokota et al., 2012; Zheng et al., 2017).

1.1.2.3. Colonization resistance

The gastrointestinal tract provides a dynamic environment for a diverse community of bacteria, allowing both opportunistic and pathogenic microbes to inhabit. The host can directly regulate the microbial population using chemical defense mechanisms, such as hydrochloric acid in the stomach, and a physical barrier in the intestines (Waterman and Small, 1998; Odenwald and Turner, 2017). However, the host itself cannot fully prevent gut pathogenicity.

Gut microbiota influences a healthy gut. This has been greatly investigated in humans, in which a healthy gut is characterized to have effective digestion and feed absorption, absence of gut illnesses, and a functioning immune system (Bischoff, 2011). In pigs, a healthy gut is characterized similarly to humans, but also highlights that a healthy gut can quickly adapt with the host to sudden changes or stresses, especially when pigs are weaned (Pluske et al., 2018; Xiong et al., 2019). The presence of microbes is critical for intestinal development, nutrient digestibility, and growth of newborn piglets (Zhou et al., 2021). For example, SCFA can play an important role in promoting the health of pigs. Supplementation of butyrate promoted anti-inflammatory cytokines in weaned pigs under experimentally induced colitis and improved growth performance and increased intestinal villus height for improved nutrient absorption in healthy weaned pigs (Lu et al., 2008; Han et al., 2020). Large quantities of butyrate can be

detected in the large intestine due to acetate being utilized by bacteria that produce butyrate, such as *Roseburia* spp. (Duncan et al., 2004). In addition, gut microbes contribute to the inhibition of the growth of pathogenic microbes by directly preventing their habitation or indirectly triggering intestinal immunity. The common term used to define gut microbes preventing pathogenic overgrowth is called 'colonization resistance'.

The direct mechanisms of colonization resistance include (1) competing for nutrients and space and (2) secreting microbial metabolites that can directly harm pathogens. Freter et al. (1983) hypothesized the nutrient niche theory, asserting that nutrients are limiting factors to allow certain microbes to colonize in the intestine. Proline, for example, is an important nutrient for bacterial growth. Commensal *E. coli* has been shown to outcompete proline resources against pathogenic *E. coli* in mice intestines (Momose et al., 2008). Diets can greatly affect nutritional availability for colonization resistance. Carbohydrates are one of the main drivers in modulating the gut microbiota and the wide range of structural complexity causes high variability in the gut microbiota. *Clostridium difficile* was observed to proliferate much slower when competing for carbohydrate sources against microbes in a pre-established cecal microbiota of mice on culture plates (Wilson and Perini, 1988). Sorghum arabinoxylan, a complex polysaccharide, has been shown to sustain a diverse microbial consortium in comparison to the low-complex oligosaccharide inulin in a sequential batch fermentation *in vitro* (Yao et al., 2020). Aside from nutrients, commensal *Enterobacteriaceae* and spore-forming bacteria in neonatal chicks compete for oxygen against *Salmonella* enteritidis which can induce intestinal inflammation (Litvak et al., 2019).

Microbial metabolites promote microbe-to-microbe interaction in colonization resistance. For example, SCFAs can directly reduce pathogenic colonization. Propionate has been shown to

disrupt intracellular pH homeostasis and prevent *Salmonella* growth *in vitro* (Jacobson et al., 2018). Butyrate has also been shown to down-regulate genes associated with intestinal pathogenicity in *Salmonella* in an *in vitro* study (Gantois et al., 2006). Gut microbes can also utilize metabolites as signaling peptides to coordinate neighboring microbes to eradicate pathogens. *Lactobacillus* spp. has been shown to release bacteriocins in the presence of acetate, killing *Staphylococcus aureus* (Meng et al., 2021). Plant-expressed colicins released by *E. coli* can rapidly disrupt their genetic information *in vitro* and immobilize foodborne pathogenic *E. coli* (Cascales et al., 2007; Schulz et al., 2015). In addition, bile acids secreted by the host liver can be further derived by intestinal microbes into secondary bile acids, which have been shown to reduce the virulence activity of pathogenic *Clostridium difficile* by disrupting their life cycle (Winston and Theriot, 2016).

An indirect mechanism of colonization resistance is also present and executed by luminal bacteria stimulating mucosal immunity or the intestinal barrier. Secretory IgA (sIgA) resides in the mucosal layer of the intestines and can block the colonization receptors expressed on pathogens (Mantis et al., 2011; Rogier et al., 2014). Pattern recognition receptors such as toll-like receptors (TLRs) are crucial to induce the innate immune response such as nuclear factor-κB (NF-κB) to produce inflammatory chemokines (Miura et al., 2017). Commensal microbes such as *Bacteroidetes fragilis* can activate TLR2 on $CD4^+$ T cells to promote *Bacteroidetes fragilis* to colonize the intestine without being eliminated by T helper cells (Round et al., 2011). Propionate and butyrate altered gene expression in neutrophils to promote migration towards inflammatory sites (Vinolo et al., 2009). Feed additives, such as probiotics, are supplemented to animal feed to target the indirect mechanism of colonization resistance. For example, supplementation of

probiotic *Limosilactobacillus reuteri* R2LC triggered B cells in Peyer's patches, increasing IgA production to prevent inflammation induced by colitis (Liu et al., 2021).

1.2. Post-weaning diarrhea

Between 21 and 28 days of age, pigs are weaned from their mother in the swine industry in the U.S. and experience multiple events that influence their health (Varley and Wiseman, 2001). Newly weaned pigs undergo sudden changes in diet, social separation from their mother and possibly their littermates, and housing environment. With all these events happening simultaneously, weaning becomes one of the most stressful stages for pigs in the swine industry. Stress can reduce feed intake, leading to increased vulnerability to pathogens and causing microbial unbalance in the gut. Newly weaned pigs have been shown to have poor feed intake, deteriorating health, and increased diarrhea severity. Therefore, strategies must be employed to overcome these challenges.

1.2.1. Weaning stress

Intestinal development can be initially observed when newborn pigs are fed with colostrum (Zhang et al., 1998). Piglets provided with colostrum were observed to have a significant increase in mucosal DNA content in the large intestine and an increase in digestive enzymes, including lactase and alkaline phosphatase activities, compared to piglets that were not provided with colostrum (Wang and Xu, 1996). Suckling pigs also absorb immunoglobulin G from sow colostrum to facilitate their intestinal immune development and disease resistance (Jensen et al., 2001). In addition, digestive functions within the intestines change over time within suckling pigs, which indicates a sign of intestinal maturation (Zhang et al., 1997). Intestinal maturation is a gradual process, however, and the sudden changes in diet and

environment during weaning significantly challenge weaned pigs, thus leading to disrupted intestinal development.

Actions taken during weaning predispose pigs to a high amount of stress which can initiate a cascading effect on their health. Weaning may necessitate transporting pigs away from their mother to a nursery room or to a new facility. During transportation, pigs can experience sudden temperature fluctuations, hear unfamiliar sounds from vehicles, and overcrowding (Roldan-Santiago et al., 2013). Cortisol, a stress hormone, was observed to increase in newly weaned pigs undergoing transportation (Cooper et al., 2009). Upon arrival to their new environment, weaned pigs are fed with plant-based ingredients, such as corn and soybean meal, instead of highly digestible sow milk. Research reports that the digestibility of fat in sow milk is 17% greater than that of fat from solid feed for weaned pigs, and fat in solid diets is less able to be emulsified than in sow milk (Zheng et al., 2021). Although soybean meal is a high-quality protein ingredient, the presence of trypsin inhibitors can reduce the digestibility of amino acids and other anti-nutritional factors in soybean meal may cause diarrhea in newly weaned pigs (Herkelman et al., 1992; Stein et al., 2013). With the changes in environment and diet, reduced feed intake is commonly observed in newly weaned pigs.

Under weaning stress, the intake of metabolizable energy from the dry diet of weaned pigs was observed to be up to 40% less than in pre-weaning milk (Sève, 2000). The reduced energy intake has substantial impacts on intestinal morphology, as observed by shorter villous height and induction of villous atrophy (van Beers-Schreurs et al., 1998; Marion et al., 2002). The disturbance in intestinal morphology leads to reduced activities of digestive enzymes, further decreasing the digestibility of other nutrients, such as proteins (Miller et al., 1986; Sun et al., 2009). Recovery of intestinal morphology and enzymatic activities may take more than one-

week post-weaning in pigs (Hedemann et al., 2003). Weaning stress also induces changes in gut lining permeability due to the release of corticotrophin-releasing factors (Moeser et al., 2017). Disruptions in goblet cells and reduced mucin secretion are also observed in weaned pigs, which allows microbes to directly interact with the intestinal epithelial cells, increasing the susceptibility to enteric infection (Madara and Trier, 1982; Larauche et al., 2009; (Hollander and Kaunitz, 2020). For instance, research reported that weaning stress led to an increased population of opportunistic pathogens including *Camplylobacteraceae* and *Campylobacter* in the gut (Li et al., 2018). Weaning temporarily increases intestinal immune response, as indicated by the recruitment of more IgA-producing cells and increased pro-inflammatory cytokines in the small intestine for at least 15 days after weaning (de Groot et al., 2021). Under diarrheal stress, numerous studies have observed piglets undergoing microbial imbalance due to increased Firmicutes:Bacteroidetes (Stojanov et al., 2020;Bin et al., 2018; Meng et al., 2020).

1.2.2. Enterotoxigenic *E. coli*

Prolonged stress from weaning leaves pigs in a vulnerable position, increasing susceptibility to pathogens. Post-weaning diarrhea (PWD) is a condition commonly associated with the proliferation of enterotoxigenic *E. coli* (ETEC) in affected weaned pigs and can be observed within the first weeks after weaning (Le Dividich and Herpin, 1994). ETEC is a gram-negative bacteria structured with heat-stable somatic antigens composed of polysaccharide chains linking to a lipopolysaccharide (LPS) complex (Evans and Evans, 1996). ETEC is also structured with H antigens which impose ETEC to have flagella (Wolf, 1997). The source of ETEC infection can be found in the environment and infected pigs can transmit ETEC through the oral-fecal route (Hampson et al., 1987). PWD caused by ETEC infection occurs when ETEC binds to enterocytes and releases enterotoxins (Evans and Evans, 1996; U and Ra, 2008).

1.2.2.1. Intestinal colonization

The initial step of ETEC pathogenicity is to colonize the intestinal epithelial cells (Sheikh et al., 2017). Upon ingestion, ETEC can withstand gastric acidity and bile acid by maintaining their cytosolic pH and eventually invade the small intestine as their major virulence site (Lund et al., 2014; Gonzales-Siles and Sjöling, 2016). In the small intestine, ETEC adheres to the mucin layer using colonization factors. Colonization factors are usually in the form of fimbriae, with F4 and F18 being commonly associated with PWD (Madhavan and Sakellaris, 2015; Dubreuil et al., 2016). On the surface of ETEC, colonization factor antigen I is also observed to play an important role in colonizing the intestinal lining (Fleckenstein and Sheikh, 2021). After degrading mucin using proteases such as EatA and metalloprotease YghJ, ETEC can surpass the mucus layer to colonize the host epithelial cells (Kumar et al., 2014; Luo et al., 2014). In addition, one of the enterotoxins secreted by ETEC, heat-labile toxin (LT), may also inhibit mucin secretion from goblet cells (Verbrugghe et al., 2015; Pothuraju et al., 2022). Without the presence of LT, piglets had reduction in ETEC count in the ileum, signifying the importance of LT for ETEC colonization (Berberov et al., 2004).

1.2.2.2. Secretory diarrhea

Heat-stabile toxin (ST) and LT are secreted from adhered ETEC and initiate secretory diarrhea. Both ST and LT are comprised of A and B subunits. Activated enterotoxins induce severe watery diarrhea by promoting water and electrolyte imbalance, causing fluid accumulation in the intestines. STa and STb are responsible for diarrhea induction. STa targets the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel regulated by cyclic nucleotides and protein kinase C (PKC), and the activation of CFTR helps maintain an optimal, alkaline environment for ETEC to survive (Crane et al., 2006; Gonzales-Siles et al.,

2017). STa binds to the guanylate cyclase C receptor and leads to an accumulation of cyclic GMP (cGMP) from hydrolyzing guanosine triphosphate (Wang et al., 2019a). Then, cGMP stimulates CFTR in three different ways: (1) directly activating cGMP-dependent protein kinase II (PKGII), (2) activating protein kinase A (PKA) to activate PKGII, and (3) inhibiting phosphodiesterase 3 (PDE3) to activate PKA (Wang et al., 2019a). PKA can further phosphorylate sodium/hydrogen ion exchangers (NHEs) and inhibit sodium reabsorption by the enterocytes, allowing more electrolytes and fluid to accumulate in the lumen (He and Yun, 2010; Chen et al., 2019). STa can also induce bicarbonate secretion by stimulating tyrosine kinase activity, thus increasing a chloride ion/bicarbonate exchange and further luminal fluid accumulation (Sellers et al., 2005). STb can also stimulate fluid efflux by directly and indirectly activating CFTR. STb first binds to sulfatide to activate GTP-binding regulatory protein (Gαi3) and elevates calcium uptake from the intestinal lumen into the enterocytes (Beausoleil et al., 2002; Butt et al., 2020). Once calcium is accumulated, it activates calmodulin-dependent protein kinases II (CAMKII) to open calcium-activated chloride channel (CaCC) and CFTR to secrete chloride ion,sodium bicarbonate and water. Calcium influx can also activate PKC to open CFTR and inhibit NHE3 that regulates sodium uptake into the enterocytes (Butt et al., 2020).

In addition to aiding in colonization, LT inhibits intestinal fluid uptake by the enterocytes. Once the A subunit in LT (LTa) cleaves from the B subunit (LTb), LTa is internalized by enterocytes and activates adenylate cyclase by transferring adenosine diphosphate-ribose from NAD+ to guanyl nucleotide-dependent regulatory component (Gill and Richardson, 1980). Adenylate cyclase then accumulates cyclic AMP (cAMP), which will increase chloride and potassium ion efflux into the intestinal lumen by activating CFTR and potassium channels, respectively (Pongkorpsakol et al., 2014). ETEC toxicity can be observed

when LTb binds to the host GM1 ganglioside receptors; however, the exact mechanism is not well understood (Johnson et al., 2009; Mudrak and Kuehn, 2010).

1.2.2.3. Leaky gut

The intestinal barrier is permeable to small molecules such as digested nutrients with the presence of paracellular and transcellular pathway transporter proteins, but non-permeable to large molecules and bacteria (Wijtten et al., 2011). When the intestinal barrier is disrupted, however, intestinal permeability greatly increases and is often linked to chronic inflammation and functional disorders, known as leaky gut (Fasano, 2012). Unlike other *E. coli* pathotypes such as enteropathogenic *E. coli* and enterohemorrhagic *E. coli*, ETEC does not disrupt the intestinal barrier by manipulating the actin cytoskeleton (Pollard and Cooper, 2009; Navarro-Garcia et al., 2013). Instead, increased intestinal permeability is caused by STb that disrupts tight junctions between epithelial cells (Mukiza and Dubreuil, 2013; Nassour and Dubreuil, 2014). Tight junctions are composed of transmembrane proteins including occludin and claudin family proteins and are structured by scaffold proteins including zonula occludens (ZO) (Paradis et al., 2021).

1.2.2.4. Host immune response

The intestinal microbiota is involved in host immune responses during ETEC infection. For example, ETEC-induced intestinal dysbiosis can activate γ-aminobutyric acid (GABA), which further stimulates the expression of IL-17 and the recruitment of neutrophils and macrophages to the ETEC infected sites (Ren et al., 2017; Dubreuil, 2021). Secretory IgA performs immune exclusion by blocking opportunistic pathogens and their enterotoxins from attaching directly to the intestinal epithelial cells to damage the intestinal barrier and results in pathogen colonization (Corthesy, 2013). Therefore, vaccines targeting sIgA have been developed

for preventative measurements against PWD for weaned pigs; however, issues arise as the vaccines administered during early weaning are considered too late as a preventative measure (Melkebeek et al., 2013).

ETEC infection can induce pro-inflammatory responses in the intestines (Melkebeek et al., 2013). Weaned pigs challenged with ETEC expressed more pro-inflammatory cytokines, IL-6 and IL-8, in the intestines (McLamb et al., 2013; Wan et al., 2019). ETEC flagellin is the major stimulator to induce intestinal inflammation (Devriendt et al., 2010). LPS on ETEC interacts with TLR4 on myeloid cells to activate the nuclear factor-κb (NF-κB) pathway to induce pro-inflammatory cytokines in the intestines (Raetz and Whitfield, 2002). STa can also promote the secretion of pro-inflammatory cytokines and chemokines in the small intestine but the mechanism is not well understood (Wang et al., 2019a).

However, ETEC enterotoxins are capable of subverting pro-inflammatory responses. ETEC can be eradicated by the host adaptive immune system, thus ETEC suppresses local immunity to allow time for proliferation and to induce pathogenicity (Glenn et al., 2009; Read et al., 2014). In the host, IL-12 induces a pro-inflammatory response when macrophages are in contact with LPS (Saito et al., 2006). However, when monocytes were stimulated by LPS from *E. coli*, IL-12 secretion was inhibited with LTb and IL-10 interaction, minimizing inflammatory response to LPS (Turcanu et al., 2002). Both ST and LT toxigenicity have been shown to suppress inflammation by manipulating host regulatory T cells (Basset et al., 2010). In an *in vivo* study with mice, accumulation of cGMP after ST toxin exposure was shown to upregulate cytokine IL-33 to suppress intestinal T helper cells and promote regulatory T cells to disrupt the host innate immune system (Motyka et al., 2021). LT can also repress the host innate immune response by interfering with the cyclic AMP-dependent pathway, inhibiting cytokine production

and preventing host immune cells from disrupting ETEC virulence activity (Bagley et al., 2002). An *ex vivo* study has shown that the presence of both ST and LT suppressed the production of IL-6, TNF-α, and IL-9 (Read et al., 2014). LT was also shown to prevent the activation of the NF-κB pathway by inhibiting the phosphorylation of the regulator IkBα *in vitro* (Wang and Hardwidge, 2012). The NF-κB pathway is responsible for pro-inflammatory responses in the host innate immune system and can be inhibited by IL-10. IL-10 has also been shown to inhibit phosphorylation of IkBα to inhibit NF-κB signaling. Hence, in Bignon et al. (2017), LTb has shown to limit T cell activation by promoting proliferation of IL-10-producing dendritic cells and promoting regulatory T cell proliferation to limit helper T cell activation in the mucosal layer of mice lungs.

SUMMARY

Gut microbes and their host generally maintain a symbiotic relationship. However, gut microbial subpopulations can be shifted due to the introduction of a new diet and opportunistic pathogens. Pigs commonly experience high amounts of stress during weaning, which leads to reduced feed intake and increased pathogenic vulnerability. Pigs under prolonged weaning stress are associated with growth retardation and weakened immune systems, and even sudden death. In the U.S., Enterotoxigenic *E. coli* (ETEC) induced diarrhea is a common disease in pre- and post-weaning pigs and F18 ETEC has recently reemerged in the Midwest. ETEC releases enterotoxins, which modulate the electrolyte and water channels in enterocytes and disrupt tight junctions between intestinal epithelial cells to increase permeability. ETEC is also capable of inducing a pro-inflammatory response due to the presence of LPS and flagellin. However, ETEC enterotoxins have been implicated in promoting anti-inflammatory responses to suppress the host immune response. Although *E. coli* diarrhea is not one of the high mortality swine diseases, it

has posed negative effects on feed intake, intestinal development, nutrient absorption, and lifelong production and welfare of pigs. Thus, dietary manipulation of gut microbiota is investigated as a means to fortify symbiotic microbial subpopulations to prevent ETEC pathogenicity by colonization resistance.

LITERATURE CITED

Agans, R., A. Gordon, D. L. Kramer, S. Perez-Burillo, J. A. Rufián-Henares, and O. Paliy. 2018. Dietary fatty acids sustain the growth of the human gut microbiota. Appl. Environ. Microbiol. 84:e01525-18. doi:10.1128/AEM.01525-18.

Akiba, Y., O. Furukawa, P. H. Guth, E. Engel, I. Nastaskin, P. Sassani, R. Dukkipatis, A. Pushkin, I. Kurtz, and J. D. Kaunitz. 2001. Cellular bicarbonate protects rat duodenal mucosa from acid-induced injury. J Clin Invest. 108:1807–1816.

Bagley, K. C., S. F. Abdelwahab, R. G. Tuskan, T. R. Fouts, and G. K. Lewis. 2002. Cholera toxin and heat-labile enterotoxin activate human monocyte-derived dendritic cells and dominantly inhibit cytokine production through a cyclic AMP-dependent pathway. Infect. Immun. 70:5533–5539. doi:10.1128/IAI.70.10.5533-5539.2002.

Bain, C. C., and A. Schridde. 2018. Origin, differentiation, and function of intestinal macrophages. Front. Immunol. 9.

Basset, C., F. Thiam, C. D. Martino, J. Holton, J. D. Clements, and E. Kohli. 2010. Cholera-like enterotoxins and regulatory T cells. Toxins (Basel). 2:1774–1795. doi:10.3390/toxins2071774.

Beausoleil, H.-E., F. Lépine, and J. Daniel Dubreuil. 2002. LC-MS analysis of pig intestine sulfatides: Interaction with Escherichia coli STb enterotoxin and characterization of molecular species present. FEMS Microbiology Letters. 209:183–188. doi:10.1111/j.1574-6968.2002.tb11129.x.

van Beers-Schreurs, H. M. G., M. J. A. Nabuurs, L. Vellenga, H. J. K. der Valk, T. Wensing, and H. J. Breukink. 1998. Weaning and the weanling diet influence the villous height and

crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. Nutr. J. 128:947–953. doi:10.1093/jn/128.6.947.

Berberov, E. M., Y. Zhou, D. H. Francis, M. A. Scott, S. D. Kachman, and R. A. Moxley. 2004. Relative importance of heat-labile enterotoxin in the causation of severe diarrheal disease in the gnotobiotic piglet model by a strain of enterotoxigenic Escherichia coli that produces multiple enterotoxins. Infect. Immun. 72:3914–3924. doi:10.1128/IAI.72.7.3914-3924.2004.

Berg, G., D. Rybakova, D. Fischer, T. Cernava, M.-C. C. Vergès, T. Charles, X. Chen, L. Cocolin, K. Eversole, G. H. Corral, M. Kazou, L. Kinkel, L. Lange, N. Lima, A. Loy, J. A. Macklin, E. Maguin, T. Mauchline, R. McClure, B. Mitter, M. Ryan, I. Sarand, H. Smidt, B. Schelkle, H. Roume, G. S. Kiran, J. Selvin, R. S. C. de Souza, L. van Overbeek, B. K. Singh, M. Wagner, A. Walsh, A. Sessitsch, and M. Schloter. 2020. Microbiome definition re-visited: old concepts and new challenges. Microbiome. 8:103. doi:10.1186/s40168-020-00875-0.

Bignon, A., A. P. Watt, and M. A. Linterman. 2017. Escherichia coli heat-labile enterotoxin B limits t cells activation by promoting immature dendritic cells and enhancing regulatory T cell function. Front. Immunol. 8.

Bin, P., Z. Tang, S. Liu, S. Chen, Y. Xia, J. Liu, H. Wu, and G. Zhu. 2018. Intestinal microbiota mediates enterotoxigenic Escherichia coli-induced diarrhea in piglets. BMC Veterinary Research. 14:385. doi:10.1186/s12917-018-1704-9.

Bischoff, S. C. 2011. “Gut health”: a new objective in medicine? BMC Med. 9:1–14. doi:10.1186/1741-7015-9-24.

Boudry, G., P.-C. Yang, and M. H. Perdue. 2004. Small Intestine, Anatomy. In: L. R. Johnson, editor. Encyclopedia of Gastroenterology. Elsevier, New York. p. 404–409. Available from: https://www.sciencedirect.com/science/article/pii/B0123868602006481

Butt, S., M. Saleh, and J. Gagnon. 2020. Impact of the Escherichia coli heat-stable enterotoxin b (STb) on Gut Health and Function. Toxins (Basel). 12:760. doi:10.3390/toxins12120760.

Cascales, E., S. K. Buchanan, D. Duché, C. Kleanthous, R. Lloubès, K. Postle, M. Riley, S. Slatin, and D. Cavard. 2007. Colicin biology. Microbiol. Mol. Biol. Rev. 71:158–229. doi:10.1128/MMBR.00036-06.

Chen, T., R. Lin, L. Avula, R. Sarker, J. Yang, B. Cha, C. M. Tse, G. McNamara, U. Seidler, S. Waldman, A. Snook, M. J. C. Bijvelds, H. R. de Jonge, X. Li, and M. Donowitz. 2019. NHERF3 is necessary for Escherichia coli heat-stable enterotoxin-induced inhibition of NHE3: differences in signaling in mouse small intestine and Caco-2 cells. Am. J. Physiol. Cell Physiol. 317:C737–C748. doi:10.1152/ajpcell.00351.2018.

Chen, W., J. Mi, N. Lv, J. Gao, J. Cheng, R. Wu, J. Ma, T. Lan, and X. Liao. 2018. Lactation stage-dependency of the sow milk microbiota. Front. Microbiol. 9:945. doi:10.3389/fmicb.2018.00945.

Cooper, T. A., M. P. Roberts, H. G. Kattesh, and C. J. Kojima. 2009. Effects of transport stress, sex, and weaning weight on postweaning performance in pigs. Prof. Anim. Sci. 25:189–194. doi:10.15232/S1080-7446(15)30700-2.

Corthesy, B. 2013. Multi-faceted functions of secretory IgA at mucosal surfaces. Front. Immunol. 4.

Crane, J. K., S. S. Choudhari, T. M. Naeher, and M. E. Duffey. 2006. Mutual enhancement of virulence by enterotoxigenic and enteropathogenic Escherichia coli. Infect. Immun. 74:1505–1515. doi:10.1128/IAI.74.3.1505-1515.2006.

Cremer, J., I. Segota, C. Yang, M. Arnoldini, J. T. Sauls, Z. Zhang, E. Gutierrez, A. Groisman, and T. Hwa. 2016. Effect of flow and peristaltic mixing on bacterial growth in a gut-like channel. Proc. Natl. Acad. Sci. U.S.A. 113:11414–11419. doi:10.1073/pnas.1601306113.

Crespo-Piazuelo, D., J. Estellé, M. Revilla, L. Criado-Mesas, Y. Ramayo-Caldas, C. Óvilo, A. I. Fernández, M. Ballester, and J. M. Folch. 2018. Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. Sci. Rep. 8:12727. doi:10.1038/s41598-018-30932-6.

Cummings, J. H., J. L. Rombeau, and T. Sakata. 2004. Physiological and clinical aspects of short-chain fatty acids. Cambridge University Press.

Dai, Z.-L., J. Zhang, G. Wu, and W.-Y. Zhu. 2010. Utilization of amino acids by bacteria from the pig small intestine. Amino Acids. 39:1201–1215. doi:10.1007/s00726-010-0556-9.

Devriendt, B., E. Stuyven, F. Verdonck, B. M. Goddeeris, and E. Cox. 2010. Enterotoxigenic Escherichia coli (K88) induce proinflammatory responses in porcine intestinal epithelial cells. Developmental & Comparative Immunology. 34:1175–1182. doi:10.1016/j.dci.2010.06.009.

Diether, N. E., and B. P. Willing. 2019. Microbial fermentation of dietary protein: An important factor in diet–microbe–host interaction. Microorganisms. 7:19. doi:10.3390/microorganisms7010019.

Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer, and R. Knight. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota

across multiple body habitats in newborns. Proc. Natl. Acad. Sci. U.S.A. 107:11971–11975.

Donaldson, G. P., S. M. Lee, and S. K. Mazmanian. 2016. Gut biogeography of the bacterial microbiota. Nat Rev Microbiol. 14:20–32. doi:10.1038/nrmicro3552.

Douglas, G. M., V. J. Maffei, J. R. Zaneveld, S. N. Yurgel, J. R. Brown, C. M. Taylor, C. Huttenhower, and M. G. I. Langille. 2020. PICRUSt2 for prediction of metagenome functions. Nat. Biotechnol. 38:685–688. doi:10.1038/s41587-020-0548-6.

Dubreuil, J. D. 2021. Pig vaccination strategies based on enterotoxigenic Escherichia coli toxins. Braz. J. Microbiol. 52:2499–2509. doi:10.1007/s42770-021-00567-3.

Dubreuil, J. D., R. E. Isaacson, and D. M. Schifferli. 2016. Animal enterotoxigenic Escherichia coli. EcoSal Plus. 7. doi:10.1128/ecosalplus.ESP-0006-2016. Available from: https://journals.asm.org/doi/10.1128/ecosalplus.ESP-0006-2016

Dukowicz, A. C., B. E. Lacy, and G. M. Levine. 2007. Small intestinal bacterial overgrowth. Gastroenterol. Hepatol. (N Y). 3:112–122.

Duncan, S. H., G. Holtrop, G. E. Lobley, A. G. Calder, C. S. Stewart, and H. J. Flint. 2004. Contribution of acetate to butyrate formation by human faecal bacteria. Br. J. Nutr.91:915–923. doi:10.1079/BJN20041150.

Durazzi, F., C. Sala, G. Castellani, G. Manfreda, D. Remondini, and A. De Cesare. 2021. Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota. Sci. Rep. 11:3030. doi:10.1038/s41598-021-82726-y.

Evans, D. F., G. Pye, R. Bramley, A. G. Clark, T. J. Dyson, and J. D. Hardcastle. 1988. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut. 29:1035–1041. doi:10.1136/gut.29.8.1035.

Evans, D. J., and D. G. Evans. 1996. Escherichia coli in diarrheal disease. In: S. Baron, editor. Medical Microbiology. 4th ed. University of Texas Medical Branch at Galveston, Galveston (TX).

Fasano, A. 2012. Leaky gut and autoimmune diseases. Clin. Rev. Allergy Immunol. 42:71–78. doi:10.1007/s12016-011-8291-x.

Fleckenstein, J. M., and A. Sheikh. 2021. Emerging themes in the molecular pathogenesis of enterotoxigenic *Escherichia coli*. Int. J. Infect. Dis. 224:S813–S820. doi:10.1093/infdis/jiab359.

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome. 3. doi:10.1186/s40168-015-0091-8. Available from: http://www.microbiomejournal.com/content/3/1/28

Freter, R., H. Brickner, J. Fekete, M. M. Vickerman, and K. E. Carey. 1983. Survival and implantation of Escherichia coli in the Intestinal Tract. Infect. Immun. 39:686–703.

Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, I. Hautefort, A. Thompson, J. Hinton, and F. Van Immerseel. 2006. Butyrate specifically down-regulates Salmonella pathogenicity island 1 gene expression. Appl. Environ. Microbiol. 72:946–949.

Gill, D. M., and S. H. Richardson. 1980. Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of Escherichia coli: Comparison with Cholera Toxin. Int. J. Infect. Dis. 141:64–70. doi:10.1093/infdis/141.1.64.

Glenn, G. M., D. H. Francis, and E. M. Danielsen. 2009. Toxin-mediated effects on the innate mucosal defenses: implications for enteric vaccines. Infect. Immun.. 77:5206–5215. doi:10.1128/IAI.00712-09.

Gonzales-Siles, L., R. Karlsson, D. Kenny, A. Karlsson, and Å. Sjöling. 2017. Proteomic analysis of enterotoxigenic Escherichia coli (ETEC) in neutral and alkaline conditions. BMC Microbiology. 17:11. doi:10.1186/s12866-016-0914-1.

Gonzales-Siles, L., and Å. Sjöling. 2016. The different ecological niches of enterotoxigenic E scherichia coli. Environ Microbiol. 18:741–751. doi:10.1111/1462-2920.13106.

de Groot, N., F. Fariñas, C. G. Cabrera-Gómez, F. J. Pallares, and G. Ramis. 2021. Weaning causes a prolonged but transient change in immune gene expression in the intestine of piglets. J. Anim. Sci. 99:skab065. doi:10.1093/jas/skab065.

Guevarra, R. B., J. H. Lee, S. H. Lee, M.-J. Seok, D. W. Kim, B. N. Kang, T. J. Johnson, R. E. Isaacson, and H. B. Kim. 2019. Piglet gut microbial shifts early in life: causes and effects. J. Anim. Sci. Biotechnol. 10:1. doi:10.1186/s40104-018-0308-3.

Gunnarsdottir, S. A., R. Sadik, S. Shev, M. Simrén, H. Sjövall, P.-O. Stotzer, H. Abrahamsson, R. Olsson, and E. S. Björnsson. 2003. Small intestinal motility disturbances and bacterial overgrowth in patients with liver cirrhosis and portal hypertension. Am. J. Gastroenterol. Suppl. 98:1362–1370. doi:10.1016/S0002-9270(03)00250-8.

Hampson, D. J., Z. F. Fu, and I. D. Robertson. 1987. Investigation of the source of haemolytic Escherichia coli infecting weaned pigs. Epidemiol. Infect. 99:149–153.

Han, Y., Q. Zhao, C. Tang, Y. Li, K. Zhang, F. Li, and J. Zhang. 2020. Butyrate mitigates weanling piglets from lipopolysaccharide-induced colitis by regulating microbiota and energy metabolism of the gut–liver axis. Front. Microbiol. 11.

He, P., and C. C. Yun. 2010. Mechanisms of the regulation of the intestinal Na+/H+exchanger NHE3. J. Biomed. Biotechnol. 2010:238080. doi:10.1155/2010/238080.

Hedemann, M. S., S. Højsgaard, and B. B. Jensen. 2003. Small intestinal morphology and activity of intestinal peptidases in piglets around weaning. J. Anim. Physiol. Anim. Nutr. (Berl). 87:32–41. doi:10.1046/j.1439-0396.2003.00405.x.

Herath, M., S. Hosie, J. C. Bornstein, A. E. Franks, and E. L. Hill-Yardin. 2020. The role of the gastrointestinal mucus system in intestinal homeostasis: Implications for neurological disorders. Front. cell. infect. 10.

Herkelman, K. L., G. L. Cromwell, T. S. Stahly, T. W. Pfeiffer, and D. A. Knabe. 1992. Apparent digestibility of amino acids in raw and heated conventional and low-trypsin-inhibitor soybeans for pigs. J. Anim. Sci. 70:818–826. doi:10.2527/1992.703818x.

Hollander, D., and J. D. Kaunitz. 2020. The "Leaky Gut": Tight junctions but loose associations? Dig Dis Sci. 65:1277–1287. doi:10.1007/s10620-019-05777-2.

Holman, D. B., B. W. Brunelle, J. Trachsel, and H. K. Allen. 2017. Meta-analysis to define a core microbiota in the swine gut. mSystems. 2. doi:10.1128/mSystems.00004-17. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5443231/

Holzer, P. 2015. Acid-sensing ion channels in gastrointestinal function. Neuropharmacology. 94:72–79. doi:10.1016/j.neuropharm.2014.12.009.

Itoh, M., and M. J. Bissell. 2003. The organization of tight junctions in epithelia: implications for mammary gland biology and breast tumorigenesis. J. Mammary. Gland Biol. Neoplasia. 8:449–462. doi:10.1023/B:JOMG.0000017431.45314.07.

Jacobson, A., L. Lam, M. Rajendram, F. Tamburini, J. Honeycutt, T. Pham, W. V. Treuren, K. Pruss, S. R. Stabler, K. Lugo, D. M. Bouley, J. G. Vilches-Moure, M. Smith, J. L.

Sonnenburg, A. S. Bhatt, K. C. Huang, and D. Monack. 2018. A gut commensal-produced metabolite mediates colonization resistance to Salmonella infection. Cell Host Microbe. 24:296-307.e7. doi:10.1016/j.chom.2018.07.002.

Jensen, A. R., J. Elnif, D. G. Burrin, and P. T. Sangild. 2001. Development of intestinal immunoglobulin absorption and enzyme activities in neonatal pigs is diet dependent. Nutr. J. 131:3259–3265. doi:10.1093/jn/131.12.3259.

Johansson, M. E. V. 2012. Fast renewal of the distal colonic mucus layers by the surface goblet cells as measured by in vivo labeling of mucin glycoproteins. PLOS ONE. 7:e41009. doi:10.1371/journal.pone.0041009.

Johnson, A. M., R. S. Kaushik, D. H. Francis, J. M. Fleckenstein, and P. R. Hardwidge. 2009. Heat-labile enterotoxin promotes Escherichia coli adherence to intestinal epithelial cells. J. Bacteriol. Res.. 191:178–186. doi:10.1128/JB.00822-08.

Kelly, S. M., J. Munoz-Munoz, and D. van Sinderen. 2021. Plant glycan metabolism by Bifidobacteria. Front. Microbiol. 12.

Kenworthy, R., and W. E. Crabb. 1963. The intestinal flora of young pigs, with reference to early weaning, Escherichia coli and scours. Journal of Comparative Pathology. 73:215–228.

Khan, I., N. Ullah, L. Zha, Y. Bai, A. Khan, T. Zhao, T. Che, and C. Zhang. 2019. Alteration of gut microbiota in inflammatory bowel disease (IBD): Cause or consequence? IBD Treatment Targeting the Gut Microbiome. Pathogens. 8. doi:10.3390/pathogens8030126. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6789542/

Kim, M., C. Galan, A. A. Hill, W.-J. Wu, H. Fehlner-Peach, H. W. Song, D. Schady, M. L. Bettini, K. W. Simpson, R. S. Longman, D. R. Littman, and G. E. Diehl. 2018. Critical

role for the microbiota in CX3CR1+ Intestinal mononuclear phagocyte regulation of intestinal T cell responses. Immunity. 49:151-163.e5. doi:10.1016/j.immuni.2018.05.009.

Kubasova, T., L. Davidova-Gerzova, E. Merlot, M. Medvecky, O. Polansky, D. Gardan-Salmon, H. Quesnel, and I. Rychlik. 2017. Housing systems influence gut microbiota composition of sows but not of their piglets. PLoS One. 12:e0170051. doi:10.1371/journal.pone.0170051.

Kumar, P., Q. Luo, T. J. Vickers, A. Sheikh, W. G. Lewis, and J. M. Fleckenstein. 2014. EatA, an immunogenic protective antigen of enterotoxigenic Escherichia coli, degrades intestinal mucin. Infect. Immun.. 82:500–508. doi:10.1128/IAI.01078-13.

Larauche, M., C. Kiank, And Y. Tache. 2009. Corticotropin releasing factor signaling in colon and ileum: Regulation by stress and pathophysiological implications. J. Physiol. Pharmacol. 60:33–46.

Lawson, M. A. E., I. J. O'Neill, M. Kujawska, S. Gowrinadh Javvadi, A. Wijeyesekera, Z. Flegg, L. Chalklen, and L. J. Hall. 2020. Breast milk-derived human milk oligosaccharides promote Bifidobacterium interactions within a single ecosystem. ISME J. 14:635–648. doi:10.1038/s41396-019-0553-2.

Le Dividich, J., and P. Herpin. 1994. Effects of climatic conditions on the performance, metabolism and health status of weaned piglets: a review. Livestock Production Science. 38:79–90. doi:10.1016/0301-6226(94)90052-3.

Li, W.-Z., K. Stirling, J.-J. Yang, and L. Zhang. 2020. Gut microbiota and diabetes: From correlation to causality and mechanism. World J. Diabetes. 11:293–308. doi:10.4239/wjd.v11.i7.293.

Li, Y., Y. Guo, Z. Wen, X. Jiang, X. Ma, and X. Han. 2018. Weaning stress perturbs gut microbiome and its metabolic profile in piglets. Sci. Rep.. 8:1–12.

Litvak, Y., K. K. Z. Mon, H. Nguyen, G. Chanthavixay, M. Liou, E. M. Velazquez, L. Kutter, M. A. Alcantara, M. X. Byndloss, C. R. Tiffany, G. T. Walker, F. Faber, Y. Zhu, D. N. Bronner, A. J. Byndloss, R. M. Tsolis, H. Zhou, and A. J. Bäumler. 2019. Commensal Enterobacteriaceae protect against Salmonella colonization through oxygen competition. Cell Host Microbe. 25:128-139.e5. doi:10.1016/j.chom.2018.12.003.

Liu, H.-Y., A. Giraud, C. Seignez, D. Ahl, F. Guo, J. Sedin, T. Walden, J.-H. Oh, J. P. van Pijkeren, L. Holm, S. Roos, S. Bertilsson, and M. Phillipson. 2021. Distinct B cell subsets in Peyer's patches convey probiotic effects by Limosilactobacillus reuteri. Microbiome. 9:198. doi:10.1186/s40168-021-01128-4.

Liu, P.-Y., W.-K. Wu, C.-C. Chen, S. Panyod, L.-Y. Sheen, and M.-S. Wu. 2020. Evaluation of Compatibility of 16S rRNA V3V4 and V4 Amplicon Libraries for Clinical Microbiome Profiling. doi:10.1101/2020.08.18.256818

Lönnerdal, B., and O. Hernell. 2016. An opinion on "staging" of infant formula: A developmental perspective on infant feeding. J. Pediatr. Gastroenterol. Nutr. 62:9–21. doi:10.1097/MPG.0000000000000806.

Lozupone, C. A., J. Stombaugh, A. Gonzalez, G. Ackermann, D. Wendel, Y. Vázquez-Baeza, J. K. Jansson, J. I. Gordon, and R. Knight. 2013. Meta-analyses of studies of the human microbiota. Genome Res. 23:1704–1714. doi:10.1101/gr.151803.112.

Lu, J. J., X. T. Zou, and Y. M. Wang. 2008. Effects of sodium butyrate on the growth performance, intestinal microflora and morphology of weanling pigs. J. Anim. Feed Sci. 17:568–578. doi:10.22358/jafs/66685/2008.

Lührmann, A., K. Ovadenko, J. Hellmich, C. Sudendey, V. Belik, J. Zentek, and W. Vahjen. 2021. Characterization of the fecal microbiota of sows and their offspring from German commercial pig farms. PLOS ONE. 16:e0256112. doi:10.1371/journal.pone.0256112.

Lund, P., A. Tramonti, and D. De Biase. 2014. Coping with low pH: molecular strategies in neutralophilic bacteria. FEMS Microbiol. Rev.38:1091–1125. doi:10.1111/1574-6976.12076.

Luo, Q., P. Kumar, T. J. Vickers, A. Sheikh, W. G. Lewis, D. A. Rasko, J. Sistrunk, and J. M. Fleckenstein. 2014. Enterotoxigenic Escherichia coli secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. Infect. Immun.. 82:509–521. doi:10.1128/IAI.01106-13.

Madara, J. L., and J. S. Trier. 1982. Structure and permeability of goblet cell tight junctions in rat small intestine. J. Membr. Biol. 66:145–157. doi:10.1007/BF01868490.

Madhavan, T. P. V., and H. Sakellaris. 2015. Colonization factors of enterotoxigenic Escherichia coli. Adv Appl Microbiol. 90:155–197. doi:10.1016/bs.aambs.2014.09.003.

Maga, E. A., P. T. Desai, B. C. Weimer, N. Dao, D. Kültz, and J. D. Murray. 2012. Consumption of Lysozyme-Rich Milk Can Alter Microbial Fecal Populations. Appl. Environ. Microbiol. 78:6153–6160. doi:10.1128/AEM.00956-12.

Maltecca, C., D. Lu, C. Schillebeeckx, N. P. McNulty, C. Schwab, C. Shull, and F. Tiezzi. 2019. Predicting growth and carcass traits in swine using microbiome data and machine learning algorithms. Sci. Rep. 9:6574. doi:10.1038/s41598-019-43031-x.

Mantis, N. J., N. Rol, and B. Corthésy. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol. 4:603–611.

Marchesi, J. R., and J. Ravel. 2015. The vocabulary of microbiome research: a proposal. Microbiome. 3:31. doi:10.1186/s40168-015-0094-5.

Marcobal, A., M. Barboza, J. W. Froehlich, D. E. Block, J. B. German, C. B. Lebrilla, and D. A. Mills. 2010. Consumption of human milk oligosaccharides by gut-related microbes. J. Agric. Food Chem. 58:5334–5340. doi:10.1021/jf9044205.

Marion, J., M. Biernat, F. Thomas, G. Savary, Y. Le Breton, R. Zabielski, I. Le Huërou-Luron, and J. Le Dividich. 2002. Small intestine growth and morphometry in piglets weaned at 7 days of age. effects of level of energy intake. Reprod. Nutr. Dev. 42:339–354. doi:10.1051/rnd:2002030.

McDermott, A. J., and G. B. Huffnagle. 2014. The microbiome and regulation of mucosal immunity. Immunology. 142:24–31. doi:10.1111/imm.12231.

McLamb, B. L., A. J. Gibson, E. L. Overman, C. Stahl, and A. J. Moeser. 2013. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic E. coli challenge and exacerbates intestinal injury and clinical disease. PLOS ONE. 8:e59838. doi:10.1371/journal.pone.0059838.

Melkebeek, V., B. M. Goddeeris, and E. Cox. 2013. ETEC vaccination in pigs. Veterinary Immunology and Immunopathology. 152:37–42. doi:10.1016/j.vetimm.2012.09.024.

Meng, F., H. Zhao, T. Nie, F. Lu, C. Zhang, Y. Lu, and Z. Lu. 2021. Acetate activates Lactobacillus bacteriocin synthesis by controlling quorum sensing. Appl. Environ. Microbiol. 87:e00720-21. doi:10.1128/AEM.00720-21.

Meng, Q., Z. Luo, C. Cao, S. Sun, Q. Ma, Z. Li, B. Shi, and A. Shan. 2020. Weaning alters intestinal gene expression involved in nutrient metabolism by shaping gut microbiota in pigs. Front. Microbiol. 11.

Mignard, S., and J. P. Flandrois. 2006. 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. J. Microbiol. Methods. 67:574–581. doi:10.1016/j.mimet.2006.05.009.

Miller, B. G., P. S. James, M. W. Smith, and F. J. Bourne. 1986. Effect of weaning on the capacity of pig intestinal villi to digest and absorb nutrients. J. Agric. Sci. 107:579–590. doi:10.1017/S0021859600069756.

Mirbagheri, S. A., and H. C. Fu. 2017. Effect of peristalsis in balance of intestinal microbial ecosystem. E5.001. Available from: https://ui.adsabs.harvard.edu/abs/2017APS..DFD.E5001M

Miura, K., M. Ishioka, and K. Iijima. 2017. The roles of the gut microbiota and toll-like receptors in obesity and nonalcoholic fatty liver disease. J. Obes. Metab. Syndr. 26:86–96. doi:10.7570/jomes.2017.26.2.86.

Moeser, A. J., C. S. Pohl, and M. Rajput. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Anim Nutr.3:313–321.

Momose, Y., K. Hirayama, and K. Itoh. 2008. Competition for proline between indigenous Escherichia coli and E. coli O157:H7 in gnotobiotic mice associated with infant intestinal microbiota and its contribution to the colonization resistance against E. coli O157:H7. Antonie van Leeuwenhoek. 94:165–171. doi:10.1007/s10482-008-9222-6.

Morikawa, M., S. Tsujibe, J. Kiyoshima-Shibata, Y. Watanabe, N. Kato-Nagaoka, K. Shida, and S. Matsumoto. 2016. Microbiota of the small intestine is selectively engulfed by phagocytes of the lamina propria and Peyer's patches. PLOS ONE. 11:e0163607. doi:10.1371/journal.pone.0163607.

Morota, G., R. V. Ventura, F. F. Silva, M. Koyama, and S. C. Fernando. 2018. Big data analytics and precision animal agriculture symposium: Machine learning and data mining advance predictive big data analysis in precision animal agriculture1. J. Anim. Sci. 96:1540–1550. doi:10.1093/jas/sky014.

Motyka, N. I., S. R. Stewart, I. E. Hollifield, T. R. Kyllo, J. A. Mansfield, E. B. Norton, J. D. Clements, and J. P. Bitoun. 2021. Elevated extracellular cGMP produced after exposure to enterotoxigenic escherichia coli heat-stable toxin induces epithelial IL-33 release and alters intestinal immunity. Infect. Immun. 89:e00707-20. doi:10.1128/IAI.00707-20.

Mudrak, B., and M. J. Kuehn. 2010. Heat-labile enterotoxin: Beyond GM1 binding. Toxins (Basel). 2:1445–1470. doi:10.3390/toxins2061445.

Mukiza, C. N., and J. D. Dubreuil. 2013. Escherichia coli heat-stable toxin b impairs intestinal epithelial barrier function by altering tight junction proteins. Infect. Immun.

Nassour, H., and J. D. Dubreuil. 2014. Escherichia coli STb enterotoxin dislodges claudin-1 from epithelial tight junctions. PLOS ONE. 9:e113273. doi:10.1371/journal.pone.0113273.

National Academies of Sciences and Medicine. 2018. Environmental chemicals, the human microbiome, and health risk: a research strategy. National Academies Press.

Navarro-Garcia, F., A. Serapio-Palacios, P. Ugalde-Silva, G. Tapia-Pastrana, and L. Chavez-Dueñas. 2013. Actin cytoskeleton manipulation by effector proteins secreted by diarrheagenic Escherichia coli pathotypes. Biomed. Res. Int. 2013:374395. doi:10.1155/2013/374395.

Nigro, G., and P. J. Sansonetti. 2015. Microbiota and gut stem cells cross-talks: A new view of epithelial homeostasis. Curr. Stem Cell Rep. 1:48–52. doi:10.1007/s40778-014-0005-x.

Odenwald, M. A., and J. R. Turner. 2017. The intestinal epithelial barrier: A therapeutic target? Nat Rev Gastroenterol. Hepatol. 14:9–21. doi:10.1038/nrgastro.2016.169.

O'Hara, A. M., and F. Shanahan. 2006. The gut flora as a forgotten organ. EMBO Rep. 7:688–693. doi:10.1038/sj.embor.7400731.

Okumura, R., and K. Takeda. 2017. Roles of intestinal epithelial cells in the maintenance of gut homeostasis.Exp. Mol. Med. 49:e338–e338.

Paradis, T., H. Bègue, L. Basmaciyan, F. Dalle, and F. Bon. 2021. Tight junctions as a key for pathogens invasion in intestinal epithelial cells. International Journal of Molecular Sciences. 22:2506.

Patel, K. S., and A. Thavamani. 2022. Physiology, peristalsis. in: StatPearls. StatPearls Publishing, Treasure Island (FL). Available from: http://www.ncbi.nlm.nih.gov/books/NBK556137/

Percy, W. H., T. H. Fromm, and C. E. Wangsness. 2003. Muscularis mucosae contraction evokes colonic secretion via prostaglandin synthesis and nerve stimulation. Am. J. Physiol. Gastrointest. Liver Physiol. 284:G213–G220. doi:10.1152/ajpgi.00179.2002.

Perez-Muñoz, M. E., M.-C. Arrieta, A. E. Ramer-Tait, and J. Walter. 2017. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. Microbiome. 5:48. doi:10.1186/s40168-017-0268-4.

Pieper, R., C. V. Tudela, M. Taciak, J. Bindelle, J. F. Pérez, and J. Zentek. 2016. Health relevance of intestinal protein fermentation in young pigs. Anim. Health Res. Rev. 17:137–147. doi:10.1017/S1466252316000141.

Pjevac, P., B. Hausmann, J. Schwarz, G. Kohl, C. W. Herbold, A. Loy, and D. Berry. 2021. An economical and flexible dual barcoding, two-step PCR approach for highly multiplexed amplicon sequencing. Front. Microbiol. 12.

Pluske, J., D. Turpin, and J. Kim. 2018. Gastrointestinal tract (gut) health in the young pig. Anim. Nutr. 4: 187–196.

Pollard, T. D., and J. A. Cooper. 2009. Actin, a central player in cell shape and movement. Science. 326:1208–1212. doi:10.1126/science.1175862.

Pongkorpsakol, P., N. Pathomthongtaweechai, P. Srimanote, S. Soodvilai, V. Chatsudthipong, and C. Muanprasat. 2014. Inhibition of cAMP-activated intestinal chloride secretion by diclofenac: Cellular mechanism and potential application in cholera. PLoS Negl. Trop. Dis. 8:e3119. doi:10.1371/journal.pntd.0003119.

Pothuraju, R., P. Pai, S. Chaudhary, J. A. Siddiqui, J. L. Cox, S. Kaur, S. Rachagani, H. K. Roy, M. Bouvet, and S. K. Batra. 2022. Depletion of transmembrane mucin 4 (Muc4) alters intestinal homeostasis in a genetically engineered mouse model of colorectal cancer. Aging (Albany NY). 14:2025–2046. doi:10.18632/aging.203935.

Pugin, B., W. Barcik, P. Westermann, A. Heider, M. Wawrzyniak, P. Hellings, C. A. Akdis, and L. O'Mahony. 2017. A wide diversity of bacteria from the human gut produces and degrades biogenic amines. Microb. Ecol. Health Dis. 28:1353881. doi:10.1080/16512235.2017.1353881.

Quigley, E. M. M. 2013. Gut bacteria in health and disease. Gastroenterol Hepatol (N Y). 9:560–569.

Raetz, C. R., and C. Whitfield. 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. 71:635.

Read, L. T., R. W. Hahn, C. C. Thompson, D. L. Bauer, E. B. Norton, and J. D. Clements. 2014. Simultaneous exposure to Escherichia coli heat-labile and heat-stable enterotoxins increases fluid secretion and alters cyclic nucleotide and cytokine production by intestinal epithelial cells. Infect. Immun. 82:5308–5316. doi:10.1128/IAI.02496-14.

Ren, M., S. H. Zhang, X. F. Zeng, H. Liu, and S. Y. Qiao. 2015. Branched-chain amino acids are beneficial to maintain growth performance and intestinal immune-related function in weaned piglets fed protein restricted diet. Asian-Australas J. Anim. Sci. 28:1742–1750. doi:10.5713/ajas.14.0131.

Ren, W., J. Yin, H. Xiao, S. Chen, G. Liu, B. Tan, N. Li, Y. Peng, T. Li, B. Zeng, W. Li, H. Wei, Z. Yin, G. Wu, P. R. Hardwidge, and Y. Yin. 2017. Intestinal microbiota-derived GABA Mediates interleukin-17 expression during enterotoxigenic Escherichia coli infection. Front. Immunol. 7.

Rezania, K. 2018. Role of inflammatory mechanisms in Guillain-Barre'Syndrome. In: Neuroinflammation. Elsevier. p. 505–516.

Rogier, E. W., A. L. Frantz, M. E. Bruno, and C. S. Kaetzel. 2014. Secretory IgA is concentrated in the outer layer of colonic mucus along with gut bacteria. Pathogens. 3:390–403.

Roldan-Santiago, P., R. Martinez-Rodriguez, A. Yanez-Pizana, and M. Trujillo-Ortega. 2013. Stressor factors in the transport of weaned piglets: a review. Veterinarni Medicina. 58:241–251. doi:10.17221/6805-VETMED.

Rose, E. C., A. T. Blikslager, and A. L. Ziegler. 2022. Porcine models of the intestinal microbiota: The translational key to understanding how gut commensals contribute to gastrointestinal disease. Front. Vet. Sci. 9.

Round, J. L., S. M. Lee, J. Li, G. Tran, B. Jabri, T. A. Chatila, and S. K. Mazmanian. 2011. The toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science. 332:974–977. doi:10.1126/science.1206095.

Rowland, I., G. Gibson, A. Heinken, K. Scott, J. Swann, I. Thiele, and K. Tuohy. 2018. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr. 57:1–24. doi:10.1007/s00394-017-1445-8.

Saito, S., M. Matsuura, and Y. Hirai. 2006. Regulation of lipopolysaccharide-induced interleukin-12 production by activation of repressor element GA-12 through hyperactivation of the ERK Pathway. Clin Vaccine Immunol. 13:876–883. doi:10.1128/CVI.00075-06.

Salcedo, J., S. A. Frese, D. A. Mills, and D. Barile. 2016. Characterization of porcine milk oligosaccharides during early lactation and their relation to the fecal microbiome. J. Dairy Sci. 99:7733–7743. doi:10.3168/jds.2016-10966.

Schoenborn, A. A., R. J. von Furstenberg, S. Valsaraj, F. S. Hussain, M. Stein, M. T. Shanahan, S. J. Henning, and A. S. Gulati. 2019. The enteric microbiota regulates jejunal Paneth cell number and function without impacting intestinal stem cells. Gut Microbes. 10:45–58. doi:10.1080/19490976.2018.1474321.

Schulz, S., A. Stephan, S. Hahn, L. Bortesi, F. Jarczowski, U. Bettmann, A.-K. Paschke, D. Tusé, C. H. Stahl, A. Giritch, and Y. Gleba. 2015. Broad and efficient control of major foodborne pathogenic strains of Escherichia coli by mixtures of plant-produced colicins. Proceedings of the Proc. Natl. Acad. Sci. U.S.A. 112:E5454–E5460. doi:10.1073/pnas.1513311112.

Sellers, Z. M., D. Childs, J. Y. C. Chow, A. J. Smith, D. L. Hogan, J. I. Isenberg, H. Dong, K. E. Barrett, and V. S. Pratha. 2005. Heat-stable enterotoxin of Escherichia coli stimulates a non-CFTR-mediated duodenal bicarbonate secretory pathway. Am. J. Physiol. Gastrointest. Liver Physiol. 288:G654–G663. doi:10.1152/ajpgi.00386.2004.

Sève, B. 2000. Effects of underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet. Domest. Anim. Endocrinol. 19:63–74.

Sheikh, A., R. Rashu, Y. A. Begum, F. M. Kuhlman, M. A. Ciorba, S. J. Hultgren, F. Qadri, and J. M. Fleckenstein. 2017. Highly conserved type 1 pili promote enterotoxigenic E. coli pathogen-host interactions. PLOS Negl. Trop. Dis. 11:e0005586. doi:10.1371/journal.pntd.0005586.

Shi, N., N. Li, X. Duan, and H. Niu. 2017. Interaction between the gut microbiome and mucosal immune system. Mil. Med. Res. 4:14. doi:10.1186/s40779-017-0122-9.

Shroyer, N. F., and S. A. Kocoshis. 2011. Anatomy and physiology of the small and large intestines. In: Pediatric Gastrointestinal and Liver Disease. Elsevier. p. 324-336. e2.

Silva, G. G. Z., K. T. Green, B. E. Dutilh, and R. A. Edwards. 2016. SUPER-FOCUS: a tool for agile functional analysis of shotgun metagenomic data. Bioinformatics. 32:354–361. doi:10.1093/bioinformatics/btv584.

Slifierz, M. J., R. M. Friendship, and J. S. Weese. 2015. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. BMC microbiology. 15:1–12.

Smythies, L. E., R. Shen, D. Bimczok, L. Novak, R. H. Clements, D. E. Eckhoff, P. Bouchard, M. D. George, W. K. Hu, S. Dandekar, and P. D. Smith. 2010. Inflammation anergy in human intestinal macrophages is due to smad-induced IκBα expression and NF-κB inactivation. J Biol Chem. 285:19593–19604. doi:10.1074/jbc.M109.069955.

Snoeck, V., B. Goddeeris, and E. Cox. 2005. The role of enterocytes in the intestinal barrier function and antigen uptake. Microbes Infect. 7:997–1004. doi:10.1016/j.micinf.2005.04.003.

Sokol, H. 2019. [Definition and roles of the gut microbiota]. Rev Prat. 69:776–782.

Stein, H. H., J.A. Roth, K. M. Sotak, and O.J. Rojas. 2013. Nutritional value of soy products fed to pigs. Swine Focus, 4.

Stojanov, S., A. Berlec, and B. Štrukelj. 2020. The influence of probiotics on the Firmicutes/Bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. Microorganisms. 8:E1715. doi:10.3390/microorganisms8111715.

Sun, Z., Q. Ma, Z. Li, and C. Ji. 2009. Effect of partial substitution of dietary spray-dried porcine plasma or fishmeal with soybean and shrimp protein hydrolysate on growth performance, nutrient digestibility and serum biochemical parameters of weanling piglets. Asian Australas. J. Anim. Sci. 22:1032–1037. doi:10.5713/ajas.2009.70107.

Taft, D. H., J. Liu, M. X. Maldonado-Gomez, S. Akre, M. N. Huda, S. M. Ahmad, C. B. Stephensen, and D. A. Mills. 2018. Bifidobacterial dominance of the gut in early life and acquisition of antimicrobial resistance. mSphere. 3:e00441-18. doi:10.1128/mSphere.00441-18.

Turcanu, V., T. R. Hirst, and N. A. Williams. 2002. Modulation of human monocytes by Escherichia coli heat-labile enterotoxin B-subunit; altered cytokine production and its functional consequences. Immunology. 106:316–325. doi:10.1046/j.1365-2567.2002.01429.x.

Turnbaugh, P. J., C. Quince, J. J. Faith, A. C. McHardy, T. Yatsunenko, F. Niazi, J. Affourtit, M. Egholm, B. Henrissat, and R. Knight. 2010. Organismal, genetic, and transcriptional

variation in the deeply sequenced gut microbiomes of identical twins. Proc. Natl. Acad. Sci. U.S.A. 107:7503–7508.

U, N., and G. Ra. 2008. Mechanisms of infectious diarrhea. Nature clinical practice. Gastroenterol. Hepatol. 5. doi:10.1038/ncpgasthep1264. Available from: https://pubmed.ncbi.nlm.nih.gov/18813221/

Varley, M. A., and J. Wiseman. 2001. The weaner pig: Nutrition and management. CABI.

Verbrugghe, E., A. Van Parys, B. Leyman, F. Boyen, S. Arnouts, U. Lundberg, R. Ducatelle, W. Van den Broeck, M. A. Yekta, E. Cox, F. Haesebrouck, and F. Pasmans. 2015. Heat-labile enterotoxin of Escherichia coli promotes intestinal colonization of Salmonella enterica. Comp. Immunol. Microbiol. Infect. Dis. 43:1–7. doi:10.1016/j.cimid.2015.09.002.

Vinolo, M. A. R., H. G. Rodrigues, E. Hatanaka, C. B. Hebeda, S. H. P. Farsky, and R. Curi. 2009. Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites. Clin Sci (Lond). 117:331–338. doi:10.1042/CS20080642.

Wan, J., J. Zhang, G. Wu, D. Chen, B. Yu, Z. Huang, Y. Luo, P. Zheng, J. Luo, X. Mao, J. Yu, and J. He. 2019. Amelioration of enterotoxigenic Escherichia coli-induced intestinal barrier disruption by low-molecular-weight chitosan in weaned pigs is related to suppressed intestinal inflammation and apoptosis. Int J Mol Sci. 20:3485. doi:10.3390/ijms20143485.

Wang, H., Z. Zhong, Y. Luo, E. Cox, and B. Devriendt. 2019. Heat-Stable enterotoxins of enterotoxigenic Escherichia coli and their impact on host immunity. Toxins (Basel). 11:24. doi:10.3390/toxins11010024.

Wang, T., and R. J. Xu. 1996. Effects of colostrum feeding on intestinal development in newborn pigs. Biol Neonate. 70:339–348. doi:10.1159/000244385.

Wang, W., H. Hu, R. T. Zijlstra, J. Zheng, and M. G. Gänzle. 2019. Metagenomic reconstructions of gut microbial metabolism in weanling pigs. Microbiome. 7:48. doi:10.1186/s40168-019-0662-1.

Wang, X., and P. R. Hardwidge. 2012. Enterotoxigenic Escherichia coli prevents host NF-κB activation by targeting IκBα polyubiquitination. Infect. Immun. 80:4417–4425. doi:10.1128/IAI.00809-12.

Ward, J. B. J., Simon J Keely, and Stephen J Keely. 2014. Oxygen in the regulation of intestinal epithelial transport. J. Physiol. 592:2473–2489. doi:10.1113/jphysiol.2013.270249.

Waterman, S. R., and P. L. C. Small. 1998. Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. Appl. Environ. Microbiol. 64:3882–3886.

Wemheuer, F., J. A. Taylor, R. Daniel, E. Johnston, P. Meinicke, T. Thomas, and B. Wemheuer. 2020. Tax4Fun2: prediction of habitat-specific functional profiles and functional redundancy based on 16S rRNA gene sequences. Environmental Microbiome. 15:11. doi:10.1186/s40793-020-00358-7.

Wijtten, P. J. A., J. van der Meulen, and M. W. A. Verstegen. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. Br. J. Nutr.105:967–981. doi:10.1017/S0007114510005660.

Wilson, K. H., and F. Perini. 1988. Role of competition for nutrients in suppression of Clostridium difficile by the colonic microflora. Infect. Immun. 56:2610–2614. doi:10.1128/iai.56.10.2610-2614.1988.

Winston, J. A., and C. M. Theriot. 2016. Impact of microbial derived secondary bile acids on colonization resistance against Clostridium difficile in the gastrointestinal tract. Anaerobe. 41:44–50. doi:10.1016/j.anaerobe.2016.05.003.

Wolf, M. K. 1997. Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic Escherichia coli. Clin. Microbiol. Rev. 10:569–584.

Xiong, X., B. Tan, M. Song, P. Ji, K. Kim, Y. Yin, and Y. Liu. 2019. Nutritional intervention for the intestinal development and health of weaned pigs. Front. Vet. Sci. 6.

Yao, T., M.-H. Chen, and S. R. Lindemann. 2020. Carbohydrate complexity structures stable diversity in gut-derived microbial consortia under high dilution pressure. bioRxiv. 2020.01.31.929760. doi:10.1101/2020.01.31.929760.

Yokota, A., S. Fukiya, K. B. M. S. Islam, T. Ooka, Y. Ogura, T. Hayashi, M. Hagio, and S. Ishizuka. 2012. Is bile acid a determinant of the gut microbiota on a high-fat diet? Gut Microbes. 3:455–459. doi:10.4161/gmic.21216.

Zhang, H., C. Malo, C. R. Boyle, and R. K. Buddington. 1998. Diet influences development of the pig (Sus scrofa) intestine during the first 6 hours after birth. J Nutr. 128:1302–1310. doi:10.1093/jn/128.8.1302.

Zhang, H., C. Malo, and R. K. Buddington. 1997. Suckling induces rapid intestinal growth and changes in brush border digestive functions of newborn pigs. Nutr. J. 127:418–426. doi:10.1093/jn/127.3.418.

Zhang, S., F. Chen, Y. Zhang, Y. Lv, J. Heng, T. Min, L. Li, and W. Guan. 2018. Recent progress of porcine milk components and mammary gland function. J. Anim. Sci. Biotechnol. 9:77. doi:10.1186/s40104-018-0291-8.

Zheng, L., M. E. Duarte, A. Sevarolli Loftus, and S. W. Kim. 2021. Intestinal health of pigs upon weaning: Challenges and nutritional intervention. Front. Vet. Sci. 8:628258. doi:10.3389/fvets.2021.628258.

Zheng, L., C. J. Kelly, and S. P. Colgan. 2015. Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A review in the theme: Cellular responses to hypoxia. Am J Physiol Cell Physiol. 309:C350–C360. doi:10.1152/ajpcell.00191.2015.

Zheng, X., F. Huang, A. Zhao, S. Lei, Y. Zhang, G. Xie, T. Chen, C. Qu, C. Rajani, B. Dong, D. Li, and W. Jia. 2017. Bile acid is a significant host factor shaping the gut microbiome of diet-induced obese mice. BMC Biology. 15:120. doi:10.1186/s12915-017-0462-7.

Zhou, H., J. Sun, B. Yu, Z. Liu, H. Chen, J. He, X. Mao, P. Zheng, J. Yu, J. Luo, Y. Luo, H. Yan, L. Ge, and D. Chen. 2021. Gut microbiota absence and transplantation affect growth and intestinal functions: An investigation in a germ-free pig model. Anim Nutr.7:295–304. doi:10.1016/j.aninu.2020.11.012.

CHAPTER 2

DIETARY SUPPLEMENTATION WITH *BACILLUS SUBTILIS* MODIFIED THE INTESTINAL MICROBIOME OF WEANED PIGS IN COMPARISON TO ANTIBIOTICS

ABSTRACT

The objective of this study was to investigate the effects of supplementing *Bacillus subtilis* on the intestinal microbiota of pigs experimentally infected with F-18 *Escherichia coli (E. coli),* in comparison to carbadox. Forty-eight weaned pigs (6.17 ± 0.36 kg BW) were individually housed and randomly allotted to one of four treatments with 12 pigs per treatment: negative control (NC), positive control (PC), antibiotics (AGP, 50 mg/kg of carbadox), and direct fed microbials (DFM, 500 mg/kg of *Bacillus subtilis*). The experiment lasted 28 days with 7 days before and 21 days after the first *E. coli* inoculation (d 0). Pigs in the PC, AGP, and DFM groups were orally inoculated with F18 *E. coli* for 3 consecutive days with a dosage of 10^{10} CFU per 3 mL. Fecal samples were collected on d -7 and 0 before *E. coli* inoculation, and d 7 and 21 post-inoculation (PI) for longitudinal investigation of the fecal microbiome changes. All pigs were euthanized on d 21 PI to collect digesta from the jejunum, ileum, and distal colon to analyze the spatial heterogeneity of microbiota across different intestinal segments. DNA was extracted from feces and intestinal contents to perform 16S rRNA amplicon sequencing of the V4 hypervariable region. Chao1 index was the greatest ($P < 0.05$) in feces collected on d 0 before *E. coli* inoculation and lowest ($P < 0.05$) in d -7 feces. Shannon and Chao1 indices were similar between feces and colon, which were higher ($P < 0.05$) than those in jejunum and ileum. Pigs supplemented with DFM had lower ($P < 0.05$) Chao1 index in colon than pigs fed with antibiotics on d 21 PI. Bray-Curtis PCoA displayed separate clusters among days, but treatments

overlapped within different intestinal segments except in the colon. Bacteroidetes and Proteobacteria were the most ($P < 0.05$) abundant in feces on d -7 and lowest ($P < 0.05$) on d 21 PI. However, Actinobacteria and Firmicutes were most ($P < 0.05$) abundant in feces on d 21 PI. Firmicutes were more ($P < 0.05$) abundant in jejunum and ileum than colon, whereas Bacteroidetes were more ($P < 0.05$) abundant in colon. Pigs in the NC and DFM groups had greater ($P < 0.05$) relative abundance of Firmicutes in feces than pigs fed AGP on d 0 and d 7 PI. Pigs supplemented with DFM had higher ($P < 0.05$) relative abundance of Firmicutes, but lower ($P < 0.05$) relative abundance of Bacteroidetes than pigs supplemented with AGP in ileum. In comparison with all treatments, AGP supplementation reduced ($P < 0.05$) the relative abundance of *Lactobacillaceae* in feces on d 0 and in ileum and colon on d 21 PI. Both age and carbadox treatments influenced the fecal microbiome of weaned pigs infected with F18 *E. coli* but *B. subtilis* treatments had limited effect on the fecal microbiome. Supplementations with *B. subtilis* and carbadox impacted the intestinal microbiome differently in weaned pigs infected with F18 *E. coli*.

Key words: Antibiotics, *Bacillus subtilis*, *Escherichia coli* challenge, Microbiome, Weaned pigs

INTRODUCTION

Transitioning from farrowing to the nursery stage is known to aggravate the stress of pigs raised for pork production. Newly weaned pigs can experience extreme discomfort when separated from their sows, as this change is also accompanied by a sudden change in diet, environment, and social life conditions (Campbell et al., 2013). Prolonged exposure to stress has been shown to adversely impact the health and performance of pigs, resulting in huge economic losses. Stress can also induce microbial imbalance in the gut and increase vulnerability to

pathogens (Madison and Kiecolt-Glaser, 2019). Enterotoxigenic *E. coli* (ETEC) is an intestinal pathogen that is commonly known to induce secretory diarrhea and intestinal inflammation in pigs under post-weaning stress (Fairbrother et al., 2005; Lallès et al., 2004). In-feed antibiotics, commonly known as antibiotic growth promoters (AGP), are used in nursery diets to alleviate post-weaning diarrhea and to promote growth in weaned pigs. Pigs on average consumed approximately 172 mg of antibiotics per kilogram body weight globally, which was greater than the amounts that cattle and chicken consumed (Boeckel et al., 2015). Boeckel et al. (2015) stated that global antimicrobial consumption is estimated to increase by 67% between 2010 and 2030. The extensive use of AGP, however, may increase the chance of dispersing antimicrobial residues and the development of antimicrobial resistance in bacteria (Menkem et al., 2019; F. Ma et al., 2021). In addition, AGP can modify the gut microbiota of pigs and essentially kill beneficial bacteria and increase their susceptibility to infections (Holman et al., 2017). The heightening concerns towards antibiotics and AGP use has led to the FDA to prohibit the use of AGP (FDA-2011-D-0889) in livestock as growth promoters and alternatives to in-feed antibiotics must be found (FDA, 2013).

The importance of the gut microbiome in human and animal health, particularly in gut health has been largely reviewed (Kamada et al., 2013; Fouhse et al., 2016; Ke et al., 2019). When weaning, intestinal tracts of pigs were reported to have a temporary loss of microbial diversity including adecrease in *Lactobacillus* spp., and an increase in a family of pathogenic bacteria, *Enterobacteriaceae (*Cao et al., 2016; Tian et al., 2017; Gresse et al., 2017). The disturbance of the gut microbiome, in combination with immature intestinal immunity, can lead to the expansion of enteric pathogens, such as ETEC, which can lead to post-weaning diarrhea. Direct-fed microbials (DFM) have been applied to nursery diets to enhance intestinal health and

performance and to maintain pig health (Liao and Nyachoti, 2017). *Bacillus*-based DFMs are spore-forming and thermostable for feed storage and processing, therefore *Bacillus*-based DFMs are commonly provided to livestock animals in-feed. In our previous study, we observed that supplementing *B. subtilis* DSM 25841 reduced diarrhea and enhanced the growth performance of weaned pigs experimentally infected with ETEC (He et al., 2020b). However, the impacts of this *Bacillus* strain on the gut microbiota of weaned pigs under post-weaning diarrhea has yet to be addressed. Thus, the major objectives of the present study are: 1) to determine the impacts of ETEC infection on the fecal and intestinal microbiome of weaned pigs; and 2) to investigate the effects of supplementing *B. subtilis* DSM 25841 on the gut microbiome of ETEC infected pigs, in comparison to antibiotics.

MATERIALS AND METHODS

Animals and study design

Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC #19322) at the University of California, Davis (UC Davis). A total of 48 weaned pigs (21 day (d) old; 6.17 ± 0.36 kg) with equal number of barrows and gilts were obtained from the UC Davis Swine Teaching and Research Center and the experiment was conducted at Cole facility at UC Davis. The current experiment was conducted for a previous study (He et al., 2020). All pigs and their sows did not receive *E. coli* vaccines, antibiotic injections, or antibiotics in feed prior to the experiment. After weaning, pigs were individually housed (pen size: 0.61 m × 1.22 m) and assigned to one of 4 treatment groups using a randomized complete block design with sex normalized by body weight and litter as blocks and pig as experimental unit. Four treatments were: (1) negative control (NC): control diet and without *E. coli* challenge, (2) positive control (PC): control diet and with ETEC challenge, (3) AGP:

inclusion of 50 mg/kg carbadox with ETEC challenge, (4) DFM: inclusion of 500 mg/kg *Bacillus subtilis* DSM 25841 (2.56×10^9 CFU/kg) with ETEC challenge. There were 12 replicate pigs per treatment. The experiment lasted 28 days with first 2 weeks as phase 1 and last 2 weeks as phase 2. Therefore, 6 diets were prepared and all diets met the current estimates for nutrient requirements for nursery pigs (NRC, 2012; Table 2-1).

The experiment included a 7-day habituation period and 21 days after the first ETEC F18 inoculation (d 0). Pigs in the ETEC challenge groups received 3 oral doses of ETEC F18 at 10^{10} CFU per dose, while pigs in NC group were orally inoculated with 3 mL phosphate-buffered saline per day. The ETEC F18 were cultured at the Western institute for Food Safety & Security at UC Davis. The bacterial strain was originally isolated from a field disease outbreak by the University of Illinois Veterinary Diagnostic Lab (isolate number: U.IL-VDL # 05-27242) and the strain expresses heat-labile toxin, heat-stable toxin b, and Shiga-like toxins. Our previous research confirmed the current ETEC challenge dosage induced mild diarrhea in weaned pigs (Liu et al., 2013; Kim et al., 2019a; Kim et al., 2019b). The detailed animal study procedures and data for growth performance and diarrhea were reported in He et al. (2020).

Sample collection

Prior to weaning, tail samples were collected from all piglets to assess their susceptibility to ETEC F18 using the genotyping analysis described in Kreuzer et al. (2013). All pigs used in the present study were susceptible to ETEC F18. Fresh fecal samples were collected at the beginning of the experiment (d -7), d 0 before ETEC inoculation, and d 7 and 21 post-inoculation (PI). Samples were immediately stored at −80°C until further analysis. At the termination of the experiment (d 21 PI), all pigs were euthanized. For euthanasia, pigs were anesthetized by intramuscularly injecting a 1 mL mixture of telazol (100 mg) ketamine (50 mg), and xylazine (50

mg) prior to an intracardiac injection of 78 mg sodium pentobarbital (Vortech Pharmaceuticals, Ltd., Dearborn, MI) per 1 kg of body weight. Digesta was collected from the middle of the jejunum, ileum, and distal colon and was immediately frozen into liquid nitrogen and stored at −80°C until further analysis.

Library preparation

Bacterial DNA was extracted from fecal samples and intestinal digesta using the Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA samples were amplified by PCR at the V4 region of the 16S rRNA gene using primers 515F (5′-XXXXXXXX**GT**GTGCCAGCMGCCGCGGTAA-3′), which included an 8-nt barcode (X) unique to each sample followed by a 2-nt Illumina adapter (bold), and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). Samples were PCR amplified in duplicats, and each PCR reaction was comprised of 2 µL template DNA, 9.5 µL nuclease free water, 12.5 µL GoTaq 2× Master Mix (Promega, Madison, WI, USA), 0.5 µL V4 reverse primer (10 µM), and 0.5 µL barcoded forward primer (10 µM). Amplification was performed in a thermocycler with the following settings: 94°C for 3 min for initial denaturation; followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1.5 min; and 72°C for 10 min for final elongation. Agarose gel electrophoresis was used to verify the amplicon size for each sample and band brightness was observed to subjectively quantify the amount of sample to be added when pooling PCR products. Pooled samples were then purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and submitted to the UC Davis Genome Center DNA Technologies Core for 250 bp paired-end sequencing on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA).

Microbiota analysis

Raw fastq files were first demultiplexed and barcode sequences were removed using the software sabre (https://github.com/najoshi/sabre) and demultiplexed sequences were then imported into Quantitative Insights Into Microbial Ecology 2 (QIIME2; version 2019.4) (Bolyen et al., 2019: 2). Using the DADA2 plugin, primers and lower quality reads were removed and the paired end reads were denoised and merged (Callahan et al., 2016). Chimeras were removed after merging and amplicon sequence variants (ASVs) were constructed. Representative sequences for each ASV were aligned using MAFFT and masked alignments were used to generate phylogenetic trees using FastTree2 (Price et al., 2010; Katoh and Standley, 2013). Python library scikit-learn was used to assign taxonomy based on representative sequences against Silva (version 138), which was pre-trained in QIIME2 and clipped to only the V4 hypervariable region and clustered at 99% sequence identity (Pedregosa et al., 2011; Quast et al., 2012; Bokulich et al., 2018).

Shannon and Chao1 indices were measured for alpha diversity by using the estimate_richness function in phyloseq (McMurdie and Holmes, 2013). To compare community composition among treatments and day or intestinal site, the Bray-Curtis matrix was used to calculate beta diversity. The principal coordinate analysis (PCoA) plot was used to visualize dissimilarities between samples employing the Bray-Curtis distance matrix. Relative abundance of each taxon in each sample was calculated by dividing the taxa count by total number of filtered reads within each sample.

Statistical analyses

Files were exported from QIIME2 and imported into R 4.1.0 for data visualization and statistical analysis (Team, 2021). All microbiota analyses was performed using the phyloseq

package and data were visualized using the ggplot2 package (Wickham, 2011). Normality and homoscedasticity were tested using the Shapiro Wilks test and Bartlett test, respectively. A linear mixed-effect model was fitted using the lme4 package with treatment and site or day and interaction as fixed effects while pig as random effect (Bates et al., 2014). Significance of each term in the model was determined using the F-test as type 3 analysis of variance using the Anova function in the car package, followed by group comparison using the cld function in the emmeans package (Fox and Weisberg, 2018; Lenth, 2021). When normality or homoscedasticity was not observed, non-parametric testing was performed using the Kruskal-Wallis sum-rank test using the agricolae package (de Mendiburu and de Mendiburu, 2019). Bray-Curtis dissimilarity was first tested for homoskedasticity using the betadisper function and the statistical significance was tested using PERMANOVA and the vegan package (Oksanen et al., 2013). Statistical significance was assessed at $\alpha = 0.05$ and statistical tendency at $\alpha = 0.10$, and *P*-values were adjusted for multiple comparisons using false discovery rate (FDR).

RESULTS

Shifts in fecal microbiota with age

For feces, the mean sampling read per sample was 13,974 and the total number of taxa identified was 2,814 in the sequence data. No significant difference in Shannon index was observed among treatments throughout the experiment (Figure 2-1A). Sampling day influenced ($P < 0.05$) Chao1 index and an increase ($P < 0.05$) in Chao1 index was observed in all treatments from d -7 to d 0 (Figure 2-1B). Feces collected from pigs in the PC and DFM groups had decreased ($P < 0.05$) Chao1 index on d 7 PI, compared with feces collected on d 0. In beta diversity, the samples grouped according to sampling days, as indicated by the statistical difference using adonis2 ($R^2 = 0.20$; $P < 0.05$) (Figure 2-2A). No distinctive clusters were

observed among treatments on d -7 and d 7 PI. However, a separated cluster between AGP and DFM was observed on d 0 and d 21 PI (Figure 2-2B).

Relative abundances of various phyla and families are presented in Table 2-2. Firmicutes and Bacteroidetes were the two most abundant phyla in feces throughout the experiment, accounting for more than 75% in relative abundance per treatment group on each sampling day. No difference was observed in the relative abundance of the top 6 phyla in feces among all treatments on d -7. The relative abundance of Firmicutes was increased ($P < 0.05$), while the relative abundance of Bacteroidetes was decreased ($P < 0.05$) in NC as pig age increased. No difference was observed in the relative abundance of Spirochaetes, Actinobacteria, and Euryarchaeota among treatments and sampling days. On d 0 and d 7 PI, pigs in NC and DFM had greater ($P < 0.05$) relative abundance of Firmicutes in feces than pigs in PC and AGP. On the concluding day of the experiment (d 21 PI), pigs in the AGP group had lower (77.22% vs 82.36%, $P < 0.05$) relative abundance of Firmicutes in feces than pigs in NC. No difference was observed in the relative abundance of Bacteroidetes between NC and PC on any sampling date. The relative abundance of Bacteroidetes was lower (10.03% vs. 18.31%, $P < 0.05$) in feces of pigs fed with DFM than pigs supplemented with AGP on d 7 PI. Pigs in PC had greater ($P < 0.05$) relative abundance of Proteobacteria in feces than pigs in NC on d 7 PI. No difference was observed in the relative abundance of Proteobacteria between AGP and DFM throughout the experiment.

Within the Firmicutes phylum, the relative abundance of *Bacillaceae* was the greatest ($P < 0.05$) in DFM among all treatment on d 0, 7, and 21 PI. The relative abundance of *Lactobacillaceae* in NC was increased ($P < 0.05$) in feces on d 0 but decreased ($P < 0.05$) on d 21 PI, compared with feces collected on d -7 (Table 2-2). In addition, pigs supplemented with

AGP had reduced ($P < 0.05$) relative abundance of *Lactobacillaceae* in feces on d 0 and d 21. The relative abundance of *Lactobacillaceae* was lower ($P < 0.05$) in feces of pigs fed with AGP than that in DFM (4.44% vs. 23.98%) on d 21 PI. Pigs in PC, AGP, and DFM had increased ($P < 0.05$) relative abundance of *Lachnospiraceae* between d -7 and 0. *Bacteroidaceae* was the most abundant family in Bacteroidetes phylum on d -7, while *Prevotellaceae* was the most abundant one on d 0, 7, 21 PI (Table 2-2). Pigs in AGP had greater ($P < 0.05$) relative abundance of *Bacteroidaceae* in feces than pigs in NC and DFM on d 7 and 21 PI. Within Proteobacteria phylum, the relative abundance of *Enterobacteriaceae* was decreased ($P < 0.05$) but the relative abundance of *Succinivibrionaceae* was increased ($P < 0.05$), as pig age increased (Table 2-2). Pigs in PC and AGP had greater ($P < 0.05$) relative abundance of *Enterobacteriaceae* than NC on d 7 PI. However, no difference was observed in the relative abundance of *Enterobacteriaceae* among other treatments on d 7 PI and other sampling dates. The relative abundance of *Succinivibrionaceae* was higher ($P < 0.05$) in PC and AGP than pigs in NC on d 21 PI.

The top 11 most abundant genera in feces are presented in Figure 2-3. Between d -7 and 21 PI, all treatments increased ($P < 0.05$) the relative abundance of *Blautia* and reduced ($P < 0.05$) the relative abundance of *Bacteroides* and *Escherichia-Shigella*. All treatments except AGP reduced ($P < 0.05$) the relative abundance of *Megasphaera* between d -7 and 21 PI. The most abundant genus was *Lactobacillus*. In NC pigs, the relative abundance of *Lactobacillus* was increased ($P < 0.05$) between d -7 and 0 but then decreased ($P < 0.05$) by d 21 PI. Pigs in AGP had the lowest abundance ($P < 0.05$) of *Lactobacillus* on d 0 and pigs in DFM had the greatest abundance ($P < 0.05$) of *Lactobacillus* on d 21 PI. In addition, the relative abundance of *Lactobacillus* was greater in DFM (27.91% vs. 20.73%, $P < 0.05$) than in AGP on d 7 PI. The relative abundance of *Blautia* was greater ($P < 0.05$) in feces collected on d 0, 7 PI, and 21 PI than

in feces collected on d -7. The relative abundance of *Bacteroides* was greater ($P < 0.05$) in AGP than in PC on d 0 and was greater ($P < 0.05$) in AGP than in DFM on d 7 and 21 PI.

Shifts in gut microbiota within different intestinal sites

The mean sampling read was 14,939 per sample and the total number of taxa identified was 1,854 in the sequence data. The microbial composition of the digesta of the jejunum, ileum, and colon was also investigated at the conclusion of this experiment (d 21 PI). Significant interaction between treatment and intestinal site was observed ($P < 0.001$) in both Shannon and Chao1 diversities (Figure 2-4). Shannon and Chao1 indices in colon digesta were greater ($P < 0.05$) than in jejunal and ileal digesta. No difference was observed in Shannon and Chao1 indices between NC and PC in any of intestinal sites. Pigs supplemented with AGP had greater ($P < 0.05$) Shannon index value than other treatments in the ileum (Figure 2-4A) and had greater ($P < 0.05$) Chao1 index than pigs in NC in the jejunum (Figure 2-4B). Supplementation of DFM reduced ($P < 0.05$) Chao 1 index in ileal digesta when compared with NC.

The adonis2 test demonstrated significance in treatment ($R^2 = 0.10$, $P < 0.05$), intestinal site ($R^2 = 0.18$, $P < 0.05$), and treatment and intestinal site interaction ($R^2 = 0.05$, $P < 0.05$). In Figure 2-5A, all colon samples were clustered together and were separated from jejunal and ileal samples, whereas clusters for the ileum and jejunum were indistinguishable from each other. No clear separation was observed among treatments in jejunum, while the AGP cluster was distinct from DFM cluster in ileum (Figure 2-5B). Within colon samples, the AGP cluster partially overlapped with the DFM cluster, while NC and PC clusters were overlapping with each other.

The relative abundance of phyla, families, and genera are presented in Table 2-3. Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria were the top four abundant phyla in the intestinal tract of weaned pigs. Firmicutes was the most abundant phylum in jejunum,

ileum, and colon in all pigs. Unlike fecal samples, Bacteroidetes was second most abundant phylum in colon, Actinobacteria was the second most abundant phylum in jejunum, and Proteobacteria was the second most abundant phylum in ileum. No difference was observed in the relative abundance of phyla between NC and PC in all intestinal sites. The relative abundance of Bacteroidetes in ileal digesta was greater ($P < 0.05$) in AGP than DFM. The relative abundance of Actinobacteria in ileal digesta was greater ($P < 0.05$) in PC than AGP. Pigs in AGP had the greatest ($P < 0.05$) relative abundance of Proteobacteria in jejunal digesta among all treatments and had greater ($P < 0.05$) relative abundance of Proteobacteria in ileal digesta than pigs in DFM (4.12% vs. 0.22%).

At the family level, jejunal digesta contained the highest ($P < 0.05$) relative abundance of *Lactobacillaceae, Actinomycetaceae,* and *Micrococcaceae* among the three intestinal sites. Ileal digesta had more ($P < 0.05$) relative abundance of *Clostridiaceae1* and *Enterobacteriaceae* than jejunal and colon digesta. Colon digesta contained more ($P < 0.05$) *Lachnospiraceae, Ruminococcaceae*, *Veillonellaceae,* the top 4 families under Bacteroidetes, *Coriobacteriaceae*, and *Succinivibrionaceae* than jejunal and ileal digesta. The relative abundance of *Lactobacillaceae* was greater ($P < 0.05$) in DFM than NC and AGP in ileal digesta and was greater ($P < 0.05$) in DFM than AGP in colon digesta. ETEC F18 challenge increased ($P < 0.05$) the relative abundance of *Actinomycetaceae* and *Micrococcaceae* in jejunal and ileal digesta, *Lachnospiraceae* in ileal digesta and *Succinivibrionaceae* in colon digesta, but reduced ($P < 0.05$) *Streptococcaceae* in colon digesta. Compared with PC, supplementation of AGP enhanced ($P < 0.05$) the relative abundance of *Peptostreptococcaceae* and *Pasteurellaceae* in jejunal digesta, *Muribacelaceae* in ileal digesta, and *Bacteroidaceae* and *Rikenellacea*e in colon digesta. In addition, inclusion of AGP reduced ($P < 0.05$) the relative abundance of *Veillonellaceae*,

Actinomycetaceae, *Atopobiaceae*, *Bifidobacteriaceae*, *Eggerthellaceae* in jejunal digesta, *Lachnospiraceae*, *Actinomycetaceae*, *Bifidobacteriaceae* in ileal digesta, and *Veillonellaceae* and *Coriobacteriaceae* in colon digesta. In comparison to PC, supplementation of DFM reduced ($P < 0.05$) the relative abundance of *Actinomycetaceae* in jejunal digesta and *Lachnospiraceae* in ileal digesta. Pigs fed with DFM had greater ($P < 0.05$) the relative abundance of *Ruminococcaceae*, *Veillonellaceae*, *Bifidobacteriaceae* in jejunal digesta, *Lactobacillaceae* in both ileum and colon, and *Bifidobacteriaceae* in colon when compared with pigs in AGP. However, pigs fed with DFM had reduced ($P < 0.05$) the relative abundance of *Pasteurellaceae* in jejunum, *Ruminococcaceae*, *Rikenellaceae*, *Pasteurellaceae*, *Succinivibrionaceae* in ileum, and *Bacteroidaceae* in colon, compared with pigs fed with AGP. In all intestinal sites, pigs in DFM had the highest ($P < 0.05$) relative abundance of *Bacillaceae* among all treatments.

The relative abundances of the top abundant genera are presented in Figure 2-6. Relative abundance of *Lactobacillus* was greater ($P < 0.05$) but the relative abundance of *Clostridium sensu stricto 1* was less ($P < 0.05$) in ileal and colon digesta of pigs fed with DFM than in AGP. ETEC infection reduced the relative abundance of *Streptococcus* in ileum and colon, however, pigs supplemented with AGP had more ($P < 0.05$) *Streptococcus* in jejunum but lower ($P < 0.05$) *Streptococcus* in ileum and colon when compared with NC.

DISCUSSION

Our previous studies reported that dietary supplementation of *B. subtilis* promoted growth performance, reduced diarrhea, and enhanced intestinal immunity in weaned piglets under ETEC challenge (Kim et al., 2019b; He et al., 2020b). However, the potential mechanisms of the beneficial effects of *B. subtilis* on swine health and the impacts of *B. subtilis* on intestinal microbiota remain unclear. In the present study, we characterized the impacts of ETEC infection,

DFM, and AGP on the dynamics of microbial composition in feces during the weaning transition period. The microbiota in different segments of the intestine were also investigated as ETEC mainly targets the small intestine of weaned pigs. Identifying shifts in microbial communities in the gut could facilitate the development of nutritional and microbial interventions to control post-weaning diarrhea when the use of AGP is restricted, and to understand the impacts of currently available DFM on swine health.

Fecal microbiota

Temporal analysis of fecal microbiota was performed using fecal samples collected throughout the experiment. Richness in microbial populations was measured with Chao1 and richness and evenness were measured with the Shannon index (Shannon, 1948; Chao, 1984). Lack of significant treatment effect was observed in the Shannon diversity metric in fecal samples of pigs throughout the experiment. Microbial richness was increased during the habituation period (from d -7 to 0) but decreased from d 0 to d 7 PI in feces collected from pigs in the positive control, which indicated that ETEC F18 challenge reduced microbial richness in feces. Beta diversity measures the variability in microbial community composition among samples. We observed distinctive clusters between d 0 and d 7 PI; however, it was not clearly evident that ETEC F18 affected the variability in microbial community composition. These results indicate that the microbiota was shifted due to ETEC challenge, thus the modulation in gut microbiota when supplemented with antibiotics or *B. subtilis* was observed to be limited. Therefore, future studies should implement either AGP or DFM supplementation on weaned pigs when not challenged with ETEC.

Firmicutes and Bacteroidetes were the top two most abundant phyla in fecal samples throughout the experiment. As the pigs got older, the relative abundance of Firmicutes increased

and the relative abundance of Bacteroidetes decreased. These findings were consistent with previous publications (Mach et al., 2015; Chen et al., 2017) and indicate that the fecal microbiota shifts during the post-weaning period likely due to the sudden change in diet from sow milk to solid and plant-based meal and the changes in environment after sow separation. At the family level, weaning reduced the relative abundance of *Peptostreptococcaceae*, *Streptococcaceae*, and *Bacteroidaceae*, but increased the relative abundance of *Lactobacillaceae* in feces from NC when comparing d 0 with d -7. As the pigs recovered from weaning stress, the relative abundance of *Clostridiaceae1*, *Peptostreptococcaceae*, *Streptococcaceae*, and *Lactobacillaceae* in feces was reversed by d 21 PI, while the relative abundance of *Bacteroidaceae* and *Enterobacteriaceae* remained low and *Lachnospiraceae* gradually increased as the age of pigs increased. Most of the influenced bacterial families are considered part of the swine core gut microbiome (Mach et al., 2015; Chen et al., 2017; Pollock et al., 2019).These results suggest that the impacts of weaning stress on gut microbiota are significant but temporary. When pigs adapt to their new diets and environment, diets can be a main driver of regulating gut microbial composition.

The present study observed that ETEC inoculation also affected the fecal microbiome when comparing PC and NC groups on d 7 and 21 PI. Reduced relative abundance of Firmicutes and increased relative abundance of Proteobacteria were observed in pigs challenged with ETEC on d 7 PI. At the family level, both *Bacteroidaceae* and *Enterobacteriaceae* were increased in feces due to ETEC infection. ETEC infection in pigs can reduce pig appetite, disrupt intestinal barrier, and induce intestinal inflammation, all of which may contribute to the imbalance of the microbiota to promote favorable condition for ETEC to further proliferate (Pollock et al., 2019). ETEC is a Proteobacteria in the family of *Enterobacteriaceae*, which is likely the reason for the

increased relative abundance of Proteobacteria and *Enterobacteriaceae*, congruent with clinical signs of ETEC infection seen in the pigs (He et al., 2020). Similar results were also reported in mice inoculated with ETEC (Wang et al., 2018). Growing evidence suggests that an increased abundance of Proteobacteria might be associated with dysbiosis (Holman et al., 2017). The impacts of ETEC inoculation on the fecal microbiome were gradually reduced when pigs recovered from ETEC infection by d 21 PI. Our previous research reported that ETEC infected pigs were fully recovered on d 21 PI, with no diarrhea and no β-hemolytic coliforms present in feces (He et al., 2020).

The use of carbadox in this experiment also resulted in changes in the fecal microbiota. During the habituation period, supplementation with carbadox reduced relative abundance of Firmicutes and *Lactobacillaceae* in feces. The relative abundance of Bacteroidetes was also numerically higher than pigs in negative and positive control groups, despite a lack of statistical significance. These observations could indicate the impacts of in-feed antibiotics on the gut microbiome were immediate (Lourenco et al., 2021). The reduced *Lactobacillaceae* suggests that carbadox may reduce the favorable bacteria in the intestine of weaned pigs. Although ETEC infection temporarily increased the relative abundance of *Lactobacillaceae* in feces of pigs fed with antibiotics, the abundance was sharply reduced when pigs were recovered from ETEC infection.

Compared with carbadox, the influences of *B. subtilis* on the fecal microbiome of weaned pigs was limited throughout the experiment. The relative abundance of *Bacillaceae* was the greatest in DFM among all treatments on d 0 and d 21 PI, which is likely due to *B. subtilis* belonging to the family *Bacillaceae.* Supplementation of *B. subtilis* significantly enhanced the relative abundance of Firmicutes, but numerically reduced Proteobacteria and

Enterobacteriaceae in feces at the peak of ETEC infection. This observation is consistent with fecal culture results with the same pigs used in He et al. (2020), in which pigs fed with *B. subtilis* had a lower percentage of β-hemolytic coliforms in feces on d 7 PI. Our previous research revealed that both carbadox and *B. subtilis* supplementation were effective at enhancing growth performance and reducing diarrhea in weaned pigs challenged with ETEC F18 (He et al., 2020). However, results here suggest that the impacts of dietary supplementation of *B. subtilis* and antibiotics on the fecal microbiome of weaned pigs were different. The major highlights were the modulation of the Firmicutes phylum. Overall, pigs fed with *B. subtilis* contained relatively higher *Lactobacillaceae* than pigs fed with antibiotics throughout the experiment, although ETEC infection temporarily reduced this difference on d 7 PI. In addition, pigs supplemented with *B. subtilis* also had lower relative abundance of Bacteroidetes and *Bacteroidaceae* than pigs fed with carbadox during the peak of ETEC infection. Carbadox is an oxidative DNA-damaging agent which can disturb bacterial DNA synthesis and induce the breakdown of chromosomes in many gram-negative bacteria, including ETEC (Suter et al., 1978; Cheng et al., 2015). However, B. subtilis as a probiotic strain must colonize temporarily into the intestines of pigs in order to perform have beneficial effects on the gut ecosystem. Thus, it is not supersizing surprising to observe the different impacts of these two supplements on the fecal microbiome.

Intestinal microbiota

In the current study, the intestinal microbiota changes were analyzed in digesta samples collected at the end of experiment when pigs were fully recovered from ETEC infection. Similar to the results shown in Crespo-Piazuelo et al. (2018), Shannon and Chao1 index values in the colon were higher than in the jejunum and ileum. Correspondingly, beta diversity results showed clear separation between colon digesta vs. jejunal and ileal digesta, indicating different microbial

diversity is present within the different intestinal sites as expected. Supplementation of carbadox or *B. subtilis* had more influence on microbial diversity and composition in the ileum.

Ileal and jejunal digesta had greater relative abundance of Firmicutes and lowest relative abundance of Bacteroidetes compared to colon digesta, suggesting the luminal environment remarkably modulates the digesta microbiome (Gao et al., 2018; Pollock et al., 2019). The acidic environment and high oxygen level prevents anaerobes from colonizing in the small intestine (Donaldson et al., 2016). In addition, the small intestinal digesta contains more simple carbohydrates that could be utilized by the host and the microbiota, while the large intestine lumen comprises more complex carbohydrates for microbial fermentation (Zoetendal et al., 2012). Enrichment in *Lachnospiraceae*, *Ruminococcaceae*, and *Prevotellaceae* enables the large intestine's capacity to degrade complex carbohydrates (Duncan et al., 2007; Arumugam et al., 2011; Holman et al., 2017). The ileum is the major intestinal site where ETEC colonizes , which is probably the major reason that more abundant *Enterobacteriaceae* were still observed in ileal digesta than in the jejunum and colon (Nagy et al., 1992). However, limited changes were observed in the microbial composition in different intestinal segments when comparing positive control vs. negative control pigs on d 21 PI, which was likely due to the recovery of weaned pigs from ETEC F18 infection (He et al., 2020). The relative abundance of *Micrococcaceae* proliferated in jejunal and ileal digesta of pigs infected with ETEC. *Micrococcaceae* are relatively abundant in newborn pigs, but are gradually reduced during the pre-weaning period (Pena Cortes et al., 2018). The increased abundance of *Micrococcaceae* were also observed in diarrheal fecal samples in a human study (De et al., 2020). However, the reason for this change is still unclear.

In comparison to the positive control, carbadox supplementation had more influences on the intestinal microbiota than did *B. subtilis*. In addition, carbadox supplementation altered the jejunal and ileal microbiota more than the colon microbiota of weaned pigs, which is in close agreement with a previous study using a mixed antibiotic supplementation (Mu et al., 2017). This observation also indicates that antibiotics treatment may mainly target the microbiota in the small intestine. At the taxonomic level, pigs fed with carbadox had greater relative abundance of gram-negative bacteria, including S*uccinivibrionaceae* and *Pasteurellaceae* in the jejunum and *Muribaculaceae* in the ileum, but lower relative abundance of *Lactobacillaceae* and *Bifidobacteriaceae* in the ileum. This observation suggests that gram-negative bacteria may be more tolerant to carbadox due to their complicated outer membrane structure, which contains lipopolysaccharides and peptidoglycans to reduce the chance of carbadox penetrating into the bacterial membranes (Breijyeh et al., 2020). Gram-negative bacteria are more resistant to commonly used antibiotics by developing antibiotics resisting enzymes and undergoing mutations to increase resistance against antibiotics (Miller, 2016; Sumi et al., 2019). Therefore, the accumulation of gram-negative bacteria in the small intestine when carbadox is applied may increase the risk of developing antimicrobial resistance. Pigs supplemented with *B. subtilis* had increased relative abundance of gram-positive bacteria compared to pigs supplemented with carbadox, including *Lactobacillaceae*, *Bacillaceae*, and *Bifidobacteriaceae* in ileal digesta. The increased abundance of *Bacillaceae* was likely due to the colonization of *B. subtilis* in the intestinal tract (Pollock et al., 2019). However, the more enriched *Lactobacillaceae* and *Bifidobacteriaceae* suggest that supplementing *B. subtilis* may help to maintain the desirable bacteria in the small intestine of weaned pigs, which may contribute to the concomitant decrease in enteric bacterial infection. Previous research reported that *B. subtilis* supplementation could

decrease luminal pH and oxidation potential of the gut matrix, which may provide a favorable condition for *Lactobacillaceae* to thrive but inhibit the growth of the pathogenic bacteria (Yang et al., 2015). Therefore, results from the current study indicate that the modulation of the gut microbiome by *B. subtilis* supplementation also contributes to the reduced diarrhea and enhanced intestinal health of ETEC infected pigs.

CONCLUSION

Our previous study reported that supplementation with *B. subtilis* improved growth performance and disease resistance of weaned pigs under ETEC challenge (He et al., 2020). The efficacy of *B. subtilis* DSM 25841 was comparable to an in-feed antibiotic, carbadox. Current results indicate that weaning stress, age of pigs, ETEC infection, and dietary supplements all contributed to the fecal and intestinal microbiota changes in weaned pigs. Supplementation with carbadox and *B. subtilis* differentially modified fecal and intestinal microbiota when pigs were challenged with ETEC. Pigs supplemented with *B. subtilis* had higher abundance of *Bacillaceae* than pigs supplemented with carbadox in fecal and intestinal microbiota. Supplementation of carbadox increased the relative abundance of gram-negative bacteria including Bacteroidetes and Proteobacteria and may impose a risk of increasing antibiotics resistance. Supplementation with *B. subtilis* was associated with an increase or maintenance of the relative abundance of beneficial bacteria including *Lactobacillaceae* and *Bifidobacteriaceae* in the ileum and colon. Future research is needed to quantify *B. subtilis* colonization in the gastrointestinal tract and to investigate the interactions of *B. subtilis* with other bacteria, especially ETEC.

LITERATURE CITED

Arumugam, M., J. Raes, E. Pelletier, D. Le Paslier, T. Yamada, D. R. Mende, G. R. Fernandes, J. Tap, T. Bruls, and J.-M. Batto. 2011. Enterotypes of the human gut microbiome. Nature. 473:174–180.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2014. Fitting linear mixed-effects models using lme4. arXiv preprint arXiv:1406.5823.

Boeckel, T. P. V., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. PNAS. 112:5649–5654. doi:10.1073/pnas.1503141112.

Bokulich, N. A., B. D. Kaehler, J. R. Rideout, M. Dillon, E. Bolyen, R. Knight, G. A. Huttley, and J. Gregory Caporaso. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 6. doi:10.1186/s40168-018-0470-z. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5956843/

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, and F. Asnicar. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37:852–857.

Breijyeh, Z., B. Jubeh, and R. Karaman. 2020. Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. Molecules. 25:1340. doi:10.3390/molecules25061340.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High resolution sample inference from Illumina amplicon data. Nat. Methods. 13:581–583. doi:10.1038/nmeth.3869.

Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. J. Anim. Sci. Biotechnol. 4:19. doi:10.1186/2049-1891-4-19.

Cao, K. F., H. H. Zhang, H. H. Han, Y. Song, X. L. Bai, and H. Sun. 2016. Effect of dietary protein sources on the small intestine microbiome of weaned piglets based on high-throughput sequencing. Lett. Appl. Microbiol. 62:392–398. doi:10.1111/lam.12559

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. J. ISME 6:1621–1624. doi:10.1038/ismej.2012.8.

Chao, A. 1984. Nonparametric estimation of the number of classes in a population. Scandinavian J. Stat.265–270.

Chen, L., Y. Xu, X. Chen, C. Fang, L. Zhao, and F. Chen. 2017. The maturing development of gut microbiota in commercial piglets during the weaning transition. Front. Microbiol. 8:1688. doi:10.3389/fmicb.2017.01688.

Cheng, G., B. Li, C. Wang, H. Zhang, G. Liang, Z. Weng, H. Hao, X. Wang, Z. Liu, M. Dai, Y. Wang, and Z. Yuan. 2015. Systematic and molecular basis of the antibacterial action of quinoxaline 1,4-di-N-oxides against Escherichia coli. PLoS One. 10:e0136450. doi:10.1371/journal.pone.0136450.

Crespo-Piazuelo, D., J. Estellé, M. Revilla, L. Criado-Mesas, Y. Ramayo-Caldas, C. Óvilo, A. I. Fernández, M. Ballester, and J. M. Folch. 2018. Characterization of bacterial microbiota

compositions along the intestinal tract in pigs and their interactions and functions. Sci. Rep. 8:12727. doi:10.1038/s41598-018-30932-6.

De, R., A. K. Mukhopadhyay, and S. Dutta. 2020. Metagenomic analysis of gut microbiome and resistome of diarrheal fecal samples from Kolkata, India, reveals the core and variable microbiota including signatures of microbial dark matter. Gut Pathog. 12:32. doi:10.1186/s13099-020-00371-8.

Donaldson, G. P., S. M. Lee, and S. K. Mazmanian. 2016. Gut biogeography of the bacterial microbiota. Nat. Rev. Microbiol. 14:20–32. doi:10.1038/nrmicro3552.

Duncan, S. h., P. Louis, and H. j. Flint. 2007. Cultivable bacterial diversity from the human colon. Lett. Appl. Microbiol.44:343–350. doi:10.1111/j.1472-765X.2007.02129.x.

Fairbrother, J. M., É. Nadeau, and C. L. Gyles. 2005. Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. 6:17–39. doi:10.1079/AHR2005105.

FDA, U. 2013. Guidance for industry# 213, new animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: recommendations for drug sponsors for voluntarily aligning product use conditions with GFI# 209. Center for Veterinary Medicine, Rockville, MD. http://www. fda. gov/downloads/Animal Veterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM299624. pdf.

Fouhse, J. M., R. T. Zijlstra, and B. P. Willing. 2016. The role of gut microbiota in the health and disease of pigs. Anim. Fron. 6:30–36. doi:10.2527/af.2016-0031.

Fox, J., and S. Weisberg. 2018. An R companion to applied regression. Sage publications.

Gao, J., K. Xu, H. Liu, G. Liu, M. Bai, C. Peng, T. Li, and Y. Yin. 2018. Impact of the Gut Microbiota on Intestinal Immunity Mediated by Tryptophan Metabolism. Front. Cell Infect. Microbiol. 8:13. doi:10.3389/fcimb.2018.00013.

Gresse, R., F. Chaucheyras-Durand, M. A. Fleury, T. Van de Wiele, E. Forano, and S. Blanquet-Diot. 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol. 25:851–873. doi:10.1016/j.tim.2017.05.004.

He, Y., K. Kim, L. Kovanda, C. Jinno, M. Song, J. Chase, X. Li, B. Tan, and Y. Liu. 2020. Bacillus subtilis: a potential growth promoter in weaned pigs in comparison to carbadox. J. Anim. Sci. 98. doi:10.1093/jas/skaa290. Available from: https://doi.org/10.1093/jas/skaa290

Holman, D. B., B. W. Brunelle, J. Trachsel, and H. K. Allen. 2017. Meta-analysis to define a core microbiota in the swine gut. mSystems. 2. doi:10.1128/mSystems.00004-17. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5443231/

Kamada, N., S.-U. Seo, G. Y. Chen, and G. Núñez. 2013. Role of the gut microbiota in immunity and inflammatory disease. Nat. Rev. Immunol. 13:321–335. doi:10.1038/nri3430.

Katoh, K., and D. M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. Molecular Biology and Evolution. 30:772–780. doi:10.1093/molbev/mst010.

Ke, S., S. Fang, M. He, X. Huang, H. Yang, B. Yang, C. Chen, and L. Huang. 2019. Age-based dynamic changes of phylogenetic composition and interaction networks of health pig gut microbiome feeding in a uniformed condition. BMC Vet. Res. 15:1–13.

Kim, K., A. Ehrlich, V. Perng, J. A. Chase, H. Raybould, X. Li, E. R. Atwill, R. Whelan, A. Sokale, and Y. Liu. 2019a. Algae-derived β-glucan enhanced gut health and immune

responses of weaned pigs experimentally infected with a pathogenic E. coli. Anim. Feed Sci. Technol. 248:114–125. doi:10.1016/j.anifeedsci.2018.12.004.

Kim, K., Y. He, X. Xiong, A. Ehrlich, X. Li, H. Raybould, E. R. Atwill, E. A. Maga, J. Jørgensen, and Y. Liu. 2019b. Dietary supplementation of Bacillus subtilis influenced intestinal health of weaned pigs experimentally infected with a pathogenic E. coli. J. Animal Sci. Biotechnol. 10:52. doi:10.1186/s40104-019-0364-3.

Kreuzer, S., M. Reissmann, and G. A. Brockmann. 2013. New fast and cost-effective gene test to get the ETEC F18 receptor status in pigs. Vet. Microbiol. 163:392–394. doi:10.1016/j.vetmic.2012.12.040.

Lallès, J.-P., G. Boudry, C. Favier, N. L. Floc'h, I. Luron, L. Montagne, I. P. Oswald, S. Pié, C. Piel, and B. Sève. 2004. Gut function and dysfunction in young pigs: physiology. Anim. Res. 53:301–316. doi:10.1051/animres:2004018.

Lenth, R. V. 2021. Emmeans: estimated marginal means, aka least-squares means. R package version 1.6. 1.

Liao, S. F., and M. Nyachoti. 2017. Using probiotics to improve swine gut health and nutrient utilization. Anim. Nutr. 3:331–343. doi:10.1016/j.aninu.2017.06.007.

Liu, Y., M. Song, T. M. Che, J. a. S. Almeida, J. J. Lee, D. Bravo, C. W. Maddox, and J. E. Pettigrew. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. J. Anim. Sci. 91:5294–5306. doi:10.2527/jas.2012-6194.

Lourenco, J. M., R. S. Hampton, H. M. Johnson, T. R. Callaway, M. J. Rothrock, and M. J. Azain. 2021. The effects of feeding antibiotic on the intestinal microbiota of weanling pigs. Front. Vet. Sci. 8:131. doi:10.3389/fvets.2021.601394.

Ma, F., S. Xu, Z. Tang, Z. Li, and L. Zhang. 2021. Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. Biosafety and Health. 3:32–38. doi:10.1016/j.bsheal.2020.09.004.

Mach, N., M. Berri, J. Estellé, F. Levenez, G. Lemonnier, C. Denis, J.-J. Leplat, C. Chevaleyre, Y. Billon, J. Doré, C. Rogel-Gaillard, and P. Lepage. 2015. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ. Microbiol. Rep. 7:554–569. doi:10.1111/1758-2229.12285.

Madison, A., and J. K. Kiecolt-Glaser. 2019. Stress, depression, diet, and the gut microbiota: human–bacteria interactions at the core of psychoneuroimmunology and nutrition. Curr. Opin. Behav. Sci. 28:105–110. doi:10.1016/j.cobeha.2019.01.011.

McMurdie, P. J., and S. Holmes. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE. 8:e61217. doi:10.1371/journal.pone.0061217.

de Mendiburu, F., and M. F. de Mendiburu. 2019. Package 'agricolae.' R Package, Version. 1–2.

Menkem, Z. E., B. L. Ngangom, S. S. A. Tamunjoh, and F. F. Boyom. 2019. Antibiotic residues in food animals: Public health concern. Acta Ecologica Sinica. 39:411–415. doi:10.1016/j.chnaes.2018.10.004.

Miller, S. I. 2016. Antibiotic resistance and regulation of the gram-negative bacterial outer membrane barrier by host innate immune molecules. mBio. doi:10.1128/mBio.01541-16. Available from: https://journals.asm.org/doi/abs/10.1128/mBio.01541-16

Mu, C., Y. Yang, Y. Su, E. G. Zoetendal, and W. Zhu. 2017. Differences in microbiota membership along the gastrointestinal tract of piglets and their differential alterations

following an early-life antibiotic intervention. Front. Microbiol. 8. Available from: https://www.frontiersin.org/article/10.3389/fmicb.2017.00797

Nagy, B., L. H. Arp, H. W. Moon, and T. A. Casey. 1992. Colonization of the small intestine of weaned pigs by enterotoxigenic Escherichia coli that lack known colonization factors. Vet. Pathol. 29:239–246. doi:10.1177/030098589202900308.

National Research Council (NRC). 2012. Nutrient requirements of swine: eleventh revised edition. Washington, DC: The National Academies Press.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2013. Package 'vegan.' Community ecology package, version. 2.

Pedregosa, F., G. Varoquaux, A. Gramfort, V. Michel, B. Thirion, O. Grisel, M. Blondel, P. Prettenhofer, R. Weiss, and V. Dubourg. 2011. Scikit-learn: Machine learning in Python. J. Mach. Learn. Res. 12:2825–2830.

Pena Cortes, L. C., R. M. LeVeque, J. A. Funk, T. L. Marsh, and M. H. Mulks. 2018. Development of the tonsil microbiome in pigs and effects of stress on the microbiome. Front. Vet. Sci. 5. Available from: https://www.frontiersin.org/article/10.3389/fvets.2018.00220

Pollock, J., M. R. Hutchings, K. E. K. Hutchings, D. L. Gally, and J. G. M. Houdijk. 2019. Changes in the ileal, but not fecal, microbiome in response to increased dietary protein level and enterotoxigenic Escherichia coli exposure in pigs. 85:e01252-19. doi:10.1128/AEM.01252-19.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2010. FastTree 2 – Approximately maximum-likelihood trees for large alignments. PLOS ONE. 5:e9490. doi:10.1371/journal.pone.0009490.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research. 41:D590–D596.

Shannon, C. E. 1948. A mathematical theory of communication. Bell system technical journal. 27:379–423.

Sumi, C. D., A. J. Heffernan, J. Lipman, J. A. Roberts, and F. B. Sime. 2019. What antibiotic exposures are required to suppress the emergence of resistance for gram-negative bacteria? A systematic review. Clin. Pharmacokinet. 58:1407–1443. doi:10.1007/s40262-019-00791-z.

Suter, W., A. Rosselet, and F. Knüsel. 1978. Mode of action of quindoxin and substituted quinoxaline-di-N-oxides on Escherichia coli. Antimicrob. Agents Chemother. 13:770–783. doi:10.1128/AAC.13.5.770.

Team, R. C. 2021. R: A language and environment for statistical computing.

Tian, L., G. Bruggeman, M. van den Berg, K. Borewicz, A. J. W. Scheurink, E. Bruininx, P. de Vos, H. Smidt, H. A. Schols, and H. Gruppen. 2017. Effects of pectin on fermentation characteristics, carbohydrate utilization, and microbial community composition in the gastrointestinal tract of weaning pigs. Mol. Nutr. Food Res. 61. doi:10.1002/mnfr.201600186.

Wang, T., K. Teng, G. Liu, Y. Liu, J. Zhang, X. Zhang, M. Zhang, Y. Tao, and J. Zhong. 2018. Lactobacillus reuteri HCM2 protects mice against Enterotoxigenic Escherichia coli through modulation of gut microbiota. Sci. Rep. 8:17485. doi:10.1038/s41598-018-35702-y.

Wickham, H. 2011. ggplot2. Wiley interdisciplinary reviews: Computational statistics. 3:180–185.

Yang, J. J., C. C. Niu, and X. H. Guo. 2015. Mixed culture models for predicting intestinal microbial interactions between Escherichia coli and Lactobacillus in the presence of probiotic Bacillus subtilis. Benef. Microbes. 6:871–877. doi:10.3920/BM2015.0033.

Zoetendal, E. G., J. Raes, B. van den Bogert, M. Arumugam, C. C. Booijink, F. J. Troost, P. Bork, M. Wels, W. M. de Vos, and M. Kleerebezem. 2012. The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME J. 6:1415–1426. doi:10.1038/ismej.2011.212.

Table 2-1. Ingredient compositions of experimental diets[1]

Ingredient, %	Control, phase I	Control, phase II
Corn	44.41	57.27
Dried whey	15.00	10.00
Soybean meal	18.00	22.00
Fish meal	10.00	7.00
Lactose	6.00	-
Soy protein concentrate	3.00	-
Soybean oil	2.00	2.00
Limestone	0.56	0.70
L-Lysine·HCl	0.21	0.23
DL-Methionine	0.08	0.05
L-Threonine	0.04	0.05
Salt	0.40	0.40
Vit-mineral, Sow 6[2]	0.30	0.30
Total	100.00	100.00
Calculated energy and nutrient		
Metabolizable energy, kcal/kg	3,463	3,429
Net energy, kcal/kg	2,601	2,575
Crude protein, %	22.27	20.80
Arg,[3] %	1.23	1.15
His,[3] %	0.49	0.47
Ile,[3] %	0.83	0.76
Leu,[3] %	1.62	1.55
Lys,[3] %	1.35	1.23
Met,[3] %	0.45	0.39
Thr,[3] %	0.79	0.73
Trp,[3] %	0.23	0.21
Val,[3] %	0.91	0.84
Met + Cys,[3] %	0.74	0.68
Phe + Tye,[3] %	1.45	1.38
Ca, %	0.80	0.70
Total P, %	0.68	0.59

Digestible P, %	0.47	0.37
Analyzed nutrient, as-is		
Dry matter, %	90.70	89.90
Crude protein, %	23.13	21.30
ADF, %	7.26	9.35
NDF, %	2.54	3.60
Ca, %	0.96	0.88
P, %	0.71	0.59

[1]In each phase, two additional diets were formulated by adding probiotics or Carbadox to the control diet, respectively. The dose for probiotics was 500 mg/kg, which was equal to 2.56×10^9 CFU *Bacillus subtilis*/kg diet. The dose for carbadox was 50 mg/kg diet.

[2]Provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B12, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.

[3]Amino acids were indicated as standardized ileal digestible AA.

Table 2-2. Relative abundance (%) of Firmicutes, Bacteroidetes, and Proteobacteria and their top families in the feces of enterotoxigenic *Escherichia coli* challenged pigs fed diets supplemented with antibiotics (AGP) or *Bacillus subtilis* (DFM)

	day -7				day 0				day 7 post-inoculation				day 21 post-inoculation			
	Negative control	Positive control	AGP	DFM	Negative control	Positive control	AGP	DFM	Negative control	Positive control	AGP	DFM	Negative control	Positive control	AGP	DFM
Firmicutes	55.00^{g}	54.71^{g}	61.76^{fg}	56.61^{g}	77.84^{abcd}	72.20^{bcdef}	64.34^{fg}	75.93^{abcde}	79.17^{abc}	71.11^{def}	65.40^{efg}	80.11^{ab}	82.36^{a}	76.09^{abcd}	71.99^{cdef}	77.22^{abcd}
Bacillaceae	0.00^{bc}	0.00^{bc}	0.00^{bc}	0.00^{bc}	0.00^{bc}	0.00^{bc}	0.00^{bc}	0.03^{a}	0.00^{bc}	0.00^{bc}	0.00^{bc}	0.01^{c}	0.00^{bc}	0.00^{bc}	0.00^{c}	0.05^{ab}
Christensenellaceae	0.38^{b}	1.53^{ab}	2.15^{ab}	4.18^{a}	1.27^{ab}	1.73^{ab}	3.09^{ab}	1.72^{ab}	1.23^{ab}	0.90^{ab}	1.14^{ab}	2.28^{ab}	0.71^{b}	0.95^{ab}	0.76^{ab}	0.48^{b}
Clostridiaceae1	2.53^{abc}	3.68^{ab}	4.01^{ab}	6.74^{a}	0.24^{c}	0.49^{bc}	2.73^{abc}	1.04^{abc}	1.69^{bc}	1.14^{bc}	4.29^{ab}	3.55^{abc}	6.73^{ab}	1.93^{abc}	12.47^{ab}	2.22^{bc}
Lachnospiraceae	16.59^{bcde}	10.26^{de}	11.94^{cde}	6.58^{e}	17.98^{bcd}	26.26^{ab}	26.87^{ab}	23.64^{ab}	27.21^{a}	23.07^{ab}	20.04^{abcd}	23.88^{ab}	21.83^{ab}	21.32^{abc}	18.69^{bcd}	20.06^{abc}
Lactobacillaceae	11.55^{cde}	16.92^{bcde}	21.54^{bcd}	15.93^{bcde}	39.56^{a}	24.55^{abc}	6.42^{de}	27.09^{abc}	23.10^{abc}	28.21^{ab}	20.73^{bc}	27.91^{ab}	14.63^{cde}	14.24^{cde}	4.44^{e}	23.98^{bc}
Peptostreptococcaceae	1.46^{abcd}	2.29^{ab}	0.93^{abcd}	1.92^{ab}	0.16^{e}	0.12^{e}	0.14^{e}	0.38^{de}	0.65^{bcd}	0.53^{cde}	3.33^{abc}	1.69^{abc}	3.81^{a}	1.10^{abcd}	6.91^{a}	1.21^{bcd}
Ruminococcaceae	14.27	12.96	12.58	10.48	11.23	12.55	17.97	13.88	18.63	10.89	11.30	12.94	15.07	16.62	15.29	14.57
Streptococcaceae	0.64^{bc}	1.11^{ab}	0.39^{bcd}	0.61^{bcd}	0.05^{d}	0.08^{cd}	0.02^{d}	0.2^{cd}	0.18^{cd}	1.05^{bcd}	0.38^{bcd}	2.97^{bcd}	10.44^{a}	6.27^{ab}	0.10^{cd}	1.90^{b}
Veillonellaceae	2.32^{def}	0.67^{f}	1.22^{ef}	1.21^{ef}	4.08^{cdef}	3.41^{bcd}	2.64^{def}	3.46^{bcde}	3.77^{bcd}	2.38^{def}	1.87^{def}	2.56^{def}	6.69^{abc}	10.93^{a}	9.11^{bcd}	9.99^{ab}
Bacteroidetes	29.43^{ab}	31.97^{a}	21.97^{abc}	20.62^{abcd}	11.12^{cde}	14.20^{cde}	17.96^{abcd}	16.04^{bcde}	12.27^{cde}	12.65^{cde}	18.31^{abc}	10.03^{e}	9.70^{e}	10.25^{de}	15.85^{bcde}	13.73^{cde}
Bacteroidaceae	18.07^{a}	15.14^{ab}	8.57^{abc}	6.87^{abc}	0.65^{cde}	0.51^{ef}	1.00^{bcd}	0.68^{cde}	0.11^{fgh}	0.85^{de}	2.2^{cd}	0.28^{efg}	0.01^{h}	0.22^{gh}	0.16^{efg}	0.01^{h}
Muribaculaceae	0.50	1.83	0.79	0.79	2.13	3.75	4.20	3.63	2.50	1.83	2.36	1.24	2.40	1.64	3.05	2.52
Prevotellaceae	6.19	5.87	4.91	5.34	6.38	6.99	8.07	8.32	7.87	8.02	11.54	7.36	6.54	7.62	10.00	10.15
Rikenellaceae	3.51^{ab}	5.88^{a}	5.36^{ab}	5.39^{ab}	1.12^{bcde}	2.08^{abc}	3.92^{ab}	2.80^{abc}	1.31^{bcde}	1.49^{bcde}	1.63^{bcde}	0.87^{de}	0.61^{e}	0.65^{e}	2.27^{abcd}	0.97^{cde}
Tannerellaceae	0.41^{ab}	1.88^{a}	1.36^{a}	1.17^{ab}	0.32^{ab}	0.31^{bcd}	0.26^{bcd}	0.47^{ab}	0.13^{cde}	0.03^{e}	0.12^{bcde}	0.11^{bcd}	0.04^{de}	0.05^{de}	0.22^{bc}	0.03^{de}
Proteobacteria	8.21^{abc}	6.72^{ab}	3.78^{abc}	5.92^{ab}	1.52^{abc}	1.86^{abc}	2.82^{abc}	2.72^{abc}	1.18^{c}	7.38^{a}	8.88^{abc}	2.45^{abc}	2.25^{bc}	6.60^{ab}	5.26^{abc}	2.46^{bc}
Desulfovibrionaceae	0.60^{ab}	1.14^{a}	1.46^{a}	1.09^{ab}	0.34^{abcd}	0.32^{bcde}	0.33^{bcde}	0.38^{abc}	0.17^{def}	0.30^{bcde}	0.24^{cdef}	0.22^{def}	0.16^{ef}	0.14^{ef}	0.16^{def}	0.13^{f}
Enterobacteriaceae	6.07^{bcd}	4.23^{abc}	1.97^{abc}	3.60^{ab}	0.49^{de}	0.79^{cd}	0.60^{bcd}	1.75^{abc}	0.69^{cd}	6.49^{a}	8.35^{ab}	1.94^{abc}	0.10^{de}	0.03^{e}	0.01^{e}	0.01^{e}
Pasteurellaceae	0.22^{ab}	1.27^{abcd}	0.09^{abc}	0.19^{a}	0^{d}	0^{d}	0^{cd}	0^{cd}	0^{cd}	0.01^{bcd}	0^{d}	0^{cd}	0^{cd}	0.01^{bcd}	0^{d}	0^{d}

Succinivibrionaceae	0.58^{ef}	0^{f}	0.01^{f}	0.87^{ef}	0.65^{cde}	0.52^{bcd}	1.76^{cde}	0.54^{bcde}	0.28^{def}	0.50^{cde}	0.17^{def}	0.20^{def}	1.56^{cde}	6.12^{a}	5.07^{ab}	2.30^{abc}

$^{a-g}$Means without a common superscript are different ($P < 0.05$). Each mean represents 12 observations.

Table 2-3. Relative abundance (%) of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria and their top families in intestinal digesta of enterotoxigenic *Escherichia coli* challenged pigs fed diets supplemented with antibiotics (AGP) or *Bacillus subtilis* (DFM)

	Jejunal digesta				Ileal digesta				Colon digesta			
	Negative control	Positive control	AGP	DFM	Negative control	Positive control	AGP	DFM	Negative control	Positive control	AGP	DFM
Firmicutes	93.20^{ab}	84.11^{bcde}	89.51^{abc}	91.85^{abc}	93.28^{a}	92.77^{abc}	86.08^{abcd}	95.67^{a}	85.64^{cde}	80.75^{de}	74.89^{e}	81.23^{de}
Bacillaceae	0.03^{cde}	0.07^{cd}	0.27^{b}	0.64^{a}	0.00^{e}	0.06^{cde}	0.02^{cde}	0.18^{ab}	0.00^{e}	0.00^{e}	0.00^{de}	0.02^{c}
Clostridiaceae1	2.89^{bc}	1.78^{c}	4.91^{bc}	3.59^{bc}	11.89^{a}	11.96^{ab}	22.22^{a}	2.88^{abc}	7.22^{ab}	1.46^{bc}	13.56^{ab}	2.35^{bc}
Lachnospiraceae	0.46^{bc}	1.52^{b}	0.43^{bc}	2.55^{bc}	0.13^{d}	0.42^{bc}	2.04^{cd}	0.09^{d}	23.19^{a}	23.15^{a}	21.93^{a}	24.36^{a}
Lactobacillaceae	76.09^{a}	60.26^{ab}	59.44^{ab}	70.54^{a}	47.48^{b}	56.22^{ab}	22.98^{c}	77.12^{a}	15.09^{cd}	14.42^{cd}	4.11^{d}	21.20^{c}
Peptostreptococcaceae	3.82^{bc}	0.06^{c}	3.50^{ab}	2.14^{bc}	9.59^{a}	5.24^{ab}	15.18^{a}	3.82^{ab}	3.96^{a}	0.86^{ab}	7.48^{a}	1.42^{ab}
Ruminococcaceae	0.06^{cde}	0.44^{bc}	0.13^{b}	1.55^{cde}	0.01^{de}	0.04^{cde}	2.59^{bcd}	0.01^{e}	15.52^{a}	18.15^{a}	15.24^{a}	15.75^{a}
Streptococcaceae	4.93^{abc}	9.61^{ab}	18.40^{a}	5.69^{abc}	19.42^{a}	12.98^{abc}	11.01^{abc}	8.94^{abc}	10.78^{a}	6.60^{bcd}	0.09^{d}	3.21^{cd}
Veillonellaceae	4.40^{cd}	6.30^{abcd}	0.09^{g}	3.85^{cde}	1.38^{efg}	1.98^{def}	1.21^{fg}	0.93^{fg}	6.63^{abc}	12.32^{a}	8.09^{bcd}	9.81^{ab}
Bacteroidetes	0.03^{bc}	0.02^{bc}	0.01^{bc}	1.08^{bc}	0^{bc}	0.03^{bc}	4.16^{b}	0^{c}	7.25^{a}	7.35^{a}	12.41^{a}	9.96^{a}
Bacteroidaceae	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0.06^{b}	0^{b}	0.01^{b}	0.11^{b}	0.16^{a}	0.01^{b}
Muribaculaceae	0^{c}	0.01^{c}	0.01^{c}	0.11^{bc}	0^{c}	0^{c}	0.49^{b}	0^{c}	2.22^{a}	1.74^{a}	1.94^{a}	2.09^{a}
Prevotellaceae	0.03^{bc}	0.01^{b}	0^{c}	0.94^{bc}	0^{c}	0.03^{bc}	2.98^{b}	0^{bc}	4.64^{a}	5.09^{a}	8.93^{a}	7.23^{a}
Rikenellaceae	0^{d}	0^{cd}	0^{d}	0.03^{cd}	0^{d}	0.01^{cd}	0.54^{c}	0^{d}	0.28^{ab}	0.35^{b}	1.17^{a}	0.57^{ab}
Actinobacteria	4.76^{a}	9.43^{a}	1.79^{abc}	4.37^{a}	2.93^{abc}	4.38^{ab}	0.36^{c}	3.35^{abc}	2.26^{abc}	2.99^{abc}	0.5^{bc}	2.48^{ab}
Actinomycetaceae	0.01^{bc}	0.14^{a}	0.07^{b}	0.05^{b}	0^{c}	0.06^{b}	0^{c}	0.01^{bc}	0^{c}	0^{c}	0^{c}	0^{c}
Atopobiaceae	0.43^{ab}	0.54^{a}	0^{b}	0.32^{ab}	0.13^{ab}	0.17^{ab}	0.07^{ab}	0.09^{ab}	0.36^{ab}	1.45^{ab}	0.28^{ab}	1.01^{ab}
Bifidobacteriaceae	4.22^{a}	8.21^{a}	0.04^{b}	3.81^{a}	2.76^{a}	3.94^{a}	0.11^{b}	3.20^{a}	1.67^{a}	1.10^{ab}	0.01^{b}	1.22^{a}
Coriobacteriaceae	0^{c}	0^{c}	0^{c}	0.02^{c}	0^{c}	0^{c}	0.05^{c}	0^{c}	0.21^{ab}	0.38^{a}	0.20^{b}	0.22^{ab}
Eggerthellaceae	0.06^{ab}	0.21^{a}	0^{b}	0.05^{ab}	0.03^{ab}	0.05^{ab}	0^{b}	0.02^{ab}	0.02^{ab}	0.05^{ab}	0.01^{ab}	0.03^{ab}
Micrococcaceae	0.04^{c}	0.32^{ab}	1.51^{a}	0.11^{bc}	0.01^{d}	0.15^{bc}	0.12^{bc}	0.03^{c}	0^{d}	0^{d}	0^{d}	0^{d}

Proteobacteria	1.89^{b}	0.4^{b}	4.12^{a}	0.22^{b}	3.77^{ab}	2.21^{ab}	7.61^{a}	0.92^{b}	1.69^{ab}	4.84^{a}	4.6^{a}	2.08^{a}
Burkholderiaceae	0^{a}	0^{a}	0.04^{a}	0^{a}	0^{a}	0^{a}	0.41^{a}	0^{a}	0^{a}	0.01^{a}	0^{a}	0^{a}
Desulfovibrionaceae	0^{b}	0^{b}	0^{b}	0.01^{b}	0^{b}	0^{b}	0.04^{b}	0^{b}	0.07^{a}	0.1^{a}	0.14^{a}	0.11^{a}
Enterobacteriaceae	0.17^{c}	0.02^{abc}	0.04^{abc}	0.01^{c}	3.56^{a}	0.64^{ab}	0.71^{bc}	0.04^{bc}	0.07^{abc}	0.01^{c}	0.01^{c}	0.01^{c}
Pasteurellaceae	1.7^{bc}	0.29^{bcd}	3.75^{a}	0.02^{bcd}	0.21^{bcd}	1.54^{ab}	4.85^{a}	0.87^{bc}	0^{cd}	0.01^{bcd}	0^{cd}	0^{d}
Succinivibrionaceae	0^{d}	0.01^{cd}	0.09^{bc}	0.15^{cd}	0^{d}	0^{cd}	1.5^{bc}	0^{d}	1.41^{b}	4.71^{a}	4.45^{a}	1.94^{a}

$^{a-g}$Means without a common superscript are different ($P < 0.05$). Each mean represents 12 observations.

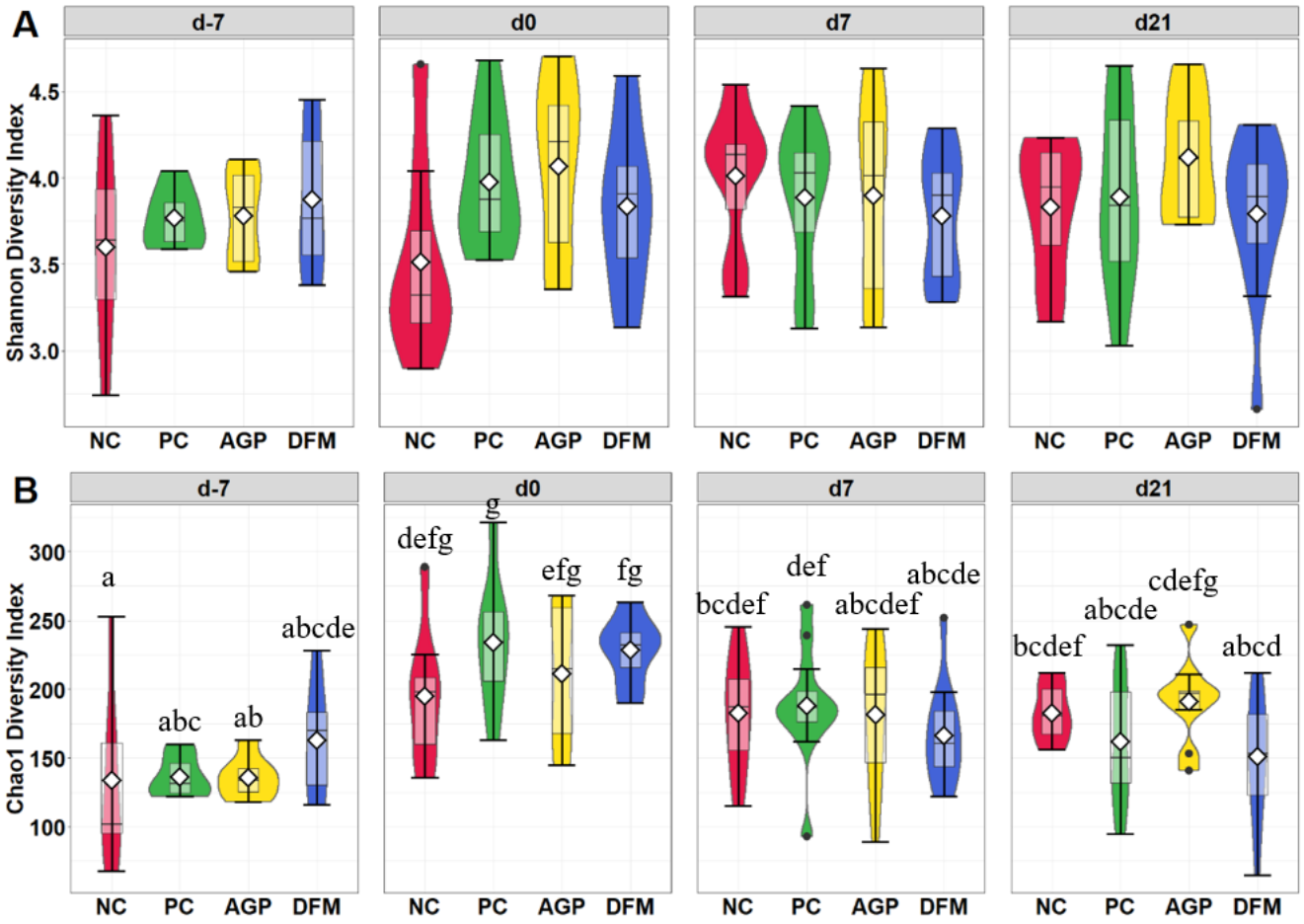


Figure 2-1. Alpha diversity as indicated by Shannon (A) and Chao1 (B) indices in feces collected from enterotoxigenic *Escherichia coli* challenged pigs fed diets supplemented with antibiotics (AGP) or *Bacillus subtilis* (DFM) at the beginning of the experiment (d -7), on d 0 before inoculation, on d 7 and 21 post-inoculation. No difference was observed in Shannon diversity (A). NC = negative control, PC = positive control, AGP = antibiotics, DFM = *B. subtilis*. Violin plots are colored by diet. Data are expressed as mean (diamond) ± SEM. [a-g]Means without a common superscript are different ($P < 0.05$).

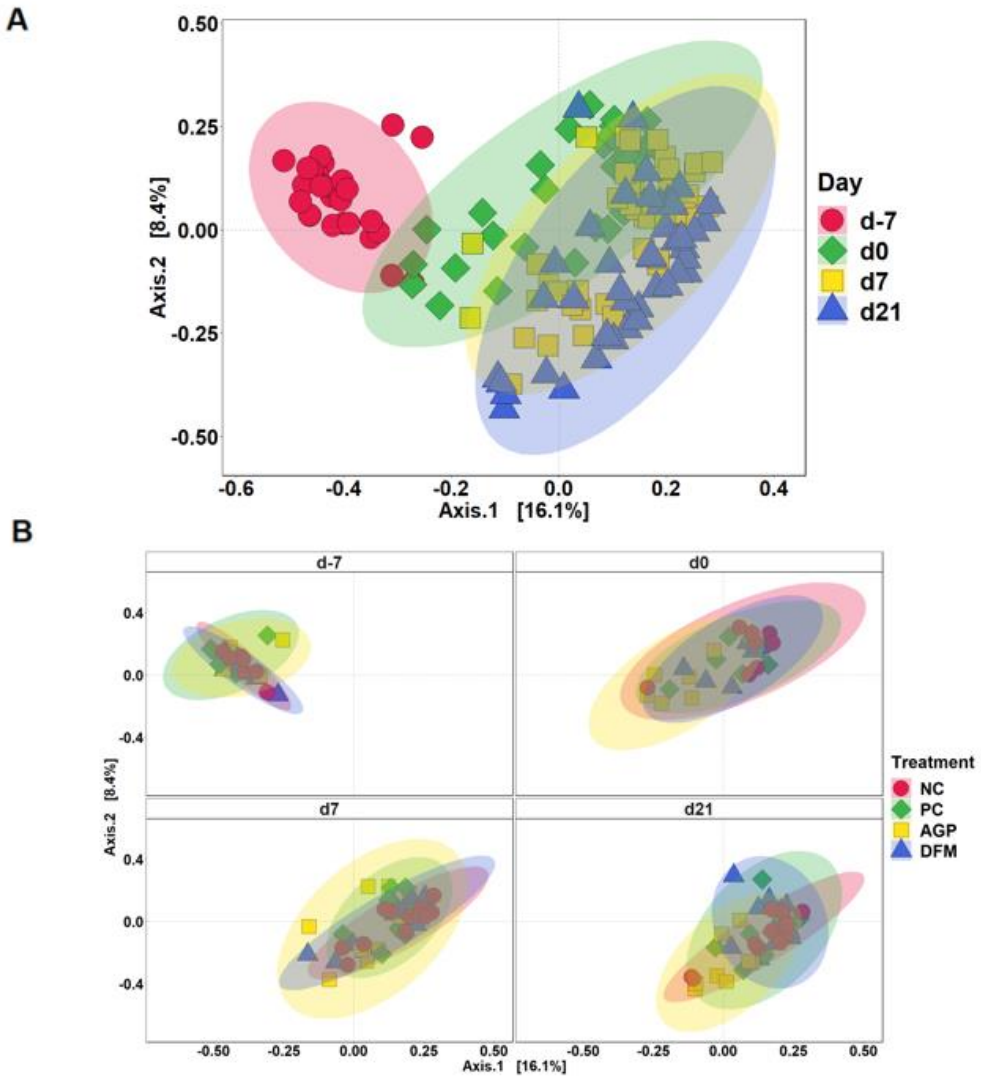


Figure 2-2. Beta diversity of fecal microbiota in enterotoxigenic *E. coli* F18 challenged pigs at the beginning of the experiment (d -7), on d 0 before inoculation, on d 7 and 21 post-inoculation by day (A) and treatment (B). Data were analyzed by principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity. NC = negative control, PC = positive control, AGP = antibiotics, DFM = *Bacillus subtilis.*

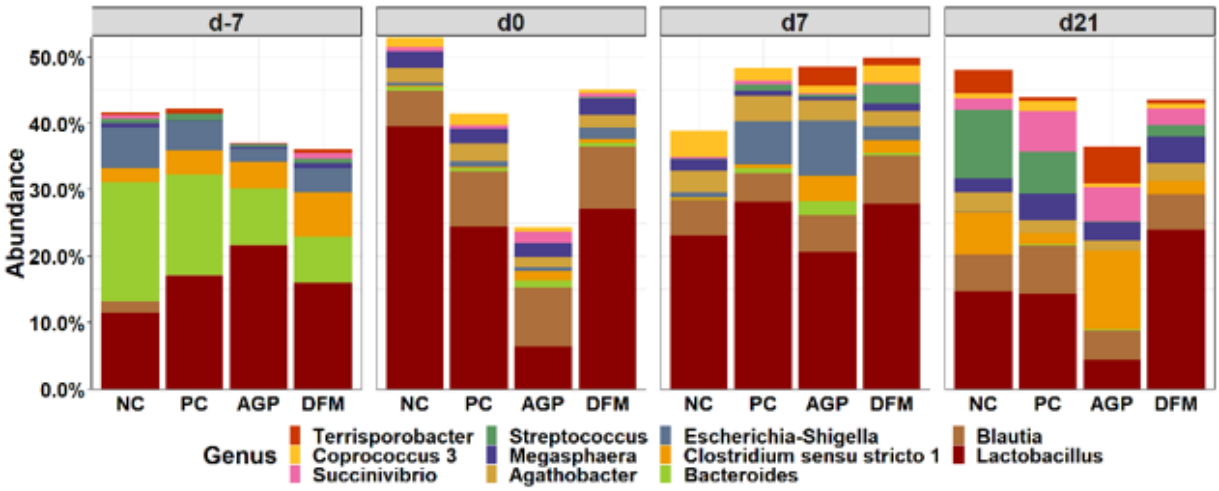


Figure 2-3. Relative abundance of genera that were most abundant in the fecal microbiota of piglets visualized by bar plot. Data are shown as means in each group (n = 12).

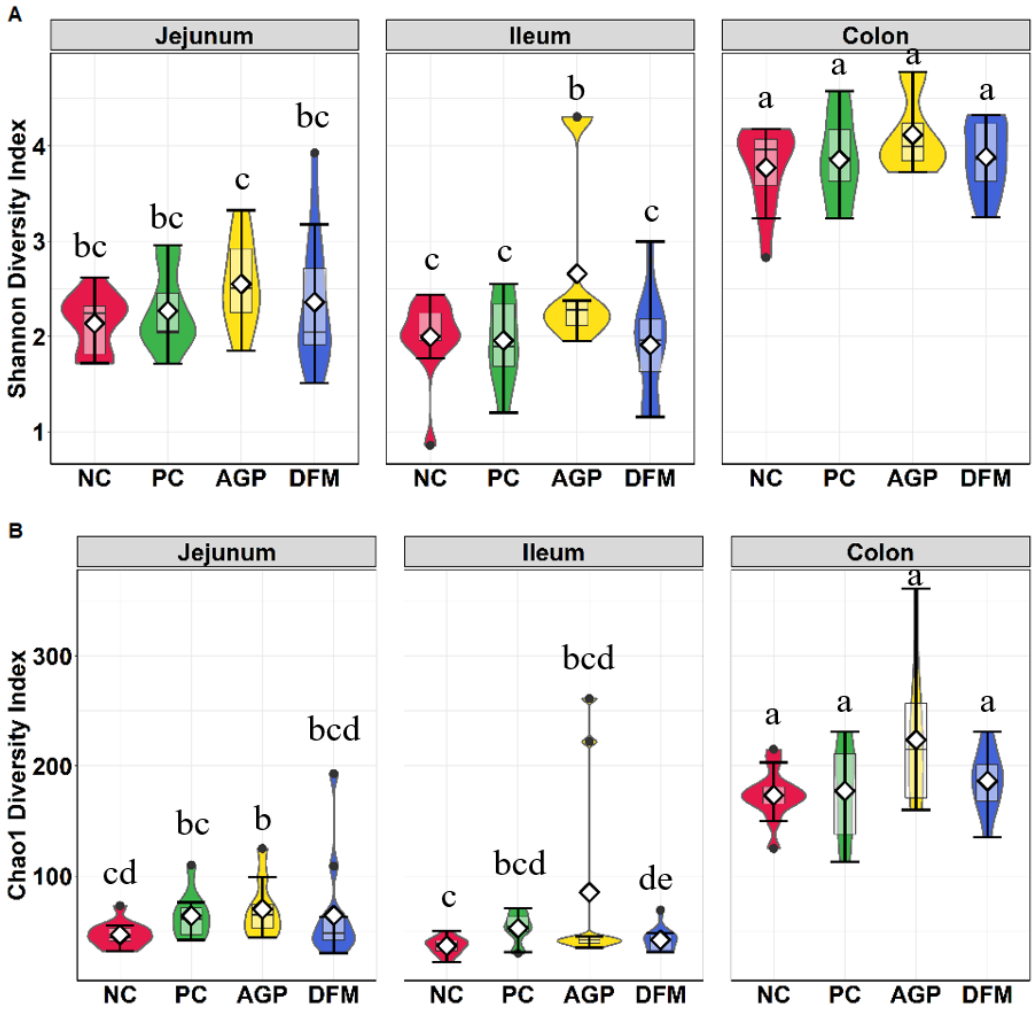


Figure 2-4. Alpha diversity as indicated by Shannon (A) and Chao1 (B) indices in intestinal digesta collected from weaned pigs challenged with enterotoxigenic *Escherichia coli*. Pigs were supplemented with antibiotics (AGP) or *Bacillus subtilis* (DFM). NC = negative control, PC = positive control. Violin plots are colored by diet. Data are expressed as mean (diamond) ± SEM. [a-g]Means without a common superscript are different ($P < 0.05$).

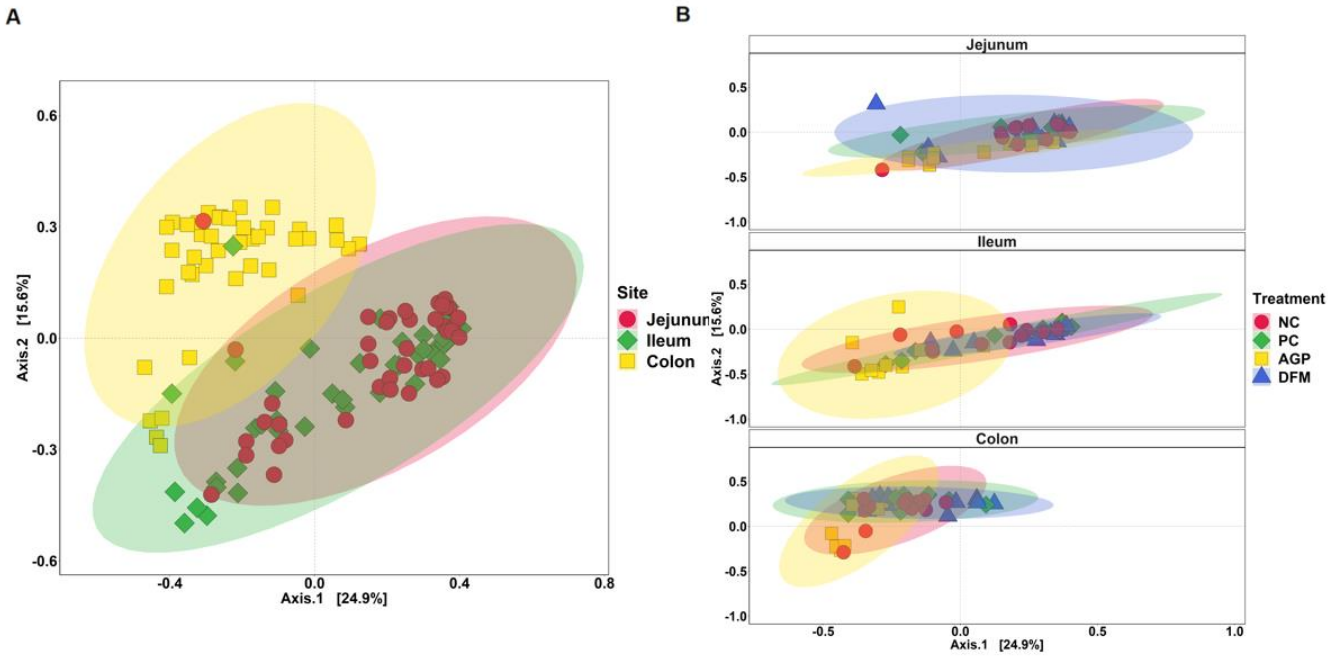


Figure 2-5. Beta diversity of intestinal digesta microbiota in pigs challenged with enterotoxigenic *E. coli* by intestinal site (**A**) and treatment (**B**). Data were analyzed by principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity. NC = negative control, PC = positive control, AGP = antibiotics, DFM = *Bacillus subtilis*.

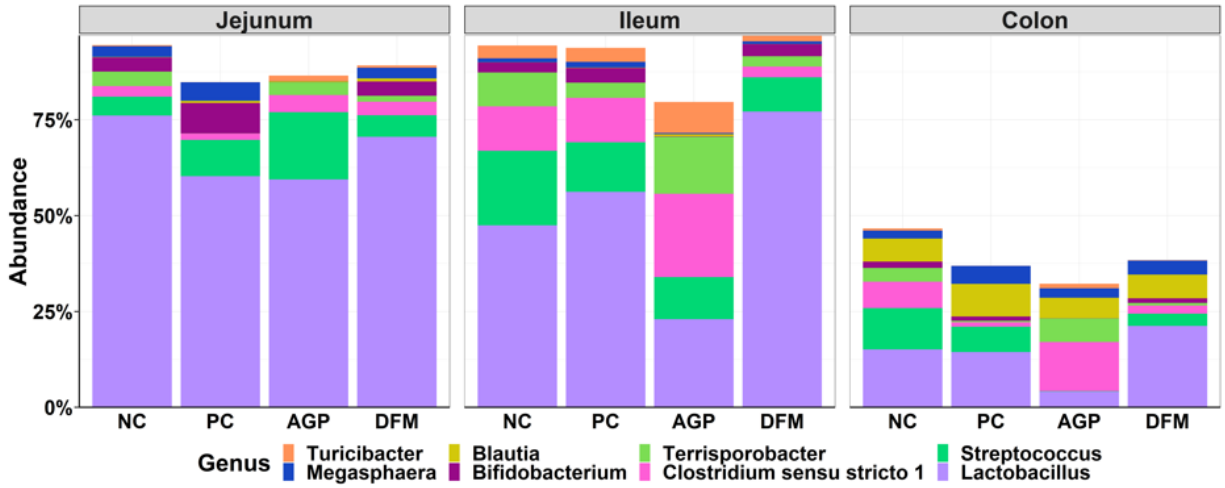


Figure 2-6. Relative abundance of the top 8 genera in the intestinal microbiota of piglets visualized by bar plot. Data are shown as means in each group (n = 12).

CHAPTER 3

EFFECTS OF *BACILLUS AMYLOLIQUEFACIENS* SUPPLEMENTATION ON PERFORMANCE, SYSTEMIC IMMUNITY, AND INTESTINAL MICROBIOTA OF WEANED PIGS EXPERIMENTALLY INFECTED WITH A PATHOGENIC ENTEROTOXIGENIC *E. COLI* F18

ABSTRACT

The objective of this study was to investigate the effects of dietary supplementation of *Bacillus amyloliquefaciens* on growth performance, diarrhea, systemic immunity, and intestinal microbiota of weaned pigs experimentally infected with F18 enterotoxigenic *Escherichia coli* (ETEC). Fifty weaned pigs (7.41 ±1.35 kg BW) were individually housed and randomly allotted to one of five treatments: sham control (CON-), sham *B. amyloliquefaciens* (BAM-), challenged control (CON+), challenged *B. amyloliquefaciens* (BAM+) and challenged carbadox (AGP+). The experiment lasted 28 days with seven days of adaptation and 21 days after the first ETEC inoculation. ETEC challenge reduced ($P < 0.05$) average daily gain (ADG) of pigs. Compared with CON+, AGP+ enhanced ($P < 0.05$) ADG, while *B. amyloliquefaciens* supplementation tended ($P < 0.10$) to increase ADG of pigs from d 0 to 21 post-inoculation (PI). ETEC challenge increased ($P < 0.05$) white blood cell (WBC) count on d 7 and 21 PI, while BAM+ pigs tended ($P < 0.10$) to have lower WBC on d 7 PI and had lower ($P < 0.05$) WBC on d 21 PI compared with CON+. In comparison to AGP+ fecal microbiota, BAM+ had lower ($P < 0.05$) relative abundance of *Lachnospiraceae* on d 0 and *Clostridiaceae* on d 21 PI, but higher ($P < 0.05$) relative abundance of *Enterobacteriaceae* on d 0. In ileal digesta, Shannon index was higher ($P < 0.05$) in BAM+ than AGP+. Bray-Curtis PCoA showed differences in bacterial community composition of ileal digesta collected from sham pigs vs. ETEC infected pigs on d 21 PI. Pigs in

BAM+ had greater ($P < 0.05$) relative abundance of Firmicutes, but lower ($P < 0.05$) relative abundance of Actinomycetota and Bacteroidota in ileal digesta than pigs in AGP+. Ileal digesta from AGP+ had greater ($P < 0.05$) abundance of *Clostridium sensu stricto* 1 but lower ($P < 0.05$) *Bifidobacterium* than pigs in BAM+. In general, supplementation with *B. amyloliquefaciens* tended to increase ADG and had limited effects on diarrhea of ETEC infected pigs. However, pigs fed with *B. amyloliquefaciens* exhibit milder systemic inflammation than control. *B. amyloliquefaciens* differently modified intestinal microbiota of weaned pigs, compared with carbadox.

Key words: *Bacillus amyloliquefaciens*, *Escherichia coli* challenge, Microbiome, Performance, Systemic immunity, Weaned pigs

INTRODUCTION

Newly weaned pigs experience a period of high stress from sudden environmental changes in housing and dietary changes from sow milk to solid diet, which increases the risk of pigs to experience post-weaning diarrhea induced by enterotoxigenic *Escherichia coli* (ETEC) (Fairbrother et al., 2005). Weaned pigs can experience watery diarrhea from ETEC disrupting the osmotic pressure in the intestines, leading pigs to undergo dehydration and reduced feed efficiency (Lee et al., 2016). Reduced feed intake corresponds to reduced energy intake, which results in less growth and lower immunity in weaned pigs (Amezcua et al., 2002). Post-weaning diarrhea in pigs that is untreated can eventually lead to death, and the increased mortality rate negatively impacts the economics and animal welfare within the swine industry. Therefore, ETEC pathogenicity must be suppressed within early on.

In the swine industry, in-feed antibiotics were administered to treat post-weaning diarrhea and to prevent the spread of ETEC in weaned pigs. Some antibiotics including carbadox can be

administered in-feed at a low dose to additionally promote pig growth and are commonly referred to as antibiotic growth promoters (AGP) (Lekagul et al., 2019). The continuous use of AGP increases the risk of antibiotic resistance, which can be transmitted zoonotically from pigs to humans, leading to increasing concerns regarding public health (Aarestrup, 2005). Thus, regulations and legislations have been applied in countries such as the European Union to ban or reduce the use of antibiotics in animal production and the World Health Organization has assembled a global action plan to increase the awareness of using antibiotics in human health and for livestock purposes (Casewell et al., 2003; World Health Organization, 2015). Although approximately 30 countries have restricted or banned the use of AGP, many other countries are still yet administering AGP in swine diet to prevent diarrhea and promote growth (Liao and Nyachoti, 2017). Hence, the swine industry is currently challenged to maintain health while improving growth of newly weaned pigs without the use of AGP.

Categorized as direct-fed microbials, *Bacillus* spp. have been shown to secrete secondary metabolites that may contribute antimicrobial factors (Sansinenea and Ortiz, 2011). In our previous study, *B. subtilis* DSM 25841 supplementation was shown to reduce diarrhea and improve growth performance in weaned pigs experimentally infected with ETEC (He et al., 2020b). *Bacillus* spp. have been also shown to modify the intestinal microbiota when supplemented in pigs (Fouhse et al., 2016; Lee et al., 2019). Comparing their whole genomes, *B. subtilis* and *B. amyloliquefaciens* have similar genes for antibacterial synthetases, which lead *B. amyloliquefaciens* to be also categorized as a direct-fed microbial (Koumoutsi et al., 2004). Salazar et al. (2017) has also identified bacteriocin-like substances in *B. amyloliquefaciens* that could inhibit the growth of pathogenic bacteria and it has shown high temperature resistance and pH stability, which may imply high survivability in the swine gastrointestinal tract. Findings in

these previous studies suggest as *B. amyloliquefaciens* as direct fed microbials may have the potential to alleviate post-weaning diarrhea and enhance the growth performance of weaned pigs. However, limited research has been reported utilizing dietary *B. amyloliquefaciens* as an in-feed supplement for pig performance and intestinal health. Therefore, the present study aimed to investigate the effects of supplementing *B. amyloliquefaciens* on growth performance and systemic immunity of newly weaned pigs with or without ETEC challenge, and to compare the effects of *B. amyloliquefaciens* with carbadox on the fecal and ileal microbiota of weaned pigs.

MATERIALS AND METHODS

Animals and study design

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC# 20809) at the University of California, Davis (UC Davis). A total of 50 weaned pigs (21 day (d) old, 7.41 ±1.35 kg body weight (BW)) with equal number of barrows and gilts were obtained from the UC Davis Swine Teaching and Research Center and the experiment was conducted at Cole facility at UC Davis. All pigs and their sows did not receive *E. coli* vaccines, antibiotic injections, or antibiotics in feed prior to the experiment. After weaning, pigs were individually housed (pen size: 0.61 m × 1.22 m) and assigned to one of five treatment groups with 10 replicate pigs per treatment using a randomized complete block design with sex normalized by BW and litter as blocks and pig as experimental unit. Four treatments were in a 2 × 2 factorial arrangement with 2 diets (control (CON) vs. 0.10% inclusion rate of 10^9 CFU/kg *B. amyloliquefaciens* (BAM) in the complete feed) and 2 challenges (sham (-) vs. ETEC (+)). The fifth treatment was an antibiotic (50 mg/kg of carbadox in the complete feed) treatment with ETEC challenge (AGP+). Prior to weaning, tail samples were collected from all piglets to

assess their susceptibility to ETEC F18 using the genotyping analysis described in Kreuzer et al. (2013). All pigs used in the present study were susceptible to ETEC F18.

The experiment included a 7-day habituation period and 21 days after the first ETEC F18 inoculation (d 0). A two-phase feeding program was used with weeks 1 and 2 as Phase I and weeks 3 and 4 as Phase II. Hence 6 diets were prepared. Spray-dried plasma, antibiotics, and high levels of zinc oxide were not included in the diets. All diets were formulated to meet pig nutritional requirements (NRC, 2012; Table 3-1).

Pigs in the ETEC challenge groups received three oral doses of ETEC F18 at 10^{10} CFU per dose. The F18 ETEC was cultured in at the Western Institute for Food Safety & Security at UC Davis. The bacterial strain was originally isolated from a field disease outbreak by the University of Illinois Veterinary Diagnostic Lab (isolate number: U.IL-VDL # 05-27242) and the strain expresses heat-labile toxin, heat-stable toxin b, and Shiga-like toxins. On the final day of the experiment, all pigs were anesthetized by intramuscularly injecting a 1 mL mixture of telazol (100 mg), ketamine (50 mg), and xylazine (50 mg) prior to an intracardiac injection of 78 mg sodium pentobarbital (Vortech Pharmaceuticals, Ltd., Dearborn, MI) per 1 kg of BW for euthanasia.

Clinical observations and sample collection

Fecal and alertness scores were recorded twice daily from d 0 to 21 post-inoculation (PI). Fecal score was measured by two independent evaluators with scores ranging from 1 to 5 (1, normal feces; 2, moist feces; 3, mild diarrhea; 4, severe diarrhea; and 5, watery diarrhea). Alertness score of each pig was also assessed visually with the score ranging from 1 to 3 (1, normal; 2, slightly depressed or listless, and 3, severely depressed or recumbent). Frequency of

diarrhea was calculated by quantifying the number of pigs and days with fecal score ≥ 3 or ≥ 4, respectively.

Fecal samples were collected from the rectum of each pig at the beginning of experiment (d -7), d 0 before ETEC inoculation, d 2, 7, 14, and 21 PI to perform fecal culture. Whole blood samples were collected from the jugular vein of all pigs on d -7, d 0, and d 7, 14, and 21 PI. Fresh blood samples were submitted to Comparative Pathology Laboratory at the University of California, Davis to measure total and differential blood cell count. Feeder weights, feed allowance, and pig BWs were recorded weekly to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed ration (G:F) from d -7 to 0, d 0 to d 7 PI, d 7 to d 14 PI, and d 14 to d 21 PI. Additional batches of fecal samples collected from d -7 and 0 before ETEC inoculation, and d 7, 14, and 21 PI and ileal digesta collected on d 21 PI were immediately frozen into liquid nitrogen and stored at −80°C until microbiota analysis.

Fecal culture

ETEC used in this experiment possesses β-hemolysis and lactose fermentation, thus Columbia blood agar with 5% sheep blood and MacConkey agar were used to identify the percentage of β-hemolytic coliforms in feces. Fecal samples collected from the rectum of all pigs using cotton swabs on d 2, 7, 14, and 21 PI were used for fecal cultures. Briefly, fecal swabs were plated on Columbia blood agar and MacConkey agar using the quadrant streak plate method, and all plates were cultured in an air incubator at 37˚C for 24 hours. Total coliforms from both agars and β-hemolytic coliforms from blood agar were assessed visually using a score system ranging from 0 to 8 (0 = no bacterial growth, 8 = very heavy bacterial growth). The percentage of β-hemolytic coliforms in feces were calculated.

Microbiota analysis

Bacterial DNA was extracted from fecal samples and ileal digesta using the Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions and diluted to use the same amount of DNA in each PCR. DNA samples were amplified in duplicate by PCR targeting the V4 region of the 16S rRNA gene using primers 515F (5′-XXXXXXXX**GT**GTGCCAGCMGCCGCGGTAA-3′), which included an 8-bp barcode (X) unique to each sample followed by a 2 nt Illumina adapter (bold), and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). PCR reactions were comprised of 2 µL template DNA, 9.5 µL nuclease free water, 12.5 µL GoTaq 2× Master Mix (Promega, Madison, WI, USA), 0.5 µL V4 reverse primer (10 µM), and 0.5 µL barcoded forward primer (10 µM). Amplification was carried out in a thermocycler with the following settings: 94°C for 3 min for initializing denaturation; followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1.5 min; and 72°C for 10 min for final elongation. The amplicon size for each sample was verified using agarose gel electrophoresis, and amplified samples were then pooled together, with the amount of sample added being quantified subjectively based on the band brightness in the agarose gel. The pooled sample was then purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and submitted to the UC Davis Genome Center DNA Technologies Core for 250 bp paired-end sequencing on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA).

Raw fastq files were first demultiplexed and 8-bp barcodes were removed using sabre (https://github.com/najoshi/sabre). Demultiplexed sequences were then imported into Quantitative Insights Into Microbial Ecology 2 (QIIME2; version 2020.8) to use the DADA2 plugin, which removes primers and lower quality reads (Callahan et al., 2016; Bolyen et al.,

2019). Paired-end reads were denoised and merged and, chimeras were then removed to construct amplicon sequence variants (ASVs). Representative sequences for each ASV were aligned using MAFFT, and masked alignments were used to generate phylogenetic trees using FastTree2 (Price et al., 2010; Katoh and Standley, 2013). Python library scikit-learn was used to assign taxonomy based on representative sequences against Silva (version 138), which was pre-trained in QIIME2 to be clipped to only the V4 hypervariable region and clustered at 99% sequence identity (Pedregosa et al., 2011; Quast et al., 2012; Bokulich et al., 2018).

Statistical analyses

All data excluding microbiota were analyzed using SAS (SAS Inst. Inc., Cary, NC). Normality of all data was verified using the UNIVARIATE procedure. Values that deviated from the treatment mean by more than three times the interquartile range were assumed outliers and removed. Measurements were analyzed by ANOVA using PROC MIXED in SAS in a randomized complete block design with pig as the experimental unit. The model included treatment as main effect and blocks as random effects. LSMEANS statement and the PDIFF option of PROC MIXED were used to separate treatment means. Chi-square was used to find significance in the frequency of diarrhea. Statistical significance and tendency were assessed as $\alpha = 0.05$ and $\alpha = 0.10$, respectively.

Shannon and Chao1 indices were measured for alpha diversity by using the estimate_richness function in phyloseq (McMurdie and Holmes, 2013). The Bray-Curtis matrix was used to compare community composition among treatments and day for feces and only treatment for ileal digesta. The relative abundance of each taxon in each sample was calculated by dividing the number of taxa by the total number of filtered reads in each sample. Files were exported from QIIME2 and imported into the R 4.1.0 for data visualization and statistical

analysis (Team, 2021). All microbiota analyses was performed using the phyloseq package and data were visualized using the ggplot2 package (Wickham, 2011). Normality and homoscedasticity were tested using the Shapiro Wilks test and Bartlett test, respectively. For fecal microbiota, a linear mixed-effect model was fitted using the lme4 package with treatment and day and interaction as fixed effects while pig as random effect (Bates et al., 2014). Significance of each term in the model was determined using the F-test as a type 3 analysis of variance using the Anova function in the car package, followed by a group comparison using the cld function in the emmeans package (Fox and Weisberg, 2018; Lenth, 2021). When normality or homoscedasticity was not observed, a non-parametric test was performed using the Kruskal-Wallis sum-rank test using the agricolae package (de Mendiburu and de Mendiburu, 2019). Bray-Curtis dissimilarity was first tested for homoscedasticity using the betadisper function and confirmed with $P > 0.05$. Statistical significance for beta diversity was then tested using PERMANOVA and the vegan package (Oksanen et al., 2013). Statistical significance was assessed as $\alpha = 0.05$ and statistical tendency as $\alpha = 0.10$. The *P*-values were adjusted for multiple comparisons using false discovery rate (FDR).

RESULTS

Growth performance, diarrhea, and white blood cell profile

No difference was observed in pig BW among treatments on d -7, d 0, d 7 PI and d 14 PI (Table 2). On d 21 PI, pigs in BAM- had the heaviest BW and pigs in CON+ had the lowest BW among all treatments ($P < 0.05$). Pig's final BW was greater ($P < 0.05$) in CON- than CON+, and final BW was greater ($P < 0.05$) in AGP+ than in CON+. No difference in ADG, ADFI, and Gain:Feed was observed between pigs in between CON- and BAM- throughout the experiment. ETEC inoculation reduced ($P < 0.05$) ADG from d 0 to 21 PI and Gain:Feed from d 14 to 21 PI

when CON+ was compared with CON-. Supplementation with AGP enhanced ($P < 0.05$) ADG from d 0 to 21 PI compare with CON+. Compared with CON+, pigs fed BAM+ diet tended ($P < 0.10$) to increase ADFI and ADG of weaned pigs from d 0 to 21 PI and final BW at d 21 PI.

Pigs in the sham groups (CON- and BAM-) had the lowest fecal score throughout the experiment (Figure 3-1). Pigs in BAM+ and CON+ had greater ($P < 0.05$) fecal score from d 1 to 8 PI than pigs in CON-. While for all treatments fecal score decreased as of d 9 to a level below diarrhea, between d 11 and d 14 PI, pigs in BAM+ had the highest ($P < 0.05$) fecal score among treatments. After ETEC inoculation, the frequency of diarrhea (diarrhea score ≥ 3) was 31.36% in CON+ pigs, while the diarrhea frequency was 8.18% in CON- pigs (Figure 3-2). Pigs in CON+, BAM+, and AGP+ had higher ($P < 0.05$) frequency of diarrhea than pigs in CON- and BAM-, regardless of incidence (diarrhea score ≥ 3) and severity (diarrhea score ≥ 4). Supplementation with either BAM or AGP did not affect frequency of diarrhea throughout the experiment.

No β-hemolytic coliforms were detected in fecal samples of pigs in CON- and BAM- throughout the experiment, and no β-hemolytic coliforms were observed in all pigs on d -7, 0, 14 PI, and 21 PI. On d 2 PI, AGP+ had lower ($P < 0.05$) percentage of β-hemolytic coliforms on blood agar plates than CON+ (Figure 3-3). On d 7 PI, no difference was observed among all 3 treatments under ETEC challenge.

No difference was observed in the white blood cell profile among the five treatments on d -7 (Table 3-3). On d 0, lymphocyte count was greater ($P < 0.05$) in AGP+ than other treatments, except for BAM+. However, lymphocyte percentage was greater ($P < 0.05$) in BAM+ than in CON- and CON+. After ETEC inoculation, greater ($P < 0.05$) white blood cell and lymphocyte counts were observed in CON+ on d 7 and 21 PI, and greater ($P < 0.05$) neutrophil count was

observed in CON+ on d 14 PI, compared with CON-. Pigs in BAM+ had lower ($P < 0.05$) neutrophils on d 14 PI, and lower ($P < 0.05$) white blood cell, neutrophils, lymphocytes on d 21 PI, than pigs in CON+. Supplementation with AGP reduced ($P < 0.05$) neutrophil count on d 14 and 21 PI and increased ($P < 0.05$) lymphocyte percentage and monocyte percentage on d 21 PI compared with CON+. No difference was observed in white blood cell profile of pigs between BAM+ and AGP+, with the exception that pigs in AGP+ had greater ($P < 0.05$) monocyte percentage on d 7 and 21 PI but lower ($P < 0.05$) neutrophil percentage on d 21 PI than pigs in BAM+. No difference was observed in the white blood cell profile of pigs in BAM- and CON-. No difference was observed in the red blood cell profile of pigs among all treatments (Data not shown).

Fecal microbiota

The mean sampling read was 15,368 per sample and the total number of taxa identified was 4,410 in the sequence data. An increase ($P < 0.05$) in Shannon and Chao1 indices were observed in feces when all pigs aged between d -7 to d 21 PI. However, no significant differences in Shannon and Chao1 indices were observed in feces among treatments throughout the experiment (Figure 3-4). In beta diversity, all fecal samples collected on d -7 were clustered and separated from fecal samples collected on d 0, 7, 14, and 21 PI (Figure 3-5A). Fecal samples collected on d 0 were clustered away from fecal samples collected on d 21 PI. Fecal samples from all treatments were clustered together on d -7 (Figure 3-5B). On d 7 PI, fecal samples from AGP+ were moderately clustered away from CON- and CON+. AGP+ was clustered farther away from BAM+ and CON+ on d 21 PI.

The three most abundant phyla (most to least abundant) were Firmicutes, Bacteroidota, and Proteobacteria in fecal samples of pigs from all treatments throughout the

experiment. No difference was observed in relative abundance of phyla in fecal samples on d -7 (Table 3-4). The relative abundance of Firmicutes was the highest on d 14 PI, compared with other time points. The relative abundance of Proteobacteria was observed to be the highest ($P < 0.05$) on d 7 PI and relative abundance of Bacteroidota to be the highest ($P < 0.05$) on d 21 PI among all fecal collection days. At the family level, the relative abundance of *Bacteroidaceae* and *Clostridiaceae* was decreased ($P < 0.05$), while the relative abundance of *Prevotellaceae*, *Lachnospiraceae,* and *Lactobacillaceae* was increased ($P < 0.05$) in feces as pig age increased.

Dietary treatments had limited effects on the relative abundance of Firmicutes, Bacteroidota, and Proteobacteria in fecal samples collected on d 0, except pigs in AGP+ had greatest ($P < 0.05$) *Lachnospiraceae* but lowest ($P < 0.05$) *Enterobacteriaceae* among all treatments. Pigs in BAM- had relative lower ($P < 0.05$) abundance of *Clostridiaceae* than pigs in CON-, while pigs in BAM+ had relative higher ($P < 0.05$) abundance of *Bacteroidaceae* than pigs in CON- on d 0. ETEC inoculation increased ($P < 0.05$) the relative abundance of *Lactobacillaceae* in feces on d 7 PI when CON+ was compared with CON-. Supplementation with AGP enhanced ($P < 0.05$) the relative abundance of Bacteroidota on d 7 PI and *Clostridiaceae* on d 21 PI but AGP decreased ($P < 0.05$) the relative abundance of *Lachnospiraceae* on d 7 PI and *Lactobacillaceae* on d 21 PI, compared with CON+. Pigs in AGP+ also had greater ($P < 0.05$) relative abundance of *Clostridiaceae* in feces than pigs in BAM+ on 21 PI.

Within the 12 most abundant genera, nine genera were classified under Firmicutes, two under Bacteroidota, and one under Proteobacteria (Table 3-5). The three most abundant genera in all fecal samples throughout the experiment were *Lactobacillus, Blautia,* and *Prevotella*. The relative abundance of these three genera was increased from d -7 to d 0. However, the relative

abundance of *Lactobacillus* in fecal samples from all pigs was decreased ($P < 0.05$) from d 0 to d 7 PI. No difference was observed in the most abundant genera in fecal samples of pigs between CON- and BAM- throughout the experiment. On d 0, pigs supplemented with AGP had greater ($P < 0.05$) relative abundance of *Prevotella* than pigs in BAM+ and BAM- and had greater ($P < 0.05$) relative abundance of *Blautia* than pigs in all other treatments. Compared CON+ with CON-, ETEC infection enhanced ($P < 0.05$) the relative abundance of *Lactobacillus* on d 7 PI and *Megasphaera* on d 21 PI but reduced ($P < 0.05$) the relative abundance of *Coprococcus* on d 14 PI and *Streptococcus* on d 21 PI. Supplementation of AGP reduced ($P < 0.05$) the relative abundance of *Agathobacter* on d 7 PI, the relative abundance of *Dorea* and *Streptococcus* on d 14 PI, and the relative abundance of *Blautia*, *Dorea*, *Lactobacillus*, and *Megasphaera* on d 21 PI, compared with CON+. Compared with AGP+, pigs in BAM+ had relative higher ($P < 0.05$) abundance of *Streptococcus* on d 14 PI, and *Prevotella*, *Megasphaera*, and *Streptococcus* on d 21 PI in feces.

Ileal digesta microbiota

In ileal digesta, BAM+ had greater ($P < 0.05$) Shannon index than AGP+ (Figure 3-6A). Pigs in CON+ had a greater ($P < 0.05$) and a tendency ($P < 0.10$) to have greater Chao1 diversity than pigs in BAM- and CON- (Figure 3-6B). In beta diversity, CON- and BAM- clusters were overlapping each other and separated from ETEC infected groups, while the ETEC infected groups had ileal digesta samples more dispersed from each other (Figure 3-7). Clusters for BAM+ and CON+ were overlapping each other and the AGP+ cluster was partially isolated from other treatment clusters.

The top four most abundant phyla in ileal digesta collected on d 21 PI were Firmicutes, Proteobacteria, Actinomycetota, and Bacteroidota from most to least abundant (Table 3-6). Pigs

in CON+ had greater ($P < 0.05$) relative abundance of Bacteroidota and Proteobacteria, but lower ($P < 0.05$) relative abundance of Firmicutes than pigs in CON-. At the family level, pigs in CON+ had greater ($P < 0.05$) relative abundance of *Atopobiaceae*, *Clostridiaceae*, and *Pasteurellaceae*, but lower ($P < 0.05$) relative abundance of *Lactobacillaceae* than pigs in CON-. No difference was observed ($P > 0.05$) in ileal digesta microbiota between CON and BAM regardless of ETEC challenge. Under ETEC challenge, AGP enhanced ($P < 0.05$) the relative abundance of Firmicutes and *Clostridiaceae* but reduced ($P < 0.05$) the relative abundance of Bacteroidota and *Atopobiaceae* in ileal digesta compared with CON+. Compared with AGP+, BAM+ increased ($P < 0.05$) the relative abundance of Actinomycetota, Bacteroidota, *Atopobiaceae*, *Bifidobacteriaceae*, and *Prevotellaceae*, but reduced ($P < 0.05$) the relative abundance of Firmicutes and *Clostridiaceae* in ileal digesta.

Within the eight most abundant genera, one was under Actinomycetota, six genera were under Firmicutes and one was under Proteobacteria (Table 3-7). ETEC infection increased ($P < 0.05$) the relative abundance of *Clostridium sensu stricto* 1 and *Actinobacillus* but reduced ($P < 0.05$) relative abundance of *Lactobacillus* when comparing pigs in CON+ vs. CON-. AGP+ had greater ($P < 0.05$) relative abundance of *Clostridium sensu stricto* 1 and less ($P < 0.05$) relative abundance in *Megasphaera* than CON+. In addition, the relative abundance of *Megasphaera* and *Bifidobacterium* was greater ($P < 0.05$) in BAM+ than in AGP+, while AGP+ had greater ($P < 0.05$) relative abundance of *Clostridium sensu stricto* 1 than BAM+.

DISCUSSION

Antibiotics have been shown to induce prophages in fecal samples of pigs over time, posing the risk of developing antibiotic resistant pathogens that could be spread to humans (Allen et al., 2011). A complete eradication of antibiotics use at the post-weaning stage is

desirable yet currently not feasible, thus developing alternative practices to treat post-weaning diarrhea and enhance feed efficiency of weaned pigs is necessary (Angulo et al., 2005). Direct fed microbials are looked upon to alleviate the intestinal damage caused by ETEC and to reduce the mortality rate of weaned pigs (Buntyn et al., 2016). Although *B. amyloliquefaciens* supplementation has been tested on broilers, limited studies have investigated the effects of *B. amyloliquefaciens* in weaned pigs. The present study observed that *B. amyloliquefaciens* supplementation tended to enhance growth performance and reduce systemic inflammation of weaned pigs challenged with ETEC F18. In addition, carbadox supplementation in the present study improved feed efficiency and alleviated diarrhea of ETEC challenged weaned pigs. The gut microbiota was influenced differently between carbadox and *B. amyloliquefaciens*.

Consistent with our previous research, increased frequency of diarrhea in pigs confirmed that the ETEC F18 strain inoculated into the pigs successfully induced pathogenicity in the present study (Kim et al., 2019; He et al., 2020b). ETEC infected pigs had reduced feed intake and weight gain and experienced severe diarrhea for approximately 6 days after the first ETEC inoculation, which falls under the average number of days when weaning pigs show diarrheal symptoms (Cox et al., 2012). No difference was observed in growth performance of weaned pigs between control and *B. amyloliquefaciens* in the sham group. Supplementation of *B. amyloliquefaciens* tended to enhance body weight, average daily gain, and feed intake of ETEC infected pigs compared with control. In consistency with previous research, supplementation of mixed strains of *B. amyloliquefaciens* enhanced feed efficiency of ETEC infected pigs, while supplementation of single strain of *B. amyloliquefaciens* reduced feed conversion ratio in in broiler under necrotic enteritis challenge (Jerzsele et al., 2012; Becker et al., 2020). As expected, supplementation with carbadox in the current study reduced frequency of diarrhea and enhanced

the growth rate of ETEC challenged pigs. This observation was consistent with results by Hung et al. (2020) and was supported by our fecal culture results, in which pigs fed with carbadox had fewer β-hemolytic coliforms in feces right after ETEC inoculation.

Complete blood count is crucial to evaluate the systemic severity of inflammation induced by bacterial infection, including ETEC. Without ETEC infection, *B. amyloliquefaciens* supplementation did not impact blood counts compared with the negative control. Similar results were also observed in Tang et al. (2018), in which no difference was observed in white and red blood cell counts and lymphocyte percentage when healthy laying hens were supplemented with *B. amyloliquefaciens*. The results from the current and previous studies suggest that *B. amyloliquefaciens* may have limited impacts on systemic immunity in animals when they are healthy. Consistent with He et al. (2020a), we also observed that ETEC inoculation increased total white blood cell counts and lymphocytes within 7 days post-inoculation. Reduced counts in lymphocytes and white blood cells in pigs supplemented with *B. amyloliquefaciens* or carbadox on d 14 and 21 PI suggest that both supplements may alleviate systemic inflammation caused by ETEC. These findings were also analogous to a study where *B. amyloliquefaciens* supplementation decreased white blood cell counts when broilers were challenged with lipopolysaccharides (Li et al., 2015).

The gut microbiota plays an important role in reducing intestinal inflammation to promote a mutual relationship with the host (Lawley and Walker, 2013). Watery diarrhea induced by ETEC infection can cause perturbance to the gut microbiota, leading to difficulty suppressing inflammation (Bin et al., 2018). The alpha diversity in the present study did no present changes in microbial diversity and richness in fecal samples of ETEC challenged pigs compared to sham pigs. This observation was contradicted with the findings in the previous

chapter and in Pollock et al. (2018), in which fecal microbial diversity decreased over time when pigs were challenged with ETEC. The decreased microbial diversity observed in Pollock et al. (2018) may have occurred due to pigs being inoculated with ETEC at five different time points throughout the experiment, whereas pigs in the present study were inoculated with ETEC for three consecutive days after seven days of adaptation period. However, the alpha diversity results in the present study agree with another study, in which microbial diversity and richness in feces were not affected by ETEC challenge likely due to the short sampling intervals to observe changes in fecal microbiota over time (Pollock et al., 2019). The increase in microbial diversity and richness in fecal samples over time and the overlapping samples in beta diversity between d 14 and 21 PI can be an indicator of maturity and stability in microbial diversity in pigs over time (Chen et al., 2017).

Firmicutes and Bacteroidota were the most abundant phyla in fecal microbiota throughout the experiment, which was expected in weaned piglets (Pajarillo et al., 2014; Yue et al., 2020; Luise et al., 2021). As expected, ETEC infection altered the microbial composition in the feces of pigs. The relative abundance of *Escherichia-Shigella* peaked seven days after first ETEC inoculation, and then was decreased on d 14 PI. This observation was supported by a gradual decrease in diarrhea severity of ETEC infected pigs in the present study, and agrees with the results in Kim et al. (2022) that pigs undergo recovery from ETEC infection around 11 days after inoculation. Carbadox has been shown to decrease the relative abundance of taxa that are noted to be highly abundant in the fecal microbiota of pigs, which includes *Lachnospiraceae*, *Blautia* and *Lactobacillus.* Carbadox is known to be bactericidal primarily active against gram-positive bacteria, however, the mechanism behind it is unknown (Constable et al., 2017). In agreement with Lourenco et al. (2021), carbadox supplementation in the current study had

significantly decreased the relative abundance of *Agathobacter* on d 7 PI, *Dorea* and *Streptococcus* on d 14 PI, and *Blautia* and *Dorea* on d 21 PI, which are all gram-positive bacteria. Although some taxa including *Enterobacteriaceae* and *Clostridiaceae* are abundant after carbadox exposure as other treatment groups, the changes in gut bacterial biomass are unknown due to the limits of 16S rRNA sequencing. Moreover, the decrease of these taxa was observed on d 7 and d 21 PI, indicating that carbadox may induce short- and long-term shifts in the fecal microbiota of weaned pigs by reducing microbial diversity (Holman et al., 2017). Supplementation with *B. amyloliquefaciens* had limited effects on fecal microbiota in pigs under sham group. However, supplementation of *B. amyloliquefaciens* moderately affected fecal microbiota composition compared with control or carbadox under ETEC challenge. On d 14 PI, pigs fed with *B. amyloliquefaciens* had less abundant *Dorea* than pigs in the positive control but had more abundant *Streptococcus* than pigs fed with carbadox. Supplementation of *B. amyloliquefaciens* also increased the relative abundance of *Prevotella*, *Megasphaera*, and *Streptococcus* compared with pigs fed with carbadox on d 21 PI.

Microbiota changes in the ileal digesta on d 21 PI were expected when pigs were challenged with ETEC or fed different diets. Unlike proximal sites of the digestive system that have more bacterial barrier including stomach acid and bile salts, the ileum provides an optimal environment for ETEC proliferation in pigs (Gonzales et al., 2013; Roussel et al., 2020). ETEC infection tended to increase microbial diversity and richness in ileum. These findings were also concurrent with the findings in Pollock et al. (2019). ETEC challenge in the current study decreased the relative abundance of Firmicutes but increased the relative abundance of Bacteroidota and Proteobacteria phyla in ileum. The ratio of Firmicutes to Bacteroidota is widely accepted to have an important influence in maintaining normal intestinal homeostasis.and a

decrease in Firmicutes:Bacteroidota ratio is usually observed in inflammatory bowel disease (Shen et al., 2018). However, this ratio can be also affected by an increase in other phyla during dysbiosis, such as the change of Proteobacteria. A growing evidence suggests that Proteobacteria is the most variable phylum, which contributes to microbial perturbation and may lead to increased disease risks (Morgan et al., 2012; Shin et al., 2015). The changes in ileal phyla are also explained by the changes of microbiota composition at family and genera levels. ETEC infection reduced the relative abundance of *Lactobacillus* (26.22% in positive control vs. 70.92% in negative control) but increased the relative abundance of *Clostridium sensu stricto* 1 and *Actinobacillus* in ileal digesta. Various *Lactobacillus* species have shown beneficial impacts on overall intestinal ecology, thus the species are commonly being investigated as probiotic candidates in humans and pigs (de Vries et al., 2006; Suo et al., 2012; Sayan et al., 2018). It was also reported that commensal *Lactobacillus* can activate the host immunity to promote overall health of mice (Holman et al., 2017; Nakamoto et al., 2017; Qi et al., 2019). *Actinobacillus* are gram-negative bacteria with most of the species are characterized as commensals, but some species are considered as pathogens in animal disease and the abundance was reported to increase in human disease as well (Rycroft and Garside, 2000; Denoth et al., 2021). *Clostridium sensu stricto* 1 is characterized as an opportunistic pathogen, which was reported to induce intestinal inflammation and reduced short chain fatty acids production in pigs and poultry (Yang et al., 2019; Li et al., 2020; Hu et al., 2021). Thus, a reduction in *Lactobacillus* abundance and an increase in *Actinobacillus* and *Clostridium sensu stricto* 1 in the ileal digesta of ETEC infected pigs confirms that ETEC infection remarkably disturbs intestinal microbiota community of weaned pigs by potentially competing colonization site or nutrients with favorable bacteria. The present results also suggest that ETEC can cause a long-term perturbation in ileal microbiota

throughout the weaning phase of pigs. Perturbation in the ileal microbiota may lead to unfavorable consequences in pig health, including immunosuppression and disrupted integrity in intestinal structure (Xia et al., 2022). In addition, more differences were observed in ileal microbiota than in fecal microbiota, which is likely due to the ileum being the major site of ETEC colonization (Martín-Rodríguez et al., 2022).

Under ETEC challenge, pigs supplemented with *B. amyloliquefaciens* had an increased microbial diversity and relative abundance of Actinomycetota, particularly *Bifidobacterium* in the ileal digesta compared to that in pigs fed with carbadox. Actinomycetota plays an important role in maintaining gut homeostasis, especially its genus *Bifidobacterium* is also commonly investigated as a potential probiotic, as it can support the host immune system by stimulating the release of immunoglobulins in the intestinal mucosa (Holman et al., 2017; Binda et al., 2018; Sun et al., 2020). The presence of *B. amyloliquefaciens* may aid in the growth of other microbes in the gut that can outperform ETEC to prevents its colonization (Dubreuil et al., 2016). Moreover, *B. amyloliquefaciens* supplementation differently modified the ileal microbiota compared with carbadox. Compared with *B. amyloliquefaciens*, carbadox further increased the abundance of *Clostridium sensu stricto* 1 and reduced abundance of *Prevotellaceae* in ileal microbiota, which was also observed in a pig study with the antibiotic growth promoter tylosin and a mice study with enrofloxacin (Kim et al., 2016; Sun et al., 2019). The increased abundance of Firmicutes with carbadox supplementation may build on existing evidence of Firmicutes developing antimicrobial resistance genes due to the consistent exposure to antibiotics (Anthony et al., 2022). Current results also suggest that carbadox supplementation may have long-term impacts on the ileal microbiota of weaned pigs, which may not be re-establish to that of healthy weaned pigs (Yue et al., 2020). Our findings highlight taxa influenced by ETEC infection and/or

dietary supplements, however future studies should consider evaluating the functional genomes from the gut microbiota and assess the relationship among growth performance and immunity of the host to their microbiota.

CONCLUSIONS

As the use of antibiotics growth promoters becomes less desirable globally, alternative practices are imperative to enhance growth and reduce post-weaning diarrhea in weaned pigs. The present study ultimately observed that supplementing *B. amyloliquefaciens* tended to increase feed intake and weight gain but had limited impacts on diarrhea of weaned pigs infected with ETEC. However, pigs fed with *B. amyloliquefaciens* had relatively milder systemic inflammation than control under disease challenge conditions. In addition, pigs supplemented with *B. amyloliquefaciens* had relative higher abundance of *Bifidobacterium* but lower *Clostridium sensu stricto* 1 than carbadox treated pigs when the pigs were challenged with ETEC in ileal digesta. The modulatory effects of *B. amyloliquefaciens* on immunity and ileal microbiota in pigs warrant further investigation. Taken altogether, supplementation of *B. amyloliquefaciens* solely may not provide weaned pigs with the growth enhancement and acute diarrheal alleviation similar to what is seen when supplemented with carbadox. Although various *Bacillus* spp. have shown the potential for promoting animal health and performance, findings in the present study suggest that there are variations in which *Bacillus* spp. is used and how they impact different animal species. Nevertheless, manipulating the gut microbiota to overall improve pig health and treat ETEC pathogenicity is currently a key interest in the swine industry. To further assess the importance of gut microbiota to alleviate post-weaning diarrhea, future studies are suggested to employ metagenomics for functional profiling, and to investigate

correlations among gut microbiota, immunity, and growth performance of pigs undergoing diarrhea in a larger scale study.

LITERATURE CITED

Aarestrup, F. M. 2005. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. Basic Clin. Pharmacol. Toxicol. 96:271–281. doi:10.1111/j.1742-7843.2005.pto960401.x.

Allen, H. K., T. Looft, D. O. Bayles, S. Humphrey, U. Y. Levine, D. Alt, and T. B. Stanton. 2011. Antibiotics in feed induce prophages in swine fecal microbiomes. mBio. 2:e00260-11. doi:10.1128/mBio.00260-11.

Amezcua, R., R. M. Friendship, C. E. Dewey, C. Gyles, and J. M. Fairbrother. 2002. Presentation of postweaning Escherichia coli diarrhea in southern Ontario, prevalence of hemolytic E. coli serogroups involved, and their antimicrobial resistance patterns. Can. J. Vet. Res. 66:73.

Angulo, F. J., P. Collignon, H. C. Wegener, P. Braam, and C. D. Butler. 2005. The routine use of antibiotics to promote animal growth does little to benefit protein undernutrition in the developing world. Clin. Infect. Dis. 41:1007–1013. doi:10.1086/433191.

Anthony, W. E., B. Wang, K. V. Sukhum, A. W. D'Souza, T. Hink, C. Cass, S. Seiler, K. A. Reske, C. Coon, E. R. Dubberke, C.-A. D. Burnham, G. Dantas, and J. H. Kwon. 2022. Acute and persistent effects of commonly used antibiotics on the gut microbiome and resistome in healthy adults. Cell Reports. 39:110649. doi:10.1016/j.celrep.2022.110649.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2014. Fitting linear mixed-effects models using lme4. arXiv preprint arXiv:1406.5823.

Becker, S. L., Q. Li, E. R. Burrough, D. Kenne, O. Sahin, S. A. Gould, and J. F. Patience. 2020. Effects of an F18 enterotoxigenic Escherichia coli challenge on growth performance, immunological status, and gastrointestinal structure of weaned pigs and the potential

protective effect of direct-fed microbial blends. J. Anim. Sci. 98:skaa113. doi:10.1093/jas/skaa113.

Bin, P., Z. Tang, S. Liu, S. Chen, Y. Xia, J. Liu, H. Wu, and G. Zhu. 2018. Intestinal microbiota mediates Enterotoxigenic Escherichia coli-induced diarrhea in piglets. BMC Veterinary Research. 14:385. doi:10.1186/s12917-018-1704-9.

Binda, C., L. R. Lopetuso, G. Rizzatti, G. Gibiino, V. Cennamo, and A. Gasbarrini. 2018. Actinobacteria: A relevant minority for the maintenance of gut homeostasis. Digestive and Liver Disease. 50:421–428. doi:10.1016/j.dld.2018.02.012.

Bokulich, N. A., B. D. Kaehler, J. R. Rideout, M. Dillon, E. Bolyen, R. Knight, G. A. Huttley, and J. Gregory Caporaso. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 6. doi:10.1186/s40168-018-0470-z. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5956843/

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, and F. Asnicar. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37:852–857.

Buntyn, J. O., T. Schmidt, D. J. Nisbet, and T. R. Callaway. 2016. The role of direct-fed microbials in conventional livestock production. Annu. Rev. Anim. Biosci. 4:335–55.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High resolution sample inference from Illumina amplicon data. Nat. Methods. 13:581–583. doi:10.1038/nmeth.3869.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. J. ISME 6:1621–1624. doi:10.1038/ismej.2012.8.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother.. 52:159–161. doi:10.1093/jac/dkg313.

Chen, L., Y. Xu, X. Chen, C. Fang, L. Zhao, and F. Chen. 2017. The maturing development of gut microbiota in commercial piglets during the weaning transition. Front. Microbiol. 8:1688. doi:10.3389/fmicb.2017.01688.

Constable, P. D., K. W. Hinchcliff, S. H. Done, and W. Grünberg, eds. 2017. 6 - Practical Antimicrobial Therapeutics. In: Veterinary Medicine (Eleventh Edition). W.B. Saunders. p. 153–174. Available from: https://www.sciencedirect.com/science/article/pii/B9780702052460000061

Cox, E., M. Loos, A. Coddens, B. Devriendt, M. Vesna, D. Vanrompay, and B. Goddeeris. 2012. Post-weaning E. coli infections in pigs and importance of the immune system. Actes des Journées AFMVP: Maisons-Alfort, France.

Denoth, L., P. Juillerat, A. E. Kremer, G. Rogler, M. Scharl, B. Yilmaz, S. Bluemel, and on behalf of the Swiss IBD Cohort Study. 2021. Modulation of the mucosa-associated microbiome linked to the PTPN2 risk gene in patients with primary sclerosing cholangitis and ulcerative colitis. Microorganisms. 9:1752. doi:10.3390/microorganisms9081752.

Dubreuil, J. D., R. E. Isaacson, and D. M. Schifferli. 2016. Animal enterotoxigenic Escherichia coli. EcoSal Plus. 7:10.1128/ecosalplus.ESP-0006–2016. doi:10.1128/ecosalplus.ESP-0006-2016.

Fairbrother, J. M., É. Nadeau, and C. L. Gyles. 2005. Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. 6:17–39. doi:10.1079/AHR2005105.

Fouhse, J. M., R. T. Zijlstra, and B. P. Willing. 2016. The role of gut microbiota in the health and disease of pigs. Anim. Fron. 6:30–36. doi:10.2527/af.2016-0031.

Fox, J., and S. Weisberg. 2018. An R companion to applied regression. Sage publications.

Gonzales, L., Z. B. Ali, E. Nygren, Z. Wang, S. Karlsson, B. Zhu, M. Quiding-Järbrink, and Å. Sjöling. 2013. Alkaline pH Is a Signal for Optimal Production and Secretion of the Heat Labile Toxin, LT in Enterotoxigenic Escherichia Coli (ETEC). PLoS One. 8:e74069. doi:10.1371/journal.pone.0074069.

He, Y., C. Jinno, K. Kim, Z. Wu, B. Tan, X. Li, R. Whelan, and Y. Liu. 2020a. Dietary Bacillus spp. enhanced growth and disease resistance of weaned pigs by modulating intestinal microbiota and systemic immunity. J. Animal Sci. Biotechnol. 11:101. doi:10.1186/s40104-020-00498-3.

He, Y., K. Kim, L. Kovanda, C. Jinno, M. Song, J. Chase, X. Li, B. Tan, and Y. Liu. 2020b. Bacillus subtilis: a potential growth promoter in weaned pigs in comparison to carbadox. J. Anim. Sci. 98. doi:10.1093/jas/skaa290. Available from: https://doi.org/10.1093/jas/skaa290

Holman, D. B., B. W. Brunelle, J. Trachsel, and H. K. Allen. 2017. Meta-analysis to define a core microbiota in the swine gut. mSystems. 2. doi:10.1128/mSystems.00004-17. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5443231/

Hu, C., X. Niu, S. Chen, J. Wen, M. Bao, S. G. Mohyuddin, Y. Yong, X. Liu, L. Wu, Z. Yu, X. Ma, and X. Ju. 2021. A comprehensive analysis of the colonic flora diversity, short chain fatty acid metabolism, transcripts, and biochemical indexes in heat-stressed pigs. Front. Immunol. 12. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2021.717723

Hung, Y.-T., Q. Hu, R. J. Faris, J. Guo, P. E. Urriola, G. C. Shurson, C. Chen, and M. Saqui-Salces. 2020. Analysis of gastrointestinal responses revealed both shared and specific targets of zinc oxide and carbadox in weaned pigs. Antibiotics. 9:463. doi:10.3390/antibiotics9080463.

Jerzsele, A., K. Szeker, R. Csizinszky, E. Gere, C. Jakab, J. J. Mallo, and P. Galfi. 2012. Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and Bacillus amyloliquefaciens spore suspension against artificially induced necrotic enteritis in broilers. Poultry Science. 91:837–843. doi:10.3382/ps.2011-01853.

Katoh, K., and D. M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. Molecular Biology and Evolution. 30:772–780. doi:10.1093/molbev/mst010.

Kim, J., R. B. Guevarra, S. G. Nguyen, J.-H. Lee, D. K. Jeong, and T. Unno. 2016. Effects of the antibiotics growth promoter tylosin on swine gut microbiota. J. Microbiol. Biotechnol. 26:876–882. doi:10.4014/jmb.1512.12004.

Kim, K., Y. He, C. Jinno, L. Kovanda, X. Li, D. Bravo, E. Cox, and Y. Liu. 2022. Supplementation of oligosaccharide-based polymer enhanced growth and disease resistance of weaned pigs by modulating intestinal integrity and systemic immunity. J. Anim. Sci. Biotechnol. 13:10. doi:10.1186/s40104-021-00655-2.

Kim, K., Y. He, X. Xiong, A. Ehrlich, X. Li, H. Raybould, E. R. Atwill, E. A. Maga, J. Jørgensen, and Y. Liu. 2019. Dietary supplementation of Bacillus subtilis influenced intestinal health of weaned pigs experimentally infected with a pathogenic E. coli. J Animal Sci Biotechnol. 10:52. doi:10.1186/s40104-019-0364-3.

Koumoutsi, A., X.-H. Chen, A. Henne, H. Liesegang, G. Hitzeroth, P. Franke, J. Vater, and R. Borriss. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in Bacillus amyloliquefaciens strain FZB42. J. Bacteriol. 186:1084–1096. doi:10.1128/JB.186.4.1084-1096.2004.

Kreuzer, S., M. Reissmann, and G. A. Brockmann. 2013. New fast and cost-effective gene test to get the ETEC F18 receptor status in pigs. Vet. Microbiol. 163:392–394. doi:10.1016/j.vetmic.2012.12.040.

Lawley, T. D., and A. W. Walker. 2013. Intestinal colonization resistance. Immunology. 138:1–11. doi:10.1111/j.1365-2567.2012.03616.x.

Lee, I. K., Y. C. Kye, G. Kim, H. W. Kim, M. J. Gu, J. Umboh, K. Maaruf, S. W. Kim, and C.-H. Yun. 2016. Stress, nutrition, and intestinal immune responses in pigs — A review. Asian-Australas J. Anim. Sci. 29:1075–1082. doi:10.5713/ajas.16.0118.

Lee, N.-K., W.-S. Kim, and H.-D. Paik. 2019. Bacillus strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. Food Sci. Biotechnol. 28:1297–1305. doi:10.1007/s10068-019-00691-9.

Lekagul, A., V. Tangcharoensathien, and S. Yeung. 2019. Patterns of antibiotic use in global pig production: A systematic review. Vet. Anim. Sci.7:100058. doi:10.1016/j.vas.2019.100058.

Lenth, R. V. 2021. Emmeans: estimated marginal means, aka least-squares means. R package version 1.6. 1.

Li, Y., H. Zhang, Y. P. Chen, M. X. Yang, L. L. Zhang, Z. X. Lu, Y. M. Zhou, and T. Wang. 2015. Bacillus amyloliquefaciens supplementation alleviates immunological stress in lipopolysaccharide-challenged broilers at early age. Poultry Science. 94:1504–1511. doi:10.3382/ps/pev124.

Li, Y.-J., J. Li, and C. Dai. 2020. The role of intestinal microbiota and mast cell in a rat model of visceral hypersensitivity. J. Neurogastroenterol. Motil. 26:529–538. doi:10.5056/jnm20004.

Liao, S. F., and M. Nyachoti. 2017. Using probiotics to improve swine gut health and nutrient utilization. Anim. Nutr. 3:331–343. doi:10.1016/j.aninu.2017.06.007.

Luise, D., M. L. Sciellour, A. Buchet, R. Resmond, C. Clement, M.-N. Rossignol, D. Jardet, O. Zemb, C. Belloc, and E. Merlot. 2021. The fecal microbiota of piglets during weaning transition and its association with piglet growth across various farm environments. PLOS ONE. 16:e0250655. doi:10.1371/journal.pone.0250655.

Martín-Rodríguez, A. J., E. Joffré, and Å. Sjöling. 2022. Bacterial gastrointestinal infections. In: N. Rezaei, editor. Encyclopedia of Infect. Immun. Elsevier, Oxford. p. 72–81. Available from: https://www.sciencedirect.com/science/article/pii/B9780128187319001051

McMurdie, P. J., and S. Holmes. 2013. phyloseq: An R Package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE. 8:e61217. doi:10.1371/journal.pone.0061217.

de Mendiburu, F., and M. F. de Mendiburu. 2019. Package 'agricolae.' R Package, Version. 1–2.

Morgan, X. C., T. L. Tickle, H. Sokol, D. Gevers, K. L. Devaney, D. V. Ward, J. A. Reyes, S. A. Shah, N. LeLeiko, S. B. Snapper, A. Bousvaros, J. Korzenik, B. E. Sands, R. J. Xavier, and C. Huttenhower. 2012. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol. 13:R79. doi:10.1186/gb-2012-13-9-r79.

Nakamoto, N., T. Amiya, R. Aoki, N. Taniki, Y. Koda, K. Miyamoto, T. Teratani, T. Suzuki, S. Chiba, P.-S. Chu, A. Hayashi, A. Yamaguchi, S. Shiba, R. Miyake, T. Katayama, W. Suda, Y. Mikami, N. Kamada, H. Ebinuma, H. Saito, M. Hattori, and T. Kanai. 2017. Commensal Lactobacillus controls immune tolerance during acute liver injury in mice. Cell Reports. 21:1215–1226. doi:10.1016/j.celrep.2017.10.022.

National Research Council (NRC). 2012. Nutrient requirements of swine: eleventh revised edition. Washington, DC: The National Academies Press.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2013. Package 'vegan.' Community ecology package, version. 2.

Pajarillo, E. A. B., J.-P. Chae, M. P. Balolong, H. B. Kim, and D.-K. Kang. 2014. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. J. Gen. Appl. Microbiol. 60:140–146. doi:10.2323/jgam.60.140.

Pedregosa, F., G. Varoquaux, A. Gramfort, V. Michel, B. Thirion, O. Grisel, M. Blondel, P. Prettenhofer, R. Weiss, and V. Dubourg. 2011. Scikit-learn: Machine learning in Python. 12:2825–2830.

Pollock, J., D. L. Gally, L. Glendinning, R. Tiwari, M. R. Hutchings, and J. G. M. Houdijk. 2018. Analysis of temporal fecal microbiota dynamics in weaner pigs with and without exposure to enterotoxigenic Escherichia coli,. J. Anim. Sci. 96:3777–3790. doi:10.1093/jas/sky260.

Pollock, J., M. R. Hutchings, K. E. K. Hutchings, D. L. Gally, and J. G. M. Houdijk. 2019. Changes in the ileal, but not fecal, microbiome in response to increased dietary protein level and enterotoxigenic Escherichia coli exposure in pigs. Appl. Environ. Microbiol. 85:e01252-19. doi:10.1128/AEM.01252-19.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2010. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLOS ONE. 5:e9490. doi:10.1371/journal.pone.0009490.

Qi, H., Y. Li, H. Yun, T. Zhang, Y. Huang, J. Zhou, H. Yan, J. Wei, Y. Liu, Z. Zhang, Y. Gao, Y. Che, X. Su, D. Zhu, Y. Zhang, J. Zhong, and R. Yang. 2019. Lactobacillus maintains healthy gut mucosa by producing L-Ornithine. Commun. Biol. 2:1–14. doi:10.1038/s42003-019-0424-4.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research. 41:D590–D596.

Roussel, C., K. De Paepe, W. Galia, J. De Bodt, S. Chalancon, F. Leriche, N. Ballet, S. Denis, M. Alric, T. Van de Wiele, and S. Blanquet-Diot. 2020. Spatial and temporal modulation

of enterotoxigenic E. coli H10407 pathogenesis and interplay with microbiota in human gut models. BMC Biology. 18:141. doi:10.1186/s12915-020-00860-x.

Rycroft, A. N., and L. H. Garside. 2000. Actinobacillus species and their role in animal disease. Vet. J. 159:18–36. doi:10.1053/tvjl.1999.0403.

Salazar, F., A. Ortiz, and E. Sansinenea. 2017. Characterisation of two novel bacteriocin-like substances produced by Bacillus amyloliquefaciens ELI149 with broad-spectrum antimicrobial activity. J. Glob. Antimicrob. Resist. 11:177–182. doi:10.1016/j.jgar.2017.08.008.

Sansinenea, E., and A. Ortiz. 2011. Secondary metabolites of soil Bacillus spp. Biotechnol Lett. 33:1523–1538. doi:10.1007/s10529-011-0617-5.

Sayan, H., P. Assavacheep, K. Angkanaporn, and A. Assavacheep. 2018. Effect of *Lactobacillus salivarius* on growth performance, diarrhea incidence, fecal bacterial population and intestinal morphology of suckling pigs challenged with $F4^{+}$ enterotoxigenic *Escherichia coli*. Asian-Australas J. Anim. Sci. 31:1308–1314. doi:10.5713/ajas.17.0746.

Shin, N.-R., T. W. Whon, and J.-W. Bae. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 33:496–503. doi:10.1016/j.tibtech.2015.06.011.

Sun, L., X. Zhang, Y. Zhang, K. Zheng, Q. Xiang, N. Chen, Z. Chen, N. Zhang, J. Zhu, and Q. He. 2019. Antibiotic-induced disruption of gut microbiota alters local metabolomes and immune responses. Front. Cell Infect. Microbiol. 9:99. doi:10.3389/fcimb.2019.00099.

Sun, S., L. Luo, W. Liang, Q. Yin, J. Guo, A. M. Rush, Z. Lv, Q. Liang, M. A. Fischbach, J. L. Sonnenburg, D. Dodd, M. M. Davis, and F. Wang. 2020. Bifidobacterium alters the gut microbiota and modulates the functional metabolism of T regulatory cells in the context

of immune checkpoint blockade. Proceedings of the National Academy of Sciences. 117:27509–27515. doi:10.1073/pnas.1921223117.

Suo, C., Y. Yin, Xiaona Wang, X. Lou, D. Song, Xin Wang, and Q. Gu. 2012. Effects of lactobacillus plantarumZJ316 on pig growth and pork quality. BMC Veterinary Research. 8:89. doi:10.1186/1746-6148-8-89.

Tang, R. Y., Z. L. Wu, G. Z. Wang, and W. C. Liu. 2018. The effect of Bacillus amyloliquefaciens on productive performance of laying hens. Italian J. Anim. Sci. 17:436–441. doi:10.1080/1828051X.2017.1394169.

Team, R. C. 2021. R: A language and environment for statistical computing.

de Vries, M. C., E. E. Vaughan, M. Kleerebezem, and W. M. de Vos. 2006. Lactobacillus plantarum—survival, functional and potential probiotic properties in the human intestinal tract. Int. Dairy J. 16:1018–1028. doi:10.1016/j.idairyj.2005.09.003.

Wickham, H. 2011. ggplot2. Wiley Interdisciplinary Reviews: Computational Statistics. 3:180–185.

World Health Organization. 2015. Antimicrobial resistance–SEA/RC68/R3. World Health Organization.

Xia, B., W. Wu, W. Fang, X. Wen, J. Xie, and H. Zhang. 2022. Heat stress-induced mucosal barrier dysfunction is potentially associated with gut microbiota dysbiosis in pigs. Anim. Nutr.8:289–299. doi:10.1016/j.aninu.2021.05.012.

Yang, W.-Y., Y. Lee, H. Lu, C.-H. Chou, and C. Wang. 2019. Analysis of gut microbiota and the effect of lauric acid against necrotic enteritis in Clostridium perfringens and Eimeria side-by-side challenge model. PLoS One. 14:e0205784. doi:10.1371/journal.pone.0205784.

Yue, S., Z. Li, F. Hu, and J.-F. Picimbon. 2020. Curing piglets from diarrhea and preparation of a healthy microbiome with Bacillus treatment for industrial animal breeding. Sci. Rep. 10:19476. doi:10.1038/s41598-020-75207-1.

Table 3-1. Ingredient composition of experimental diets, as fed basis[1]

Ingredient, %	Control, phase I	Control, phase II
Corn	42.50	48.48
Dried whey	15.00	10.00
Soybean meal	20.00	24.00
Fish meal	4.00	3.00
Barley	10.00	10.00
Soy protein concentrate	3.00	-
Soybean oil	2.10	1.30
Limestone	0.95	0.95
DCP	0.55	0.52
L-Lysine·HCl	0.49	0.46
DL-Methionine	0.26	0.21
L-Threonine	0.22	0.20
L-Tryptophan	0.09	0.08
L-Valine	0.14	0.10
Salt	0.40	0.40
Vit-mineral, Sow 6[2]	0.30	0.30
Total	100.00	100.00
Calculated energy and nutrient		
Metabolizable energy, kcal/kg	3,364	3,310
Net energy, kcal/kg	2,526	2,480

Crude protein, %	20.54	19.77
Arg,[3] %	1.14	1.11
His,[3] %	0.47	0.46
Ile,[3] %	0.76	0.72
Leu,[3] %	1.50	1.44
Lys,[3] %	1.42	1.32
Met,[3] %	0.56	0.50
Thr,[3] %	0.89	0.83
Trp,[3] %	0.31	0.29
Val,[3] %	0.97	0.89
Met + Cys,[3] %	0.85	0.79
Phe + Tyr,[3] %	1.36	1.32
Ca, %	0.83	0.75
Total P, %	0.66	0.60
Digestible P, %	0.43	0.36

[1]In each phase, two additional diets were formulated by adding 10^9 CFU/kg *B. amyloliquefaciens* or 50 mg/kg of carbadox to the control diet, respectively.

[2]Provided by United Animal Health (Sheridan, IN). The premix provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride,

0.24 mg; vitamin B12, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.

[3]Amino acids are indicated as standardized ileal digestible AA.

Table 3-2. Growth performance of weaned pigs fed a control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens* (BAM) or antibiotics (AGP)

	Sham		*Escherichia coli*				
Item[1]	CON-	BAM-	CON+	BAM+	AGP+	SEM	*P*-value
BW, kg							
d -7	7.44	7.44	7.39	7.40	7.42	0.44	0.99
d 0	8.34	8.31	8.32	8.16	8.46	0.45	0.81
d 7 PI	9.75	9.60	9.15	8.95	9.62	0.47	0.18
d 14 PI	13.15	13.74	12.69	12.96	13.46	0.66	0.45
d 21 PI	17.92^{ab}	18.09^{a}	16.31^{c}	16.63^{bc}	17.65^{ab}	0.67	<0.05
ADG, g							
d -7 to 0	128	140	130	108	149	16.22	0.53
d 0 to 7 PI	202^{a}	184^{a}	117^{c}	132^{bc}	163^{ab}	16.59	<0.05
d 7 to 14 PI	486	594	506	570	552	36.36	0.094
d 14 to 21 PI	681^{a}	622^{a}	515^{c}	525^{bc}	603^{ab}	30.97	<0.01
d 0 to 14 PI	344	390	312	365	360	22.12	0.102
d 0 to 21 PI	456^{a}	467^{a}	379^{c}	403^{bc}	441^{ab}	22.14	<0.05
ADFI, g							
d -7 to 0	326	264	266	295	347	25.99	0.104
d 0 to 7 PI	716^{ab}	829^{a}	616^{bc}	679^{bc}	588^{c}	49.36	<0.01
d 7 to 14 PI	843	867	751	897	831	50.42	0.31
d 14 to 21 PI	1,066	1,084	856	1,052	1,091	67.17	0.38
d 0 to 14 PI	779^{ab}	881^{a}	702^{b}	786^{ab}	746^{b}	37.95	<0.05

d 0 to 21 PI	875[ab]	927[a]	788[b]	879[ab]	816[b]	30.90	<0.05
Gain:Feed							
d -7 to 0	0.38	0.40	0.49	0.37	0.42	0.047	0.46
d 0 to 7 PI	0.29	0.23	0.20	0.18	0.24	0.040	0.37
d 7 to 14 PI	0.60	0.69	0.68	0.69	0.67	0.035	0.35
d 14 to 21 PI	0.62[a]	0.63[a]	0.53[b]	0.51[b]	0.56[ab]	0.025	<0.01
d 0 to 14 PI	0.45	0.46	0.45	0.46	0.50	0.036	0.75
d 0 to 21 PI	0.53	0.51	0.48	0.48	0.52	0.021	0.29

[1]BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; PI = post-inoculation. Each least squares mean represents 9-10 observations. [a,b,c]Means without a common superscript are different ($P < 0.05$).

Table 3-3. Total and differential white blood cells in weaned pigs fed a control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens* (BAM) or antibiotics (AGP)

	Sham		*Escherichia coli*				
Item[1]	CON-	BAM-	CON+	BAM+	AGP+	SEM	*P*-value
d -7							
WBC, 10^3/μL	9.86	7.94	8.66	8.91	9.88	1.55	0.82
Neu, 10^3/μL	3.51	2.91	3.83	3.57	4.14	0.58	0.52
Lym, 10^3/μL	6.10	4.52	4.22	4.77	5.06	0.85	0.58
Mono, 10^3/μL	0.57	0.45	0.57	0.53	0.53	0.12	0.96
Eos, 10^3/μL	0.027	0.041	0.048	0.033	0.092	0.023	0.35
Baso, 10^3/μL	0.017	0.009	0.016	0.010	0.032	0.011	0.57
Neu, %	33.51	37.38	44.90	40.08	41.61	2.39	0.051
Lym, %	60.62	56.02	47.88	53.26	51.48	2.78	0.067
Mono, %	5.58	5.93	6.39	6.02	5.79	1.01	0.98
Eos, %	0.19	0.52	0.61	0.45	0.85	0.24	0.47
Baso, %	0.116	0.093	0.235	0.126	0.292	0.098	0.58
Neu:Lym	0.57	0.72	0.95	0.79	0.88	0.095	0.12
d 0							
WBC, 10^3/μL	10.41	9.17	11.25	11.21	13.20	1.14	0.13
Neu, 10^3/μL	4.86	3.82	5.45	4.26	5.36	0.62	0.21
Lym, 10^3/μL	4.86^{b}	4.75^{b}	5.23^{b}	6.31^{ab}	7.14^{a}	0.64	<0.05
Mono, 10^3/μL	0.60	0.56	0.46	0.56	0.55	0.095	0.87
Eos, 10^3/μL	0.067	0.045	0.078	0.061	0.095	0.020	0.50
Baso, 10^3/μL	0.021	0.008	0.035	0.022	0.046	0.011	0.16
Neu, %	46.59	42.18	47.80	37.21	40.37	3.10	0.067
Lym, %	46.73^{b}	51.36^{ab}	46.96^{b}	57.28^{a}	54.45^{ab}	3.08	<0.05
Mono, %	5.65	5.90	4.14	4.77	4.21	0.68	0.17
Eos, %	0.74	0.48	0.74	0.56	0.66	0.20	0.86
Baso, %	0.29	0.077	0.37	0.19	0.31	0.12	0.43
Neu:Lym	1.17	0.86	1.05	0.70	0.83	0.15	0.15

d 7 PI							
WBC, $10^3/\mu L$	11.45^{b}	13.17^{ab}	17.03^{a}	15.92^{ab}	16.14^{a}	1.18	<0.05
Neu, $10^3/\mu L$	5.34	5.74	7.15	6.76	6.50	0.76	0.34
Lym, $10^3/\mu L$	5.28^{b}	6.21^{ab}	8.71^{a}	8.26^{a}	8.22^{a}	0.94	<0.05
Mono, $10^3/\mu L$	0.81	1.00	0.93	0.67	1.03	0.146	0.38
Eos, $10^3/\mu L$	0.052	0.139	0.186	0.161	0.311	0.074	0.15
Baso, $10^3/\mu L$	0.021	0.022	0.050	0.057	0.062	0.016	0.17
Neu, %	46.06	44.16	42.44	42.15	40.77	2.40	0.55
Lym, %	46.21	47.06	50.65	52.71	50.59	2.50	0.33
Mono, %	7.11^{a}	7.46^{a}	5.66^{ab}	3.94^{b}	6.39^{a}	0.76	<0.05
Eos, %	0.47	1.06	1.00	0.92	1.81	0.43	0.22
Baso, %	0.19	0.17	0.27	0.35	0.35	0.085	0.24
Neu:Lym	1.03	0.99	0.88	0.83	0.84	0.096	0.44
d 14 PI							
WBC, $10^3/\mu L$	16.29	16.86	19.19	17.26	18.25	1.51	0.49
Neu, $10^3/\mu L$	7.62^{b}	7.22^{b}	9.49^{a}	7.92^{b}	7.27^{b}	0.51	<0.05
Lym, $10^3/\mu L$	7.79	8.36	8.75	8.21	9.48	1.18	0.65
Mono, $10^3/\mu L$	0.67	0.67	0.63	0.54	0.80	0.086	0.32
Eos, $10^3/\mu L$	0.24	0.49	0.28	0.55	0.62	0.15	0.28
Baso, $10^3/\mu L$	0.019	0.043	0.043	0.092	0.090	0.022	0.071
Neu, %	47.24	43.76	49.56	46.10	40.99	3.23	0.12
Lym, %	47.33	49.18	45.34	47.24	50.81	3.24	0.47
Mono, %	4.07	4.02	3.41	3.13	4.53	0.46	0.20
Eos, %	1.29	2.78	1.44	3.03	3.24	0.80	0.28
Baso, %	0.11	0.23	0.23	0.52	0.45	0.15	0.087
Neu:Lym	1.02	0.93	1.17	0.99	0.85	0.15	0.30
d 21 PI							
WBC, $10^3/\mu L$	11.86^{b}	12.06^{ab}	14.53^{a}	10.25^{b}	12.05^{ab}	1.19	<0.05
Neu, $10^3/\mu L$	5.45^{ab}	5.49^{ab}	6.33^{a}	4.13^{bc}	4.07^{c}	0.54	<0.01
Lym, $10^3/\mu L$	5.59^{b}	5.63^{b}	7.30^{a}	5.36^{b}	6.79^{ab}	0.69	<0.05
Mono, $10^3/\mu L$	0.52	0.58	0.50	0.38	0.69	0.081	0.18

Eos, $10^3/\mu L$	0.26	0.33	0.32	0.29	0.43	0.086	0.70
Baso, $10^3/\mu L$	0.044	0.040	0.076	0.079	0.067	0.018	0.20
Neu, %	45.39^{a}	45.11^{a}	43.59^{a}	41.07^{a}	34.15^{b}	1.96	<0.01
Lym, %	47.63^{b}	46.91^{b}	50.39^{b}	51.83^{ab}	56.28^{a}	1.88	<0.01
Mono, %	4.40^{abc}	4.88^{ab}	3.28^{c}	3.72^{bc}	5.74^{a}	0.57	<0.05
Eos, %	2.18	2.77	2.18	2.75	3.33	0.60	0.66
Baso, %	0.39^{b}	0.34^{b}	0.51^{ab}	0.76^{a}	0.51^{ab}	0.10	<0.05
Neu:Lym	0.98^{a}	0.99^{a}	0.89^{a}	0.82^{ab}	0.62^{b}	0.072	<0.01

[1]PI = post-inoculation; WBC = white blood cell; Neu = neutrophil; Lym = lymphocyte; Mono = monocyte; Eos = eosinophil; Baso = basophil. Each least squares mean represents 9-12 observations. [a,b,c]Means without a common superscript are different ($P < 0.05$).

Table 3-4. Relative abundance (%) of Bacteroidota, Firmicutes, and Proteobacteria and their top families in feces of weaned pigs fed control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens (*BAM) or antibiotics (AGP)

	Sham		*Escherichia coli*		
	CON-	BAM-	CON+	BAM+	AGP+
d -7					
Bacteroidota	16.78	13.05	14.35	14.68	12.09
Bacteroidaceae	6.11	3.59	6.40	5.35	3.64
Muribaculaceae	1.41	1.93	1.84	1.18	1.47
Prevotellaceae	3.96	2.29	2.31	2.75	2.36
Rikenellaceae	3.35	2.79	2.39	2.67	2.63
Firmicutes	58.19	65.1	63.87	61.37	68.43
Clostridiaceae	11.42	10.56	7.93	8.96	10.31
Lachnospiraceae	8.36	8.45	10.45	10.24	9.38
Lactobacillaceae	2.80	5.45	5.39	3.08	8.10
Oscillospiraceae	5.58	6.01	10.49	7.27	8.26
Ruminococcaceae	10.8	11.78	6.03	7.22	10.33
Proteobacteria	3.29	4.54	2.33	4.18	2.28
Enterobacteriaceae	1.31	3.26	1.79	3.04	1.66
Succinivibionaceae	1.83	1.03	0.38	1.01	0.48
d 0					
Bacteroidota	13.67	9.67	12.38	14.04	15.02
Bacteroidaceae	0.41^{ab}	0.36^{ab}	0.19^{b}	0.46^{a}	0.34^{ab}
Muribaculaceae	3.13	1.92	2.60	3.09	2.35
Prevotellaceae	8.09	5.92	7.60	7.39	10.67
Rikenellaceae	1.33	1.12	1.52	2.11	1.30
Firmicutes	76.45	79.61	76.99	75.61	76.59
Clostridiaceae	1.16^{a}	0.13^{b}	0.51^{a}	0.15^{ab}	0.33^{ab}
Lachnospiraceae	18.08^{b}	18.91^{b}	19.61^{ab}	16.50^{b}	27.29^{a}
Lactobacillaceae	29.36	36.43	32.27	35.40	25.55
Oscillospiraceae	5.44	4.22	4.66	4.72	3.38
Ruminococcaceae	4.46	4.62	4.90	4.52	5.68
Proteobacteria	1.52	1.96	0.90	1.44	0.39
Enterobacteriaceae	1.21^{a}	0.65^{ab}	0.61^{ab}	1.21^{a}	0.20^{b}
Succinivibionaceae	0.26	0.88	0.15	0.16	0.16

d 7 PI					
Bacteroidota	17.2^{ab}	15.75^{ab}	12.82^{b}	15.27^{ab}	18.61^{a}
Bacteroidaceae	0.27^{ab}	0.17^{b}	0.32^{ab}	0.64^{ab}	1.34^{a}
Muribaculaceae	4.80	3.64	3.16	2.66	4.72
Prevotellaceae	9.34	9.62	7.84	9.57	8.84
Rikenellaceae	1.60	1.56	1.10	1.39	1.40
Firmicutes	67.71	69.08	69.46	64.55	56.64
Clostridiaceae	0.21	0.11	0.16	0.45	0.69
Lachnospiraceae	29.76^{a}	28.23^{ab}	30.83^{a}	25.14^{ab}	16.48^{b}
Lactobacillaceae	7.99^{b}	11.59^{ab}	17.39^{a}	16.05^{ab}	17.01^{a}
Oscillospiraceae	6.40	3.16	4.19	4.57	6.72
Ruminococcaceae	5.89	8.17	4.17	5.5	3.39
Proteobacteria	5.42	7.24	9.86	9.72	16.54
Enterobacteriaceae	1.82^{b}	5.68^{ab}	8.84^{ab}	7.49^{ab}	15.9^{a}
Succinivibionaceae	3.36	1.06	0.87	1.65	0.50
d 14 PI					
Bacteroidota	15.64	12.19	17.71	17.93	17.53
Bacteroidaceae	0.02^{ab}	0.03^{ab}	0.00^{b}	0.34^{a}	0.13^{ab}
Muribaculaceae	3.3	2.61	2.56	1.16	3.20
Prevotellaceae	11.10^{ab}	8.44^{b}	14.08^{a}	15.95^{a}	12.47^{ab}
Rikenellaceae	0.82	0.89	0.99	0.39	1.39
Firmicutes					
Clostridiaceae	77.87	78.45	72.09	71.78	73.29
Lachnospiraceae	0.74	0.45	0.57	0.31	3.24
Lactobacillaceae	26.08	27.29	23.64	24.70	25.49
Oscillospiraceae	10.89	15.79	11.75	15.42	15.26
Ruminococcaceae	3.63	2.51	4.03	1.59	5.74
Proteobacteria	1.24	0.95	2.55	1.35	1.14
Enterobacteriaceae	0.10	0.24	0.03	0.15	0.02
Succinivibionaceae	1.11	0.68	2.37	1.17	1.03
d 21 PI					
Bacteroidota	20.21	22.24	16.50	19.47	18.34
Bacteroidaceae	0.01	0.01	0.01	0.01	0.16
Muribaculaceae	2.98	3.03	4.19	3.19	5.28
Prevotellaceae	15.97^{ab}	17.91^{a}	10.78^{b}	15.09^{ab}	11.33^{b}
Rikenellaceae	1.05	1.11	1.36	1.10	1.26

Firmicutes	67.12[ab]	61.62[b]	73.47[a]	68.29[ab]	66.89[ab]
Clostridiaceae	1.39[b]	1.08[b]	0.73[b]	4.12[b]	18.48[a]
Lachnospiraceae	20.98	18.42	23.19	18.5	17.16
Lactobacillaceae	9.12[ab]	7.33[ab]	12.38[a]	8.75[ab]	6.20[b]
Oscillospiraceae	3.63	3.00	4.28	4.04	7.38
Ruminococcaceae	6.91	7.69	7.00	7.74	5.36
Proteobacteria	3.27	3.36	1.61	2.7	6.41
Enterobacteriaceae	0.05	0.05	0.01	0.03	0.02
Succinivibionaceae	2.89	3.20	1.56	2.61	6.28

[a,b]Means without a common superscript are different ($P < 0.05$). Each mean represents 9-10 observations.

Table 3-5. Relative abundance (%) of the most abundant genera from Bacteroidota, Firmicutes, and Proteobacteria in feces of weaned pigs fed control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens (*BAM) or antibiotics (AGP)

	Sham		*Escherichia coli*		
	CON-	BAM-	CON+	BAM+	AGP+
d -7					
Bacteroidota					
Muribaculaceae	1.41	1.93	1.84	1.18	1.47
Prevotella	0.76	0.33	0.28	0.86	0.71
Firmicutes					
Agathobacter	0.01	0.06	0.01	0.17	0.00
Blautia	0.07	0.16	0.09	0.11	0.16
Coprococcus	0.01	0.18	0.00	0.18	0.04
Dorea	0.00	0.01	0.00	0.00	0.00
Faecalibacterium	0.01	0.03	0.00	0.00	0.00
Lactobacillus	2.8	5.45	5.39	3.08	8.1
Megasphaera	0.33	0.63	0.08	0.91	0.24
Ruminococcus	9.76	10.07	4.52	5.92	7.82
Streptococcus	0.18	0.86	0.66	0.68	0.4
Proteobacteria					
Escherichia-Shigella	1.31	3.26	1.79	3.04	1.66
d 0					
Bacteroidota					
Muribaculaceae	3.13	1.92	2.6	3.09	2.35
Prevotella	4.52^{ab}	2.65^{b}	3.92^{ab}	3.19^{b}	6.74^{a}
Firmicutes					
Agathobacter	0.91	1.75	1.74	3.31	3.56
Blautia	4.51^{b}	4.41^{b}	4.81^{b}	2.73^{b}	12.00^{a}
Coprococcus	1.49	1.39	1.50	1.78	2.33
Dorea	1.09	0.47	0.65	0.34	0.34
Faecalibacterium	1.39	1.39	1.34	1.61	1.49
Lactobacillus	29.36	36.43	32.27	35.4	25.51
Megasphaera	3.04	5.59	3.65	3.96	5.66
Ruminococcus	0.84	0.69	0.63	0.81	0.94
Streptococcus	1.00^{a}	0.16^{ab}	0.25^{b}	0.12^{b}	0.18^{ab}

Proteobacteria					
Escherichia-Shigella	1.21[a]	0.65[a]	0.61[a]	1.21[a]	0.2[b]
d 7 PI					
Bacteroidota					
Muribaculaceae	4.80	3.64	3.16	2.66	4.72
Prevotella	4.21	5.32	3.49	5.00	3.36
Firmicutes					
Agathobacter	2.59[a]	4.29[a]	2.06[a]	1.89[ab]	0.9[b]
Blautia	11.37[a]	9.19[a]	9.57[ab]	6.00[b]	3.17[b]
Coprococcus	2.10	1.95	3.04	3.40	1.78
Dorea	4.11[a]	3.19[a]	2.97[ab]	1.20[b]	1.08[b]
Faecalibacterium	2.57	4.50	1.78	3.14	1.18
Lactobacillus	7.99[b]	11.59[ab]	17.39[a]	16.05[ab]	17.01[a]
Megasphaera	1.35[ab]	3.77[a]	1.16[ab]	1.61[ab]	0.65[b]
Ruminococcus	0.47	0.62	0.53	0.60	0.74
Streptococcus	0.72	0.11	0.32	0.11	0.22
Proteobacteria					
Escherichia-Shigella	1.82[b]	5.68[ab]	8.84[ab]	7.49[ab]	15.90[a]
d 14 PI					
Bacteroidota					
Muribaculaceae	3.30	2.61	2.56	1.16	3.20
Prevotella	7.10[abc]	5.30[c]	10.66[a]	12.40[a]	8.16[ab]
Firmicutes					
Agathobacter	1.55	2.07	1.06	2.39	2.65
Blautia	11.75	11.07	7.51	8.79	8.22
Coprococcus	2.27[a]	2.93[ab]	1.17[b]	1.55[ab]	2.66[ab]
Dorea	2.39[ab]	2.08[abc]	3.26[a]	1.58[bc]	1.37[c]
Faecalibacterium	3.74	4.19	3.72	4.77	4.65
Lactobacillus	10.89	15.79	11.75	15.42	15.26
Megasphaera	1.67	3.53	3.46	2.39	2.02
Ruminococcus	1.00	1.76	1.53	1.83	1.79
Streptococcus	9.77[a]	3.88[a]	2.89[a]	5.21[a]	0.73[b]
Proteobacteria					
Escherichia-Shigella	0.10	0.24	0.03	0.15	0.02
d 21 PI					
Bacteroidota					

Muribaculaceae	2.98	3.03	4.19	3.19	5.28
Prevotella	9.86[a]	13.25[a]	8.59[ab]	12.37[a]	5.55[b]
Firmicutes					
Agathobacter	1.29	1.50	1.10	1.12	0.46
Blautia	6.90[ab]	5.08[b]	9.15[a]	6.38[ab]	5.20[b]
Coprococcus	2.41	2.25	1.36	1.29	1.58
Dorea	1.73[ab]	1.22[ab]	2.31[a]	1.71[ab]	0.82[b]
Faecalibacterium	2.66	2.37	2.31	3.25	1.72
Lactobacillus	9.11[ab]	7.33[ab]	12.38[a]	8.75[ab]	6.20[b]
Megasphaera	1.50[bc]	2.39[ab]	5.08[a]	4.73[a]	0.28[c]
Ruminococcus	1.48	1.98	1.49	2.24	1.61
Streptococcus	6.91[a]	5.96[ab]	2.41[bc]	4.66[ab]	0.18[c]
Proteobacteria					
Escherichia-Shigella	0.05	0.05	0.01	0.03	0.02

[a-g]Means without a common superscript are different ($P < 0.05$). Each mean represents 9-10 observations.

Table 3-6. Relative abundance (%) of Actinomycetota, Bacteroidota, Firmicutes, and Proteobacteria and their top families in ileal digesta of weaned pigs fed a control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens* (BAM) or antibiotics (AGP)

	Ileal digesta				
	Sham		*Escherichia coli*		
	CON-	BAM-	CON+	BAM+	AGP+
Actinomycetota	3.38^{ab}	3.85^{ab}	4.62^{ab}	9.84^{a}	1.39^{b}
Atopobiaceae	0.04^{bc}	0.17^{ab}	0.25^{a}	2.11^{a}	0.00^{c}
Bifidobacteriaceae	3.28^{ab}	3.58^{ab}	4.02^{ab}	7.50^{a}	1.30^{b}
Bacteroidota	0.02^{b}	0.33^{ab}	0.12^{a}	1.80^{a}	0.01^{b}
Prevotellaceae	0.02^{ab}	0.31^{ab}	0.11^{ab}	1.78^{a}	0.01^{b}
Firmicutes	94.52^{a}	93.51^{a}	76.48^{b}	76.63^{b}	92.68^{a}
Clostridiaceae	2.14^{cd}	3.71^{d}	16.49^{b}	16.4^{bc}	49.23^{a}
Erysipelotrichaceae	3.03	6.99	4.21	4.98	0.56
Lactobacillaceae	70.95^{a}	69.31^{a}	26.23^{b}	31.51^{b}	30.80^{b}
Peptostreptococcaceae	6.73	3.58	4.60	6.76	1.81
Streptococcaceae	8.43	3.43	19.28	4.89	9.22
Proteobacteria	1.52^{b}	2.07^{b}	17.81^{a}	10.93^{ab}	4.35^{ab}
Enterobacteriaceae	0.38	0.28	0.07	0.70	0.04
Pasteurellaceae	0.93^{c}	1.78^{bc}	17.58^{a}	10.20^{a}	4.00^{ab}

$^{a-d}$Means without a common superscript are different ($P < 0.05$). Each mean represents 9-10 observations.

Table 3-7. Relative abundance (%) of the most abundant genera from Firmicutes and Proteobacteria in ileal digesta of weaned pigs fed a control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens (*BAM) or antibiotics (AGP)

	Ileal digesta				
	Sham		*Escherichia coli*		
	CON-	BAM-	CON+	BAM+	AGP+
Actinomycetota					
Bifidobacterium	3.26[ab]	3.42[ab]	4.02[ab]	7.45[a]	1.30[b]
Firmicutes					
Clostridium sensu stricto 1	2.07[c]	3.69[c]	16.46[b]	16.03[b]	49.06[a]
Lactobacillus	70.92[a]	69.31[a]	26.22[b]	31.51[b]	30.80[b]
Megasphaera	2.14[a]	3.99[a]	1.90[a]	5.18[a]	0.30[b]
Streptococcus	8.43	3.43	19.28	4.89	9.22
Terrisporobacter	4.71	2.63	2.23	3.93	1.80
Turicibacter	3.02	6.97	4.14	4.90	0.53
Proteobacteria					
Actinobacillus	0.90[c]	1.76[bc]	17.28[a]	10.00[a]	3.99[ab]

[a-d]Means without a common superscript are different ($P < 0.05$). Each mean represents 9-10 observations.

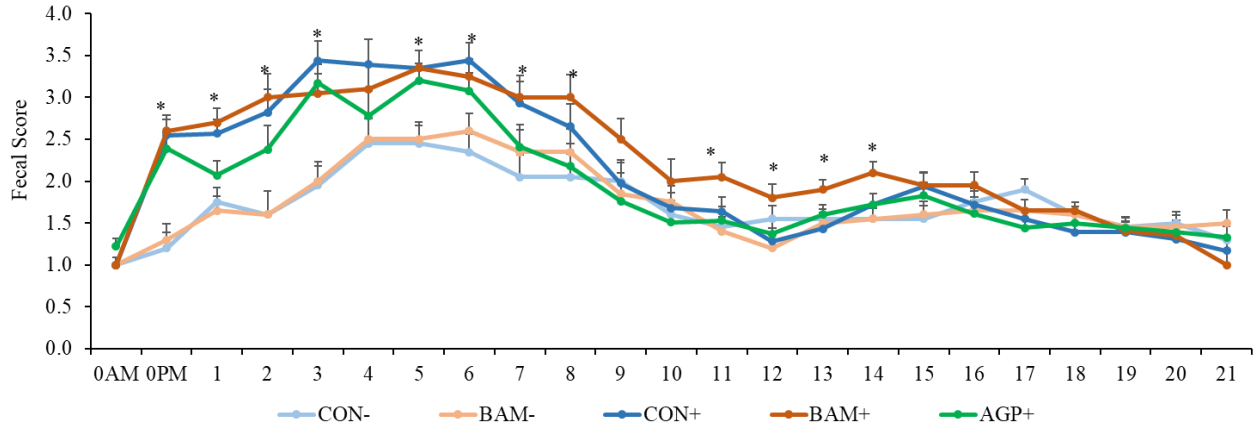


Figure 3-1. Daily fecal score of weaned pigs fed diets supplemented with *Bacillus amyloliquefaciens* (BAM) or antibiotics (AGP) with (+) or without (-) enterotoxigenic *Escherichia coli* challenge. Fecal score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea. PI = post-inoculation. *$P < 0.05$, indicating diarrhea scores were different among treatments. Each least squares mean represents 9-10 observations.

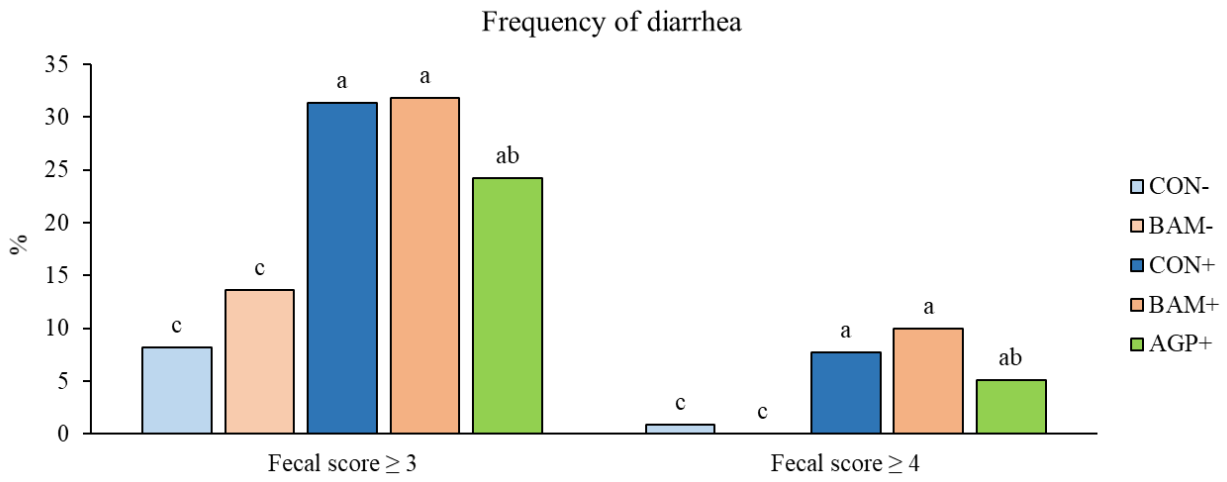


Figure 3-2. Frequency of diarrhea of weaned pigs fed diets supplemented with *Bacillus amyloliquefaciens* (BAM) or antibiotics (AGP). Frequency of diarrhea was calculated as the percentage of pig days with fecal score ≥ 3 or ≥ 4 in the total of pig days. Each least squares mean represents 9-10 observations. [a,b,c]Means without a common superscript are different ($P < 0.05$).

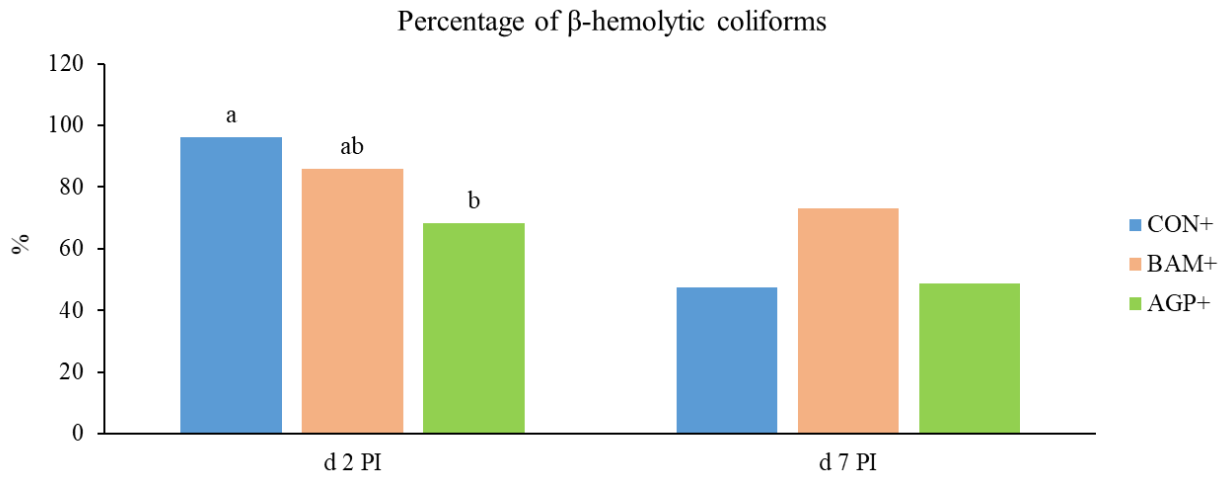
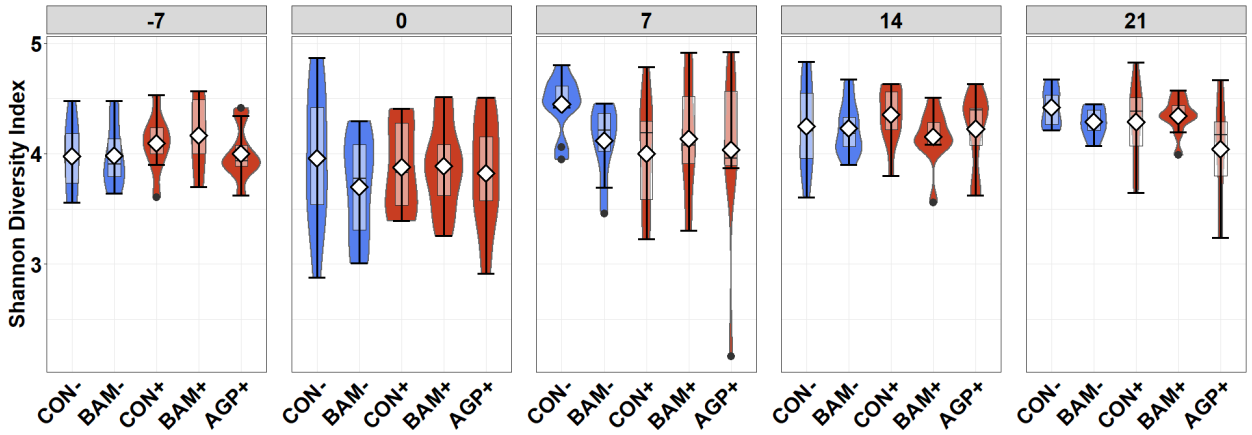


Figure 3-3. The percentage (%) of β-hemolytic coliforms in fecal samples of *Escherichia coli* challenged weaned pigs fed a control diet (CON+), or diets supplemented with *Bacillus amyloliquefaciens* (BAM+) or antibiotics (AGP+). No β-hemolytic coliforms were observed in the fecal samples of pigs in the sham groups. No β-hemolytic coliforms were observed in the fecal samples of pigs before *E. coli* (ETEC) challenge and d 14 and 21 post-inoculation (PI). Each least squares mean represents 9-10 observations. [a,b]Means without a common superscript are different ($P < 0.05$).

A.



B.

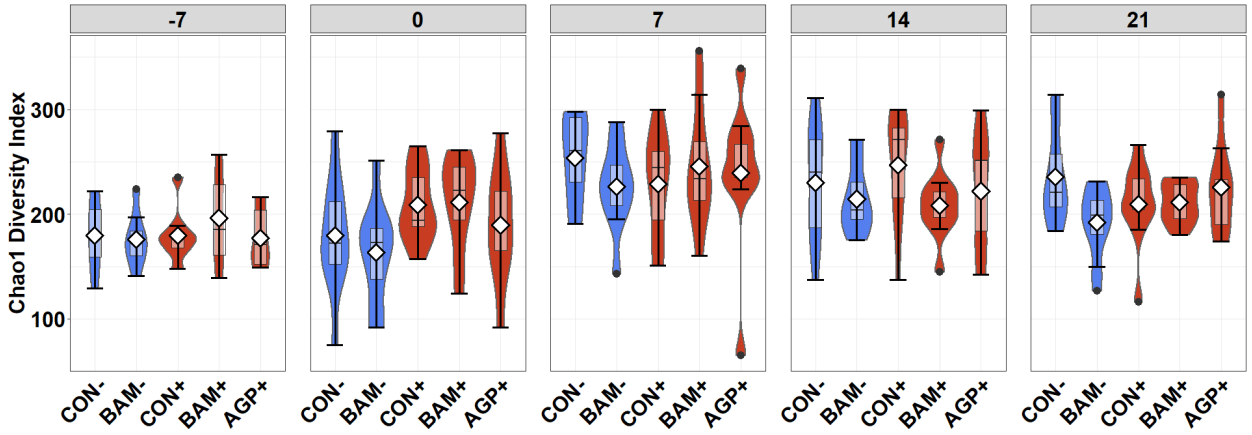
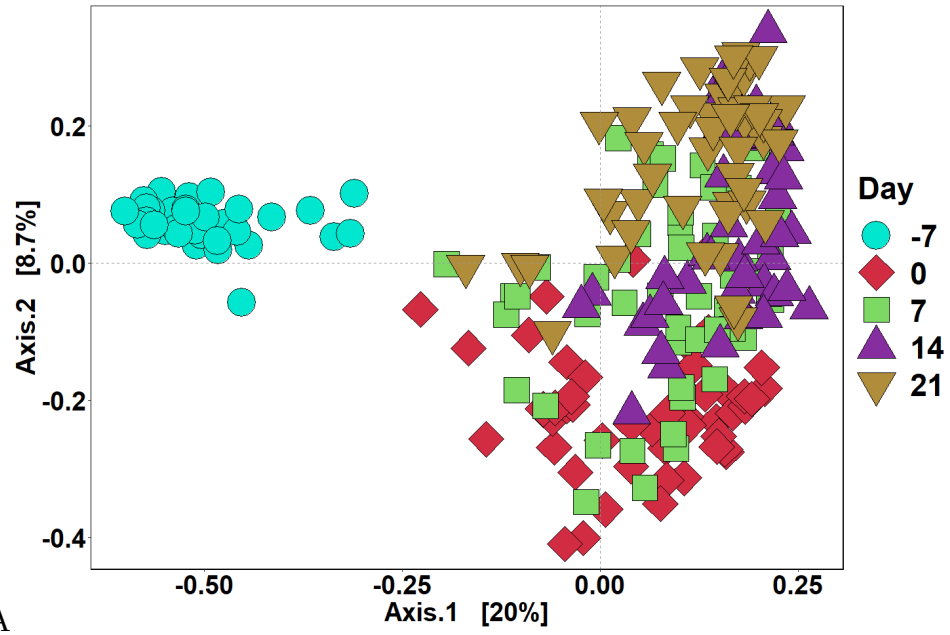
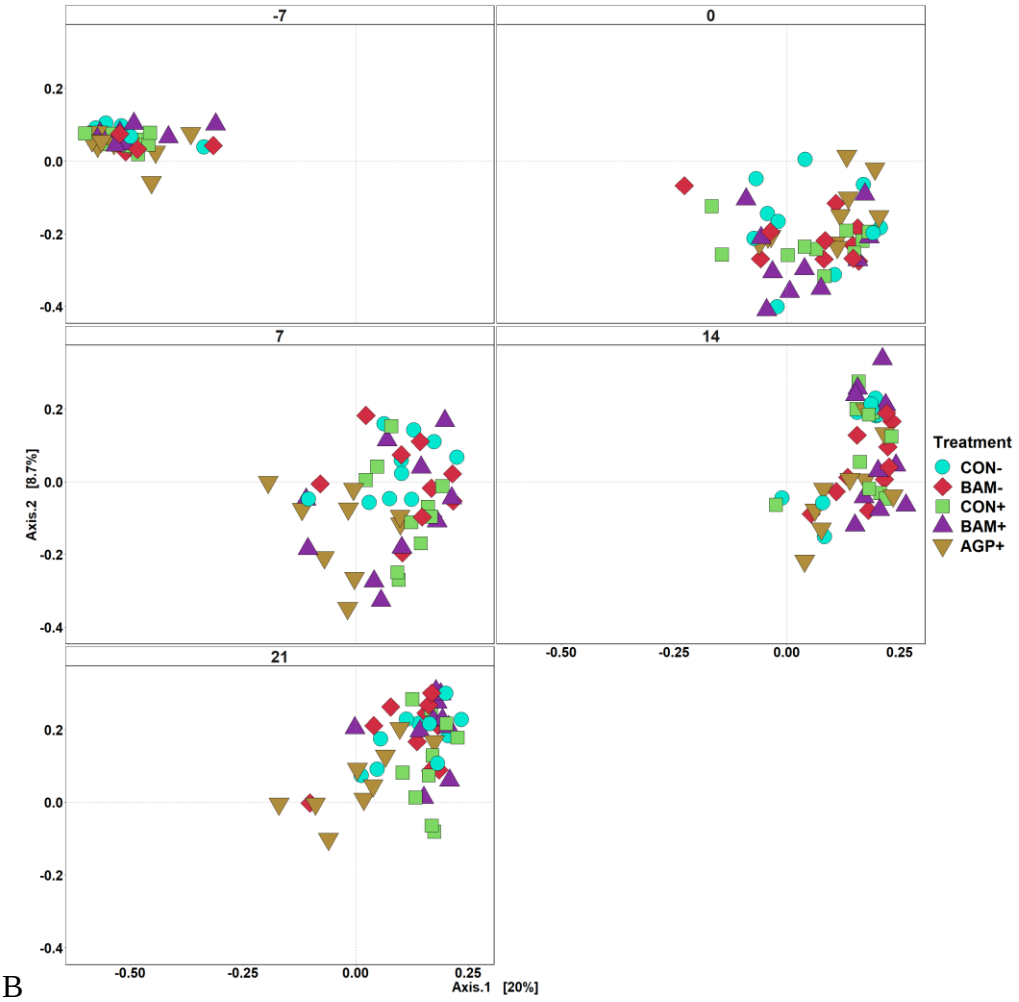


Figure 3-4. Alpha diversity as indicated by Shannon (**A**) and Chao1 (**B**) indices in feces of weaned pigs fed with a control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens* (BAM), or antibiotics (AGP) at the beginning of the experiment (d -7), first day of *E. coli* (ETEC) inoculation (d 0), and d 7, 14, and 21 post-inoculation. No difference was observed in Shannon (A) and Chao1 (B) indices among treatments. Violin plots are colored whether not infected (blue) or infected with *E. coli* (red). Data are expressed as mean (diamond) ± SEM.


0.2
0.0
-0.2
-0.4
Axis.2 [8.7%]
-0.50
-0.25
0.00
0.25
Axis.1 [20%]
Day
-7
0
7
14
21

A


-7
0
7
14
21
0.2
0.0
-0.2
-0.4
Axis.2 [8.7%]
-0.50
-0.25
0.00
0.25
Axis.1 [20%]
Treatment
CON-
BAM-
CON+
BAM+
AGP+

B

Figure 3-5. Principal coordinate analysis (PCoA) based on Bray-Curtis distance for beta diversity of fecal samples of weaned pigs fed with a control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens* (BAM), or antibiotics (AGP). Different symbols and shapes represent day fecal samples collected on d -7 and 0 before *E. coli* (ETEC) inoculation and d 7, 14, and 21 post-inoculation (**A**). Different symbols and shapes represent treatments (**B**). Each treatment has 9-10 observations.

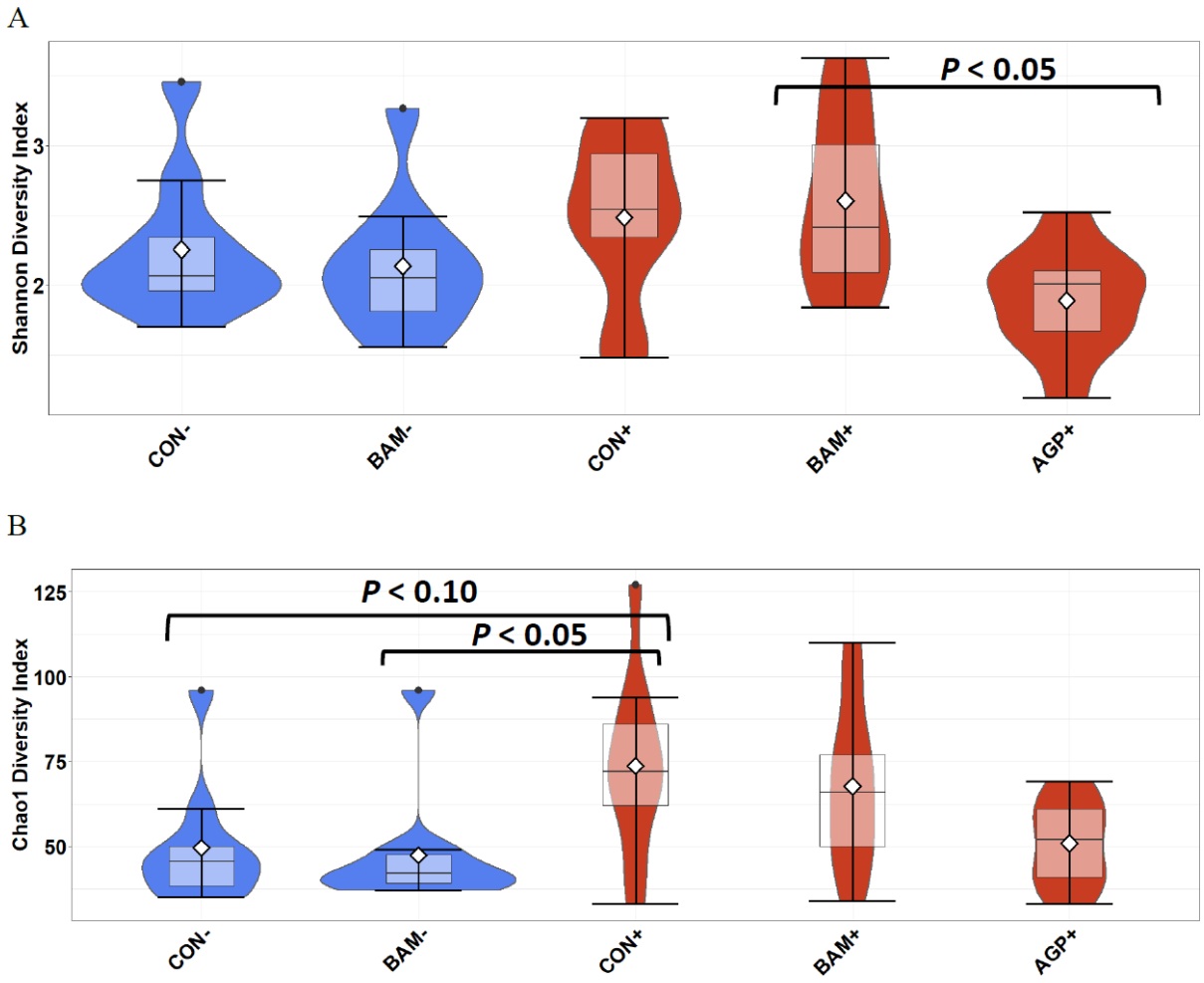


Figure 3-6. Alpha diversity as indicated by Shannon (**A**) and Chao1 (**B**) indices in ileal digesta collected from weaned pigs fed with a control (CON) diet, or diets supplemented with *Bacillus amyloliquefaciens* (BAM), or antibiotics (AGP) on 21 days after enterotoxigenic *E. coli* (ETEC) inoculation. Violin plots are colored by ETEC infected (red) or not (blue). Data are expressed as mean (diamond) ± SEM.

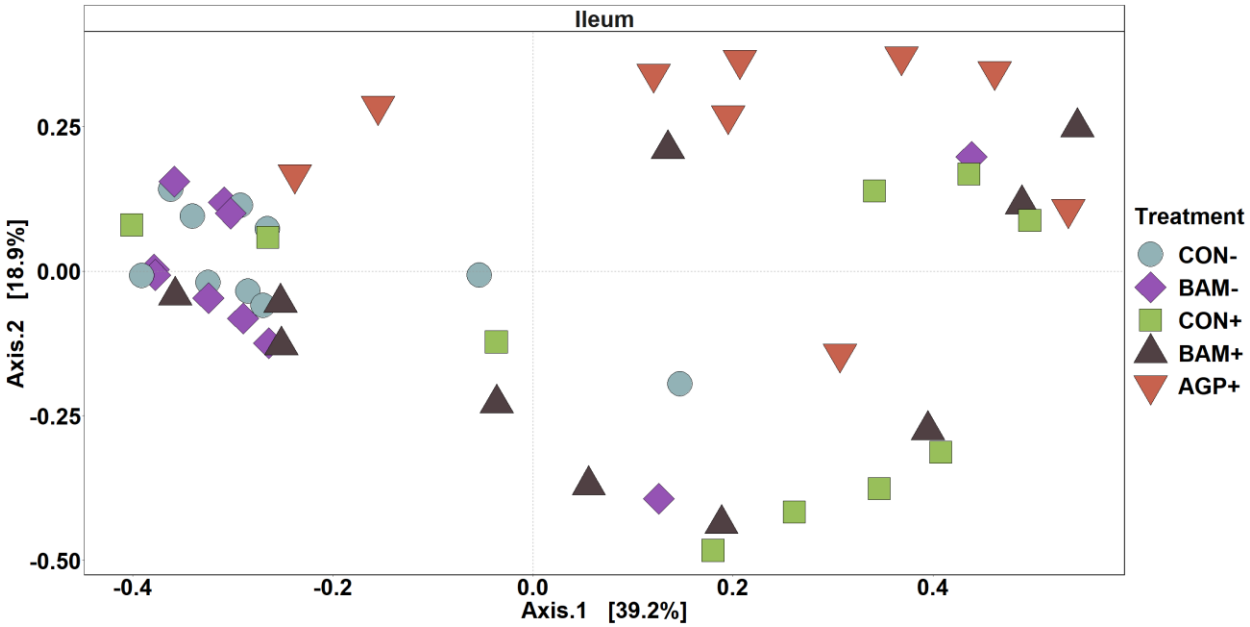


Figure 3-7. Principal coordinate analysis (PCoA) based on Bray-Curtis distance for beta diversity of ileal digesta of weaned pigs fed with a control (CON) diet, or diets supplemented with *Bacillus amyliloquefaciens* (BAM), and antibiotics (AGP). Different symbols and shapes represent treatment. Each treatment has 9-10 observations.

CHAPTER 4

DIETARY SUPPLEMENTATION WITH BOTANICAL BLENDS MODIFIED FECAL MICROBIOTA AND METABOLOMICS OF WEANED PIGS EXPERIMENTALLY INFECTED WITH ENTEROTOXIGENIC E. COLI

ABSTRACT

The objective of this study was to investigate the gut microbiota and metabolomic profiles in serum and ileal mucosa of weaned pigs supplemented with two types of botanical blends (BB) when pigs were experimentally challenged with enterotoxigenic *E. coli* (ETEC). Sixty weaned pigs (7.17 ± 0.97 kg body weight) were assigned to one of five dietary treatments, with weight and gender as blocks. Five treatments were as followed: negative control (CON-), positive control (CON+), dietary supplementation of 100 ppm BB1, and dietary supplementation of 50 ppm or 100 ppm BB2. The BB treatments were comprised of 0.3% capsicum oleoresin and 12% garlic oil extracted from different sources. All pigs, except CON-, were orally inoculated with 10^{10} CFU F18 ETEC/3-mL dose for 3 consecutive days. The experiment had a 7 d adaption period before and 21 d after the first ETEC inoculation (d 0), totaling 28 d. Fecal samples were collected on d -7, 0, 5, and 21 PI, and ileal digesta and cecal content were collected on d 5 and 21 PI for intestinal microbiota analysis using 16S rRNA amplicon sequencing. Excluding the pigs in BB1 group, serum samples from other treatments (6 pigs/treatment) on d 4 and 21 PI and ileal mucosa on d 5 PI underwent untargeted metabolomics analysis. Beta diversity displayed a shift in microbial composition based on age. Throughout the experiment, the relative abundance of *Lachnoclostridium* was decreased ($P < 0.05$) in feces as the age of pigs increased. On d 5 and 21 PI, the relative abundance of fecal *Faecalibacterium* and *Prevotella* were greater ($P < 0.05$) in CON- than in CON+. On d 21 PI, the relative abundance of Firmicutes and *Lactobacillaceae*

were greater ($P < 0.05$) in feces of pigs in CON+ than pigs in 100 ppm BB1. Pigs supplemented with 100 ppm BB2 had greater ($P < 0.05$) relative abundance of *Lachnospiraceae* in feces than pigs supplemented with 100 ppm BB1 on d 21 PI. Pigs in CON+ had the highest ($P < 0.05$) Shannon and Chao1 indices in ileal digesta, but no difference was observed among all treatments in alpha diversity in cecal content on d 5 PI. Pigs supplemented with 100 ppm BB1 had greater ($P < 0.05$) abundances of *Enterobacteriaceae* and *Escherichia-Shigella* in ileal digesta than pigs in CON+ on d 5 PI. The relative abundance of *Prevotellaceae* was greater ($P < 0.05$) in cecum of pigs supplemented with 100 ppm BB1 than CON+ pigs on d 5 PI. On d 21 PI, relative abundance of *Streptococcaceae* in ileal digesta was greater ($P < 0.05$) in CON+ than in CON-. The metabolomics analysis revealed that ETEC infection downregulated pinitol, malic acid, and methionine on d 4 PI and upregulated methionine and guanosine on d 21 PI when CON+ was compared with CON-. Supplementation with 100 ppm BB2 upregulated serum pinitol on d 4 PI and serum cholesterol and aminomalonic acids on d 21 PI compared with CON+. In ileal mucosa, asparagine was downregulated by 50 ppm BB2 in comparison to CON+. The present study indicated that supplementation with botanical blends modulated ileal microbiota and serum metabolomics profiles in weaned pigs under ETEC challenge.

Key words: Botanical blends, *Escherichia coli* challenge, Metabolomics, Microbiome, Weaned pigs

INTRODUCTION

Feed additives are often incorporated into swine feed to improve nutrient digestibility, disease resistance, and animal performance (Zheng et al., 2021). In-feed antibiotics were commonly supplemented to newly weaned pigs to prevent diarrhea induced by F18 enterotoxigenic *Escherichia coli* (ETEC) when pigs are under weaning stress (Sun and Kim,

2017). However, gut microbes can develop antibiotics resistance and be excreted in urine or feces, which can then be transmitted towards the human population (Aubry-Damon et al., 2004; Frey et al., 2022). Alternative practices are currently under demand to prevent pathogenic activities and diarrhea when antibiotics in-feed is restricted, and common alternatives include acidifiers, pharmacological levels of minerals, probiotics, and phytochemicals (Liu et al., 2018).

Phytochemicals are founded in botanical extracts and they are plant-derived materials that can possess a large variety of biological activities including antimicrobial and anti-inflammatory properties, which may promote intestinal health and performance of newly weaned pigs under stress (Cowan, 1999). Botanical extracts have been shown to elicit antimicrobial activity by directly disrupting bacterial structures in *E. coli* cells (Gonelimali et al., 2018). Study has also presumed that botanical extracts can modify gut microbiota in such a way that gut microbes release beneficial metabolites to promote health and increase anti-inflammatory effects (Sudheer et al., 2022). In our previous study, two botanical blends comprised of 0.3% capsicum oleoresin and 12% garlic oil extracted from different sources were supplemented to newly weaned pigs challenged with ETEC F18. Results of this study indicated that supplementation of botanical blends reduced frequency of diarrhea and enhanced intestinal morphology of weaned pigs compared with control pigs (Wong et al., 2022).

However, the effects of these botanical blends on gut microbiota and metabolomic profiles of serum and intestinal mucosa of weaned pigs were not investigated. Gut microbiota plays a crucial role in regulating host health. A successful manipulation of gut microbiota with dietary constituents is likely to enhance diarrhea resistance by utilizing the antimicrobial activity or colonization resistance by gut microbes (Patangia et al., 2022). The changes of gut microbiota by diets or disease conditions could further impact their metabolites and the host metabolic

profile (Goldansaz et al., 2017). Therefore, the objectives of this study were: (1) to characterize the gut microbiota of weaned pigs supplemented with botanical blends under enterotoxigenic ETEC infection and (2) to assess the impacts of botanical blend supplementation on the metabolomic profile of serum and ileal mucosa of weaned pigs.

MATERIALS AND METHODS

Animals and experimental design

The study was conducted at the University of California, Davis (UC Davis) and the protocol was review and approved by the Institutional Animal Care and Use Committee (IACUC #20809). A total of 60 weaning crossbred pigs (body weight (BW): 7.17 ± 0.97 kg) were selected from the Swine Teaching and Research Center at UC Davis. Piglets and their sows were not vaccinated for ETEC and not supplemented with antibiotics prior to the study. Piglets used in this study were also used in Braden et al. (2022). Around 21 days (d) old, piglets were weaned from their sows and were housed in individual pens (0.61 × 1.22 m) for 28 days, including 7 days before and 21 days after the first ETEC challenge, at the Cole A facility at UC Davis. Equal number of gilts and barrows were assigned to one of the five treatments in a randomized complete block design with weight within sex, litter as blocks, and pigs as experimental unit. With 12 replicates per treatment, the five dietary treatments included: (1) negative control (CON-): basal nursery diets without ETEC challenge, (2) positive control (CON+): basal nursery diets with ETEC challenge, (3) supplementation of 100 ppm of botanical blend (BB) type 1 (BB1) with ETEC challenge, (4) supplementation of 50 ppm of botanical blend type 2 (BB2) with ETEC challenge, and (5) supplementation of 100 ppm of BB2 with ETEC challenge. BB1 and BB2 had similar proprietary formulation of botanical actives, including 0.3% capsicum oleoresin and 12% garlic extracts. Synthetic garlic oil was used in BB1, while garlic oil in BB2

was extracted by subjecting ground garlic bulbs to a steam distillation process. Hydrogenated vegetable oil was used to encapsulate BB1 and BB2. The dosage of BB1 was based on our previous studies, in which capsicum oleoresin and garlic extract were supplemented individually to weaning pigs (Liu et al., 2013; Liu et al., 2014). Limited studies have investigated the optimal dosage to supplement natural garlic extract to weaned pigs, hence, two doses of BB2 were used as treatments in the present study.

A two-phase feeding program was used with d -7 to 14 as phase I and d 14 to 28 as phase II, thus, eight diets were formulated for the study. Spray-dried plasma, high levels of zinc oxide, and antibiotics were not included in the diets. All formulated diets meet the nutrient requirements of weaned pigs according to the National Research Council (Table 4-1, NRC, 2012). All pigs were fed with these experimental diets in a mash form throughout the experiment.

After 7 days of adaptation, pigs in all treatment groups except the negative control were inoculated with 3 mL of F 18 ETEC for three consecutive days starting d 0 post-inoculation (PI). Each dose was provided at 10^{10} CFU per 3 mL in phosphate buffer saline. The ETEC inoculums were prepared by the Western Institute for Food Safety and Security at UC Davis. The F18 ETEC was isolated from a field disease outbreak by the University of Illinois Veterinary Diagnostic Lab (isolate number: U.IL-VDL #05-27242) and expresses heat-labile toxin, heat-stabile toxins, and Shiga-like toxins. The dosage has been shown to cause mild diarrhea in previous studies (Liu et al., 2013; Kim et al., 2019a; Kim et al., 2019b).

Sample collection

Tail samples were collected from all piglets to test for their susceptibility to F18 ETEC. Tails were genotyped using a method described in Kreuzer et al. (2013) and confirmed that all pigs used in this study were susceptible to F18 ETEC. Fresh fecal samples were collected at the

beginning of the experiment (d -7), d 0 before ETEC inoculation, and d 5 and 21 PI for fecal microbiota analysis using 16S rRNA amplicon sequencing (Mon et al., 2015; Jinno et al., 2019). On d 5 PI, 30 pigs (six pigs per treatment) were euthanized near peak infection while the remaining 30 pigs were euthanized on the terminating day of the study, d 21 PI, during the recovery period from ETEC infection. For euthanasia, pigs were first anaesthetized with a 1 mL mixture of telazol (100 mg), ketamine (50 mg), and xylazine (50 mg) by intramuscular injection. Anesthetized pigs were then euthanized with an intracardiac injection of 78 mg sodium pentobarbital (Vortech Pharmaceuticals, Ltd., Dearborn, MI). After euthanasia, ileal digesta and cecal contents were collected from all pigs and snap-frozen in liquid nitrogen for gut microbiota analysis.

Excluding pigs in the 100 ppm BB1 group, blood samples were collected from 24 pigs (six pigs per treatment) on d 4 and 21 PI for untargeted metabolomic analysis. Ileal mucosa was collected on d 5 PI and immediately stored in liquid nitrogen for untargeted metabolic analysis.

Microbiota analysis

The 16S rRNA amplicon sequencing was used to identify and quantify microbial communities in ileal digesta, cecal content, and fecal samples. Bacterial DNA was extracted from all samples using the Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA samples were quantified and standardized prior to amplification. Duplicate DNA samples were amplified using PCR of the V4 hypervariable region of the 16S rRNA gene using primers 515F (5′-XXXXXXXX**GT**GTGCCAGCMGCCGCGGTAA-3′), including an 8-bp barcode (X) unique to each sample followed by a 2 nt Illumina adapter (bold), and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). Each PCR reaction was

comprised of 2 µL template DNA, 9.5 µL nuclease free water, 12.5 µL GoTaq 2× Master Mix (Promega, Madison, WI, USA), 0.5 µL V4 reverse primer (10 µM), and 0.5 µL barcoded forward primer (10 µM). Amplification was carried out using the following setting: 94°C for 3 min for initializing denaturation; followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1.5 min; and 72°C for 10 min for final elongation. Agarose gel electrophoresis was used to verify amplicon size for each sample, and amplified samples were then pooled together with the amount of sample added being quantified subjectively based on band brightness in the agarose gel. The pooled sample was then purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and submitted to the UC Davis Genome Center DNA Technologies Core for 250 bp paired-end sequencing on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA).

Barcode sequences were removed and the raw fastq files were demultiplexed in sabre (https://github.com/najoshi/sabre). Demultiplexed sequences were imported into Quantitative Insights Into Microbial Ecology 2 (QIIME2; version 2020.8) to remove primers and lower quality reads using the DADA2 plugin (Callahan et al., 2016; Bolyen et al., 2019). Paired-end reads were denoised and merged, and chimeras were removed to construct amplicon sequence variants (ASVs). Representative sequences for each ASV were aligned using MAFFT, and masked alignments were used to generate phylogenetic trees using FastTree2 (Price et al., 2010; Katoh and Standley, 2013). Python library scikit-learn was used to assign taxonomy based on representative sequences against Silva (version 138), which was pre-trained in QIIME2 to be clipped in to only the V4 hypervariable region and clustered at 99% sequence identity (Pedregosa et al., 2011; Quast et al., 2012; Bokulich et al., 2018).

Untargeted metabolomics analysis

Untargeted metabolomics analysis was performed using gas chromatography (Agilent 6890 gas chromatograph controlled using Leco ChromaTOF software version 2.32, Agilent, Santa Clara, CA, USA) coupled with time-of-flight mass spectrometry (GC/TOF-MS) (Leco Pegasus IV time-of-flight mass spectrometer controlled using Leco ChromaTOF software version3 2.32, Leco, Joseph, MI, USA) by the NIH West Coast Metabolomics Center. Metabolite extraction method was derived from (Fiehn et al., 2008). Approximately 30 μL of serum and 10 mg of ileal mucosa samples were first homogenized using a Retsch ball mill (Retsch, Newtown, PA, USA) for 30 s at 25 times per second. Samples were then vortexed and shaken with an extraction solution prechilled at −20 °C, in which the extraction solution consisted of isopropanol, acetonitrile, and water at a ratio 3:3:2 and degassed with liquid nitrogen. Samples were then centrifuged at 12,800 × g for 2 min to collect the supernatant and divide into two equal aliquots. Aliquots were concentrated at room temperature for 4 hours in a cold-trap vacuum concentrator (Labconco Centrivap, Kansas City, MO, USA). Residues were then re-suspended in 500 μL of 50% aqueous acetonitrile and centrifuged at 12,800 × g for 2 min to separate complex lipids and waxes. Resultant supernatant was collected and concentrated in a vacuum compressor. Dried sample extracts were derivatized and mixed with internal retention index markers, fatty acid methyl esters with chain lengths of C8 to C30. Samples were injected for GC/TOF-MS analysis, and all samples were analyzed in a single batch. Data was acquired for MS and mass calibration using FC43 (perfluorotributylamine) prior analysis sequencing. Metabolite identification was performed based on two parameters: (1) Retention index window ± 2000 U (around ± 2 sec retention time deviation), and (2) Mass spectral similarity plus additional confidence criteria that were based on Fiehn et al. (2008).

Raw data was preprocessed directly in Leco ChromaTOF software (v.2.32) for automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1. The BinBase algorithm was then used to further annotate the peaks within the deconvoluted data (Fiehn et al., 2005). The BinBase algorithm also identified derivatized metabolites by matching the spectral data again the Fiehn mass spectral library and the NIST spectral library based on retention index, validation of unique ions and apex masses, and mass spectrum similarity. InChI key, PubChem ID and KEGG ID were incorporated to name BinBase compounds. Mass/charger ratio (m/z) value of ions in MS was detected.

Statistical analyses

Sequence files for gut microbiota analysis were exported from QIIME2 and imported into R 4.1.0 for data visualization and statistical analysis (Team, 2021). Shannon and Chao1 indices were measured for alpha diversity by using the estimate_richness function in phyloseq (McMurdie and Holmes, 2013). The Bray-Curtis matrix was used to compare communities composition among treatments and days in feces and to compare community among treatments and intestinal segments (ileum vs. cecum). The relative abundance of each taxon in each sample was calculated by dividing the number of taxa by the total number of filtered reads in each sample. All microbiota analyses was performed using the phyloseq package and data were visualized using the ggplot2 package (Wickham, 2011). Normality and homoscedasticity were tested using the Shapiro Wilks test and Bartlett test, respectively. A linear mixed-effect model was fitted using the lme4 package with treatment and site or day and interaction as fixed effects and pig as random effect (Bates et al., 2014). Significance of each term in the model was determined using the F-test as a type 3 analysis of variance using the Anova function in the car package, followed by a group comparison using the cld function in the emmeans package (Fox

and Weisberg, 2018; Lenth, 2021). When normality or homoscedasticity was not observed, a non-parametric test was performed using the Kruskal-Wallis sum-rank test using the agricolae package (de Mendiburu and de Mendiburu, 2019). Bray-Curtis dissimilarity was first tested for homoscedasticity using the betadisper function and confirmed with $P > 0.05$. Statistical significance for beta diversity was then tested using PERMANOVA and the vegan package (Oksanen et al., 2013). Statistical significance was assessed as $\alpha = 0.05$ and statistical tendency as $\alpha = 0.10$. The *P*-values were adjusted for multiple comparisons using false discovery rate (FDR).

Metabolomics data was analyzed using different modules of the web-based platform MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) (Pang et al., 2021). Peaks were filtered from data. Logarithmic transformation and auto-scaling were applied to normalize data. Fold change analysis and *t*-test were conducted to determine fold change and significance of each identified metabolite. Statistical significance was adjusted with false discovery rate (FDR) with $q < 0.2$, fold change < 2.0, and Variable Importance in Projection (VIP) score > 1.

RESULTS

Fecal microbiota

Within fecal microbiota sequence data, the mean number of reads was 14,530 per sample and the total number of taxa identified was 4,134. Both Shannon and Chao1 indices decreased ($P < 0.05$) in feces as pigs aged from d -7 to 21 PI (Figure 4-1). On d -7, pigs fed with 50 ppm BB2 had lower ($P < 0.05$) Chao1 index than CON+, otherwise no difference was observed among treatments in both Shannon and Chao1 indices throughout the experiment. The principal coordinate analysis based on Bray-Curtis displayed that the fecal samples collected on d -7 were

clustered tightly and away from fecal samples collected on d 0, 5 and 21 PI (Figure 4-2). Clusters of all treatments were overlapping each other within day on d 0, 5, and 21 PI.

The three most abundant phyla in fecal samples were Firmicutes, Bacteroidota, and Proteobacteria from all treatments throughout the experiment (Table 4-2). The relative abundance of Bacteroidota, *Bacteroidaceae*, *Muribaculaceae*, *Rikenellaceae*, and *Lactobacillaceae* was decreased ($P < 0.05$) over time in fecal samples of pigs. However, the relative abundance of Firmicutes, *Lachnospiraceae*, *Streptococcaceae*, and *Veillonellaceae* was increased ($P < 0.05$) when pig age increased. ETEC infection did not affect the relative abundance of Bacteroidota, Firmicutes, and Proteobacteria on d 5 and 21 PI when CON+ was compared with CON-. Supplementation with 100 ppm BB1 or 50 ppm BB2 enhanced ($P < 0.05$) the relative abundance of Bacteroidota and Proteobacteria but reduced ($P < 0.05$) the relative abundance of Firmicutes on d 5 PI, compared with CON-. Supplementation was 100 ppm BB2 also reduced ($P < 0.05$) the relative abundance of Firmicutes on d 5 PI, compared with CON-. At the family level, pigs fed with 50 ppm BB2 or 100 ppm BB1 reduced ($P < 0.05$) the relative abundance of *Lachnospiraceae* on d 5 or 21 PI, respectively, compared with CON-.

At the genus level, *Lactobacillus*, *Streptococcus,* and *Blautia* were the three most abundant genera in fecal samples throughout the experiment (Table 4-3). Throughout the experiment, the relative abundance of *Prevotella*, *Agathobacter*, *Blautia*, *Faecalibacterium*, *Lactobacillus*, *Megasphaera*, and *Streptococcus* was increased ($P < 0.05$), but the relative abundance of *Clostridium sensu stricto* and *Lachnoclostridium* was decreased in feces over time. ETEC infection reduced ($P < 0.05$) the relative abundance of fecal *Faecalibacterium* on d 5 PI and fecal *Prevotella* on d 21 PI when CON+ was compared with CON-. Pigs supplemented with 100 ppm BB1 had lower ($P < 0.05$) relative abundance of *Blautia* in feces on d 5 and 21 PI and

had higher ($P < 0.05$) relative abundance of *Escherichia-Shigella* on d 5 PI, than pigs in CON-. Supplementation with 50 ppm BB2 reduced ($P < 0.05$) the relative abundance of Blautia and increased ($P < 0.05$) the relative abundance of *Escherichia-Shigella* on d 5 PI compared with CON-.

Intestinal digesta microbiota on F18 ETEC peak infection

Within ileal digesta and cecal contents of weaned pigs collected on d 5 PI, the mean sampling depth was 21,432 reads and the total number of identified taxa was 2,061. In alpha diversity, no difference was observed in both Shannon and Chao1 diversities in cecal contents among treatments (Figure 4-3). However, CON- was observed to have the highest diversity index in Shannon and Chao1 diversities among all treatment in ileal digesta. In beta diversity, the cluster formed by ileal digesta from CON- was distant from other treatments, while BB clusters were overlapping with each other (Figure 4-4). In cecal digesta, all treatment clusters overlapped.

In ileal digesta and cecal contents, the three most abundance phyla were Firmicutes, Proteobacteria, and Bacteroidota (Table 4-4). The relative abundance of Bacteroidota, *Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* was lower ($P < 0.05$) in the ileum than in the cecum. In ileal digesta, the relative abundance of Bacteroidota and its families *Muribaculaceae* and *Prevotellaceae*, and the relative abundance of Firmicutes families *Ruminococcaceae* and *Selemonadaceae* and Proteobacteria family *Succinivibrionaceae* were lower ($P < 0.05$) in CON+ than in CON-. The relative abundance of *Pasteurellaceae* was greater ($P < 0.05$) in CON+ than in CON-. No difference was observed in cecal content between CON- and CON+. Pigs supplemented with 100 ppm BB1 increased ($P < 0.05$) the relative abundance of *Enterobacteriaceae* in the ileum compared with CON+. In cecal content, pigs fed with 100 ppm BB1 had greater ($P < 0.05$) relative abundance of Bacteroidota and *Prevotellaceae* than CON+,

while the relative abundance of *Veillonellaceae* was greater ($P < 0.05$) in CON+ than in 100 ppm BB1. The relative abundance of *Enterobacteriaceae* was higher ($P < 0.05$) in cecum of pigs supplemented with BB1 and BB2 than CON+. No difference was observed in ileal and cecal microbiota composition among BB treatments on d 5 PI.

Lactobacillus and *Streptococcus* were the two most abundant genera in ileal digesta and cecal contents (Table 4-5). Cecal content had greater ($P < 0.05$) relative abundance of *Agathobacter*, *Blautia*, and *Faecalibacterium* than ileal digesta. No difference was observed in the relative abundance of the most abundant genera in the ileum and cecum between CON- and CON+. In ileal digesta, the relative abundance of *Escherichia-Shigella* was greater ($P < 0.05$) in 100 ppm BB1 than in CON+. In cecal content, the relative abundance of *Megasphaera* was greater ($P < 0.05$) in 100 ppm BB1 than in CON+ and the relative abundance of *Escherichia-Shigella* was greater ($P < 0.05$) when pigs were supplemented with BBs than pigs in CON+.

Intestinal digesta microbiota during the recovery period of F18 ETECS infection

The mean number of reads was 24,588 per sample and the total number of identified taxa was 1,202 in intestinal digesta from pigs collected on d 21 PI. Supplementing with BB and challenged with F18 ETEC did not affect the Shannon and Chao1 indices in ileal digesta and cecal content (Figure 4-5). For beta diversity, the 50 ppm BB2 cluster had overlap with the CON- cluster, while the 50 ppm BB2 samples clustered away from CON+ and 100 ppm BB1 clusters in ileal digesta (Figure 4-6). In cecal content, 100 ppm BB1 was moderately clustered away from the cluster for CON-.

The three most abundant phyla were Firmicutes, Bacteroidota, and Proteobacteria in ileal digesta and cecal content samples from all pigs on d 21 PI (Table 4-6). The relative abundance of Bacteroidota, *Muribaculaceae*, *Prevotellaceae*, *Lachnospiraceae*, and *Succinivibrionaceae* was

lower ($P < 0.05$) in the ileum than in the cecum. F18 ETEC inoculation increased ($P < 0.05$) relative abundance of *Streptococcaceae* and *Pasteurellaceae* in the ileum when comparing CON+ with CON-. Supplementation with 50 ppm BB2 reduced ($P < 0.05$) the relative abundance of *Streptococcaceae* in ileal digesta in comparison to CON+. In cecal content, supplementation with 100 ppm BB2 increased ($P < 0.05$) the relative abundance of *Muribaculaceae* when compared with CON+. *Lactobacillus* and *Streptococcus* were the most abundance genera in ileal digesta and cecal content on d 21 PI (Table 4-7). The relative abundance of *Blautia* was higher ($P < 0.05$), but the relative abundance of *Clostridium sensu stricto* and *Turicibacter* was lower ($P < 0.05$) in cecum than in ileum. The relative abundance of *Strepptococcus* in ileal digesta was greater ($P < 0.05$) in CON+ than in CON-.

Metabolomic profiles

A total of 221 metabolites (117 identified and 104 unidentified) were detected in serum samples on d 4 and 21 PI. VIP scores were computed to assess discriminatory variables in the dataset. On d 4 PI, F18 ETEC infection downregulated methionine, malic acid, galactonic acid, and pinitol and upregulated oleic acid, arachidonic acid, and lauric acid when CON+ was compared with CON- (Table 4-8). Supplementation with 100 ppm BB2 upregulated pinitol in comparison with CON+ on d 4 PI. No differential metabolites were identified when pairwise comparing CON+ and 50 ppm BB2, and 50 ppm BB2 vs. 100 ppm BB2 on d 4 PI. On d 21 PI, mannose was downregulated and guanosine and methionine and were upregulated in CON+ in comparison to CON-. Supplementation with 100 ppm BB2 upregulated cholesterol and aminomalonic acid, but downregulated heptanoic acid compared with CON+.

A total of 291 metabolites, including 162 identified and 129 unidentified metabolites were detected in ileal mucosa samples collected on d 5 PI. Asparagine was upregulated in ileal

mucosa by supplementing 50 ppm BB2 compared with CON+. No differential metabolites were identified in ileal mucosa when comparing CON- vs. CON+, 50 ppm BB2 vs. CON+, and 50 ppm vs. 100 ppm BB2.

DISCUSSION

Newly weaned pigs are highly stressed due to sudden dietary and environmental changes, and more are susceptible to ETEC induced post-weaning diarrhea (Kim et al., 2022). Our previous study reported that supplementation with botanical blends could alleviate diarrheal severity and regulate the local and systemic immunity of weaned pigs under ETEC challenge (Wong et al., 2022). However, limited studies have investigated the effects of botanical blends on gut microbiota and metabolomic profiles in serum and ileal mucosa of weaned pigs infected with ETEC. Results of the present study indicate that ETEC modified the intestinal microbiota and moderately modified the profile of serum metabolites of weaned pigs. Supplementation with botanical blends also influenced intestinal microbiota composition, but their effects on ileal mucosal metabolites were limited.

Fecal microbiota

In the present study, fecal microbiota was shifted in all treatments throughout the study. Decreased Shannon and Chao1 indices indicate that microbial diversity was reduced due to a decrease in microbial richness in fecal samples. Reduced microbial diversity in fecal microbiota was also reported during the early stage of weaning in pigs (Hu et al., 2016). Other studies have reported that microbial diversity in weaned pigs increased over time when feces were sampled at monthly intervals (Chen et al., 2017; Massacci et al., 2020). However, the present study thoroughly investigated the fecal microbiota changes in pigs in negative control by covering the entire post-weaning period with a shorter sampling interval. Our results suggest that the

microbial diversity initially decreases soon after weaning, but microbial diversity gradually increases as the pigs mature likely due to a dietary change from sow milk to plant based dry feed (Frese et al., 2015). Principal coordinate analysis (PCoA) plots in the current study also support that age is likely the main driver for fecal microbiota shifts (Cho et al., 2020). The two most abundant phyla in fecal samples of all weaned pigs were Firmicutes and Bacteroidota, which was consistent with the observations in previous studies (Dou et al., 2017; Cremonesi et al., 2022). Decreased abundance in Bacteroidota was observed throughout the experiment in the present study and Ma et al. (2022), but Lim et al. (2019) reported that the relative abundance of Bacteroidota was increased by age in healthy weaned pigs. The present study also observed an increase in the relative abundance of Firmicutes in control groups throughout the experiment. Increased abundance of Firmicutes and decreased abundance of Bacteroidota are often observed as a potential indicator for dysbiosis (Stojanov et al., 2020). This result may imply that weaning stress has potentially induced a microbial imbalance during the early stage of weaning, but the result does not explain if weaning stress causes a long-term effect on the gut microbiota.

Fecal microbiota was also modified by the presence of ETEC and dietary supplementation with botanical blends. Supplementation with 50 ppm botanical blend 2 increased the relative abundance of *Lachnoclostridium* in feces on d 5 and 21 PI, compared with the positive control. Previous research reported that *Lachnoclosridium* could produce butyrate, which helps maintain energy homeostasis and stimulates immune response in the small intestine of pigs (Gutiérrez and Garrido, 2019; Amiri et al., 2022). The source of garlic oil had limited effects on fecal microbiota, except for the relative abundance of *Lachnospiraceae* that was greater in pigs fed with 100 ppm botanical blend 2 than botanical blend 1. Overall, fecal microbiota was mainly impacted by age rather than botanical supplementations.

Intestinal digesta during peak ETEC infection

Our previous research reported that the peak of ETEC infection in post-weaning pigs is approximately day 5 to 7 post-inoculation (Kim et al., 2022). In the present study, ETEC infection reduced microbial richness and evenness in ileal digesta of weaned pigs during the peak of ETEC infection. However, no difference in alpha diversity and beta diversity was observed in cecal contents when pigs in the positive control were compared with pigs in the negative control. ETEC colonize in the small intestine, thus these results indicate that ETEC perturb the gut microbial community more in the ileum than in the cecum (García et al., 2020). Our results agree with findings of a previous study that Firmicutes and Proteobacteria are predominantly abundant in ileal digesta of weaned pigs (Pollock et al., 2021). With reduced microbial richness and evenness in ileal digesta of pigs infected with ETEC, differences in microbial taxa abundance were expected between sham and ETEC infected pigs. ETEC infection reduced the relative abundance of *Ruminococcaceae* and *Prevotellaceeae*, which are associated with producing butyrate and contributing to antimicrobial activity in the intestines (Yap et al., 2014; Esquivel-Elizondo et al., 2017). Moreover, *Pasteurellaceae* was also more abundant in infected pigs than in sham pigs, which was also observed by Li et al. (Li et al., 2020). This observation is in close agreement with a previous study that reported the increase in *Pasteurellaceae* might be correlated with an increase in ETEC (Higginson et al., 2022). This result also implied that pigs with ETEC infection are potentially undergoing dysbiosis, as increased *Pasteurellaceae* is an indicator of gut dysbiosis in humans with inflammatory bowel disease (Gevers et al., 2014). It was expected that greater microbial diversity would be observed in the cecal contents than in ileal digesta because the large intestine is a major site for microbial colonization while the small intestine is mainly responsible for nutrient digestion and absorption. In the present study, we did

not observe difference in taxa abundance in cecal contents between the negative control and positive control on d 5 PI. This result suggests that the high microbial diversity in the cecum may increase colonization resistance, which prevents ETEC from colonizing further into the large intestine (Saavedra and Dattilo, 2013). In addition, the relatively high amount of short-chain fatty acids produced in the large intestine might be another reason for the increased colonization resistance of ETEC (Binder, 2010).

Supplementation with botanical blends modified the intestinal microbiota of weaned pigs during the peak of ETEC infection. Pigs supplemented with 100 ppm botanical blend 1 had greater abundances of *Enterobacteriaceae* and *Escherichia-Shigella* in ileal digesta than pigs in the positive control. The performance and clinical data from these pigs reported that supplementation with botanical blends reduced diarrheal frequency in weaned pigs infected with ETEC, thus there might be other reasons for the increased abundance of *Enterobacteriaceae* in the ileal digesta of pigs in the botanical blend groups (Wong et al., 2022). The relative abundance of *Prevotellaceae* was greater in cecum when pigs were supplemented with 100 ppm botanical blend 1 than pigs in the positive control. *Prevotella* was likely responsible for the increase in *Prevotellaceae*. Similar results were also observed when growing pigs were supplemented with essential oil blends (Ruzauskas et al., 2020). Li et al. (2018) have observed an increase of *Veillonellaceae* and *Megasphaera* in the cecal microbiota of weaned pigs when supplemented with essential oils. The present study also observed an increased abundance of *Megasphaera* in the cecum when pigs were supplemented with 100 ppm botanical blend 1. *Megasphaera* can utilize dietary protein and aid in amino acid metabolism in the small intestine of pigs (Dai et al., 2010), however, research on the role of *Megasphaera* in the large intestine is limited. In the present study, pigs supplemented with botanical blends had lower abundance of

Veillonellaceae than control pigs, which was different from the observations in Li et al. (2018). This is likely due to the different compositions of plant extract and oils that were used in these studies, indicating that the impacts of botanical blend vary due to their major active components. The present study also indicates that the source of garlic oil has no effects on intestinal microbiota composition of weaned pigs when challenged with ETEC.

Intestinal digesta during ETEC recovery period

Weaned pigs were reported to fully recover from ETEC infection by d 21 PI (He et al., 2020). In the present study, no difference in microbial evenness and richness was observed in the ileum and cecum of pigs during the recovery phase of ETEC infection. The PCoA plot suggests that the overall bacterial community was similar between negative control and 50 ppm BB2 in ileal digesta of weaned pigs, while pigs fed with 100 ppm BB1 had a different bacterial community in ileal digesta and cecal content compared with the negative control. Looking into the taxonomic abundance, *Streptococcaceae* and *Pasteurellaceae* were more abundant in ileal digesta of pigs in the positive control than negative control. These results may imply that ETEC has a long-term effect on the ileal microbiota of weaned pigs. A high abundance of *Streptococcaceae* is correlated with the high abundance of *Streptococcus*. Other research also reported that pigs under ETEC challenge had high abundance of *Streptococcus* in their gut microbiota (Higginson et al., 2022). Pathogenic *Streptococcus* spp. are known to disrupt immunoglobulins from eliminating pathogens that invade the intestines (Pietrzak et al., 2020). Since 50 ppm BB2 reduced the abundance of *Streptococcaceae* in ileal digesta of weaned pigs challenged with ETEC, this result might suggest that 50 ppm BB2 may modify the intestinal environment to prevent other opportunistic pathogens from invading the gut. However, the present study did not characterize the gut microbiota to the species levels, thus future studies are

needed to quantify microbiota at the species level and confirm the potential pathogenicity of *Streptococcus*. In addition, supplementation with 100 ppm botanical blend 2 increased the relative abundance of *Muribaculaceae* in the cecum compared with the positive control. *Muribaculaceae* is often known to be one of the predominant families found in mouse cecal microbiota and their abundance can be altered by diets (Bowerman et al., 2021; Y. Ma et al., 2021). However, the major function of *Muribaculaceae* in the intestine of pigs is not well understood.

Metabolomic profile

Untargeted metabolomics was performed in serum samples collected on d 4 and 21 PI to identify metabolic changes in weaned pigs infected with ETEC. During the acute response of ETEC (d 4 PI), ETEC challenge downregulated pinitol, malic acid, galactonic acid, and methionine. Pinitol, malic acid, and galactonic acid are reported to have anti-inflammatory effects via inhibiting the NF-κB activation pathway, thus, suppressing inflammatory cellular responses (Sethi et al., 2008; Tang et al., 2013; Kong et al., 2020). The reduced methionine might be related to the generation of L-cysteine, one of the important substrates required to synthesize glutathione against increased oxidative stress induced by ETEC infection (Ji et al., 2019). In addition, ETEC infection upregulated oleic acid, arachidonic acid and lauric acid, which have been shown to induce inflammation by activating prostaglandins and indirectly activating the NF-κB pathway (Lee et al., 2001; Jang et al., 2020). The results of the serum metabolic profile are consistent with the high concentration of serum TNFα and haptoglobin observed in the present study (Wong et al., 2022), confirming the ongoing confirming the ongoing systemic inflammation in the pigs on d 4 PI.

During the recovery period of ETEC infection, ETEC challenged pigs had lower mannose and higher methionine and guanosine in serum samples. The high mannose concentration is associated with increased mannose glycosylation, which was reported to be negatively correlated with intestinal permeability (Park et al., 2017). On d 21 PI, most of the pigs in the present study were recovered from ETEC infection, as indicated by reduced diarrhea and the absence of β-hemolytic coliforms in feces (Wong et al., 2022). Thus, the downregulation of mannose also supports that pigs were undergoing intestinal repair with reduced intestinal permeability. Previous research in rats reported that guanosine could alleviate colonic inflammation during colitis challenge (Zizzo et al., 2019). The increased serum guanosine in ETEC challenged pigs also suggests that the intestinal and systemic inflammation was reduced on d 21 PI (Wong et al., 2022).

On d 4 PI, upregulation of pinitol was observed in pigs fed with 100 ppm botanical blend 2, compared with the positive control. This observation is consistent with the cytokine data published by Wong et al. (2022), in which botanical blend supplementation reduced the concentration of TNFα and haptoglobin in serum compared with the positive control. On d 21 PI, supplementation with 100 ppm botanical blend 2 increased aminomalonic acid in comparison to pigs in the positive control. Aminomalonic acid is utilized for iron metabolism and previous studies have reported that other plant extracts could regulate iron metabolism and reverse oxidative damage caused by pathogens (Kang et al., 2003; Imam et al., 2017). This observation indicates that botanical blend supplementation may speed up the recovery from intestinal damage caused by ETEC pathogenicity in weaned pigs.

Limited changes in the metabolomic profile were observed in the ileal mucosa of ETEC infected pigs on d 4 PI. The major finding was that supplementation with 50 ppm of BB 2

reduced serum asparagine compared with the positive control. Asparagine is a metabolite from aspartate metabolism. Large amounts of evidence indicate that aspartate could promote a macrophage-mediated inflammatory response and attenuate intestinal damage caused by endotoxins in weaned pigs (Pi et al., 2014; Wang et al., 2021). No differences were observed in serum and ileal mucosa metabolites in pigs fed with different doses of botanical blend 2, which is consistent with intestinal microbiota data and indicates no dose response was observed in botanical blend 2 regarding intestinal microbiota and metabolomics.

CONCLUSION

Results of the present study indicated that age was the major factor to shift fecal microbiota, but ETEC infection and botanical blend supplementation both affected ileal and cecal microbiota composition in weaned pigs. Moreover, the present study observed some changes within serum metabolites when pigs were supplemented with 100 ppm BB2, while ETEC challenge modified the serum metabolomic profile. Limited differences were observed between the two botanical blends. Taken altogether, the present study provided a wider insight on how botanical blends may have reduced inflammation by altering the serum metabolomic profile while minimally altering the gut microbiota of weaned pigs with diarrhea induced by ETEC. Manipulation of gut microbiota is seen as a potential to alleviate postweaning diarrhea of weaned pigs, but the botanical blends used in the present study could alleviate inflammatory response with limited modulation to the gut microbiota. Future studies are suggested to assess the metabolomic profiles of intestinal digesta to further investigate the impacts of botanical blends on pig gut microbiota.

LITERATURE CITED

Amiri, P., S. A. Hosseini, Samad Ghaffari, H. Tutunchi, Shamsi Ghaffari, E. Mosharkesh, S. Asghari, and N. Roshanravan. 2022. Role of butyrate, a gut microbiota derived metabolite, in cardiovascular diseases: A comprehensive narrative review. Front. Pharmacol.12.

Aubry-Damon, H., K. Grenet, P. Sall-Ndiaye, D. Che, E. Cordeiro, M.-E. Bougnoux, E. Rigaud, Y. Le Strat, V. Lemanissier, and L. Armand-Lefèvre. 2004. Antimicrobial resistance in commensal flora of pig farmers. Emerg. Infect. Dis. 10:873.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2014. Fitting linear mixed-effects models using lme4. arXiv preprint arXiv:1406.5823.

Binder, H. J. 2010. Role of colonic short-chain fatty acid transport in diarrhea. Annu. Rev. Physiol. 72:297–313. Doi:10.1146/annurev-physiol-021909-135817.

Bokulich, N. A., B. D. Kaehler, J. R. Rideout, M. Dillon, E. Bolyen, R. Knight, G. A. Huttley, and J. G. Caporaso. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 6. doi:10.1186/s40168-018-0470-z.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, and F. Asnicar. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37:852–857.

Bowerman, K. L., S. C. L. Knowles, J. E. Bradley, L. Baltrūnaitė, M. D. J. Lynch, K. M. Jones, and P. Hugenholtz. 2021. Effects of laboratory domestication on the rodent gut microbiome. ISME Commun. 1:1–14. Doi:10.1038/s43705-021-00053-9.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High resolution sample inference from Illumina amplicon data. Nat. Methods. 13:581–583. Doi:10.1038/nmeth.3869.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. J. ISME 6:1621–1624. Doi:10.1038/ismej.2012.8.

Chen, L., Y. Xu, X. Chen, C. Fang, L. Zhao, and F. Chen. 2017. The maturing development of gut microbiota in commercial piglets during the weaning transition. Front. Microbiol. 8:1688. Doi:10.3389/fmicb.2017.01688.

Cho, H. M., G. González-Ortiz, D. Melo-Durán, J. M. Heo, G. Cordero, M. R. Bedford, and J. C. Kim. 2020. Stimbiotic supplementation improved performance and reduced inflammatory response via stimulating fiber fermenting microbiome in weaner pigs housed in a poor sanitary environment and fed an antibiotic-free low zinc oxide diet. PLOS ONE. 15:e0240264. Doi:10.1371/journal.pone.0240264.

Cowan, M. M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev.12:564–582. Doi:10.1128/CMR.12.4.564.

Cremonesi, P., F. Biscarini, B. Castiglioni, C. A. Sgoifo, R. Compiani, and P. Moroni. 2022. Gut microbiome modifications over time when removing in-feed antibiotics from the prophylaxis of post-weaning diarrhea in piglets. PLOS ONE. 17:e0262199. Doi:10.1371/journal.pone.0262199.

Dai, Z.-L., J. Zhang, G. Wu, and W.-Y. Zhu. 2010. Utilization of amino acids by bacteria from the pig small intestine. Amino Acids. 39:1201–1215. Doi:10.1007/s00726-010-0556-9.

Dou, S., P. Gadonna-Widehem, V. Rome, D. Hamoudi, L. Rhazi, L. Lakhal, T. Larcher, N. Bahi-Jaber, A. Pinon-Quintana, A. Guyonvarch, I. L. E. Huërou-Luron, and L. Abdennebi-Najar. 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLOS ONE. 12:e0169851. Doi:10.1371/journal.pone.0169851.

Esquivel-Elizondo, S., Z. E. Ilhan, E. I. Garcia-Peña, and R. Krajmalnik-Brown. 2017. Insights into butyrate production in a controlled fermentation system via gene predictions. mSystems. 2:e00051-17. Doi:10.1128/mSystems.00051-17.

Fiehn, O., G. Wohlgemuth, and M. Scholz. 2005. Setup and annotation of metabolomic experiments by integrating biological and mass spectrometric metadata. In: B. Ludäscher and L. Raschid, editors. Data Integration in the Life Sciences. Springer, Berlin, Heidelberg. P. 224–239.

Fiehn, O., G. Wohlgemuth, M. Scholz, T. Kind, D. Y. Lee, Y. Lu, S. Moon, and B. Nikolau. 2008. Quality control for plant metabolomics: reporting MSI-compliant studies. Plant J. 53:691–704. Doi:10.1111/j.1365-313X.2007.03387.x.

Fox, J., and S. Weisberg. 2018. An R companion to applied regression. Sage publications. Thousand Oaks, CA.

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome. 3:28. doi:10.1186/s40168-015-0091-8.

Frey, L., B. Tanunchai, and B. Glaser. 2022. Antibiotics residues in pig slurry and manure and its environmental contamination potential. A meta-analysis. Agron. Sustain. Dev. 42:31. doi:10.1007/s13593-022-00762-y.

García, V., M. Gambino, K. Pedersen, S. Haugegaard, J. E. Olsen, and A. Herrero-Fresno. 2020. F4-and F18-positive enterotoxigenic Escherichia coli isolates from diarrhea of postweaning pigs: genomic characterization. Appl. Environ. Microbiol. 86:e01913-20.

Gevers, D., S. Kugathasan, L. A. Denson, Y. Vázquez-Baeza, W. Van Treuren, B. Ren, E. Schwager, D. Knights, S. J. Song, M. Yassour, X. C. Morgan, A. D. Kostic, C. Luo, A. González, D. McDonald, Y. Haberman, T. Walters, S. Baker, J. Rosh, M. Stephens, M. Heyman, J. Markowitz, R. Baldassano, A. Griffiths, F. Sylvester, D. Mack, S. Kim, W. Crandall, J. Hyams, C. Huttenhower, R. Knight, and R. J. Xavier. 2014. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe. 15:382–392. Doi:10.1016/j.chom.2014.02.005.

Goldansaz, S. A., A. C. Guo, T. Sajed, M. A. Steele, G. S. Plastow, and D. S. Wishart. 2017. Livestock metabolomics and the livestock metabolome: A systematic review. PloS one. 12:e0177675.

Gonelimali, F. D., J. Lin, W. Miao, J. Xuan, F. Charles, M. Chen, and S. R. Hatab. 2018. Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. Front. Microbiol. 9. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01639

Gutiérrez, N., and D. Garrido. 2019. Species deletions from microbiome consortia reveal key metabolic interactions between gut microbes. mSystems. 4:e00185-19. Doi:10.1128/mSystems.00185-19.

He, Y., C. Jinno, K. Kim, Z. Wu, B. Tan, X. Li, R. Whelan, and Y. Liu. 2020. Dietary Bacillus spp. enhanced growth and disease resistance of weaned pigs by modulating intestinal

microbiota and systemic immunity. J. Animal Sci. Biotechnol. 11:101. Doi:10.1186/s40104-020-00498-3.

Higginson, E. E., M. A. Sayeed, J. Pereira Dias, V. Shetty, M. Ballal, S. K. Srivastava, I. Willis, F. Qadri, G. Dougan, and A. Mutreja. 2022. Microbiome profiling of enterotoxigenic Escherichia coli (ETEC) carriers highlights signature differences between symptomatic and asymptomatic individuals. Mbio. 13:e00157-22.

Hu, J., Y. Nie, J. Chen, Y. Zhang, Z. Wang, Q. Fan, and X. Yan. 2016. Gradual changes of gut microbiota in weaned miniature piglets. Front. Microbiol. 7.

Imam, M. U., S. Zhang, J. Ma, H. Wang, and F. Wang. 2017. Antioxidants mediate both iron homeostasis and oxidative stress. Nutrients. 9:E671. Doi:10.3390/nu9070671.

Jang, Y., M. Kim, and S. W. Hwang. 2020. Molecular mechanisms underlying the actions of arachidonic acid-derived prostaglandins on peripheral nociception. J. Neuroinflammation 17:30. Doi:10.1186/s12974-020-1703-1.

Ji, J., Y. Xu, M. Zheng, C. Luo, H. Lei, H. Qu, and D. Shu. 2019. Methionine attenuates lipopolysaccharide-induced inflammatory responses via DNA methylation in macrophages. ACS Omega. 4:2331–2336. Doi:10.1021/acsomega.8b03571.

Jinno, C., K. Kim, M. Song, P. Ji, E. Maga, and Y. Liu. 2019. 141 Supplementation of Bacillus subtilis modified fecal microbiota of weaning pigs experimentally infected with pathogenic E. coli. J. Anim. Sci. 97:81–81. Doi:10.1093/jas/skz122.147.

Kang, D.-K., J. Jeong, S. K. Drake, N. B. Wehr, T. A. Rouault, and R. L. Levine. 2003. Iron regulatory protein 2 as iron sensor: Iron-dependent oxidative modification of cysteine*. J. Biol. Chem. 278:14857–14864. Doi:10.1074/jbc.M300616200.

Katoh, K., and D. M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. Mol. Biol. Evol. 30:772–780. Doi:10.1093/molbev/mst010.

Kim, K., A. Ehrlich, V. Perng, J. A. Chase, H. Raybould, X. Li, E. R. Atwill, R. Whelan, A. Sokale, and Y. Liu. 2019a. Algae-derived β-glucan enhanced gut health and immune responses of weaned pigs experimentally infected with a pathogenic E. coli. Anim. Feed Sci. Technol. 248:114–125. Doi:10.1016/j.anifeedsci.2018.12.004.

Kim, K., Y. He, X. Xiong, A. Ehrlich, X. Li, H. Raybould, E. R. Atwill, E. A. Maga, J. Jørgensen, and Y. Liu. 2019b. Dietary supplementation of Bacillus subtilis influenced intestinal health of weaned pigs experimentally infected with a pathogenic E. coli. J. Animal Sci. Biotechnol. 10:52. Doi:10.1186/s40104-019-0364-3.

Kim, K., M. Song, Y. Liu, and P. Ji. 2022. Enterotoxigenic Escherichia coli infection of weaned pigs: Intestinal challenges and nutritional intervention to enhance disease resistance. Front. Immunol. 13.

Kong, J., Z. Du, and L. Dong. 2020. Pinitol prevents lipopolysaccharide (LPS)-induced inflammatory responses in BV2 microglia mediated by TREM2. Neurotox Res. 38:96–104. Doi:10.1007/s12640-020-00187-z.

Kreuzer, S., M. Reissmann, and G. A. Brockmann. 2013. New fast and cost-effective gene test to get the ETEC F18 receptor status in pigs. Vet. Microbiol. 163:392–394. Doi:10.1016/j.vetmic.2012.12.040.

Lee, J. Y., K. H. Sohn, S. H. Rhee, and D. Hwang. 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4 *. J. Biol. Chem. 276:16683–16689. Doi:10.1074/jbc.M011695200.

Lenth, R. V. 2021. Emmeans: estimated marginal means, aka least-squares means. R package version 1.6. 1.

Li, Q., X. Peng, E. R. Burrough, O. Sahin, S. A. Gould, N. K. Gabler, C. L. Loving, K. S. Dorman, and J. F. Patience. 2020. Dietary soluble and insoluble fiber with or without enzymes altered the intestinal microbiota in weaned pigs challenged with enterotoxigenic E. coli F18. Front. Microbiol. 11.

Li, Y., X. Fu, X. Ma, S. Geng, X. Jiang, Q. Huang, C. Hu, and X. Han. 2018. Intestinal microbiome-metabolome responses to essential oils in piglets. Front. Microbiol. 9:1988. Doi:10.3389/fmicb.2018.01988.

Lim, M. Y., E.-J. Song, K. S. Kang, and Y.-D. Nam. 2019. Age-related compositional and functional changes in micro-pig gut microbiome. Geroscience. 41:935–944.

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, and H. H. Stein. 2018. Non-antibiotic feed additives in diets for pigs: A review. Anim. Nutr.4:113–125. Doi:10.1016/j.aninu.2018.01.007.

Liu, Y., M. Song, T. Che, J. Lee, D. Bravo, C. Maddox, and J. Pettigrew. 2014. Dietary plant extracts modulate gene expression profiles in ileal mucosa of weaned pigs after an Escherichia coli infection. J. Anim. Sci. 92:2050–2062.

Liu, Y., M. Song, T. M. Che, J. a. S. Almeida, J. J. Lee, D. Bravo, C. W. Maddox, and J. E. Pettigrew. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. J. Anim. Sci. 91:5294–5306. Doi:10.2527/jas.2012-6194.

Ma, J., J. Chen, M. Gan, L. Chen, Y. Zhao, Y. Zhu, L. Niu, S. Zhang, L. Zhu, and L. Shen. 2022. Gut microbiota composition and diversity in different commercial swine breeds in early and finishing growth stages. Animals. 12:1607.

Ma, Y., Q. Zhang, W. Liu, Z. Chen, C. Zou, L. Fu, Y. Wang, and Y. Liu. 2021. Preventive effect of depolymerized sulfated galactans from Eucheuma serra on enterotoxigenic Escherichia coli-caused diarrhea via modulating intestinal flora in mice. Marine Drugs. 19 :80. Doi :10.3390/md19020080.

Massacci, F. R., M. Berri, G. Lemonnier, E. Guettier, F. Blanc, D. Jardet, M. N. Rossignol, M.-J. Mercat, J. Doré, P. Lepage, C. Rogel-Gaillard, and J. Estellé. 2020. Late weaning is associated with increased microbial diversity and Faecalibacterium prausnitzii abundance in the fecal microbiota of piglets. Animal Microbiome. 2:2. Doi:10.1186/s42523-020-0020-4.

McMurdie, P. J., and S. Holmes. 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE. 8 :e61217. Doi :10.1371/journal.pone.0061217.

de Mendiburu, F., and M. F. de Mendiburu. 2019. Package 'agricolae.' R Package, Version. 1–2.

Mon, K. K. Z., P. Saelao, M. M. Halstead, G. Chanthavixay, H.-C. Chang, L. Garas, E. A. Maga, and H. Zhou. 2015. Salmonella enterica Serovars Enteritidis infection alters the indigenous microbiota diversity in young layer chicks. Front. Vet. Sci. 2. doi :10.3389/fvets.2015.00061.

National Research Council (NRC). 2012. Nutrient requirements of swine: eleventh revised edition. Washington, DC: The National Academies Press.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2013. Package 'vegan.' Community ecology package, version. 2.

Pang, Z., J. Chong, G. Zhou, D. A. de Lima Morais, L. Chang, M. Barrette, C. Gauthier, P.-É. Jacques, S. Li, and J. Xia. 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. Nucleic Acids Res. 49:W388–W396. Doi:10.1093/nar/gkab382.

Park, D., G. Xu, M. Barboza, I. M. Shah, M. Wong, H. Raybould, D. A. Mills, and C. B. Lebrilla. 2017. Enterocyte glycosylation is responsive to changes in extracellular conditions: implications for membrane functions. Glycobiology. 27:847–860. Doi:10.1093/glycob/cwx041.

Patangia, D. V., C. Anthony Ryan, E. Dempsey, R. Paul Ross, and C. Stanton. 2022. Impact of antibiotics on the human microbiome and consequences for host health. MicrobiologyOpen. 11:e1260.

Pedregosa, F., G. Varoquaux, A. Gramfort, V. Michel, B. Thirion, O. Grisel, M. Blondel, P. Prettenhofer, R. Weiss, and V. Dubourg. 2011. Scikit-learn: Machine learning in Python. J. Mach. Learn. Res. 12:2825–2830.

Pi, D., Y. Liu, H. Shi, S. Li, J. Odle, X. Lin, H. Zhu, F. Chen, Y. Hou, and W. Leng. 2014. Dietary supplementation of aspartate enhances intestinal integrity and energy status in weanling piglets after lipopolysaccharide challenge. J. Nutr. Biochem. 25:456–462. Doi:10.1016/j.jnutbio.2013.12.006.

Pietrzak, B., K. Tomela, A. Olejnik-Schmidt, A. Mackiewicz, and M. Schmidt. 2020. Secretory IgA in intestinal mucosal secretions as an adaptive barrier against microbial cells. Int. J. Mol. Sci. 21:9254. Doi:10.3390/ijms21239254.

Pollock, J., L. Glendinning, L. A. Smith, H. Mohsin, D. L. Gally, M. R. Hutchings, and J. G. M. Houdijk. 2021. Temporal and nutritional effects on the weaner pig ileal microbiota. Animal Microbiome. 3:58. Doi:10.1186/s42523-021-00119-y.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2010. FastTree 2 – Approximately maximum-likelihood trees for large alignments. PLOS ONE. 5:e9490. Doi:10.1371/journal.pone.0009490.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590–D596.

Ruzauskas, M., E. Bartkiene, A. Stankevicius, J. Bernatoniene, D. Zadeike, V. Lele, V. Starkute, P. Zavistanaviciute, J. Grigas, and E. Zokaityte. 2020. The influence of essential oils on gut microbial profiles in pigs. Animals. 10:1734.

Saavedra, J. M., and A. Dattilo. 2013. Microbiota of the Intestine: Prebiotics. In: B. Caballero, editor. Encyclopedia of Human Nutrition (Third Edition). Academic Press, Waltham. P. 168–174.

Sethi, G., K. S. Ahn, B. Sung, and B. B. Aggarwal. 2008. Pinitol targets nuclear factor-κB activation pathway leading to inhibition of gene products associated with proliferation, apoptosis, invasion, and angiogenesis. Molecular Cancer Therapeutics. 7:1604–1614. Doi:10.1158/1535-7163.MCT-07-2424.

Stojanov, S., A. Berlec, and B. Štrukelj. 2020. The influence of probiotics on the Firmicutes/Bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. Microorganisms. 8:E1715. Doi:10.3390/microorganisms8111715.

Sudheer, S., P. Gangwar, Z. Usmani, M. Sharma, V. K. Sharma, S. S. Sana, F. Almeida, N. K. Dubey, D. P. Singh, and N. Dilbaghi. 2022. Shaping the gut microbiota by bioactive phytochemicals: an emerging approach for the prevention and treatment of human diseases. Biochimie. 193:38–63.

Sun, Y., and S. W. Kim. 2017. Intestinal challenge with enterotoxigenic Escherichia coli in pigs, and nutritional intervention to prevent postweaning diarrhea. Anim Nutr. 3:322–330. doi:10.1016/j.aninu.2017.10.001

Tang, X., J. Liu, W. Dong, P. Li, L. Li, C. Lin, Y. Zheng, J. Hou, and D. Li. 2013. The Cardioprotective Effects of Citric Acid and L-Malic Acid on Myocardial Ischemia/Reperfusion Injury. Evid. Based Complement. Alternat. Med. 2013:820695. doi:10.1155/2013/820695.

Team, R. C. 2021. R: A language and environment for statistical computing.

Wang, H., X. Zheng, B. Liu, Y. Xia, Z. Xin, B. Deng, L. He, J. Deng, and W. Ren. 2021. Aspartate metabolism facilitates IL-1β production in inflammatory macrophages. Front. Immunol. 12.

Wickham, H. 2011. Ggplot2. Wiley Interdisciplinary Reviews: Computational Statistics. 3:180–185.

Wong, B. T., S. Park, L. Kovanda, Y. He, K. Kim, S. Xu, C. Lingga, M. Hejna, E. Wall, R. Sripathy, X. Li, and Y. Liu. 2022. Dietary supplementation of botanical blends enhanced

performance and disease resistance of weaned pigs experimentally infected with enterotoxigenic Escherichia coli F18. J. Anim. Sci. skac353. Doi:10.1093/jas/skac353.

Yap, G. C., P. Y. Hong, and B. W. Lee. 2014. MICROFLORA OF THE INTESTINE | The Natural Microflora of Humans. In: C. A. Batt and M. L. Tortorello, editors. Encyclopedia of Food Microbiology (Second Edition). Academic Press, Oxford. P. 634–638.

Zheng, L., M. E. Duarte, A. Sevarolli Loftus, and S. W. Kim. 2021. Intestinal Health of Pigs Upon Weaning: Challenges and Nutritional Intervention. Front. vet. sci. 8.

Zizzo, M. G., G. Caldara, A. Bellanca, D. Nuzzo, M. Di Carlo, and R. Serio. 2019. Preventive effects of guanosine on intestinal inflammation in 2, 4-dinitrobenzene sulfonic acid (DNBS)-induced colitis in rats. Inflammopharmacology. 27:349–359. Doi:10.1007/s10787-018-0506-9.

Table 4-1. Ingredient composition of experimental diets[1]

Ingredient, %	Control, phase I	Control, phase II
Corn	51.55	58.44
Dried whey	15.00	10.00
Soybean meal	21.00	24.00
Fish meal	4.00	3.00
Soy protein concentrate	3.00	-
Soybean oil	2.10	1.30
Limestone	0.95	0.95
Dicalcium phosphate	0.55	0.52
L-Lysine·HCl	0.48	0.48
DL-Methionine	0.24	0.21
L-Threonine	0.21	0.20
L-Tryptophan	0.09	0.09
L-Valine	0.13	0.11
Salt	0.40	0.40
Vitamin-mineral premix[2]	0.30	0.30
Total	100.00	100.00
Calculated energy and nutrients		
Metabolizable energy, kcal/kg	3390	3333
Net energy, kcal/kg	2545	2501
Crude protein, %	20.49	19.32
Arg,[3] %	1.15	1.09
His,[3] %	0.47	0.45
Ile,[3] %	0.78	0.72
Leu,[3] %	1.55	1.47
Lys,[3] %	1.42	1.32
Met,[3] %	0.55	0.50
Thr,[3] %	0.89	0.83
Trp,[3] %	0.31	0.29
Val,[3] %	0.97	0.89

Met + Cys,[3] %	0.85	0.79
Phe + Tye,[3] %	1.39	1.31
Ca, %	0.83	0.75
Total P, %	0.65	0.59
Digestible P, %	0.42	0.36
Lactose, %	10.80	7.20
Analyzed nutrients, %		
Dry matter	90.80	89.70
Crude protein	21.43	19.55
Acid detergent fiber	2.91	3.05
Neutral detergent fiber	6.63	7.89
Total Ca	1.00	0.95
Total P	0.69	0.62

[1]In each phase, three additional diets were formulated by adding 100 mg/kg of botanical blend type 1, or 50 or 100mg/kg of botanical blend type 2 to the control diet, respectively.

[2]Provided by United Animal Health (Sheridan, IN). The premix provided the following quantities of vitamins and micro minerals per kilogram of complete diet: Vitamin A as retinyl acetate, 11,136 IU; vitamin D3 as cholecalciferol, 2,208 IU; vitamin E as $_{DL}$-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B12, 0.03 mg; $_{D}$-pantothenic acid as $_{D}$-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese sulfate; Se, 0.3 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc sulfate.

[3]Amino acids are indicated as standardized ileal digestible amino acids.

Table 4-2. Relative abundance (%) of the most abundant families from phyla Bacteroidota, Firmicutes, and Proteobacteria in feces of weaned pigs at the beginning of the experiment (d -7), d 0 before first ETEC inoculation, and d 5, and 21 post-inoculation. Weaned pigs were fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2). Each treatment has 11-12 observations

	d -7					d 0					d 5					d 21				
	Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge			
	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm
Bacteroidota	16.51^{ab}	15.38^{abcd}	11.68^{bcde}	19.15^{a}	15.23^{abcd}	15.96^{abc}	10de	15.30^{abcd}	12.05^{bcde}	14.86^{abcd}	7.73^{e}	10.76^{cde}	14.96^{abcd}	14.04^{abcd}	12.39^{bcde}	9.40^{de}	2.18^{f}	6.10^{ef}	7.48^{ef}	6.25^{ef}
Bacteroidaceae	1.66^{a}	2.82^{a}	4.31^{a}	2.90^{a}	3.01^{a}	0.06^{bcd}	0.58^{b}	0.31^{b}	0.21^{b}	0.17^{b}	0.02^{cd}	0.06^{cd}	0.11^{c}	0.24^{bcd}	1.24^{c}	0^{cd}	0^{d}	0^{d}	0^{d}	0^{d}
Muribaculaceae	2.99^{a}	1.90^{abc}	0.86^{c}	2.30^{abc}	1.79^{abc}	2.52^{ab}	1.34^{c}	2.64^{ac}	2.23^{abc}	2.58^{abc}	1.72^{bc}	1.4^{abc}	2.42^{bc}	1.39^{cd}	1.56^{bc}	0.60^{c}	0.76^{c}	0.88^{c}	1.84^{bc}	1.84^{abc}
Prevotellaceae	6.96^{ab}	7.21^{abc}	3.82^{bc}	9.39^{a}	6.38^{abc}	10.93^{a}	7.31^{ab}	9.64^{a}	8.13^{ab}	10.30^{a}	4.91^{bc}	7.99^{abc}	10.18^{a}	10.62^{a}	8.18^{b}	8.51^{ab}	1.25^{c}	4.91^{bc}	4.97^{bc}	4.22^{abc}
Rikenellaceae	2.15^{a}	1.97^{abc}	1.73^{bcd}	1.95^{abc}	1.99^{abc}	1.76^{cd}	0.53^{hij}	2.25^{def}	1.08^{def}	1.47^{bcde}	0.78^{fghi}	1.06^{efgh}	1.40^{bcdef}	1.17^{fgh}	0.99^{efg}	0.24^{hij}	0.10^{j}	0.21^{ij}	0.63^{hij}	0.15^{ij}
Firmicutes	66.09^{hi}	68.65^{fghi}	71.11^{efghi}	62.97^{i}	67.68^{ghi}	73.69^{defgh}	75.24^{cdef}	70.30^{efghi}	71.98^{efgh}	71.83^{efgh}	84.54^{ab}	79.85^{bcde}	75.28^{cdef}	74.27^{cdefg}	74.39^{cdef}	83.84^{abcd}	91.05^{a}	75.67^{bcdef}	84.99^{abc}	82.59^{abcd}
Lachnospiraceae	10.29^{efg}	9^{efg}	8.06^{g}	10.44^{efg}	8.59^{fg}	20.29^{abc}	14.83^{cdef}	18.13^{bcd}	18.16^{bcd}	18.98^{bcd}	25.87^{a}	22.84^{ab}	23.07^{ab}	18.32^{bc}	21.77^{abc}	20.12^{abc}	14.88^{bcde}	9.88^{de}	15.03^{bcdef}	21.94^{abc}
Lactobacillaceae	24.71^{a}	31.10^{a}	23.32^{a}	27.49^{a}	23.22^{a}	17.75^{abc}	31.72^{abc}	23.14^{abc}	26.67^{abc}	16.41^{abcd}	22.35^{ab}	20.84^{ab}	19.20^{ab}	26.35^{a}	21.29^{ab}	5.16^{de}	11.51^{bcd}	2.46^{e}	5.15^{de}	9.93^{cde}
Ruminococcaceae	5.93	5.21	6.85	5.34	4.87	7.84	5.14	7.02	5.54	5.88	10.06	5.70	7.30	6.65	7.78	9.02	8.39	6.54	7.30	11.52
Streptococcaceae	0.95^{bcde}	0.87^{def}	0.60^{ef}	0.62^{cdef}	0.49^{ef}	0.22^{f}	5.71^{bcde}	2^{ef}	0.46^{ef}	2.12^{def}	1.17^{def}	4.16^{def}	3.37^{bcde}	4.93^{ef}	3.72^{bcde}	12.90^{abcde}	13.19^{ab}	19.69^{a}	12.79^{abc}	7.14^{bcd}
Veillonellaceae	1.20^{d}	0.85^{d}	0.55^{d}	0.39^{d}	0.95^{d}	3.73^{bc}	5.42^{bc}	5.78^{c}	6.49^{bc}	5.80^{bc}	6.30^{bc}	7.12^{bc}	4.25^{c}	6.52^{c}	4.87^{bc}	10.96^{a}	10.23^{a}	5.74^{c}	7.58^{c}	9.40^{b}

Proteobacteria	2.36[a] bcd	1.60[a] bcd	2.03[ab] cd	2.18 abcd	1.85[a] bcd	1.97[ab] cd	7.26[a]	6.39[ab] cd	7.77[a] b	3.36[a] bcd	0.34[e]	1.61[c] de	1.93[b] cd	5.60[ab] cd	5.45[a] bcd	1.04[cd] e	0.74[d] e	10.82 abc	0.94[cd] e	4.05[a] bcd
Enterobacteriaceae	0.75 bcd	0.87[b] cd	1.16[ab] c	0.49 bcd	0.79[b] cd	0.46[ab] c	4.32[a]	3.84[ab]	4.32[a]	2.23[a] bc	0.07 d	0.64[b] cd	1.01[a] bc	4.52[ab] c	4.75[a] bc	0.07[cd]	0.13[b] cd	1.78[ab] c	0.37[bc] d	1.42[b] cd
Succinivibrionaceae	1.22[a] b	0.44[a] b	0.70[ab]	1.27 ab	0.68[a] b	1.34[a]	1.32[a] b	0.79[ab]	2.44[a]	0.78[a] b	0.25 b	0.31[b]	0.34[a] b	0.60[ab]	0.30[b]	0.96[ab]	0.59[a] b	2.48[ab]	0.41[ab]	2.57[a] b

Table 4-3. Relative abundance (%) of families in the three most abundant phyla in feces of weaned pigs at the beginning of the experiment (d -7), d 0 before first ETEC inoculation, and d 5, and 21 post-inoculation. Weaned pigs were fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2). Each treatment had 11-12 observations

	d -7					d 0					d 5					d 21				
	Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge			
	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm
Bacteroidota																				
Prevotella	2.70^{a} b	3.20^{a} b	1.64 ab	3.05^{a} b	2.60 ab	6.75^{a}	4.79^{ab}	5.69 a	3.88 ab	5.42^{a}	3.07^{a} b	4.88^{a} b	5.04^{ab}	5.20^{a} b	4.10^{ab}	6.59^{a}	0.68^{b}	3.54^{a} b	3.51^{a} b	2.81^{ab}
Firmicutes																				
Agathobacter	0.05^{e}	0.27^{c} de	0^{e}	0.04^{e}	0.03 de	2.13^{a}	1.94^{ab}	1.79 a	3.11 a	3.33^{a}	2.21^{a}	2.44^{a}	2.10^{a}	1.79^{a} b	3.37^{a}	2.37^{ab}	1.68^{a} bcd	0.53^{b} cde	1.82^{a} bc	1.47^{ab}
Blautia	0.29^{e}	0.34^{e}	0.21 e	0.14^{e}	0.60 de	7.38^{a} b	4.51^{bc}	7.02 ab	4.56 bc	7.45^{a} b	9.19^{a}	6.66^{a} bc	5.74^{bc}	5.49^{b} c	6.67^{ab}	7.26^{ab}	4.31^{b} c	3.13^{c} d	4.87^{b} c	6.37^{ab} c
Clostridium sensu stricto 1	7.33^{a}	6.51^{a}	10.45^{a}	6.72^{a}	6.86 a	0.06^{b}	0.11^{b}	0.23 b	0.05 b	0.26^{b}	0.02^{b}	0.02^{b}	0.12^{b}	0.08^{b}	0.16^{b}	0.05^{b}	1.04^{b}	0.15^{b}	0.14^{b}	0.86^{b}
Faecalibacterium	0.03^{d}	0.20^{d}	0.38 d	0.04^{d}	0.18 d	4.19^{a} b	1.70^{c}	3.68 abc	2.75 bc	3.13^{b} c	5.63^{a}	3.21^{b} c	3.33^{ab} c	3.37^{a} b	4.52^{ab}	4.27^{ab}	3.72^{b} c	3.17^{b} c	2.86^{b} c	4.24^{ab} c
Lachnoclostridium	5.59^{a}	5.31^{a}	5.01 a	6.37^{a}	4.17 a	0.31^{b} cde	0.96^{bc} d	0.50 cde	1.05 b	0.43^{b} cd	0.88^{c} de	0.58^{b} c	0.33^{bc} de	0.19^{c} de	0.24^{bc} de	0.21^{bc} de	0.12^{d} e	0.10^{e}	0.11^{d} e	0.21^{bc} de
Lactobacillus	5.16^{d} e	11.51 bcd	2.46 e	5.15^{d} e	9.93 cde	24.71 a	31.1^{a}	23.32^{a}	27.49^{a}	23.22^{a}	22.35 ab	20.84^{ab}	19.20 ab	26.35^{a}	21.29 ab	17.75 abc	31.72 abc	23.14 abc	26.67 abc	16.41 abcd
Megasphaera	1.16^{b}	0.75^{b}	0.50 b	0.35^{b}	0.92 b	3.51^{a}	4.50^{a}	5.18 a	5.65 a	5.15^{a}	4.79^{a}	6.15^{a}	3.71^{a}	5.71^{a}	4.07^{a}	7.10^{a}	5.54^{a}	4.13^{a}	5.23^{a}	4.01^{a}
Streptococcus	0.95^{b} cde	0.87^{c} def	0.6^{cd} ef	0.62^{c} def	0.49 def	0.22^{f}	5.71^{ab} cde	2^{ef}	0.46 def	2.12^{c} def	1.17^{c} def	4.16^{c} def	3.37^{ab} cde	4.93^{c} def	3.72^{ab} cde	12.9^{ab} cde	13.19 ab	19.68 a	12.79 abc	7.14^{ab} cd
Proteobacteria																				
Escherichia-Shigella	0.75^{b} cd	0.87^{b} cd	1.16 abc	0.49^{b} cd	0.79 bcd	0.46^{a} bc	4.32^{a}	3.84 ab	4.32 a	2.23^{a} bc	0.07^{d}	0.64^{b} cd	1.01^{ab} c	4.52^{a} bc	4.75^{ab} c	0.07^{cd}	0.13^{b} cd	1.78^{a} bc	0.37^{b} cd	1.42^{bc} d

Table 4-4. Relative abundance (%) of Bacteroidota, Firmicutes, and Proteobacteria and their top families in digesta on d 5 post-inoculation of F18 ETEC infected weaned pigs. Weaned pigs were fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2). Each treatment had 11-12 observations

	Ileum					Cecum				
	Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge			
	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm
Bacteroidota	7.13^{c}	0.78^{d}	1.41^{d}	0.14^{d}	0.36^{d}	9.96^{ab}	6.46^{bc}	15.21^{a}	10.81^{ab}	12.3^{ab}
Muribaculaceae	0.51^{b}	0.17^{c}	0.02^{c}	0^{c}	0.01^{c}	0.31^{ab}	0.30^{ab}	0.52^{a}	0.39^{ab}	0.26^{ab}
Prevotellaceae	6.49^{c}	0.57^{d}	1.38^{d}	0.13^{d}	0.33^{d}	9.42^{ab}	5.98^{bc}	13.26^{a}	9.64^{ab}	10.92^{ab}
Firmicutes	83.77^{a}	86.22^{a}	67.26^{a}	74.48^{a}	74.25^{a}	81.98^{a}	84.98^{a}	71.72^{a}	76.77^{a}	70.90^{a}
Lachnospiraceae	7.34^{b}	2.97^{bc}	0.41^{c}	0.50^{bc}	0.61^{bc}	20.43^{a}	16.32^{a}	18.54^{a}	16.32^{a}	17.33^{a}
Lactobacillaceae	29.35	44.09	37.91	51.18	43.01	27.69	27.22	18.94	29.39	19.91
Ruminococcaceae	3.87^{b}	1.08^{c}	0.07^{c}	0.12^{c}	0.08^{c}	10.20^{a}	9.33^{a}	9.08^{a}	9.09^{a}	8.89^{a}
Selemonadaceae	6.23^{a}	1.16^{b}	1.45^{b}	2.34^{b}	0.79^{b}	4.81^{a}	5.78^{a}	2.97^{a}	3.29^{a}	4.82^{a}
Streptococcaceae	6.94	31.07	13.07	12.94	14.24	5.18	8.50	8.13	5.03	3.96
Veillonellaceae	8.61^{abcd}	2.48^{de}	2.78^{e}	1.99^{e}	3.31^{cde}	6.67^{abc}	11.36^{a}	4.32^{bcde}	7.53^{ab}	7.34^{ab}
Proteobacteria	3.95^{ab}	6.10^{ab}	20.10^{a}	17.69^{ab}	18.2^{ab}	1.83^{b}	2.74^{ab}	4.99^{ab}	6.06^{ab}	8.68^{ab}
Enterobacteriaceae	1.91^{bcd}	0.30^{bcd}	16.25^{a}	16.36^{ab}	16.22^{abc}	0.43^{cd}	0.07^{d}	1.93^{abc}	3.72^{abc}	5.69^{abc}
Pasteurellaceae	0.34^{d}	5.36^{ab}	3.77^{a}	1.30^{abc}	1.75^{ab}	0.01^{d}	0.20^{bcd}	0.20^{abcd}	0.18^{cd}	0.07^{bcd}
Succinivibrionaceae	1.64^{a}	0.13^{b}	0.04^{b}	0.01^{b}	0.01^{b}	1.33^{a}	1.74^{a}	1.91^{a}	1.32^{a}	2.84^{a}

Table 4-5. Relative abundance (%) of the most abundant families in the three most abundant phyla in digesta on d 5 post-inoculation of F18 ETEC infected weaned pigs. Weaned pigs were fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2). Each treatment had 11-12 observations

	Ileum					Cecum				
	Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge			
	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm
Actinobacteria										
Bifidobacterium	1.91	4.39	6.47	5.74	4.89	0.72	1.64	1.21	1.61	2.56
Firmicutes										
Agathobacter	0.76^{b}	0.28^{b}	0.04^{b}	0.02^{b}	0.03^{b}	3.08^{a}	2.94^{a}	2.92^{a}	2.59^{a}	3.16^{a}
Blautia	1.6^{b}	1.18^{bc}	0.05^{c}	0.09^{c}	0.09^{bc}	7.58^{a}	4.38^{a}	5.41^{a}	5.25^{a}	5.7^{a}
Faecalibacterium	0.89^{b}	0.46^{bc}	0.02^{c}	0.03^{c}	0.03^{c}	6.36^{a}	5.15^{a}	3.92^{a}	5.07^{a}	4.16^{a}
Lactobacillus	29.34	44.09	37.91	51.18	43	27.69	27.22	18.94	29.39	19.91
Megasphaera	5.04^{bc}	1.98^{c}	2.33^{c}	1.69^{c}	2.82^{bc}	4.1^{ab}	8.22^{a}	2.81^{bc}	5.46^{ab}	4.71^{ab}
Streptococcus	6.94	31.06	13.07	12.94	14.24	5.18	8.5	8.13	5.03	3.96
Turicibacter	6.02^{abc}	0.33^{a}	5.11^{a}	1.15^{a}	3.34^{ab}	0.11^{abcd}	0.05^{bcd}	0.09^{cd}	0.07^{d}	0.25^{bcd}
Proteobacteria										
Escherichia-Shigella	1.91^{bcd}	0.3^{bcd}	16.25^{a}	16.36^{ab}	16.22^{abc}	0.43^{cd}	0.07^{d}	1.93^{abc}	3.72^{abc}	5.69^{abc}

Table 4-6. Relative abundance (%) of Bacteroidota, Firmicutes, and Proteobacteria and their top families in digesta on d 21 post-inoculation of F18 ETEC infected weaned pigs. Weaned pigs were fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2). Each treatment had 11-12 observations

	Ileum					Cecum				
	Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge			
	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm
Bacteroidota	0.33^{b}	0.05^{b}	0.14^{b}	0.24^{b}	0.07^{b}	12.39^{a}	8.80^{a}	16.59^{a}	12.34^{a}	15.66^{a}
Muribaculaceae	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0.24^{ab}	0.25^{b}	0.27^{ab}	0.25^{ab}	0.38^{a}
Prevotellaceae	0.33^{b}	0.05^{b}	0.14^{b}	0.24^{b}	0.06^{b}	12.05^{a}	8.46^{a}	16.22^{a}	11.99^{a}	15.14^{a}
Firmicutes	91.34	82.67	72.98	74.31	75.81	78.89	82.08	72.11	77.79	69.68
Lachnospiraceae	0.16^{b}	0.52^{b}	0.38^{b}	0.63^{b}	0.85^{b}	16.18^{a}	17.20^{a}	18.87^{a}	15.39^{a}	16.19^{a}
Lactobacillaceae	48.62	17.11	36.16	43.76	31.31	27.88	12.68	14.10	24.25	14.22
Streptococcaceae	1.93^{b}	56.81^{a}	17.83^{ab}	15.63^{b}	17.98^{ab}	8.66^{b}	14.94^{ab}	13.9^{ab}	7.34^{b}	4.75^{b}
Proteobacteria	4.50	9.89	20.09	16.24	15.24	2.89	3.71	5.33	3.51	7.51
Enterobacteriaceae	3.71^{ab}	0.30^{ab}	13.74^{a}	15.61^{ab}	12.44^{ab}	0.43^{ab}	0.07^{b}	1.20^{ab}	1.69^{ab}	2.27^{ab}
Pasteurellaceae	0.68^{cde}	8.95^{a}	6.30^{ab}	0.58^{abcd}	2.40^{abc}	0^{e}	0.36^{bcde}	0.29^{cde}	0.09^{de}	0.03^{de}
Succinivibrionaceae	0.02^{b}	0.02^{b}	0.01^{b}	0.01^{b}	0.02^{b}	2.42^{a}	3.14^{a}	3.38^{a}	1.67^{a}	5.15^{a}

Table 4-7. Relative abundance (%) of the most abundant genera from Actinobacteria, Firmicutes and Proteobacteria in digesta on d 21 post-inoculation of F18 ETEC infected weaned pigs. Weaned pigs were fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2). Each treatment had 11-12 observations

	Ileum					Cecum				
	Sham	*Escherichia coli* challenge				Sham	*Escherichia coli* challenge			
	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm	Negative Control	Positive control	BB1 100 ppm	BB2 50 ppm	BB2 100 ppm
Actinobacteria										
Bifidobacterium	3.2	3.85	4.77	6.93	5.98	0.39	0.62	0.26	1.53	1.13
Firmicutes										
Blautia	0.05^{b}	0.08^{b}	0.05^{b}	0.13^{b}	0.12^{b}	6^{a}	4.99^{a}	5.3^{a}	4.65^{a}	5.3^{a}
Clostridium sensu stricto 1	1.88^{a}	0.68^{ab}	1.46^{a}	0.48^{a}	10.57^{a}	0.06^{c}	0.02^{c}	0.07^{c}	0.05^{c}	0.63^{bc}
Lactobacillus	48.59	17.11	36.16	43.75	31.31	27.88	12.68	14.1	24.25	14.22
Megasphaera	3.82^{bc}	2.91^{bc}	0.79^{c}	2.31^{bc}	4.04^{abc}	3.36^{abc}	6.81^{a}	2.63^{abc}	5.69^{a}	4.25^{ab}
Romboutsia	10.77	0.48	1.84	0.75	0.88	0.22	0.25	0.01	0.04	0.08
Streptococcus	1.93^{b}	56.79^{a}	17.83^{ab}	15.63^{b}	17.96^{ab}	8.66^{b}	14.94^{ab}	13.9^{ab}	7.34^{b}	4.75^{b}
Turicibacter	12.01^{a}	0.56^{ab}	9.56^{a}	2.16^{a}	6.59^{a}	0.18^{bc}	0.03^{c}	0.17^{c}	0.14^{c}	0.41^{c}
Proteobacteria										
Escherichia-Shigella	3.71^{ab}	0.3^{ab}	13.74^{a}	15.61^{ab}	12.44^{ab}	0.43^{ab}	0.07^{b}	1.2^{ab}	1.69^{ab}	2.27^{ab}

Table 4-8. Serum and ileal mucosa metabolites that differed among the dietary treatment groups

Metabolite	Fold Change[1]	VIP[2]	FDR[3]
CON-[4] vs. CON+[5], serum d 4 PI			
oleic acid	0.32	1.33	0.048
arachidonic acid	0.36	1.61	0.121
lauric acid	0.39	1.53	0.133
methionine	2.01	1.59	0.121
malic acid	2.32	1.68	0.100
galactonic acid	2.48	1.49	0.145
pinitol	3.15	1.89	0.048
CON+ vs. 100 ppm BB2[6], serum d 4 PI			
pinitol	0.47	2.09	0.180
CON- vs. CON+, serum d 21 PI			
guanosine	0.39	1.79	0.114
methionine	0.45	1.96	0.067
mannose	2.49	2.27	0.004
CON+ vs. 100 ppm BB2, serum d 21 PI			
cholesterol	0.36	2.02	0.190
aminomalonic acid	0.44	1.92	0.190
heptanoic acid	2.25	2.06	0.190
CON+ vs. 50 ppm BB2, ileal mucosa d 5 PI			
asparagine	0.49	2.35	0.160

[1]Fold change values less than one indicate that the differential metabolites were reduced in CON- compared with CON+, or CON+ compared with 50 ppm BB2, or CON+ compare with 100 ppm BB2, respectively.

[2]VIP = Variable Importance in Projection.

[3]FDR = False discovery rate.

[4]CON- = negative control; basal nursery experimental diet, with ETEC challenge.

[5]CON+ = positive control; basal nursery experimental diet, without ETEC challenge.

[6]BB2 = botanical blend 2.

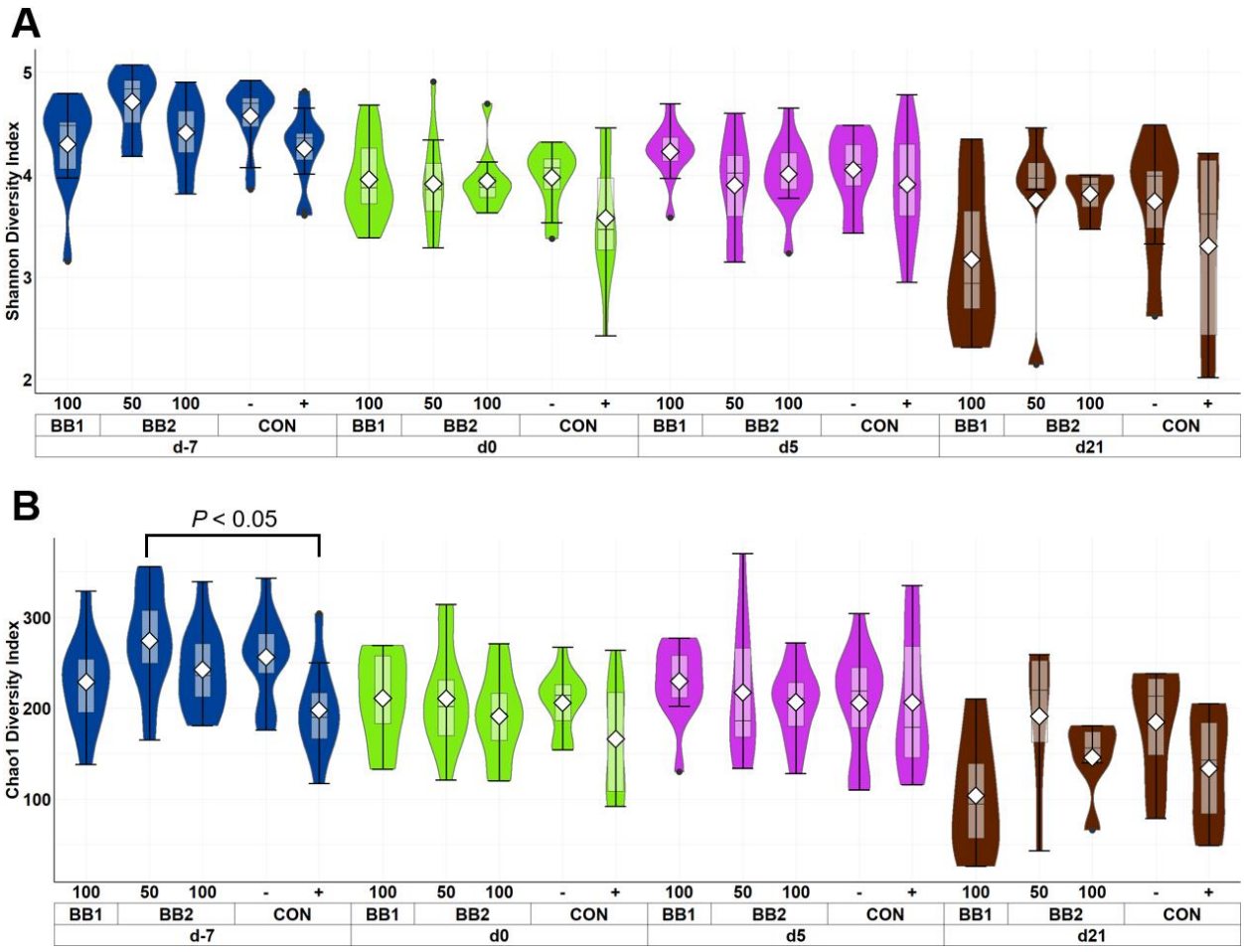


Figure 4-1. Alpha diversity as indicated by Shannon (**A**) and Chao1 (**B**) indices in feces of weaned pigs fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2) at the beginning of the experiment (d -7), first day of F18 ETEC inoculation (d 0), and d 7, 14, and 21 post-inoculation. No difference was observed in Shannon (A) index among treatments. Violin plots are colored by day. Data are expressed as mean (diamond) ± SEM. Each treatment has 11-12 observations**.**

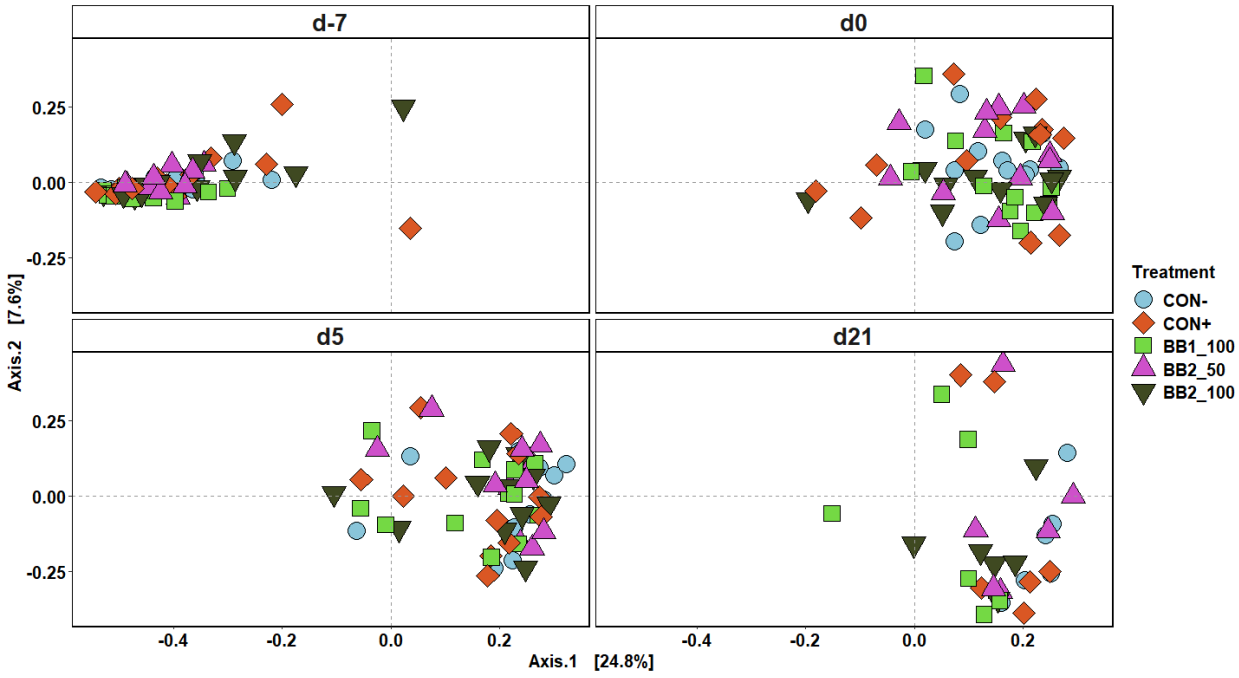


Figure 4-2. Principal coordinate analysis (PCoA) based on Bray-Curtis distance for beta diversity of fecal samples of weaned pigs fed with a control (CON) diet, or diets supplemented with two different botanical blends (BB). Different colors and shapes represent treatments. The sampling days included d -7 and 0 before ETEC inoculation and d 5 and 21 post-inoculation. CON- = negative control; CON+ = positive control; BB1_100 = 100 ppm BB1; BB2_50 = 50 ppm BB2; BB2_100 = 100 ppm BB2. Each treatment had 11-12 observations.

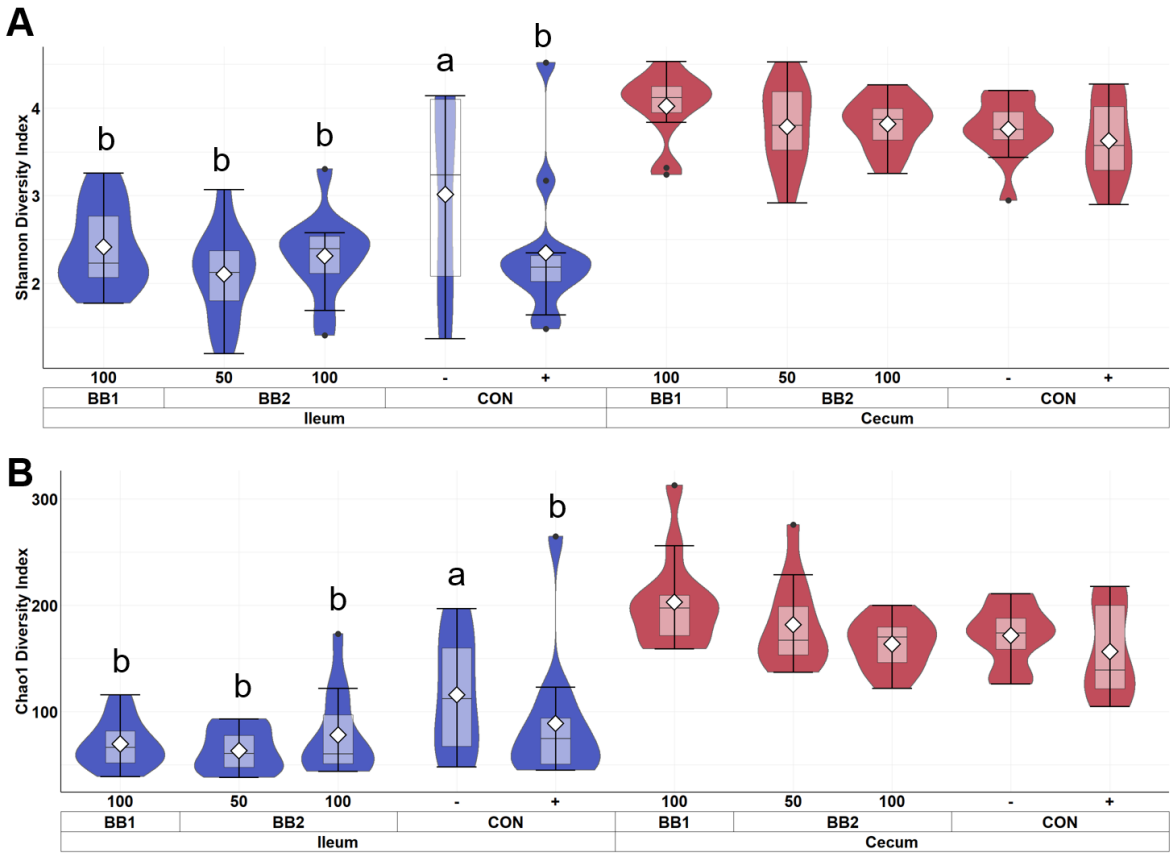


Figure 4-3. Alpha diversity as indicated by Shannon (A) and Chao1 (B) indices of ileal and cecal digesta of weaned pigs on d 5 PI fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2). Violin plots are colored by site. Data are expressed as mean (diamond) ± SEM. Each treatment had 11-12 observations.

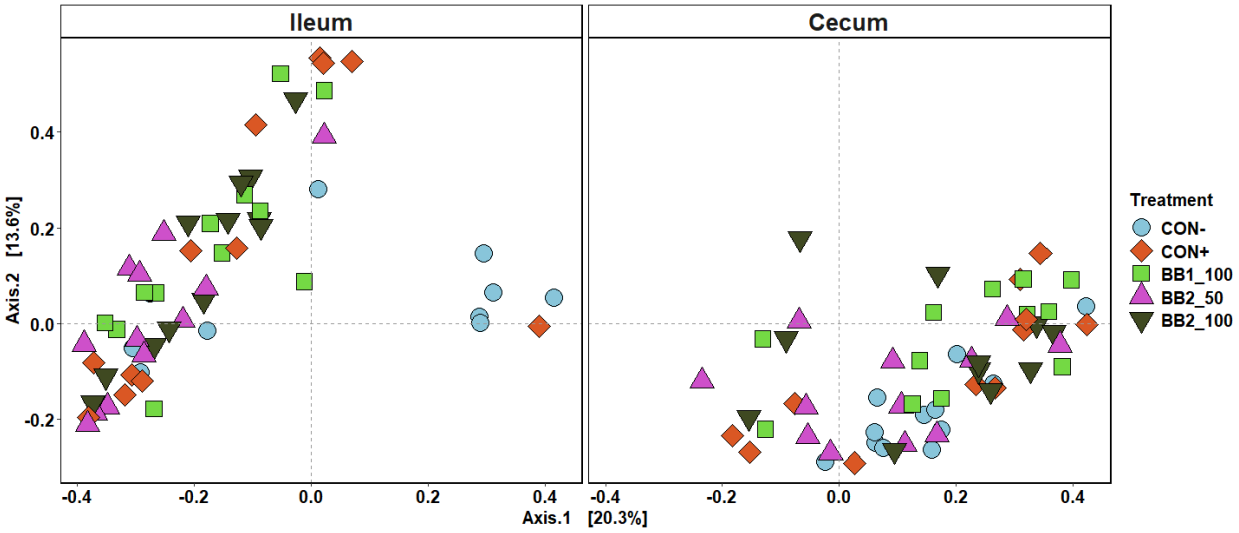


Figure 4-4. Principal coordinate analysis (PcoA) based on Bray-Curtis distance for beta diversity of ileal and cecal digesta on d 5 post-inoculation of weaned pigs fed with control (CON) diet, or diets supplemented two botanical blends. Different symbols and shapes represent treatments. CON- = negative control; CON+ = positive control; BB1_100 = 100 ppm BB1; BB2_50 = 50 ppm BB2; BB2_100 = 100 ppm BB2. Each treatment had 11-12 observations.

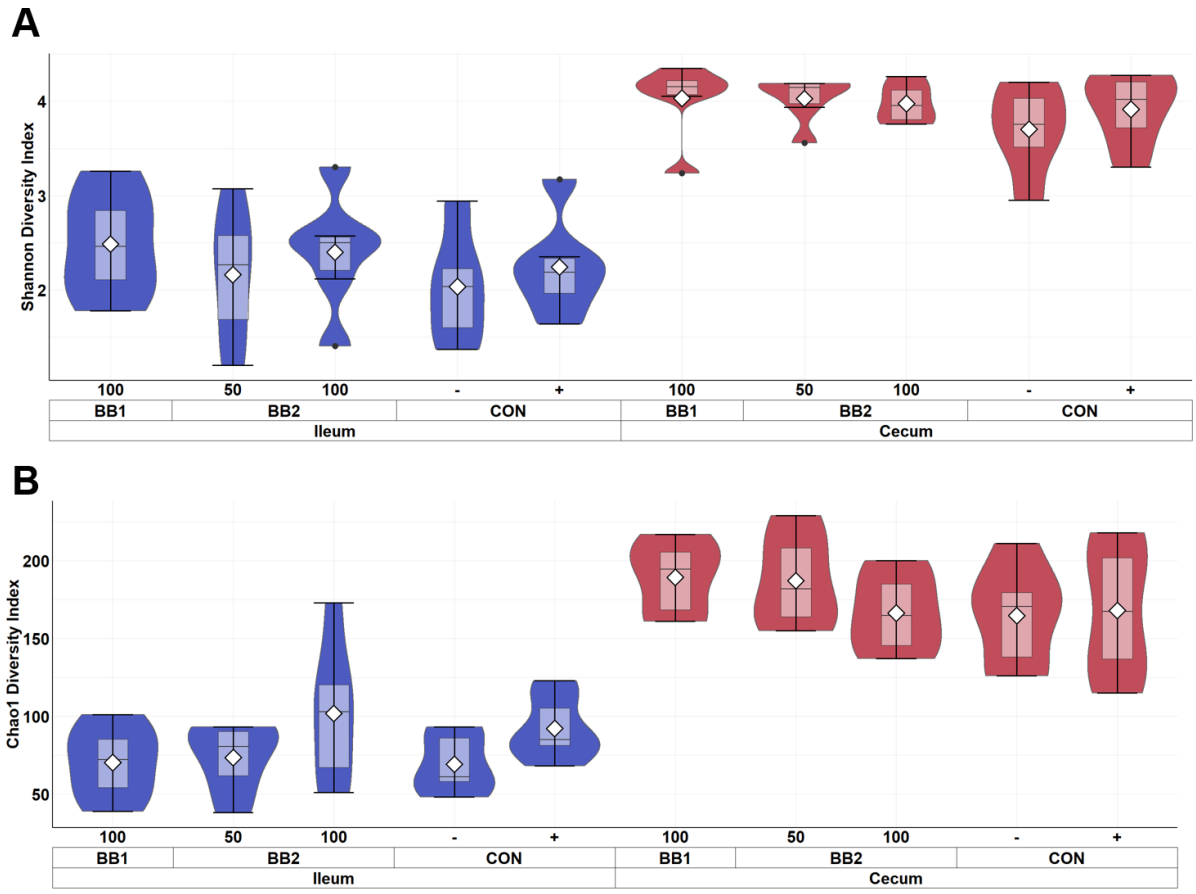


Figure 4-5. Alpha diversity as indicated by Shannon (A) and Chao1 (B) indices in ileal and cecal digesta of weaned pigs fed with 100 ppm of botanical blend 1 (BB1), or 50 ppm or 100 ppm botanical blend 2 (BB2) on d 21 post-inoculation. No difference was observed in Shannon (A) and Chao1 (B) indices among treatments. Violin plots are colored by site. Data are expressed as mean (diamond) ± SEM. Each treatment had 11-12 observations.

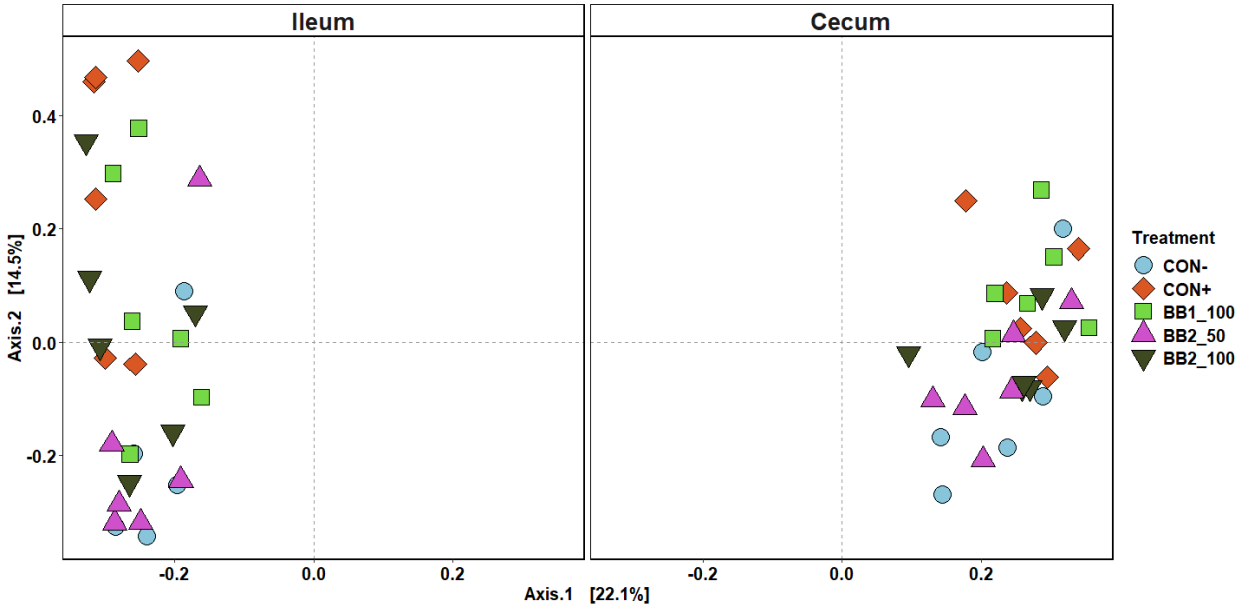


Figure 4-6. Principal coordinate analysis (PCoA) based on Bray-Curtis distance for beta diversity of ileal and cecal digesta on d 21 PI of weaned pigs fed with a control (CON) diet, or diets supplemented with two botanical blends (BB). Different symbols and shapes represent treatments. CON- = negative control; CON+ = positive control; BB1_100 = 100 ppm BB1; BB2_50 = 50 ppm BB2; BB2_100 = 100 ppm BB2. Each treatment had 11-12 observations.

CHAPTER 5

GENERAL SUMMARY, DISCUSSION, AND CONCLUSION

Currently, pigs that undergo weaning stress experience post-weaning diarrhea commonly induced by enterotoxigenic *Escherichia coli* (ETEC). Post-weaning diarrhea causes growth retardation and perturbation in intestinal development of pigs, which leads to increased mortality rate and reduced profit in the swine industry. Antibiotics are currently a remedy to alleviate post-weaning diarrhea, however, excessive use of antibiotics can lead to increased antibiotic resistance gene in opportunistic pathogens that could eventually impact the human population. Therefore, alternative practices, including nutritional intervention, are to be sought. Various feed additives are investigated upon as potential remedy to post-weaning diarrhea including direct fed microbials and botanical extracts. However, limited studies have investigated the gut microbiota of weaned pigs that were supplemented with these feed additives and assess whether the modulated gut microbiota could enhance the health benefits of weaned pigs. The gastrointestinal tract provides an optimal environment for various gut microbes to flourish and to fulfill their crucial roles in supporting nutrient utilization and immune regulation in pigs. A dynamic microbial population in the gut can be altered by age, diet, and many other factors. Characterizing the gut microbiota changes using 16S rRNA sequencing could help swine industry to better understand the impacts of disease and diet on the intestinal microbiota in order to find alternative strategies to promote pig health when the use of antibiotics in feed is restricted. In this dissertation, three studies were conducted to assess the changes of gut microbiota in weaned pigs under ETEC induced diarrhea and supplemented with one of the three feed additives: *Bacillus* (*B.*) *subtilis, B. amyloliquefaciens,* and botanical blends.

The first study characterized changes in gut microbiota of weaned pigs challenged with ETEC when supplemented with *B. subtilis* DSM 25841 or carbadox, an antibiotic growth promoter. Along with age, *B. subtilis* modified the gut microbiota of weaned pigs and altered the microbiota differently when pigs were supplemented with carbadox. *B. subtilis* supplementation increased relative abundances of *Lactobacillaceae* and *Bifidobacteriaceae,* while carbadox supplementation increased relative abundances of Bacteroidetes and Proteobacteria in ileal digesta. These results indicate that the modulation of gut microbiota may contribute to diarrhea alleviation and intestinal health enhancement of weaned pigs supplemented with *B. subtilis*, while carbadox modulated gut microbiota that may impose risk of increasing antimicrobial resistances in opportunistic pathogens.

The second study investigated the impacts of dietary supplementation of *B. amyloliquefaciens* on growth performance, diarrhea, systemic immunity, and intestinal microbiota of weaned pigs challenged with ETEC. *B. amyloliquefaciens* had limited impacts in reducing diarrheal frequency but alleviated systemic inflammation of weaned pigs when they were challenged with ETEC. Relative abundance of *Bifidobacterium* was greater and *Clostridium sensu stricto 1* was lower in pigs supplemented with *B. amyloliquefaciens* than with carbadox in ileal digesta. Results in present study imply that *B. amyloliquefaciens* supplementation had modified the ileal microbiota composition by enhancing beneficial bacteria and reducing pathogenic bacteria genera, although this benefit was not reflected in disease resistance of weaned pigs challenged with ETEC.

The third study assessed the impacts of two types of botanical blends (BB1 and BB2) on the gut microbiota and metabolomic profiles of weaned pigs when challenged with ETEC. BB treatments were comprised of 0.3% capsicum oleoresin and 12% garlic oil extracted from

different sources. Supplementation of 100 ppm BB1 increased abundances of *Enterobacteriaceae* in ileum and *Prevotellaceae* in cecum of weaned pigs during the acute phase of ETEC challenge (d 5 post-inoculation). Supplementation of 100 ppm BB2 upregulated serum pinitol during the acute phase and upregulated serum aminomalonic acids during the recovery phase (d 21 post-inoculation) of ETEC challenge. Supplementation of 50 ppm BB2 also downregulated asparagine in ileal mucosa of ETEC challenged pigs on d 5 post-inoculation. The results from the third study imply that botanical blends modulated ileal microbiota and serum metabolomic profiles in weaned pigs under ETEC challenge.

The first two studies confirm than the effects of *Bacillus* spp. supplementation on pig health, gut microbiota, and performance varied due to the variation of species. In addition, the gut microbiota modulation by *Bacillus* spp. was different as carbadox. The third study suggests that botanical blends, including capsicum oleoresin and garlic oil can modulate both gut microbiota and serum metabolomic profile, which might be associated with their antimicrobial and immunoregulatory effects *in vivo*. All three studies confirm that age and ETEC infection also have significant impacts on the intestinal microbiota of weaned pigs. The findings in this dissertation help us to better understand the major factors that are involved in gut microbiota modulation during post-weaning period, which will benefit to future research focusing on enhancing growth and strengthening immunity of weaned pigs via microbiota modulation. It is also important to note that 16S rRNA sequencing has limitation to quantify gut microbiota. Thus, future research is suggested to characterize functional profiling within the gut microbiota and profile metabolites released into the intestinal digesta by gut microbes.

BIOGRAPHY

Cynthia Jinno was born and raised in West Hollywood, CA. She acquired her Bachelor's degree in Animal Science at the University of California, Davis (UC Davis) in 2017 where she enjoyed studying animal nutrition and physiology. She then acquired her Master's degree in Animal Biology with her thesis entitled "Utilization of Enzymatically Digested Food Waste as Feed for Growing-Finishing Pigs" from UC Davis under Dr. Yanhong Liu's supervision in 2019. Afterwards, she continued her Ph.D. research in Dr. Liu's lab by focusing on the gut microbiota of weaned pigs under diarrheal stress, which drove her great interest in computational biology. Throughout her time in Dr. Liu's lab, she has authored 2 peer-reviewed articles and 13 abstracts, co-authored 5 peer-reviewed articles, gave 5 oral presentations and 11 poster presentations in 3 major American Society of Animal Science conferences and annual department colloquium. She developed a 16S sequence analysis pipeline and data visualization that has been used to support numerous studies in her lab and enjoyed giving a quarter-long seminar in Fall 2021 to teach her lab and interested parties how to utilize it. She has received the Microbiome Graduate Research Award from the Microbiome Research Working Group in UC Davis in 2019 and was awarded an Animal Science Young Scholar issued by American Society of Animal Science - Midwest section in 2022. Her hobbies are swimming, drawing, painting, and spending time with her 4 adoring dogs Quu, Maru, Coco, and Chacha.