Investigation of an ETEC challenge and supplementation of direct-fed microbials in weaned pigs

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Economic losses in the nursery phase of pork production can largely be attributed to the underdeveloped gastrointestinal tract (GIT) and immune system of weaned pigs, which greatly increases their susceptibility to potential pathogens. Enterotoxigenic *Escherichia coli* (ETEC) is a common enteric pathogen that results in secretory diarrhea and leads to reductions in growth performance and increases in mortality, morbidity, and treatment costs. Vaccinations and antibiotics have traditionally been used as means of prevent or mitigating ETEC infections; however, with the increase in antibiotic resistant pathogens and pressure from the consumer population, pork producers are in need of alternative strategies. Dietary inclusion of direct-fed microbials (DFM) have been proposed as a potential dietary strategy to prevent or mitigate the negative effects associated with weaning and ETEC infections due to their suggested ability to exert beneficial effects on the host's GIT, immune system, and microbial population. Nonetheless, having an understanding of how disease physiologically impacts the pig is essential to developing effective alternative dietary technologies to prevent or mitigate disease. Therefore, the objectives of this thesis were to characterize the impact of an ETEC challenge in weaned pigs on growth, intestinal function, immune response, and mucosal microbiota, and to evaluate the potential beneficial effects of *Bacillus*-based DFMs under normal physiological and ETEC challenge conditions.

To achieve our objective, two experiments were conducted. The results of Experiment 1 (Chapter 2) revealed that *Bacillus*-based DFMs did not affect body weight or growth performance of healthy nursery pigs housed in a commercial-like environment compared to the control diet, which may be a result of contrasting effects of the DFM

products and dietary ingredients, such as zinc oxide. Experiment 2 (Chapters 3 and 4) investigated the impact of an F18 ETEC challenge on growth performance, intestinal function, immune response, and mucosal microbiota of weaned pigs, as well as evaluated the potential protective effects of two *Bacillus*-based DFMs (DFM1 And DFM2). The ETEC challenge increased fecal scores, reduced rectal temperature, impaired intestinal barrier integrity, altered immune response, resulting in reduced growth performance during 10-d post-challenge period. The ETEC challenge also induced dysbiosis in the mucosa-associated microbiota by increasing the abundance of potentially pathogenic bacteria and reducing the abundance of beneficial microbes. Our results suggest that the supplementation of DFM2 may potentially alleviate negative impacts of an ETEC challenge by improving intestinal barrier integrity and reducing bacterial pathogen load in the GIT microbial population.

CHAPTER I

LITERATURE REVIEW

Introduction

Weaning is one of the most significant events in a pig's life and represents major challenges in developing their intestinal epithelial barrier and immune system. Abrupt removal from the sow, transportation and handling, changes in diet and social and physical environment, and exposure to new pathogens all contribute to poor performance and increased mortality following weaning. The gastrointestinal tract (GIT) provides a physical and immunological barrier for piglets subjected to pathogens. This barrier is a complex but elegant system composed of defense mechanisms provided by epithelial cells and the mucosal immune system acting to regulate normal GIT barrier function, immunological responses, and homeostasis.

Pathogens, such as enterotoxigenic *Escherichia coli* (ETEC), breakdown these components of GIT function causing decreases in feed intake and growth and reducing overall animal health and survivability. Inflammatory responses and interactions between the GIT mucosa and lymphoid tissues are essential in the host defense against ETEC.

Concerns regarding antimicrobial resistance and consumer demand has led to reduced antibiotic use in swine production. Consequently, alternative non-antibiotic products to mitigate ETEC are currently being investigated; however, in order to develop such strategies to mitigate this disease, a concrete understanding of ETEC's mechanism of action is required. The objective of this review is to discuss the impact of post-weaning diarrhea caused by ETEC on growth performance, intestinal integrity, and gut microbiota

in nursery pigs. The utilization of non-antibiotic feed additives, especially direct fed microbials (DFMs), in the prevention and mitigation of post-weaning diarrhea will also be reviewed.

Gastrointestinal tract function

The gastrointestinal tract is responsible for digestion and absorption of many nutrients, including water, lipids, proteins, carbohydrates, vitamins, and minerals. In addition, the GIT serves as the largest immune organ in the body and provides a barrier between the lumen and systemic circulation. Maintaining a healthy GIT is essential for the productivity and longevity of an animal. The term 'gut health' has been increasingly used across both human and animal health industries, and is an area of study that has gained significant interest around the world. This term encompasses a wide variety of meanings, making it difficult to establish a consensus definition. Gut health comprises multiple physiological and functional properties and their interactions among each other, including, but not limited to, intestinal structure and integrity, digestion and absorption of nutrients, mucosal immunity, and a stable microbial population (Moeser et al., 2017; Pluske et al., 2018).

General description

The GIT barrier is a multi-layer system comprised of both physical and chemical defense mechanisms to prevent and alleviate pathogen infiltration. A single layer of selectively permeable intestinal epithelial cells (IECs) line the GIT and serves as the primary border between the pig and its external environment. The maintenance of the IEC layer is critical for disease mitigation and prevention of whole-body systemic immune

stimulation. Both the apical and basolateral membranes of the intestinal epithelium are home to several receptors, enzymes, transporters, channels, and pumps that are essential to facilitating the breakdown and uptake of important nutrients needed by the pig. Furthermore, the GIT also has a large excretory function and is responsible for preventing absorption of unneeded or harmful substances.

Defense mechanisms

Preserving the physical barrier and intestinal integrity of the GIT is largely dependent upon junction complexes connecting the enterocytes of the intestinal epithelium. These complexes are comprised of multiple intercellular membrane proteins including tight junctions (TJ), adhesion junctions, desmosomes, and the gap junction. In particular, paracellular permeability between enterocytes is largely maintained by interactions between the TJ proteins: claudins (CLDN), occludin (OCLN), and zonula occludens (ZO) (Tsukita et al., 2001). These proteins play a significant role in preventing harmful bacteria and their products (e.g. endotoxin) from translocating across the intestine and activating an immune response (Mukiza et al., 2013).

The mucosal immune system is essential in balancing the competing demands of pathogenic antigens derived from the environment and harmless antigens, including nutrients and commensal organisms. Secretory immunoglobulin A (sIgA) is an anti-inflammatory compound that is the predominant class of antibody found in the mucosal immune system and provides important humoral protection against pathogenic infection in the GIT. sIgA is the first line of barrier defense by the intestinal mucosa and has an important role in maintaining homeostasis of the host's commensal bacteria. In the event of an enteric infection, luminal sIgA is absorbed on the layer of mucus covering the

epithelium and binds via their carbohydrate component. Once bound, it aims to prevent bacterial adherence to the intestinal epithelium. If the bacteria are able to penetrate the barrier, sIgA has the ability to neutralize the lipopolysaccharide component of the gram-negative bacteria within the endosome of the epithelial cells (Cerutti and Rescigno, 2008; Corthesy, 2013).

Microbiota and microbial metabolites

A substantial and diverse population of bacteria comprise the pig's GIT, all of which have a key role in maintaining an animal's overall health. As culture-independent analysis of intestinal microbiota has progressed with the development of high-throughput sequencing technology, including 16s rRNA sequencing, research has shown that the gut microbiota impacts multiple physiological traits in the host (Camarinha-Silva et al., 2017; Leulier et al., 2017; Han et al., 2018). An estimated 100 trillion bacterial cells inhabit the mammalian gut and support host health through beneficial metabolite production, such as short-chain fatty acids (SCFA), inhibition of pathogen colonization, and immune system preservation (Holman et al., 2017). Although it is difficult to define a healthy gut microbiota, it is important to note that several species have multiple strains, some of which are commensal and others pathogenic. The balance of the microbial ecosystem is achieved through commensal, neutral, antagonistic, and symbiotic relationships between bacterial species (Boon et al., 2014).

It is well established that diet largely influences gut microbe composition, as it is the main source of growth substrate (Frese et al, 2015; Heinritz et al., 2016). The microbes are also very important for digesting dietary compounds left undegraded by endogenous enzymes. Carbohydrates are the major energy substrate for GIT microbes. Fermentation of

undigested carbohydrates results in the production of SCFAs, which are rapidly absorbed and utilized as an energy source for the enterocytes (den Besten et al., 2013). Gut microbes can also utilize nitrogen from dietary compounds. This mainly occurs in the large intestine, where many bacteria utilize peptides and amino acids as carbon, nitrogen, and energy sources (Dai et al., 2010).

Given that diet plays an essential role in shaping the gut microbial population, the relationship between age and intestinal microbiota is an important consideration, as growth stage and dietary composition are directly associated with one another. Stress during the weaning period results in substantial disruptions to the GIT microbial environment (Konstantinov et al., 2006), partly due to the introduction of cereal-based diets and reductions in feed and water intake (Mach et al., 2015). This increases the pig's susceptibility to pathogenic infections from organisms such as *Salmonella, Escherichia coli,* and Clostridia. Mucin production by intestinal goblet cells is critical to prevent pathogen adherence and penetration. Beneficial microbes, such as *Lactobacillus,* improve mucin production and are a key factor in disease prevention (Fouhse et al., 2016). It has also been shown that increases in *Prevotella* are positively correlated with sIgA concentrations and improved growth performance (Mach et al., 2015). Other factors including genetics, environment, and health status have been shown to influence changes across microbial communities. Kubasova et al. (2018) evaluated the fecal microbial composition of high or low residual feed intake (HRFI and LRFI) pig lines via 16s rRNA sequencing and observed a greater abundance of *Megasphaera* and *Lactobacillus* in HRFI pigs. Additionally, microbial adaptation to new diets following weaning was slower in LRFI pigs, indicating that genetic selection influences selection of microbiota. Le Floc'h

et al. (2014) reported differences in fecal microbial communities of pigs housed in either good or poor hygiene conditions.

Post-weaning diarrhea

Despite numerous vaccines and dietary interventions, post-weaning diarrhea (**PWD**) resulting from *E. coli* infection continues to have a considerable negative impact on global swine production. Increases in mortality, morbidity, and treatment costs coupled with decreases in growth performance cause significant economic losses for pork producers and the industry (Fairbrother and Gyles, 2012). Post-weaning diarrhea commonly occurs between 4 and 14 days following weaning. Susceptibility to PWD is intensified by multi-factorial stressors at weaning, including sudden changes in environment, social interactions, and diet, as well as immature intestinal and immune system development in newly weaned pigs (Campbell et al., 2013; Heo et al., 2013).

While there are many classifications of *E. coli*, the most common is ETEC (Zhang et al., 2007). Additionally, ETEC carries the greatest economic importance among pathogenic *E. coli* in pigs (Gyles and Fairbrother 2010). Environmental ETEC enters the pig GIT orally. Several factors including poor hygiene, insufficient disinfection, low environmental temperature, and excessive air flow promote development of diarrhea and accumulation of ETEC in the environment (Gyles and Fairbrother 2010).

Pathogenesis

Fimbrial adhesins and enterotoxins are the major virulence factors of ETEC induced diarrhea (Zhang et al., 2007). ETEC adhere to the small intestinal mucosa and colonize through fimbrial adhesins, which are rod-like appendages on the surface of ETEC

that facilitate bacterial attachment to the host's receptors. The fimbrial adhesins most commonly associated with PWD in pigs are F4 (K88) and F18 (Zhang et al., 2007).

Susceptibility to ETEC is determined by expression of fimbrial adhesin binding receptors in the pig small intestine. (Frydendahl et al., 2003; Roubos-van den Hil et al., 2017). F4 receptor expression is present both in neonatal and weaned pigs, whereas F18 receptors, whose expression are controlled by the α (1,2)-fucosyltransferase gene (FUT1), are absent in neonatal piglets and not fully expressed until approximately 3 weeks of age. (Fairbrother et al., 2005; Coddens et al., 2007). Following adhesion and colonization of the small intestine, secreted enterotoxins can be translocated to the small intestine. Enterotoxins disrupt water and electrolyte balance in the small intestine resulting in increased secretion of chloride and carbonate ions, inhibition of sodium and water absorption, and eventually secretory diarrhea. Continual secretion leads to dehydration, metabolic acidosis, and death. ETEC strains produce several enterotoxins, including heat labile, heat stable, and enteroaggregative heat-stable enterotoxins (Sun and Kim, 2017).

Impact of post-weaning diarrhea in pigs

Experimental induction of PWD has been achieved through ETEC challenge models in order to evaluate immunological, intestinal barrier, and growth performance responses in weaned pigs. Challenges can induce either subclinical or clinical symptoms of ETEC infection. Pigs with subclinical expression of disease lack visual symptoms, such as watery diarrhea, but still have an immune system response and often reductions in growth performance (Martin et al., 1987).

In addition to clinical symptoms, ETEC infections in pigs are known to increase intestinal permeability by altering the structure of the TJ complex through ZO-1

redistribution and dissociation of diarrhea (Berkes et al., 2003; Roselli et al., 2007). ETEC enterotoxins can stimulate secretion of pro-inflammatory cytokines (TNF-α, IL-1β, etc.), which triggers phosphorylation of the myosin light chain, resulting in contraction and opening of tight junction proteins (Gao et al., 2013; Turner et al., 1997). In ETEC challenged pigs, decreases in ileal claudin-1, claudin-2, occludin, and ZO-1 TJ proteins have been reported (Ewaschuk et al., 2011; Gao et al., 2013; Li et al, 2019). Ewaschuk et al. (2011) also observed an increase in mannitol flux across the jejunum, further validating the increase in intestinal permeability following an ETEC infection.

When the tight junctions are disrupted, paracellular transport of luminal contents and pathogenic material into circulation can increase, activating an immune response and intestinal inflammation through TLR4 and CD14 binding (Guo et al., 2013). TLR4 and CD14 are co-receptors of LPS, a powerful innate immune system stimulus produced by gram-negative bacteria (Alexander and Rietschel, 2001). This is in agreement with Li et al., (2019), who reported an increase in ileal TLR4 and CD14 mRNA in ETEC challenged pigs compared to a non-challenged control, indicating an immune system activation followed by an ETEC challenge. Studies have also described changes in localized immune responses in the small intestine due to an ETEC challenge, as supported by increased ileal macrophages and neutrophils (Liu et al., 2013), an upregulation in cytokines *IL-1B*, *IL-8*, *IL-17A, TNF-α,* and *IFN-γ* mRNA (Loos et al., 2012; Liu et al., 2014; Li et al., 2019), and increased sIgA (Gao et al., 2013, Zhang et al., 2013). Previous research has shown that an ETEC challenge also results in systemic immune system activation. Liu et al. (2013) reported increased levels of serum TNF-α and haptoglobin and decreased white blood cell counts. Zhang et al. (2013) observed an increase in serum IgA, IgM, and IgG. These results

are in agreement with Sugiharto et al. (2014), who found elevated plasma IgA, neutrophils, and lymphocytes following an F18 ETEC challenge.

Intestinal morphology is a key marker that reflects GIT development. ETEC attachment to the small intestine without destruction of the microvilli is critical for pathogen elaboration; however, research evaluating the influence of ETEC on intestinal structure in pigs is inconsistent. Villus atrophy and reductions in villus height:crypt depth in the small intestine following an ETEC challenge have been described (Yi et al., 2005; Gao et al., 2013), while others reported no association between an ETEC challenge and changes in intestinal morphology (Liu et al., 2013). Changes in villus height and crypt depth are often correlated with digestive and absorptive capacities of the GIT (Pluske et al., 1997; Tsukahara et al., 2012). While the effects of weaning on digestion and absorption have been well described (Montagne et al., 2007; Wijtten et al., 2011), there is a paucity of literature describing the impact of an ETEC challenge in pigs on these functions.

Pathogenic bacterial infections cause proliferation of harmful bacteria, resulting in dysbiosis of the intestinal microbiota (Burrough et al., 2017; Gresse et al, 2017). Post-weaning diarrhea is often characterized by a decline in lactic acid-producing bacteria (*Lactobacillus* spp.) and the increase in clostridia and pathogenic *E. coli* in the GIT (Konstantinov et al., 2006); however, a greater understanding of microbiota changes following a direct ETEC challenge in weaned pigs is needed. The continued development of new technologies, such as Next-Generation Sequencing (NGS), offers means to investigate microbial communities and their interactions with the host immune system, genetics, environment, and diet (Holman et al., 2017; Pollock et al., 2018).

Alternatives to the use of sub-therapeutic antibiotics

As our understanding of potential adverse consequences of including antibiotics in pig diets has increased, there is a growing interest in antibiotic-free and reduced antibiotic pork production, particularly in weaned pigs. Removal of antibiotics from newly weaned pigs has greater negative implications compared to other production stages due to large growth performance reductions, high mortalities, and increased disease incidence (Hao et al., 2014), all resulting from the stressors associated with weaning, as previously discussed. Historically, vaccines and antibiotics have been utilized to prevent and control ETEC infections in commercial nurseries. ETEC outbreaks are still present worldwide, and with increasing limitations on antibiotic use in pork production, concerns of antimicrobial resistance, as well as consumer demand to reduce antibiotic use, alternatives to these treatments are necessary. Nutritional intervention is largely being investigated as a means of preventing and/or mitigating ETEC. Commonly investigated feed additives include direct-fed microbials, acidifiers, enzymes, plant extracts, fiber, prebiotics, and antimicrobial peptides. (Pettigrew, 2006; Stein and Kil 2006; Kil and Stein, 2010). The objective of these feed additives in nursery pigs is to prevent or mitigate post-weaning diarrhea and subsequent economic losses. This objective is often met by developing the pigs' ability to prevent colonization of pathogenic bacteria via alteration of the gut microbiota and environment, improved immunological response, or enhanced intestinal barrier function. The strengths, weaknesses, and potential modes of action of these alternative feed additives have been previously reviewed. (Thacker, 2013; Liu et al., 2018). The current review will focus on the use of direct-fed microbial blends in swine diets.

Direct-fed microbials

Direct-fed microbials, often referred to as probiotics, have been utilized in both human nutrition and livestock production for many years. The Food and Drug Administration implemented that the label "probiotic" only be applied to microbial products for human use; consequently, the label "DFM" is utilized in the U.S. feed industry. Direct-fed microbials are defined as products that "contain live naturally occurring microorganisms" (FDA, 2015). Bacterial strains including *Bacillus, Lactobacillus, Bifidobacterium, Enterococcus,* and *Streptococcus* are common in commercial products, which are often comprised of multiple species (Yirga, 2015). Their effectiveness is dependent upon their ability to be prepared on a large scale, provide beneficial effects on the host, and their capacity to tolerate various environmental pressures during processing, storage, and passage through the gastrointestinal tract of the animal (Bajagai, 2016). These bacteria must also be resistant to gastric acid, digestive enzymes, and bile acids to survive in the gastrointestinal tract (Larsen et al., 2014). Several mechanisms of action have been proposed by which DFMs exert effects on the animal and improve growth performance, including, but not limited to: production of antimicrobial compounds and enzymes (Kunst et al., 1997), enhanced nutrient digestibility (Meng et al., 2010; Giang et al., 2010; Jørgensen et al., 2016), promotion of beneficial bacteria growth (Baker et al., 2013; Hu et al., 2014; Cui et al., 2013), competitive exclusion of pathogenic bacteria (Tsukahara et al., 2013), immune response modulation (Lee et al., 2014; Yang et al., 2016), and improvements in intestinal barrier integrity (Baum et al., 2002; Yang et al., 2016; Gu et al., 2014).

Direct-fed microbials are classified into 3 main categories: *Bacillus,* lactic acid-producing, and yeast (Stein and Kil, 2006, NRC, 2012). The *Bacillus* genus has been substantially researched due to their ability to form endospores, produce exogenous enzymes and antimicrobial compounds, and beneficially alter the gut microbiota (Cutting, 2011). *Bacillus* spp. Are gram-positive, facultatively anaerobic, and spore-forming organisms, making them highly stable at various temperatures and low pH (Nicholson et al., 2000; Setlow, 2006). These characteristics allow them to survive pelleting and extrusion processes, storage, as well as the unhospitable environment of the pig's stomach; however, survival through the stomach does not guarantee the DFM will exert beneficial effects on the animal. From 245 bacterial isolates, Larsen et al. (2014) identified *B. amyloliquefaciens* , *B. subtilis* and *B. mojavensis* as three strains with the best characteristics regarding pathogen inhibition, spore-forming ability, antibiotic resistance, and enzyme production, making them strong potential candidates for DFMs. For the purposes of this review, emphasis will be placed on *Bacillus*-based DFM products.

Efficacy of direct-fed microbials

The ability of *Bacillus*-based DFMs to improve ADG, ADFI, and feed efficiency in pigs has been well documented. Wang et al. (2017) reported an increase in ADG and ADFI in nursery pigs fed *B. amyloliquefaciens* compared to pigs fed an antibiotic. In weaned pigs fed a DFM blend of *B. subtilis* and *B. amyloliquefaciens*, improvements in feed efficiency have been observed (Cai et al., 2015; Jaworski et al., 2017). In a 14-d study evaluating the effects of a water-delivered *Bacillus*-based DFM, increased ADG in weaned pigs following a *Salmonella* infection was observed. (Walsh et al., 2012). Similarly, Pan et al. (2017) reported similar growth performance in ETEC challenged pigs supplemented

with *B. licheniformis and Saccharomyces cerevisiae* compared to a non-challenged control. Addition of *B. licheniformis* and *B. subtilis* in pig diets from wean to finish has been shown to reduce morbidity and mortality (Alexopoulos et al., 2004b). These improvements in performance can partly be credited to enhanced nutrient digestibility resulting from exogenous enzyme production (Ferrari et al., 1993; Jørgensen et al., 2016; Lan et al., 2016; Payling et al., 2017).

DFM supplementation in pigs has been shown to positively influence intestinal barrier function, as well as immune responses. Hu et al. (2018) reported that pigs fed *B. amyloliquefaciens* significantly increased amylase, sucrase, maltase, and Na+/K+-ATPase, indicating a positive influence on intestinal barrier integrity. Increases in serum immunoglobulins IgA and IgG in pigs fed *B. subtilis* have been observed (Kunavue and Lien, 2012; Lee et al., 2014). These immunoglobulins are known to play a critical role in clearing foreign pathogens. Results regarding intestinal morphology in nursery pigs fed a *Bacillus*-based DFM have been conflicting. Cai et al. (2015) reported a blend of 1 strain of *B. subtilis* and 2 strains of *B. amyloliquefaciens* increased villus height in the duodenum and jejunum of weaned pigs; however, others have shown no effect of DFM supplementation on villus height (Walsh et al., 2007; Choi et al., 2011). Bhandari et al. (2008) also reported no change in intestinal villus height in pigs subjected to an ETEC challenge with DFM supplementation. While limited data exist regarding *Bacillus*-based DFM supplementation in ETEC challenged pigs, Tsukahara et al. (2013) observed a reduction of pathogenic bacteria in the ileal digesta and feces of pigs fed *B. subtilis* and challenged with Shiga toxin-producing *E. coli* (STEC).

Healthy gut microbial populations contribute to enhanced performance and immunity. DFMs are known to balance the GIT microbial population dynamics in order to create a more beneficial microbial population. This beneficial modulation may begin as early as gestation, as demonstrated by Baker et al. (2013). In sows fed a diet supplemented with *B. subtilis,* nursing piglets had a greater abundance of *Lactobacillus* in the ileum and lower *E. coli* numbers in the colon at 3 days old. In *MUC4*-resistant piglets orally administered *B. licheniformis* and *B. subtilis,* attenuated growth of *Bacteroides uniformis*, *Eubacterium eligens*, *Acetanaerobacterium*, and *Sporobacter* populations was observed following an ETEC challenge, while *Clostridium, Turicibacter,* and *Lactobacillus* populations increased. An increase in ileal goblet cells and MUC2 production also occurred, indicating enhanced GIT defense and preservation of the mucosal barrier against an ETEC challenge (Zhang et al., 2017). Hu et al. (2013) observed an increase in the relative number of fecal *Lactobacillus* and a corresponding decrease in *E. coli* in weaned pigs fed *Bacillus subtilis* KN-42, as well as an increase in bacterial diversity. These results agree with Lan et al. (2016), in which supplementation of pigs with a mixture of *B. coagulance, B. lichenformis, B. subtilis,* and *Clostridium butyricum* resulted in higher fecal *Lactobacillus* counts and lower *E. coli* counts. Similarly, Payling et al. (2017) reported an increased in fecal *Lactobacillus* in growing pigs fed three *Bacillus* strains.

Sources of variability

Despite the several studies reporting beneficial effects of *Bacillus*-based DFM products, results in pigs are inconsistent. While many researchers have observed improvements in growth performance, other studies have reported no effects of DFM supplementation on growth performance, nutrient digestibility, or gut health parameters

(Chen et al., 2006; Walsh et al., 2007). Several sources of variability are observed across studies evaluating DFMs, making it difficult to compare results. Studies vary in experimental design, stage of production, DFM species, strain, dose and application, adaptation time, sample collection, and inclusion of a challenge. Dietary ingredient composition is also a key factor; however, limited research has evaluated the interactions between DFM products and feed ingredients. Additionally, there may be further inconsistencies when assessing single strain or multi-strain DFM products. Improvements in genetics and pork production practices may also influence DFM efficacy between studies. There is strong evidence that the gut microbiota are constantly evolving, and as the swine industry continues to reduce the use of antibiotics and pharmacological levels of minerals, development of new DFM products with strains that are more relevant to the pig's changing GIT environment may be necessary.

Conclusions

In piglets, gastrointestinal function following weaning is closely associated with their subsequent growth performance and economic values. GIT disruption and immunological stimulation due to ETEC infections after weaning have negative consequences on the pig's ability to intake feed and uptake nutrients, resulting in reduced growth, increased morbidity, and potentially death. The GIT and mucosal immune systems are large and complex features strongly impacted by ETEC. In order to develop interventions to control or prevent ETEC, more comprehensive research evaluating intestinal barrier function, localized and systemic immune response, and gut microbiota changes in response to an ETEC infection is needed. Including direct-fed microbial blends,

especially *Bacillus*-based DFMs, in nursery pig diets is a nutritional strategy that shows great promise to improve pig growth performance and gut health following weaning; however, a better understanding of their mode of action, particularly under pathogenic infection, is warranted. Therefore, the overall objective of this thesis research was to investigate the physiological impact of an ETEC challenge in weaned pigs and to understand the effects of supplementing *Bacillus*-based direct-fed microbial blends in both disease challenge and normal commercial-like conditions.

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CHAPTER II

IMPACT OF DIRECT-FED MICROBIAL SUPPLEMENTATION ON NURSERY PIG GROWTH PERFORMANCE

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Abstract

Direct-fed microbial blends have been proposed as non-antibiotic nutritional interventions to prevent or mitigate weaning stress. These feed additives may interact with a host's gastrointestinal tract, immune system, and microbiota, and could potentially improve animal production and minimize economic losses. The experimental objective was to evaluate the effects of two novel *Bacillus*-based direct-fed microbial blends on nursery pig growth performance. A total of 480 weaned pigs (5.6 ± 0.2 kg BW; L337 X Camborough, PIC, Inc., Hendersonville, TN) were blocked by initial BW, and pens (n = 16 per treatment) were randomly assigned to 1 of 3 dietary treatments for a 35-d experiment. The diets were fed over 3 phases and included a basal control (**CON**) and the CON supplemented with 0.03% of either direct-fed microbial blend 1 (**DFM1**; 3 strains of *Bacillus amyloliquefaciens*) or **DFM2** (1 strain of *Bacillus subtilis* and 2 strains of *Bacillus amyloliquefaciens*). Pigs and feeders were weighed on d 0 and d 35 to calculate BW, ADG, ADFI, and feed efficiency. Data were analyzed as a randomized complete block design using PROC MIXED of SAS (9.4) with pen as the experimental unit. Treatment was a

fixed effect and block was a random effect. Over the 35-d experiment, ADG, ADFI, feed efficiency, and BW did not significantly differ ($P > 0.05$). Under the experimental conditions of this study, neither DFM product affected growth performance of nursery pigs compared to pigs fed a non-supplemented control diet.

Introduction

In commercial swine production, limitations in efficiency and profitably are often related to high mortality, morbidity, and poor growth performance during the post-weaning phase. Traditionally, antibiotics have been utilized to reduce morbidity and improve growth performance post-weaning; however, due to consumer demand, potential trade restrictions, and concern for antimicrobial resistance, United States pork production has moved towards limited antibiotic use in commercial practice. As a result, various nutritional interventions, such as direct-fed microbial blends (DFMs), organic acids, enzymes, phytobiotics, nucleotides, and essential oils are being investigated as non-antibiotic alternative dietary technologies. The critical objectives of using these products in livestock feed are to promote animal productivity and growth and alleviate the negative social, environmental, and health stressors piglets face after weaning.

Direct-fed microbials, previously referred to as probiotics, contain live, viable microorganisms (FDA, 2015). Recently, direct-fed microbials have gained popularity in both the human and animal nutrition sectors due to their ability to exert beneficial health effects. Improvements in growth performance and feed efficiency have been observed with *Bacillus*-based DFM supplementation in nursery pig (Lee et al., 2014, Hu et al., 2014, Wang et al., 2017) and growing pig diets (Jørgensen et al., 2016). In addition to growth

performance benefits, *Bacillus*-based DFMs have been shown to have a favorable impact on intestinal health and function in the weaned pig (Cai et al., 2015). Though several mechanisms have been proposed by which DFMs exert their effects, the exact mode of action remains unknown. The differences in these mechanisms can be attributed to variation in DFM composition, dietary inclusion rate and composition, health status, production stage, and environment, resulting in a lack of consistent outcomes. Therefore, the objective of this study was to evaluate the effects of two novel *Bacillus*-based direct-fed microbial blends on nursery pig growth performance in a commercial-like environment.

Materials and methods

All experimental procedures adhered to guidelines for the ethical and humane use of animals for research and were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC #18-147).

Animals, housing, and experimental design

A total of 480 weanling pigs (5.6 ± 0.2 kg BW; L337 X Camborough, PIC, Inc., Hendersonville, TN) were purchased and transported to the Iowa State University Swine Nutrition Farm (Ames, IA). Upon arrival, pigs were individually weighed, ear-tagged, and vaccinated for K88+ *Escherichia coli* via a water-delivered vaccine (Arko Laboratories, Jewell, IA). Pigs were blocked by initial weight and pens were randomly assigned to 1 of 3 dietary treatments. There were 16 blocks, 10 pigs per pen, and 16 pens per treatment. Sexes were not separated, but there were the same number of barrows and gilts per pen across all treatments within each block.

Diets and feeding

Pens (1.2 m × 2.4 m) were equipped with a four-space dry self-feeder and 2 nipple waterers to provide *ad libitum* access to feed and water. Pigs were fed experimental diets in 3 phases over 35 d. Phase 1 was offered for 7 d and phases 2 and 3 were each offered for 14 d. Diets were formulated to meet or exceed NRC (2012) nutrient recommendations. Dietary treatments consisted of a basal control diet (**CON**), CON supplemented with 3 strains of *Bacillus amyloliquefaciens* (**DFM1**; Danisco Animal Nutrition, Marlborough, UK) and CON supplemented with 1 strain of *Bacillus subtilis* and 2 strains of *Bacillus amyloliquefaciens* (**DFM2**; Danisco Animal Nutrition, Marlborough, UK). The DFM products were supplemented at 0.03% of the basal control diet at the expense of corn to achieve 7.5×10^5 cfu DFM/g of complete feed and 1.5×10^5 cfu DFM/g of complete feed for DFM1 and DFM2, respectively (Table 2.1).

Sample collection

Pig BW and feed intake were measured on d 0 and 35 of the experiment to calculate ADG, ADFI, and G:F for the overall growth period. Multiple diet subsamples were collected as each was unloaded from the mixer. Samples were homogenized, subsampled, and stored at -20º C until later analysis.

Analytical methods

Diets were ground to 1 mm particle size with a Wiley Mill (Variable Speed Digital ED-5 Wiley Mill; Thomas Scientific, Swedesboro, NJ) and analyzed in duplicate for DM (method 930.15; AOAC, 2007) and acid-hydrolyzed ether extract (**aEE**; method 2003.06; AOAC, 2007). Diets were analyzed in duplicate for nitrogen (**N**; method 990.03; AOAC, 2007; TruMac; LECO Corp., St. Joseph, MI). An EDTA sample (9.56% N) was used as the

standard for calibration and was determined to contain 9.55 ± 0.01% N. Crude protein was calculated as N × 6.25. Gross energy was determined in duplicate using an isoperibolic bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL). Benzoic acid (6318 kcal GE/kg; Parr Instrument Co.) was used as the standard for calibration and was determined to contain 6319 ± 0.8 kcal GE/kg.

Statistical analysis

Data were analyzed in a randomized complete block design using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The UNIVARIATE procedure was used to check normality and equal variance of residuals. Pen was the experimental unit, treatment was a fixed effect, and block was a random effect. Differences among treatments were determined using ANOVA and means were separated using the PDIFF option of the least square means statement. Differences were considered significant if P was ≤ 0.05 and a tendency if $0.05 < P \leq 0.10$.

Results and discussion

By design, initial pig BW did not differ among treatments ($P > 0.10$; Table 2.2). Pigs fed the control diet tended to have a higher final BW compared to the other two treatments (d 35; $P = 0.090$). Average daily gain, ADFI, and feed efficiency were unaffected by DFM supplementation over the 35-d experimental period ($P > 0.10$). Several direct-fed microbial products have been researched, with variable results on growth performance in pigs. The absence of performance responses are in agreement with other studies that supplemented a DFM product in weaned pig diets (Keegan et al., 2004; LeJeune et al., 2006; Walsh et al., 2007; Kunavue and Lien, 2012).

Multiple factors possibly contribute to this lack of response to supplementation of either DFM product. First, herd health status could impact the effectiveness of DFM products. It is hypothesized that the production response to DFM supplementation would be greater in animals with a poor health status (McEwen and Fedorka-Cray, 2002). In the present study, there was no evidence of disease or diarrhea complications throughout the 35 d period.

Furthermore, DFM products contain live microorganisms whose efficacy is dependent upon longevity and proper storage conditions. The products used in this experiment were utilized and stored according to manufacturer recommendations.

Pigs experience enteric dysbiosis following weaning, which includes large fluctuations in the microbial environment. Growth promotion of beneficial microbes, such as *Lactobacilli* and *Bifidobacteria*, and competitive exclusion of harmful bacteria are proposed mechanisms by which DFMs provide a benefit to the pig (Hu et al., 2014, Lan et al., 2016, Tsukahara et al., 2013). Though environmental factors and diet composition greatly influence the gut microbiota (Mann et al., 2014, Frese et al., 2015), it has also been suggested that host genetics play a role in gut microbiome development and composition (Goodrich et al., 2014, Kubasova et al., 2018). This may partially explain the variation in the growth responses with DFM supplementation observed across studies.

Additionally, variability in diet composition could alter DFM efficacy. Minimal literature exists on the interactions of feed ingredients and DFM supplementation in pigs, specifically zinc. Addition of zinc oxide at high levels (2,000 – 4,000 mg/kg) to nursery diets is a common practice in U.S. pork production to improve growth performance and reduce the incidence of diarrhea following weaning (Hill et al., 2000, Case and Carlson,

2002). In the present study, all phase 1 and 2 diets contained pharmacological levels of zinc oxide across all treatments. There is a paucity of data regarding the interaction between zinc and DFM products; however, alterations in fecal and intestinal microbiota of the pig due to zinc oxide and DFM supplementation, independent of one another, have been widely reported. Researchers have observed reductions in *Lactobacillus*, which are considered beneficial microorganisms in the gastrointestinal tract, in weaned pigs due to zinc oxide supplementation at pharmacological levels (Højberg et al., 2005, Vahjen et al., 2011, Starke et al., 2014). Supplementation of a *Bacillus*-based product to weaned pigs has been shown to increase *Lactobacillus* (Payling et al., 2017, Hu et al., 2013). These competing effects of zinc and DFM on the microbial population may have altered the efficacy of the DFMs or competitively inhibited the DFMs ability to elicit a phenotypic response in this study.

In conclusion, under the conditions of this study, addition of either *Bacillus*-based DFM product did not alter body weight or growth performance of nursery pigs across the 35-d period compared to pigs fed the basal control diet. Research evaluating the interactions of dietary feed ingredients and feed additives, as well as the interactions of multiple feed additives, is warranted.

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Table 2.1. Ingredient composition of experimental diets (as-fed basis) for phases 1 – 3[1]

Item	Phase 1	Phase 2	Phase 3
Ingredient, %			
Corn	32.24	53.84	63.87
Soybean meal	15.00	20.00	29.95
Whey permeate	15.00	5.00	--
Oat groats	15.00	5.00	--
HP300	11.58	7.24	--
Blood plasma	4.50	2.00	--
Soybean oil	3.00	3.00	3.00
Limestone	0.95	1.00	0.86
Monocalcium phosphate	0.85	0.97	0.86
Salt	0.23	0.53	0.30
Vitamin premix[2]	0.30	0.30	0.30
Trace mineral premix[3]	0.20	0.20	0.20
L-Lysine HCL	0.45	0.46	0.41
DL-Methionine	0.20	0.16	0.13
L-Threonine	0.12	0.12	0.12
Zinc oxide, 72%[4]	0.38	0.18	--

Dietary treatments delivered in mash form with each DFM (Danisco Animal Nutrition, Wilmington, DE) included in the diet at 0.03% at the expense of corn

[1]Phase 1 = d 0 – 7; Phase 2 = d 7 – 21; Phase 3 = d 21 – 35

[2]Provided 6,614 IU vitamin A, 827 IU vitamin D, 26 IU vitamin E, 2.6 mg vitamin K, 29.8 mg niacin, 16.5 mg pantothenic acid, 5.0 mg riboflavin, and 0.023 mg vitamin B12 per kg of diet.

[3]Provided 165 mg Zn (zinc sulfate), 165 mg Fe (iron sulfate), 39 mg Mn (manganese sulfate), 17 mg Cu (copper sulfate), 0.3 mg I (calcium iodate), and 0.3 mg Se (sodium selenite) per kg of diet.

[4]Provided 3000 ppm zinc in Phase 1 and 1500 ppm in Phase 2

Table 2.2. Formulated and analyzed energy and nutrient composition (as-fed basis)

Item	Phase 1	Phase 2	Phase 3
Formulated composition			
ME, Mcal/kg	3.49	3.44	3.41
SID amino acid, %			
Lys	1.50	1.35	1.23
Met	0.47	0.44	0.41
Total sulfur AA	0.82	0.74	0.68
Thr	0.88	0.79	0.73
Trp	0.27	0.23	0.21
Ca, %	0.85	0.80	0.70
STTD P, %	0.46	0.41	0.34
Analyzed composition			
DM, %	89.90	88.48	87.69
GE, kcal/kg	4389	4389	4432
CP, %	24.97	22.49	21.68
aEE[1], %	5.81	6.18	6.29

[1]Acid-hydrolyzed ether extract (**aEE**)

Table 2.3. Effects of dietary treatment on nursery pig BW, growth rate, feed intake, and efficiency[1]

Item	Treatment			SEM	*P*-value
	Control	DFM1[2]	DFM2[3]		
d 0 BW, kg	5.65	5.63	5.64	0.28	0.421
d 35 BW, kg	17.19	16.67	16.73	0.50	0.090
ADG, kg	0.32	0.31	0.32	0.01	0.428
ADFI, kg	0.49	0.48	0.48	0.01	0.461
G:F	0.66	0.66	0.66	0.005	0.618

[1]n=16 pens per treatment (main effect)
[2]DFM1 (Danisco Animal Nutrition, Wilmington, DE) was included at 0.03% to achieve a final dose of $7.5x10^5$ cfu/g of feed
[3]DFM2 (Danisco Animal Nutrition, Wilmington, DE) was included at 0.03% to achieve a final dose of $1.5x10^5$ cfu/g of feed

CHAPTER III

EFFECTS OF DIRECT-FED MICROBIAL BLENDS ON WEANED PIGS CHALLENGED WITH F18 ENTEROTOXIGENIC *ESCHERICHIA COLI*

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Abstract

The objective of this experiment was to investigate the impact of an F18 enterotoxigenic *Escherichia coli* (**ETEC**) challenge on growth performance, intestinal function, and immune response of piglets, as well as to evaluate potential protective effects of direct-fed microbial blends. Seventy-two weaned piglets (6.4 ± 0.6 kg BW; ~21 d of age) were assigned to one of four treatments: 1) **NC**: Non-challenged (n=10), 2) **PC**: F18 ETEC challenged (n=10), 3) PC + **DFM1** (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g) or 4) PC + **DFM2** (n=8; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis*; 1.5×10^5 cfu/g). Pigs were housed two pigs per pen to record BW and feed intake on d 0, 7, and 17. Pigs were either sham-infected with 6 mL phosphate-buffered saline or orally inoculated with 6 mL hemolytic F18 ETEC (~1.9×10^9 CFU/mL) on d 7 (0 d post-inoculation, dpi). All ETEC challenged pigs were confirmed to be

genetically susceptible to F18 ETEC. Pigs had *ad libitum* access to feed and water throughout the 17-d trial. Fecal scores were visually ranked and rectal temperatures were recorded daily. To evaluate *E. coli* shedding, fecal swabs were collected on dpi 0, 1, 2, 3, 5, 7, and 10. Blood samples were collected on dpi 0, 1, 2, 4, 7, and 10. Ileal tissues were collected at necropsy. All challenged treatments had lower final BW, decreased ADG, and ADFI during the 10-d challenge period ($P < 0.01$). The DFM2 treatment increased *E. coli* shedding on dpi 2 ($P < 0.05$) and decreased shedding on dpi 7 ($P < 0.05$) compared with PC. Rectal temperature was decreased across all challenged treatments ($P < 0.01$). Ileal mRNA abundance of *occludin* (***OCLN***) and *zonula occludens*-1 (***ZO-1***) decreased in PC and DFM1 compared with NC ($P < 0.05$). Pigs fed DFM2 had intermediate ileal mRNA abundance of *OCLN* and increased *ZO-1* mRNA compared with pigs on PC ($P < 0.05$). Interleukin 8 (**IL-8**) increased in the plasma of PC and DFM2 on dpi 2 compared with NC ($P < 0.05$). Mucosal IL-8 increased in PC compared with NC ($P < 0.05$). All challenged treatments tended to have elevated *tumor necrosis factor-α* (***TNFα***) mRNA abundance compared with NC ($P < 0.10$). Challenged pigs had reduced secretory immunoglobulin A and villus height compared with non-challenged pigs ($P < 0.05$). Overall, DFM1 did not appear to attenuate the impacts of ETEC; however, DFM2 may provide some protection to nursery pigs challenged with ETEC by improving intestinal barrier integrity.

Introduction

There is a growing interest in reducing or eliminating antibiotic use in livestock production due to growing regulatory constraints, concerns about antimicrobial resistance, and consumer pressure. Antibiotic removal from weaned pig diets has substantial

consequences compared to other production stages due to social and environmental stressors adversely affecting gastrointestinal function and the immune system, leading to increased incidence of disease, including diarrhea (Hao et al., 2014). Enterotoxigenic *Escherichia coli* (**ETEC**) infections are a major cause of post-weaning diarrhea (**PWD**) in nursery piglets worldwide. ETEC attach to the intestinal epithelium via fimbria, commonly F4 or F18. This leads to subsequent pathogen proliferation and secretion of enterotoxins, resulting in secretory diarrhea in nursery pigs. A decrease in the rate and efficiency of body weight gain combined with increased death loss following an ETEC infection results in considerable economic losses. Vaccines and antibiotics have been used to prevent and control ETEC infections in nursery barns for many years; however, alternative strategies are needed. Utilization of in-feed direct-fed microbials (**DFM**) are being developed as a means of preventing and mitigating PWD. Direct-fed microbials are defined as products that "contain live naturally occurring microorganisms" (FDA, 2015). These products typically consist of single or multiple bacterial strains and often include *Bacillus* species. In pigs, DFMs have been shown to enhance growth performance (Alexopoulus et al., 2004), improve intestinal function (Scharek et al., 2007; Yang et al., 2016), and positively modulate immune responses and microbial populations (Lan et al., 2016). Despite these observed beneficial effects, results derived from swine studies are inconsistent. Currently, there is limited information regarding the impact of DFM supplementation in ETEC challenged pigs. Therefore, the objective of this study was to evaluate the impact of an ETEC challenge on growth performance, intestinal barrier function, and immune response of weaned pigs while concurrently evaluating the potential protective effects of DFMs.

Materials and methods

All experimental procedures adhered to guidelines for the ethical and humane use of animals for research and were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC #8-17-8576-S).

Animals, Diets, and Experimental Design

A total of 72 weaned pigs (6.4 ± 0.6 kg BW; ~21 d of age; L337 × Camborough, PIC, Hendersonville, TN) were individually weighed and allotted to pens such that there were one barrow and one gilt per pen. Pens were randomly assigned to one of four dietary treatments: a non-challenged control (**NC**; *n* = 10), an ETEC challenged control (**PC**; *n* = 10), PC + DFM1 (3 strains of *Bacillus amyloliquefaciens;* **DFM1**; *n* = 8), and PC + DFM2 (2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtillis;* **DFM2**; *n* = 8). The DFM1 and DFM2 were included at 0.03% of the diet to achieve a final dose of 7.5×10^5 cfu/g of feed and 1.5×10^5 cfu/g of feed, respectively (Danisco Animal Nutrition, Marlborough, UK). The control diet was presented in mash form and was primarily based on corn and soybean meal with 9.0% whey powder and 8.5% enzymatically treated soybean meal. The DFM1 and DFM2 were added to replace corn in the control diet. The diets were formulated to meet or exceed NRC (2012) nutrient recommendations of weaned pigs and did not contain antibiotics or pharmaceutical levels of copper or zinc (Table 3.1).

This trial was conducted in a biosecurity level 2 facility at Iowa State University. Pigs were housed in 1 of 2 separate rooms based on their challenge status: 1 smaller room with 20 non-challenged control pigs (10 pens) and a second larger room with 52 challenged pigs (26 pens). Room temperature, humidity, and lighting were carefully monitored throughout the trial to ensure equivalency of the room conditions. All pens were of equal

space and flooring material. A four-space polyethylene dry feeder and one nipple drinker were used to provide *ad libitum* access to feed and water throughout the 17-day experiment. To avoid ETEC contamination in the non-challenged room, strict biosecurity protocols were followed. Pigs' genetic susceptibility to F18 ETEC was tested via Sanger DNA sequencing of the α (1,2) fucosyltransferase-1 (**FUT1**) gene (Frydendahl et al. 2003). The F18 ETEC isolate used in this study was obtained from the culture collection at the Iowa State University Veterinary Diagnostic Laboratory (**ISU VDL;** Ames, IA). After 7 days of acclimation (0 day post-inoculation: **dpi**), pigs were orally gavaged with 6 mL of freshly grown F18 ETEC inoculum (approximately 1.9×10^9 cfu/mL) or sham-infected with PBS. The sows and piglets used in this experiment had not been previously vaccinated against *E. coli.*

Inoculum Preparation

A fluoroquinolone-resistant hemolytic *E. coli* isolate with an enrofloxacin minimal inhibitory concentration (**MIC**) > 2 μg/mL was used to prepare the bacterial inoculum at the ISU VDL. A resistant isolate was selected in order to improve the specificity of recovery via selective media post-inoculation. Briefly, a frozen culture stock of the isolate was grown (~ 16 h at 37°C) on blood agar (TSA with 5% sheep blood) and was used to inoculate two bottles, each containing 50 mL of sterile TSB. The bottles were incubated overnight at 37°C with shaking. The broth cultures were then transferred to two new sterile bottles each with 450 mL fresh TSB and incubated for an additional 5 h at 37°C with shaking. The bacterial culture was centrifuged and the pellet was suspended in 900 mL of sterile PBS. The OD_{600} of the culture in PBS was measured to be 4.25 using a

spectrophotometer. A viable CFU count was performed and the inoculum was determined to have approximately 1.9×10^9 cfu/mL.

Sample Collection

Pigs were individually weighed on dpi -7, 0, and 10. Feed disappearance was also recorded to calculate ADG, ADFI, and G:F for each phase. On dpi 0, 1, 2, 3, 5, 7, and 10, fecal swabs were collected from one barrow per pen to evaluate F18 ETEC shedding. Rectal temperatures were obtained daily from every pig via rapid-response digital electric thermometers (ReliOn, MABIS Healthcare Inc., Waukegan, IL). Pen fecal score was visually assessed daily by two unbiased personnel using the following scale: 0 = solid, 1 = semi-solid, 2 = semi-liquid, and 3 = liquid. Fecal score ≥ 2 was considered diarrhea. On dpi 0 (immediately before inoculation), 1, 2, 4, 7, and 10, blood samples were collected from 1 barrow per pen via jugular venipuncture into a 10 mL vacutainer tube (Bection Dickinson, Franklin Lakes, NJ). Plasma was separated by centrifugation ($2000 \times g$ for 10 min at 4°C), divided into three aliquots, and stored at -80°C for later analysis.

On dpi 10, one pig from each pen was euthanized by captive bolt stunning followed by exsanguination. Post-euthanasia, the abdomen was opened and a 30 cm segment of ileum anterior to the ileocecal junction was removed, drained of digesta, and rinsed with ice-cold PBS. Three 2 cm segments of the terminal ileum were fixed in 10% neutral buffered formalin. The remaining ileal segments were snap frozen in liquid N and stored at -80°C for later analysis. A second 20 cm segment of ileum was removed, snap frozen in liquid N, and stored at -80°C for harvesting of mucosal scrapings.

Chemical analysis

Diets were ground to 1 mm particle size with a Wiley Mill (Variable Speed Digital ED-5 Wiley Mill; Thomas Scientific, Swedesboro, NJ) and analyzed in duplicate for DM [method 930.15 (AOAC, 2007)], acid-hydrolyzed ether extract [**aEE**; method 2003.06; (AOAC, 2007)], and N [method 990.03 (AOAC, 2007); TruMac; LECO Corp., St. Joseph, MI]. An EDTA sample (9.56% N) was used as the standard for calibration and was determined to contain 9.55 ± 0.01% N. Crude protein was calculated as N x 6.25. Gross energy was determined in duplicate using an isoperibolic bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL). Benzoic acid (6,318 kcal GE/kg) was used as the standard for calibration and was determined to contain 6,319 ± 0.8 kcal GE/kg.

Fecal F18 E. coli shedding

For isolation of *E. coli* from fecal samples, fecal swabs were plated onto selective TSA agar with 5% bovine blood, 16 µg ciprofloxacin/mL, and 50 µg cycloheximide/mL and a MacConkey agar plate. Plates were incubated at 37°C for 24 h to determine hemolytic *E. coli* shedding using a semi-quantification method. ETEC shedding was measured using a 5-point scale ranging from 0 to 4 according to the number of streaked sections that had viable *E. coli*, where 0 corresponded to no growth, 1 corresponded to growth in the primary streak, 2 corresponded to compatible growth extending into the secondary streak, 3 corresponded to growth into the tertiary streak and 4 corresponded to growth of pathogenic *E. coli* into the quaternary section of the agar plate. Identification of *E. coli* isolates was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (**MALDI-TOF MS**) at the **ISU VDL**.

Ileal E. coli attachment

Formalin-fixed ileum tissues were processed and embedded in paraffin wax at the ISU VDL. Three transverse sections (5 μm) were cut from the ileum, stained with hematoxylin and eosin, and mounted on glass slides. Visualization of *E. coli* attachment to epithelial cells was accomplished using an OLYMPUS BX 53/54 microscope at 40X power. Each section was scored as either 0 if there was no attachment or 1 if there was attachment of *E. coli* on ≥ 5 villi in each section. The *E. coli* attachment frequency (%) was calculated by summing up the score of all 3 sections on each glass slide and then dividing by 3.

Intestinal Morphology

Images of ileal sections were taken using a DP80 Olympus Camera mounted on an OLYMPUS BX 53/43 microscope with a motorized stage. Whole ileal sections were scanned at 4X power, then regions containing well-orientated villus and crypt pairs were selected. These regions were rescanned at 20X power. The 20X regions were stitched together to form a composite image. Ten well-orientated villus and crypt pairs per ileal section per slide were selected and analyzed using OLYMPUS cellSens Dimension 1.16 software.

RNA isolation and quantitative PCR

Approximately 30 mg of ileal tissue was homogenized using the Qiagen Tissuelyser II (Germantown, MD, USA), then total RNA was isolated using the Qiagen Rneasy Mini Kit according to the manufacturer’s recommendations. The concentration of RNA was quantified using a spectrophotometer (ND-100; NanoDrop Technologies, Inc., Rockland, DE). All samples had 260:280 nm ratios above 1.8. The QuantiTect Reverse Transcription

Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's instructions to synthesize complementary DNA (**cDNA**) from 0.8 µg of the isolated RNA. All cDNA samples were diluted 10-fold with nuclease-free water.

Real-time quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). The gene-specific primers, shown in Table 3.2, were diluted to 10 µM with nuclease-free water. Genes were chosen to evaluate small intestinal inflammatory status and paracellular permeability. *Ribosomal protein – L19 (**RPL19**)* was included as an endogenous reference gene. Each reaction included 10 µL of SYBR Green Supermix, 1 µL of each forward and reverse primer, 5 µL of nuclease-free water, and 3 µL of cDNA, for a total of 20 µL reaction volume. Each 96-well plate contained a no-reverse transcriptase negative control and a pooled cDNA reference sample. Samples were assayed in triplicate. Fluorescence of SYBR Green was quantified with a Real-time PCR Detection System (iQ5; Bio-Rad Laboratories Inc.). Cycling conditions were as follows: 5-min initial denaturation at 95°C followed by 40 PCR cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s) and a dissociation curve to verify the amplification of a single PCR product. Optical detection was performed at 55°C. Analyses of amplification plots were performed with an Optical System Software version 2.0 (iQ5; Bio-Rad Laboratories Inc.) and cycle threshold (**Ct**) values for each reaction was obtained. The mRNA abundance for each sample was normalized to *RPL19* and the pooled sample, and fold change was calculated using the $2^{-\Delta\Delta CT}$method (Livak and Schmittgen, 2001).

Mucosal disaccharidase activity, secretory IgA, and cytokines

Ileal mucosal scrapings (0.5 g) were added to 4.5 mL of PBS containing a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and triton (0.1%). The resulting solution

was homogenized and centrifuged at 10,000 × g for 15 min at 4°C and the supernatant was stored in aliquots. Total protein concentration of hydrolyzed mucosa was quantified using a Pierce bicinchoninic acid (BCA) Protein Assay kit (Thermo Scientific, Woltham, MA). Disaccharidase activity was determined as previously described by Dahlqvist (1964) using lactose, maltose, and sucrose as substrates. Enzyme activity was expressed as μmol hydrolyzed substrate × min-1×g tissue protein-1. Concentration of secretory IgA was obtained using a porcine-specific ELISA kit following manufacturer's instructions (Bethyl Laboratories, Inc., Montegomery, TX). Homogenized mucosa and plasma subsamples were analyzed for cytokines using a Multiplex Immunoassay (Eve Technologies, Calgary, AB, Canada).

Statistical Analysis

Data were analyzed as a complete randomized design. Pen was the experimental unit and treatment was a fixed effect. Growth performance data and plasma cytokines were analyzed as repeated measures with using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with a spatial power covariance structure. Baseline (d 0) measurements of plasma cytokines were used as a covariate. ETEC shedding scores were analyzed using PROC MIXED as repeated measures using a spatial power covariance structure with pig as a random effect. Averaged fecal scores and rectal temperatures were analyzed using PROC MIXED as repeated measures with a first order autoregressive covariance structure. Mucosal cytokines, morphology, secretory IgA, disaccharidase, and mRNA abundance data were analyzed in PROC MIXED. ETEC attachment data were analyzed in PROC GLIMMIX assuming a binomial distribution. Least square means of treatments were reported. Pre-planned contrasts were performed using the ESTIMATE statement to

evaluate the effects of the ETEC challenge (NC vs. PC) and dietary treatment (PC vs. DFM1, DFM2). For each variable, normal distribution of residuals was tested using PROC UNIVARIATE. Differences were considered significant if P was ≤ 0.05 and a tendency if P was > 0.05 and ≤ 0.10.

Results

Growth Performance

Among challenged pigs, 23% mortality was observed, with no differences among treatments (data not shown). During the 7-d adaptation period, there were no significant differences in ADG or ADFI among the four treatments ($P \geq 0.466$; Table 3.3). Pigs receiving either DFM product had increased G:F during the 7-d adaptation period ($P = 0.005$). Pig BW did not differ on dpi -7 or dpi 0 ($P \geq 0.785$). As expected, the pigs receiving the PC, DFM1, and DFM2 treatments had lower final BW ($P <.0001$) and lower ADG ($P <.0001$) during the 10-d challenge period compared with the NC. The NC pigs also had a higher feed intake than pigs on the other three treatments ($P <.0001$). The G:F during post-challenge period was not different among all treatments ($P = 0.203$).

ETEC shedding, fecal score, and rectal temperature

Overall fecal score for the 10-d challenge period for NC was lower compared with the challenged treatments ($P <.0001$, Fig. 3.1). There were no differences in fecal scores among challenged treatments over the 10-d challenge period ($P > 0.10$). Pigs on NC had no ETEC shedding throughout the experiment (Fig. 3.2). The DFM2 treatment increased ETEC shedding score (SS) on dpi 2 ($P = 0.044$) and decreased SS on dpi 7 ($P = 0.003$) compared with PC. There were no differences in SS between PC and DFM1 ($P > 0.10$). On dpi 10, there were no differences in SS among all treatments ($P > 0.10$). For the overall

10-d challenge period, there was a significant reduction in rectal temperature across all challenged pigs compared with NC (P <.0001; Fig. 3.3). There were no differences in rectal temperature among challenged pigs (P > 0.10).

Mucosal and plasma cytokines

Analysis of ileal mucosa cytokines revealed no differences among treatments for interferon-γ (IFNγ), interleukin-1rα (IL-1rα), IL-1α, IL-1ß, IL-2, IL-4, IL-6, IL-10, IL-12, or IL-18 (P > 0.10; Table 3.4). Mucosal IL-8 was elevated in PC compared with NC (P = 0.011); however, there were no differences among challenged treatments. Similarly, there were no differences in plasma cytokines among treatments (data not shown) with the exception of IL-8. While there were no differences among challenged treatments, IL-8 tended to be elevated in PC vs. NC (P = 0.069; Fig. 3.4) on dpi 1 and significantly elevated on dpi 2 (P = 0.031). There were no differences in plasma IL-8 on dpi 4, 7, or 10 among all treatments (P > 0.10).

Mucosal Secretory IgA and disaccharidases

Secretory IgA was reduced in ileal mucosa of all challenged treatments compared with the NC (P = 0.011; Table 3.4). Lactase activity did not differ among treatments (P = 0.511). Sucrase activity increased in PC and DFM2 compared with NC (P = 0.003). Pigs fed DFM1 had reduced sucrase activity compared with PC. The PC and DFM2 also had increase maltase activity compared with NC (P = 0.004); there was no difference between PC and DFM1 (P > 0.10).

Ileal gene transcription

There were no differences among treatments in *toll-like receptor 4 (**TLR4**)* mRNA abundance or the tight junction proteins *claudin-1 (**CLDN1**)* or *CLDN3* (P > 0.10; Table

3.5). A trend for greater *TNFα* mRNA abundance was observed across all challenged treatments compared with NC (*P* = 0.087). *Occludin (**OCLN**)* mRNA abundance was significantly reduced in PC and DFM1 compared with NC (*P* = 0.045). Pigs receiving DFM2 had OCLN mRNA abundance intermediate of NC and PC. Lower *zonula-occludens-1 (**ZO-1**)* mRNA abundance was observed in PC and DFM1 compared with NC and DFM2 (*P* = 0.001). *Cluster of differentiation (**CD14**)* was elevated in DFM2 compared with NC and PC (*P* = 0.019). Pigs receiving DFM1 had *CD14* mRNA abundance intermediate of PC and DFM2.

Morphology and E. coli attachment to epithelial cells

Villus height in the ileum was reduced across all challenged treatments compared with NC (*P* <.0001; Table 3.6). Ileal crypts tended to be shallower in pigs fed DFM1 (*P* = 0.074). Villus height:crypt depth was reduced in DFM1 vs NC (*P* = 0.046). There were no differences in villus height:crypt depth among NC, PC, and DFM2. *E. coli* attachment to the epithelial cells in the ileum did not differ across all treatments (*P* = 0.101).

Discussion

Rate and efficiency of body weight gain in newly weaned pigs is closely associated with intestinal health and function. Enterotoxigenic *E. coli* infections after weaning reduce feed intake and negatively impact gut health and intestinal function in the pig. Limited research exists evaluating the impact of *Bacillus*-based DFM products in ETEC challenged pigs. This study evaluated the effects of two novel *Bacillus*-based DFMs on growth performance, intestinal function, and immune response in weaned pigs challenged with F18 ETEC. Following inoculation with ETEC, all challenged pigs had increased fecal

scores and shedding of the F18 ETEC strain compared with NC, confirming the challenge model was successful. Final BW, and post-challenge ADG and ADFI were reduced in ETEC challenged pigs, as expected, though this did not result in a difference in feed efficiency compared with the NC. The lack of difference in feed efficiency was likely due to the significant decreases in both ADG and ADFI. The lack of difference in feed efficiency and reductions in pig growth performance due to an ETEC challenge are in agreement with previous research (Pan et al., 2017; Li et al., 2019).

While there were no differences in F18 ETEC shedding of challenged pigs across the 10-d challenge period, pigs supplemented with DFM2 had increased ETEC shedding on dpi 2, followed by a decrease in shedding on dpi 7 compared with PC. Despite the fact that the exact modes of action of *Bacillus*-based DFMs are unknown, the decrease in *E. coli* shedding on dpi 7 is possibly due to the ability of the DFM to successfully compete for carbon and energy sources and nutrient absorption sites, thereby suppressing growth of pathogenic bacteria (Cho et al., 2011). Members of the *Bacillus* genus have also been widely reported to produce bacteriocins, which are proteins with antimicrobial properties that can inhibit activity of pathogenic bacteria (Abriouel et al., 2011; Larsen et al., 2014). The ETEC challenge also reduced rectal temperatures across all challenged treatments compared with NC. This lack of febrile response is consistent with the absence of systemic cytokine and chemokines production, which is known to initiate a febrile response (Evans et al., 2015). To our knowledge, there is no published literature reporting a decrease in rectal temperature due to ETEC challenge; however, reductions in rectal temperature have been reported due to decreased feed intake (Pearce et al., 2013), which in turn presumably reduces the heat increment of feeding.

In addition to clinical symptoms, ETEC is known to increase intestinal permeability through alterations in tight junction proteins. The reductions of *OCLN* and *ZO-1* mRNA abundance due to ETEC infection observed in this study are in agreement with other reports (Ewaschuk et al., 2011; Gao et al., 2013; Li et al, 2019). The greater mRNA abundance of *OCLN* and *ZO-1* in ETEC challenged pigs fed DFM2 compared with PC indicates an improvement in intestinal barrier integrity, potentially resulting from the initial increases and subsequent decreases in *E. coli* shedding. The ability of *Bacillus*-based products to improve tight junction protein expression has been previously described (Gu et al., 2014; Rhayat et al, 2019). Intestinal barrier preservation is partly dependent upon tight junction proteins, as they have an important role in preventing paracellular transport of harmful bacteria and toxins across the intestine (Mukiza et al., 2013). Transport of luminal contents and pathogenic material into the peripheral circulation can increase when tight junctions are disrupted, thus activating an immune response and intestinal inflammation. Activation of intestinal inflammation was indicated by elevated levels of ileal *CD14*, a co-receptor of the TLR-4 complex, which recognizes the lipopolysaccharide component of gram-negative bacteria (Guo et al., 2013). The upregulation of *CD14* possibly explains the increase in the pro-inflammatory cytokine IL-8 observed in both the intestinal mucosa and plasma of PC compared with NC, which has been previously reported following an ETEC challenge (Li et al., 2019). However, pigs receiving either DFM product had intermediate IL-8 levels in the ileal mucosa, suggesting the ability of the DFMs to blunt this response. Roselli et al. (2007) reported increased levels of IL-8 accompanied by disruption in the tight junction complex following an ETEC infection, which agrees with these results. These data provide insight into the role of IL-8 during an ETEC challenge, as it was localized in the intestine;

however, there was no difference in systemic IL-8 at dpi 10. This localized pro-inflammatory response may be associated with the negative impacts on the intestinal barrier observed in PC. The lack of differences observed in other pro-inflammatory cytokines, such as IL-6 and TNFα, in both the blood and tissue are likely due to the fact that pigs were recovering from ETEC infection by necropsy day on dpi 10, which is supported by the decreases in fecal scores, ETEC shedding, and *E. coli* attachment to the intestinal epithelium.

To further evaluate intestinal immune response, sIgA was measured in the ileal mucosa. The mucosal immune system is critical in protecting the host from pathogens. Secretory IgA is the most abundant antibody found in the intestine, serving as the first line of barrier defense of the mucosal immune system in the event of an enteric infection (Mantis et al., 2011). In the current study, sIgA levels were reduced due to ETEC challenge, which is inconsistent with previous literature (Zhang et al., 2013). This is possibly explained by a mechanism used by sIgA known as immune exclusion, which inhibits the ability of pathogens and toxins to interact with the intestinal epithelium. Once bound, sIgA facilitates bacterial clearance by increasing peristaltic movement in the GIT (Mantis et al., 2011). Thus, the majority of sIgA produced may have been shed with the GIT contents. Additionally, sIgA can intercept incoming pathogens intracellularly as it is crossing the epithelial barrier, as well as neutralize pathogens that have successfully crossed the intestinal barrier into the lamina propria (Corthésy, 2013). Due to the observed impairment in intestinal barrier integrity, sIgA may be acting at these sites to neutralize pathogenic agents instead of at the mucosal surface. Additionally, it has been established that mucosal atrophy occurs with adverse morphological changes (Shaw et al., 2012). In the present

study, villus height was significantly reduced across all challenged treatments, which has been associated with weaning and ETEC challenges (Pluske et al., 1997; Yang et al., 2014). The observed villus atrophy may have resulted in less mucosal mass in the intestine and therefore less surface area for sIgA to bind to.

Intestinal function was also assessed by measuring disaccharidase activity in the ileum. Improvements in disaccharidase activity are indicative of improved digestive capacity. While no difference in lactase activity was observed, PC and DFM2 had increased sucrase and maltase activity compared with NC. It has been shown that DFM supplementation in pig diets increases amylase, sucrase, maltase, and Na^+/K^+-ATPase activities (Hu et al., 2018), which is reflective of improved intestinal function. There is little information regarding the impact of an ETEC challenge on disaccharidase activity; however, authors reported increases in maltase activity in early weaned-pigs, which was closely correlated with corresponding mRNA levels, suggesting a large impact of transcriptional regulation of maltase after weaning (Marion et al., 2005). Increases in intestinal disaccharidase activities may also signify indirect effects of reduced feed intake.

In conclusion, these results provide insight into how a severe ETEC infection impacts growth performance, intestinal function, and immune response in weaned pigs. Weaned pigs challenged with F18 ETEC had reduced BW, ADFI, and ADG, which resulted from colonization of pathogenic *E. coli* as supported by increases in shedding and fecal scores, and reduced body temperature. While no apparent beneficial effects of DFM1 supplementation were observed, DFM2 appeared to partially attenuate the ETEC challenge by decreasing *E. coli* shedding following inoculation, which resulted in improvements in intestinal barrier integrity and function on dpi 10. The ETEC challenge also resulted in

impaired intestinal barrier integrity, shown by lower mRNA abundance of the tight junction proteins *OCLN* and *ZO-1* and reduced mucosa sIgA, as well as activation of an immune response, as evidenced by increases in both localized and systemic *IL-8* production. Overall, inclusion of DFM2 in nursery pig diets may be a useful tool to help alleviate PWD induced by an ETEC. The somewhat promising results of the DFM2 product indicates a need to conduct studies on this product on a larger scale and under a more moderate health challenge.

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Table 3.1. Ingredient and nutrient composition of the experimental diets (as-fed basis, %)

Item	Control	Control+DFM1[1]	Control+DFM2[2]
Ingredient			
Corn	58.07	58.04	58.04
Soybean meal	15.00	15.00	15.00
Whey powder	9.00	9.00	9.00
HP300[3]	8.50	8.50	8.50
Fishmeal	4.00	4.00	4.00
Soybean oil	2.00	2.00	2.00
Limestone	1.27	1.27	1.27
Monocalcium phosphate	0.10	0.10	0.10
Salt	0.68	0.67	0.67
Vitamin premix[4]	0.20	0.20	0.20
Trace mineral premix[5]	0.20	0.20	0.20
L-Lysine HCl	0.52	0.52	0.52
DL-Methionine	0.19	0.19	0.19
L-Threonine	0.16	0.16	0.16
L-Valine	0.06	0.06	0.06
L-Tryptophan	0.03	0.03	0.03
Phytase[6]	0.02	0.02	0.02
DFM1	--	0.03	--
DFM2	--	--	0.03
Calculated nutrient levels[7]			
ME, kcal/kg	3,407	3,407	3,407
NE, kcal/kg	2,559	2,559	2,559
Crude protein	20.34	20.34	20.34
Ether extract	4.92	4.92	4.92
Total P	0.53	0.53	0.53
STTD P	0.43	0.43	0.43
Calcium	0.85	0.85	0.85
SID Lys	1.40	1.40	1.40
SID Met + Cys	0.77	0.77	0.77
SID Thr	0.83	0.83	0.83
SID Trp	0.24	0.24	0.24
Analyzed nutrient levels			
Dry matter	85.52	85.63	85.45
GE, kcal/kg	4,329	4,338	4,321
Crude protein	21.51	22.32	21.68
aEE[8]	5.69	5.56	5.82

[1]Direct-fed microbial 1 (DFM1) = Three strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g of feed, Danisco Animal Nutrition, Marlborough, UK.

[2]Direct-fed microbial 2 (DFM2) = Two strains of *Bacillus amyloliquefaciens* and one strain of *Bacillus subtilis;* 1.5×10^5 cfu/g of feed, Danisco Animal Nutrition, Marlborough, UK.

[3]Enzymatically-treated soybean meal; Hamlet Protein, Findlay, OH
[4]Provided per kg of diet: 7,656 IU vitamin A, 875 IU vitamin D, 63 IU vitamin E, 4 mg vitamin K, 70 mg niacin, 34 mg pantothenic acid, 14 mg riboflavin, and 0.06 mg vitamin B_{12}.
[5]Provided per kg of diet: 165 mg Zn (zinc sulfate), 165 mg Fe (iron sulfate), 39 mg Mn (manganese sulfate), 17 mg Cu (copper sulfate), 0.3 mg I (calcium iodate), and 0.3 mg Se (sodium selenite).
[6]2,000 FTU/kg of feed provided 0.109% available P; AxtraPhy, Danisco Animal Nutrition, Marlborough, UK.
[7]STTD = standardized total tract digestible; SID = standardized ileal digestible.
[8]aEE: acid-hydrolyzed ether extract.

Table 3.2. Primer sequences used for quantitative PCR

Gene[1]	Primer sequence	Product size, bp	GenBank accession
TNFα	F: CACCACGCTCTTCTGCCTAC	132	X57321
	R: ACGGGCTTATCTGAGGTTTGAGACG		
CLDN1	F: GATTTACTCCTACGCTGGTGAC	199	AJ318102
	R: CACAAAGATGGCTATTAGTCCC		
CLDN3	F: TTGCATCCGAGACCAGTCC	85	NM_001160075
	R: AGCTGGGGAGGGTGACA		
OCLN	F: AACTCCCGTCAGCAGATCC	95	NM_001163647
	R: ATCAGTGGAAGTTCCTGAACCA		
ZO-1	F: CTCTTGGCTTGCTATTCG	197	XM_003353439
	R: AGTCTTCCCTGCTCTTGC		
TLR4	F: CAGATAAGCGAGGCCGTCATT	113	AB232527
	R: TTGCAGCCCACAAAAAGCA		
CD14	F: CCTCAGACTCCGTAATGTG	180	AB267810
	R: CCGGGATTGTCAGATAGG		
RPL19	F: AACTCCCGTCAGCAGATCC	147	AF435591
	R: AGTACCCTTCCGCTTACCG		

[1]*TNFα = tumor necrosis factor alpha; CLDN1 = claudin-1; CLDN3 = claudin-3; OCLN = occludin; ZO-1 = zonula occludens-1; TLR4 = toll-like receptor 4; CD14 = cluster of differentiation 14; RPL19 = ribosomal protein-L19.*

Table 3.3. Effects of treatment on growth performance in weaned pigs challenged with F18 ETEC

Item	Treatment[1]				SEM	*P*-value
	NC	PC	DFM1	DFM2		
BW, kg						
dpi -7	6.59	6.59	6.34	6.17	0.17	0.785
dpi 0	6.88	6.83	6.73	6.80	0.17	0.990
dpi 10	10.78[a]	9.22[b]	8.55[b]	9.17[b]	0.17	<.0001
dpi -7 to 0						
ADG, kg	0.04	0.03	0.05	0.09	0.01	0.466
ADFI, kg	0.08	0.09	0.08	0.11	0.01	0.859
G:F[2]	0.45[b]	0.34[b]	0.67[ab]	0.79[a]	0.06	0.035
dpi 1 to 10						
ADG, kg	0.39[a]	0.19[b]	0.15[b]	0.22[b]	0.01	<.0001
ADFI, kg	0.49[a]	0.33[b]	0.19[b]	0.33[b]	0.01	<.0001
G:F	0.81	0.54	0.51	0.65	0.06	0.203

[a,b]Means with differing superscripts indicate a significant ($P < 0.05$) difference.

[1]NC = non-challenged control (n=10); PC = positive challenged control (n=9); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g of feed); DFM2 = PC + DFM2 (n=7; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5×10^5 cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK).

[2]Gain:feed ratio. Interpretation of G:F should be cautious because values less than -1.4 were removed from analysis (2 numbers pre-challenge from PC and DFM1). Additionally, 3 pigs in PC had G:F ranging from -0.47 to -0.07 and 1 pig from DFM1 had a G:F = -0.56. Five pigs with G:F > 1 from both DFM treatments during dpi -7 to 0 were included in the analysis.

Table 3.4. Effect of treatment on mucosa cytokines[2], disaccharidase activity[3], and secretory immunoglobulin A[4] in the ileum of weaned pigs challenged with F18 ETEC

Item[4]	Treatment[1]				SEM	*P*-value
	NC	PC	DFM1	DFM2		
IFNγ	7.04	7.96	5.07	7.47	1.57	0.521
IL-1rα	20.56	24.06	19.21	16.48	3.69	0.828
IL-1α	1.66	1.88	2.11	1.70	0.27	0.421
IL-1ß	8.30	9.59	12.08	7.55	1.59	0.397
IL-2	0.49	0.69	0.57	0.52	0.11	0.465
IL-4	0.79	0.48	0.58	0.48	0.17	0.508
IL-6	0.35	0.57	0.86	0.24	0.20	0.212
IL-8	240.20[b]	343.06[a]	288.07[ab]	283.42[ab]	29.13	0.011
IL-10	0.30	0.25	0.27	0.25	0.04	0.302
IL-12	3.06	3.81	3.14	4.35	0.44	0.214
IL-18	220.69	227.45	223.82	229.00	27.64	0.854
Lactase	0.07	0.11	0.03	0.13	0.05	0.511
Sucrase	1.81[c]	4.49[a]	2.56[b]	4.72[a]	0.58	0.003
Maltase	7.39[c]	14.16[ab]	9.61[bc]	20.91[a]	2.64	0.004
sIgA	2.50[a]	1.11[b]	1.37[b]	0.95[b]	0.33	0.011

[a,b,c]Means with differing superscripts indicate a significant ($P < 0.05$) difference.

[1]NC = non-challenged control (n=10); PC = positive challenged control (n=9); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g of feed); DFM2 = PC + DFM2 (n=7; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5×10^5 cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK).

[2]ng/g of mucosa.

[3]U/minute/g of protein.

[4]μg/mg of protein.

[5]*IFNγ = interferon gamma; IL = interleukin.*

Table 3.5. Effect of treatment on relative ileal gene mRNA abundance in weaned pigs challenged with F18 ETEC

Gene[2]	Treatment[1]				SEM	*P*-value
	NC	PC	DFM1	DFM2		
CLDN1	1.15	0.59	0.75	1.12	0.21	0.145
CLDN3	0.98	0.64	0.65	0.95	0.34	0.332
OCLN	1.15[a]	0.48[b]	0.54[b]	0.69[ab]	0.38	0.045
ZO-1	1.05[a]	0.56[b]	0.67[b]	1.05[a]	0.10	0.001
TNFα	1.01	1.84	1.56	2.30	0.37	0.087
CD14	1.09[b]	1.52[b]	1.89[ab]	2.60[a]	0.34	0.019
TLR4	0.88	0.59	0.68	0.95	0.14	0.145

[a,b]Means with differing superscripts indicate a significant ($P < 0.05$) difference.

[1]NC = non-challenged control (n=10); PC = positive challenged control (n=10); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5 $\times 10^5$ cfu/g of feed); DFM2 = PC + DFM2 (n=8; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5 $\times 10^5$ cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK).

[2]*TNFα: tumor necrosis factor alpha; CLDN1: claudin-1; CLDN3: claudin-3; OCLN: occludin; ZO-1: zonula occludens-1; TLR4 = toll-like receptor 4; CD14 = cluster of differentiation 14.*

Table 3.6. Effect of treatment on ileal morphology and *E. coli* attachment in weaned pigs challenged with F18 ETEC

Item	Treatment[1]				SEM	*P*-value
	NC	PC	DFM1	DFM2		
Villus height, µm	344.41[a]	253.82[b]	206.92[c]	256.05[b]	14.56	<.0001
Crypt depth, µm	199.43	177.95	160.44	172.83	10.53	0.074
VH:CD[2]	1.74[a]	1.48[ab]	1.29[b]	1.54[ab]	0.11	0.046
Attachment, %	0.00	14.80	33.33	0.00	5.72	0.102

[a,b,c]Means with differing superscripts indicate a significant ($P < 0.05$) difference.

[1]NC = non-challenged control (n=10); PC = positive challenged control (n=10); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g of feed); DFM2 = PC + DFM2 (n=8; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5×10^5 cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK).

[2]Villus height:crypt depth ratio

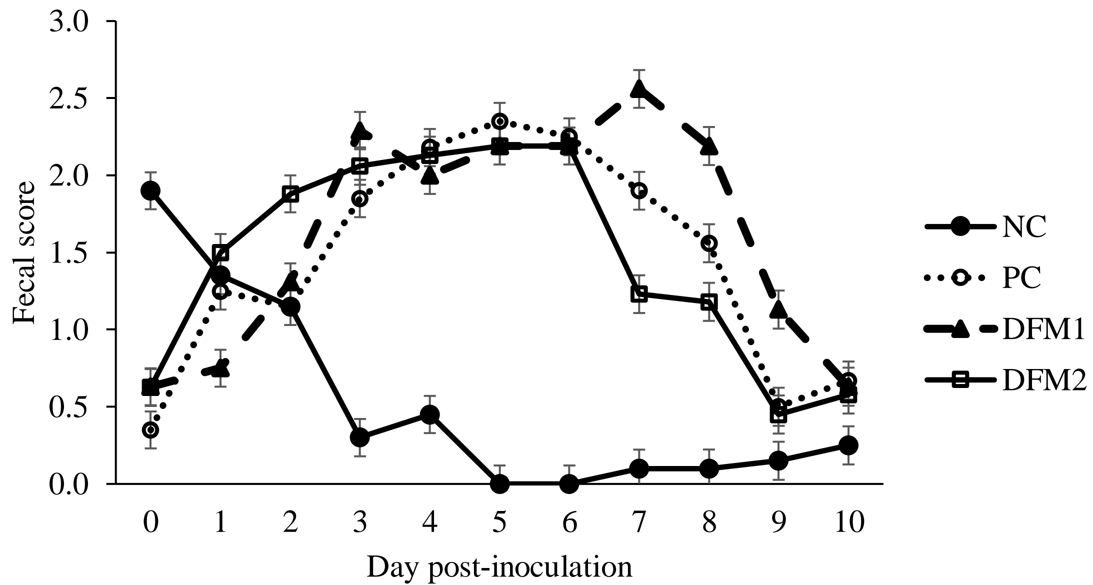

Figure 3.1. Effects of treatment on the daily fecal score of pigs challenged with F18 ETEC. NC = non-challenged control (n=10); PC = positive challenged control (n=9); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g of feed); DFM2 = PC + DFM2 (n=7; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5×10^5 cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK). *P* (NC vs. PC; dpi 4) <.0001, *P* (PC vs. DFM1, DFM2; dpi 4) > 0.10, *P* (all treatments; dpi 10) > 0.10.

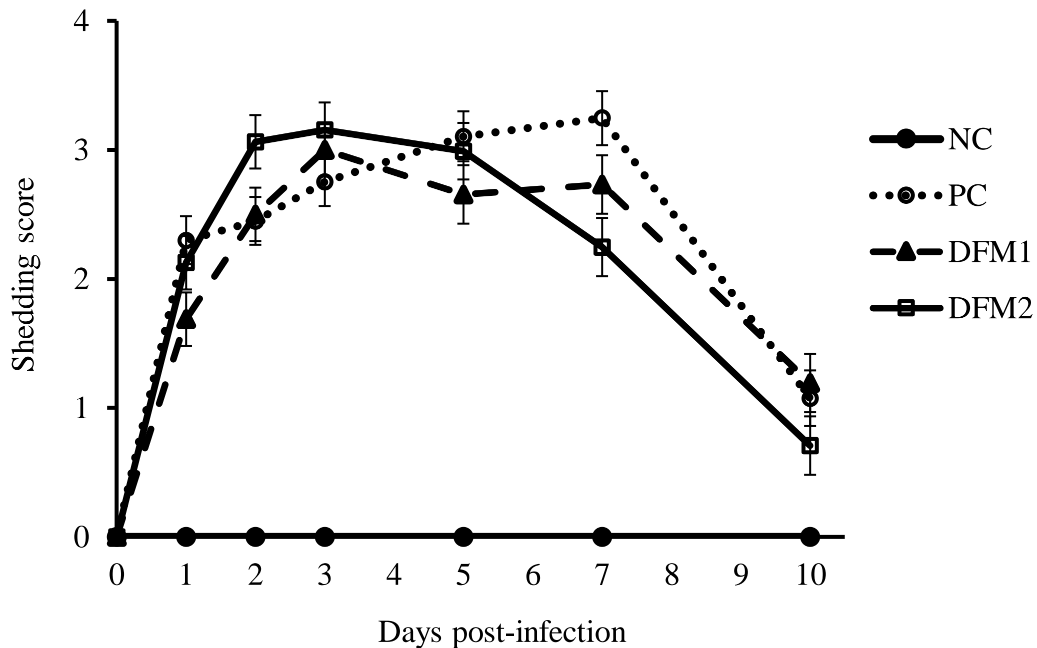

Figure 3.2. Effects of treatment on *E. coli* shedding score of pigs challenged with F18 ETEC. NC = non-challenged control (n=10); PC = positive challenged control (n=9); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5 $\times 10^5$ cfu/g of feed); DFM2 = PC + DFM2 (n=7; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5 $\times 10^5$ cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK). *P* (NC vs. PC; overall period) <.0001, *P* (PC vs. DFM1; overall period) > 0.10, *P* (PC vs. DFM2; dpi 2) = 0.044, *P* (PC vs. DFM2; dpi 7) = 0.003, *P* (all treatments; dpi 10) > 0.10.

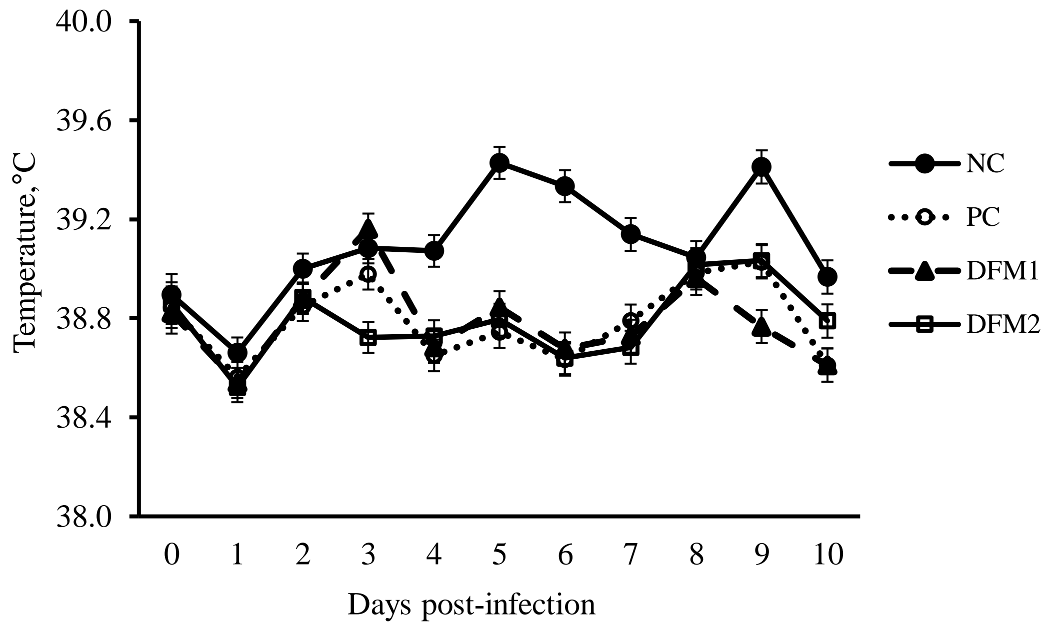

Figure 3.3. Effects of treatment on daily rectal temperature of pigs challenged with F18 ETEC. NC = non-challenged control (n=10); PC = positive challenged control (n=9); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5 $\times 10^5$ cfu/g of feed); DFM2 = PC + DFM2 (n=7; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5 $\times 10^5$ cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK). *P* (NC vs. PC; overall period) <.0001, *P* (PC vs. DFM1; overall period) = 0.962; *P* (PC vs. DFM2; overall period) = 0.947

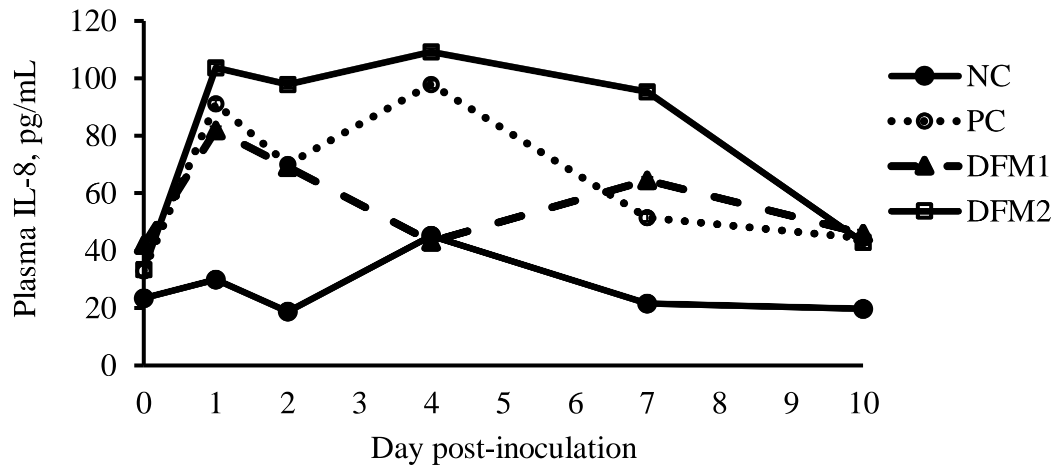

Figure 3.4. Effects of treatment on plasma IL-8 of pigs challenged with F18 ETEC. NC = non-challenged control (n=10); PC = positive challenged control (n=9); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5 $\times 10^5$ cfu/g of feed); DFM2 = PC + DFM2 (n=7; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5 $\times 10^5$ cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK). *P* (NC vs. PC; dpi 1) = 0.069; *P* (NC vs. PC; dpi 2) = 0.031; *P* (PC vs. DFM1, DFM2; overall period) > 0.10; *P* (all treatments; dpi 4, 7, 10) > 0.10.

CHAPTER IV

ALTERATION OF MUCOSA-ASSOCIATED MICROBIOTA BY ETEC CHALLENGE IN NURSERY PIGS FED DIRECT-FED MICROBIAL BLENDS

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Abstract

The objectives of this study were to investigate the impact of an F18 ETEC challenge, as well as the potential protective effects of two *Bacillus*-based DFMs, on homeostasis of the mucosa-associated microbiota. Seventy-two weaned piglets (6.4 ± 0.6 kg BW ~21 d of age) were assigned to one of four treatments: 1) **NC**: Non-challenged (n=10), 2) **PC**: F18 ETEC challenged (n=10), 3) PC + **DFM1** (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g) or 4) PC+**DFM2** (n=8; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis*; 1.5×10^5 cfu/g). Pigs were either sham-infected with 6 mL sterile PBS or orally inoculated with 6 mL hemolytic F18 ETEC (~1.9 $\times 10^9$ cfu/mL) on d 7 post-weaning. All ETEC challenged pigs were confirmed to be genetically susceptible to F18 ETEC. Pigs had *ad libitum* access to feed and water throughout the 17-d trial. Tissues from the ileum and colon were collected on day 17 and

mucosa scrapings were collected. The V4 region of the 16S rDNA was amplified and sequenced. High-quality reads (total 5,421,869) were selected and clustered into 7,570 OTUs based on 99% sequence similarity.

The ETEC challenge disrupted gut microbial homeostasis in pigs by increasing *Escherichia-Shigella, Chlamydia,* and *Actinobacillus* in the intestinal mucosa ($q < 0.05$). This was accompanied by decreases in beneficial genera, including *Streptococcus, Prevotella_9,* and *Veillonella* ($q < 0.05$). The inclusion of *Bacillus*-based DFMs altered the mucosa-associated microbiota to preserve or reestablish homeostasis in the GIT. Greater abundance of *Prevotella_9* and *Veillonella* in the colon mucosa was observed in pigs fed DFM1 than those fed PC ($q < 0.05$). Supplementation of DFM2 increased *Streptococcus* and reduced *Escherichia-Shigella, Chlamydia, Helicobacter* (OTU 140), and *Fusobacterium* in the intestinal mucosa compared with PC ($q < 0.05$). This demonstrates the ability of DFM2 to reduce pathogenic bacteria load. These results suggest that an ETEC challenge negatively alters gastrointestinal function by inducing microbial dysbiosis through the increase in potentially pathogenic bacteria and reduction in beneficial bacteria. Supplementation of *Bacillus*-based DFMs appeared to have altered the mucosa-associated microbiota to preserve or reestablish homeostasis in the GIT and may be a beneficial feeding strategy used to alleviate the effects of an ETEC challenge in weaned pigs.

Introduction

Enterotoxigenic *E. coli* (**ETEC**) in weaned pigs causes the breakdown of gastrointestinal tract functions, resulting in decreased feed intake and growth, as well as impaired health and survivability. Although vaccines and dietary feed additives have been

developed with the objective to prevent or mitigate the negative impacts of ETEC infections, the swine industry worldwide continues to experience substantial economic losses due to this pathogen. Concerns regarding antimicrobial resistance has led to reduced antibiotic use in swine production; thus, alternative products to mitigate ETEC infections in nursery pigs are needed. Direct-fed microbial blends (DFM) are products that contain live microorganisms, typically strains of *Bacillus, Lactobacillus, Bifidobacterium,* and/or *Enterococcus*. Several methods by which DFMs beneficially affect the host have been proposed, including production of antimicrobial compounds and enzymes, enhanced nutrient digestibility, improvements in intestinal barrier integrity, modulation of immune response, promotion of beneficial bacterial growth, and competitive exclusion of pathogenic bacteria (Meng et al., 2010; Hu et al., 2014; Lee et al., 2014; Yirga et al., 2015).

Healthy microbial populations in the gastrointestinal tract (GIT) contribute to enhanced performance and immunity. *Lactobacillus* is a highly abundant, beneficial bacterial group found in the GIT of pigs. After weaning, alterations in these mucosa-associated beneficial bacteria occur, allowing pathogens to colonize and proliferate, which negatively impacts intestinal health and subsequent growth performance; however, a greater understanding of microbiota changes following a direct ETEC challenge in weaned pigs is needed. DFMs are suggested to balance the GIT microbial population dynamics in order to create a more beneficial microbial population. Previous results demonstrated that an ETEC challenge reduced growth performance of weaned pigs as a result of increased diarrhea, reduced rectal temperature, secretory immunoglobulin a (sIgA) and tight junction protein gene transcription (Becker et al., 2019). The supplementation of a *Bacillus*-based DFM to ETEC-challenged pigs also improved intestinal barrier integrity and *E. coli*

shedding; thus, a better understanding of how the DFM is modifying these responses to an ETEC challenge is warranted. The continued development of new technologies, such as 16S rRNA gene sequencing, offers means to investigate microbial communities and their interactions with the host immune system, genetics, environment, and diet (Holman et al., 2017; Pollock et al., 2018).

Therefore, the objectives of this study were to investigate the impact of an F18 ETEC challenge, as well as the potential protective effects of two *Bacillus*-based DFMs, on homeostasis of the mucosa-associated microbiota.

Materials and methods

All experimental procedures adhered to guidelines for the ethical and humane use of animals for research and were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC #8-17-8576-S).

Animals, diets, and sampling

Seventy-two newly weaned pigs (6.4 ± 0.6 kg BW; ~21d of age; L337 × Camborough, PIC, Hendersonville, TN) were randomly assigned to 1 of 4 dietary treatments: a non-challenged control (**NC**; n = 10)**,** an ETEC challenged positive control (**PC**; n = 10), and PC + DFM1 (3 strains of *Bacillus amyloliquefaciens;* **DFM1**; n = 8), or PC + DFM2 (2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* **DFM2**; n = 8). DFM1 and DFM2 were included at 0.03% of the diet to achieve a final dose of 7.5×10^5 cfu/g of feed and 1.5×10^5 cfu/g of feed, respectively (Danisco Animal Nutrition, Marlborough, UK). The control diet was primarily based on corn and soybean meal with 9.0% whey powder and 8.5% enzymatically treated soybean meal (Table 4.1). The diets were formulated to meet or exceed the nutrient requirements of weaned pigs

(NRC, 2012) and did not contain antibiotics or pharmaceutical levels of copper or zinc. Pigs were housed one barrow and one gilt per pen and had free access to feed and water during the 17-d trial. The sows and piglets used in this experiment had not been vaccinated against *E. coli*.

Prior to ETEC inoculation, all challenged pigs were determined via Sanger DNA sequencing of the α (1,2) fucosyltransferase-1 (**FUT1**) gene to be genetically susceptible to F18 ETEC (Frydendahl et al., 2003). On day 7 post-weaning, pigs were orally gavaged with 6 mL of sterile PBS or freshly grown F18 ETEC inoculum (1.9×10^9 cfu/mL). A fluoroquinolone-resistant hemolytic *E. coli* isolate that had an enrofloxacin minimal inhibitory concentration (**MIC**) > 2 μg/mL was used to prepare the bacterial inoculum at the Iowa State University Veterinary Diagnostic Lab (Ames, IA).

On day 17, one pig per pen was euthanized via captive bolt stunning followed by exsanguination. Post-euthanasia, the abdomen was opened and a segment of ileum 30 cm from the ileocecal junction and the apex of the spiral colon were removed, carefully drained of digesta, rinsed with ice-cold PBS, and immediately snap frozen in liquid N. Tissue samples were stored at -80°C pending analysis.

DNA extraction

Total genomic DNA was extracted from ileal and colonic mucosa scrapings (250 mg) using Dneasy PowerLyzer PowerSoil kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Genomic DNA concentration and purity were measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). All samples had 260:280 nm ratios above 1.8. Extracted DNA was adjusted to 20 ng/μL.

Illumina MiSeq sequencing

A PCR-amplified 16S rRNA sequencing of 68 pig intestinal mucosa samples was conducted using the 16S rRNA gene Illumina Amplicon protocol designed to amplify bacteria and archaea (The Earth Microbiome Project; http://www.earthmicrobiome.org/) at the Iowa State University DNA Facility (Ames, IA). Briefly, genomic DNA from samples was amplified using Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) with one replicate per sample. Universal 16S rRNA bacterial primers [515F (5′-GTGYCAGCMGCCGCGGTAA-3′; Parada et al., 2016), and 806R (5′-GGACTACNVGGGTWTCTAAT-3′; Apprill et al., 2015)] for the variable region V4 were utilized during the sequencing procedure as previously described (Kozich et al., 2013). All the samples underwent PCR with an initial denaturation step at 94°C for 3 min, followed by 35 PCR cycles (45 s at 94°C, 20 s at 50°C, and 90 s at 72°C), and finished with a 10 min extension at 72°C. All the PCR products then were purified with the QIAquick 96 PCR Purification Kit (Qiagen Sciences Inc, Germantown, MD) according to the manufacturer’s recommendations. PCR bar-coded amplicons were mixed at equal molar ratios and used for Illumina MiSeq paired-end sequencing with 150 bp read length and cluster generation with 10% PhiX control DNA on an Illumina MiSeq platform (Illumina Inc., San Diego, CA). After sequencing, corresponding overlapping paired-end reads were stitched to obtain a final amplicon size of approximately 255 bp.

Quality filtering and sequence analysis

Samples that failed to generate enough PCR products and contained very low numbers of sequencing reads were removed. Raw sequence data in fastq format were analyzed using mothur v.1.40.4 (http://www.mothur.org/; Kozich et al., 2013). Briefly,

paired-end reads were combined into contigs and were screened for quality with the "*screen.seqs*" command excluding sequences with any ambiguities. The number of homopolymers was set to eight. Sequencing error was reduced by pre-clustering sequences with up to 2 bp differences using the "*pre.cluster*" command. Using the "*cluster.split*" command, sequences were then clustered into operational taxonomic units (OTU) with a 99% similarity cutoff (0.01 distance) based on the distance matrix generated by default. Consensus taxonomy for OTUs was assigned using the "*classify.otu*" command using the SILVA SSU reference database (version 132, Pruesse et al., 2007). Relative abundances were calculated for the top 25 OTUs across both intestinal locations.

Statistical analysis

Absolute abundances of assigned OTUs were analyzed using a negative binomial distribution in GLIMMIX of SAS (9.4, Cary, NC). Data were offset by the total library count for each sample. Treatment was a fixed effect and pen was the experimental unit. Pre-planned contrasts were performed using the ESTIMATE statement to evaluate the effects of the ETEC challenge (NC vs. PC) and dietary treatment (PC vs. DFM1, DFM2). Assigned P values were corrected for multiple comparisons using the "Qvalue" package of R statistical software. Differences were considered significant if q was ≤ 0.05 and a tendency if q was > 0.05 and ≤ 0.10.

Results

There were 68 mucosa samples, 34 each from the ileum and the colon. A total of 5,421,869 high-quality reads were obtained after size filtering and quality control, with an average of 85,515 and 73,951 sequences per colon and ileum. Based on 99% sequence

similarity, 7,570 OTUs (> 10 sequences per OTU) were classified. At the genus level, *Helicobacter* dominated the mucosal microbiota (17.49%; Table 4.2), followed by *Lactobacillus* (7.31%) and *Escherichia-Shigella* (5.28%).

Microbiota in the ileal mucosa

At the genus level, the PC increased the abundances of *Escherichia-Shigella, Turicibacter,* and *Actinobacillus* (OTU 24; $q \leq 0.036$; Table 4.3), and tended to decrease *Acidaminococcus* compared with NC ($q = 0.068$). Greater abundances of *Streptococcus,* and *Intestinibacter* were observed in the NC compared with PC ($q \leq 0.036$). There were no differences in absolute abundances between NC and PC for *Helicobacter, Enterococcus, Lactobacillus, Fusobacterium,* or *Actinobacillus* (OTU 47; $q > 0.10$). Compared with PC, DFM1 increased *Helicobacter* and *Acidaminococcus* ($q \leq 0.034$) and tended to decrease *Lactobacillus* ($q = 0.052$) and increase *Actinobacillus* (OTU 47; $q = 0.066$). The abundance of *Escherichia-Shigella, Streptococcus, Enterococcus,* and *Fusobacterium* did not differ between PC and DFM1 ($q > 0.10$). DFM1 or DFM2 did not differ from PC in the abundances of *Turicibacter, Actinobacillus* (OTU 24) or *Intestnibacter* ($q > 0.10$). DFM2 significantly reduced the abundances of *Escherichia-Shigella, Enterococcus, Lactobacillus,* and *Fusobacterium* compared with PC ($q \leq 0.034$). The abundance of *Helicobacter, Turicibacter, Actinobacillus* (OTU 24), *Acidaminococcus,* and *Intestinibacter* did not differ between PC and DFM2 ($q > 0.10$).

Microbiota in the colon mucosa

At the genus level, *Prevotella_9* was reduced in PC compared with NC ($q = 0.003$; Table 4.4). The PC increased *Chlamydia, Romboustia* and *Clostridium_sensu_stricto_1* abundance compared with NC ($q \leq 0.035$). *Veillonella* tended to be reduced in PC

compared with NC (*q* = 0.066). PC was not different from NC in *Escherichia-Shigella*, *Anaerovibrio*, *Methanobrevibacter, Desulfovibrio, Helicobacter,* and *Bacteroides* abundance (*q* > 0.10). Compared with PC, pigs receiving DFM1 did not differ in the abundance of *Escherichia-Shigella, Chlamydia, Romboutsia, Clostridium_sensu_stricto_1,* and *Helicobacter* (*q* > 0.10). *Prevotella_9* and *Veillonella,* abundances were increased in DFM1 (*q* ≤ 0.037) compared with PC, while *Anaerovibrio, Desulfovibrio, Bacteroides* and *Methanobrevibacter* abundances were decreased in pigs fed DFM1 (*q* ≤ 0.047). DFM2 had decreased abundance of *Escherichia-Shigella, Chlamydia,* and *Helicobacter* compared with PC (*q* ≤ 0.017).

Discussion

Pathogenic bacterial infections can cause proliferation of other harmful bacteria or opportunistic pathogens, resulting in dysbiosis of the intestinal microbiota (Gresse et al, 2017; Pollock et al., 2018). Our previous findings revealed that an ETEC challenge reduced growth performance of nursery pigs; however, pigs fed a *Bacillus*-based DFM had improved intestinal barrier integrity. To enhance our understanding of how an F18 ETEC challenge modifies the pig's gastrointestinal function and how DFMs alter their response to a pathogenic challenge, high-throughput 16S rRNA gene sequencing technology was used. Utilization of this technology provides great advantages in analyzing a broad range of phylotypes at the genus level; however, the species-level classification may be insufficient due to the relatively short amplicon length. Furthermore, the absence of closely-related characterized reference strains makes it challenging to classify swine-specific GIT bacteria. This study investigated the impact of an F18 ETEC challenge, as

well as the potential protective effects of *Bacillus*-based DFMs, on microbial homeostasis in the gastrointestinal tract.

The detected abundances of *Bacillus* genus in the mucosa were low regardless of treatment or GIT location, which is in agreement with recent research utilizing 16S rRNA gene sequencing on fecal and intestinal samples of pigs fed *Bacillus*-based products (Poulsen et al., 2018). This could be attributed to a variety of factors, including sample type, primer bias, and/or bias in the DNA extraction method due to the resilient spore structure. It has been shown that *Bacillus* spores have the ability to germinate in significant numbers; however, this does not correlate to colonization in the mucus layer. Previous work has demonstrated a lack of or temporary colonization of *Bacillus* spp. in the GIT (Casula and Cutting, 2002; Leser et al., 2007; Bernardeau et al., 2017).

Despite the evident lack of *Bacillus* proliferation, supplementation of the DFMs did appear to have an effect on the mucosal microbiota. As expected, the ETEC challenge increased the abundance of *Escherichia-Shigella* in the ileal mucosa compared with NC; however, differences between pathogenic and indigenous strains of *Escherichia-Shigella* cannot be distinguished. These results agree with the elevated fecal *E. coli* shedding and increased fecal scores previously reported (Becker et al., 2019). DFM2 reduced abundance of *Escherichia-Shigella* compared with PC in both the ileum and colon mucosa, which aligns with previous observations in these pigs (Becker et al., 2019) and corresponds with previous studies in which *Bacillus*-based DFMs were fed to nursery pigs (Hu et al., 2013; Zhang et al., 2017).

Prevotella_9 and *Streptococcus* are highly abundant bacteria in the gastrointestinal tract of pigs (Mach et al., 2015; Isaacson and Kim, 2012). Abundances of both *Prevotella_9*

and *Streptococcus* were reduced in PC compared to NC, representing beneficial bacteria negatively altered by ETEC challenge. *Streptococcus* spp. contribute to lactic acid production and are often used in DFM products. DFM2 increased the abundance of *Streptococcus* in the ileum, possibly indicating a beneficial alteration of the mucosal microbiota. Though certain species of *Streptococcus* are pathogenic in swine, species cannot be differentiated using the current methodology. *Prevotella* produce short-chain fatty acids (SCFA) by metabolizing non-starch polysaccharides derived from plants (Ivarrson et al., 2014). These bacteria have also been shown to produce exogenous enzymes, such as mannanase, β-glucanase, and xylanase, which aid in the degradation of plant cell wall polysaccharides (Flint and Bayer, 2008). *Prevotella* abundance has also been positively correlated with secretory IgA concentrations in the intestinal mucosa and increased animal growth (Mach et al., 2015). Pigs fed DFM1 had increased *Prevotella_9* in the colon, suggesting the ability of DFM1 to promote beneficial bacterial growth. DFM1 also increased the abundance of *Veillonella* compared with PC, while PC tended to have *Veillonella* compared with NC. These indigenous bacteria have the ability to produce acetic and propionic acids from lactic acid. High concentrations of these SCFAs can then inhibit pathogenic bacteria growth (Hinton et al., 1991; Hinton and Hume 1997).

Research has shown that *Fusobacterium* abundance is increased during enteric infections in pigs, including porcine epidemic diarrhea virus (Koh et al., 2015) and swine dysentery (Burrough et al., 2017), as well as in piglets with non-specific diarrhea (Dou et al., 2017; Yang et al, 2017). The reduced *Fusobacterium* in the ileal mucosa of DFM2 pigs compared with PC indicates a positive modulation of the microbial population. DFM2 supplementation also resulted in positive modulation of the colon microbiota, shown by

the decrease in the abundance of *Helicobacter* (OTU 140); however, DFM1 increased *Helicobacter* (OTU 11) in the ileum. *Helicobacter* is a potentially pathogenic bacterial genus that is known to associate with the mucosal microbiota population and is highly abundant in weaned pigs (Mann et al., 2014; Adhikari et al., 2019); however, the function of these particular *Helicobacter* OTUs in pigs remains unknown.

The abundance of *Chlamydia,* a known pathogen, was increased in the colon mucosa of PC compared with NC, and DFM2 had reduced abundance of *Chlamydia* compared with PC. *Chlamydia* are obligate intracellular bacteria, meaning they need to live within epithelial cells to survive (Rank and Yeruva, 2014). The increase in mucosal *Chlamydia* in PC and DFM1 pigs challenged treatments may be a result of impaired intestinal integrity previously observed in these pigs (Becker et al., 2019). These results denote an ETEC-induced disruption of microbial homeostasis in pigs, which appeared to be attenuated by DFM2. *Actinobacillus* spp. are also especially pathogenic bacteria in swine (Fairbrother and Gyles, 2012). In the ileum, abundance of *Actinobacillus* (OTU 24) increased in PC compared with NC, thus representing another potential pathogenic bacteria impacted by the ETEC challenge.

Conclusions

In this study, an ETEC challenge disrupted gut microbial homeostasis in pigs by increasing the abundance of potentially pathogenic bacteria in the ileal and colonic mucosa, including *Escherichia-Shigella,* and potentially *Chlamydia,* and *Actinobacillus.* This was accompanied by decreases in beneficial genera, including *Streptococcus, Prevotella_9,* and *Veillonella* in the intestinal mucosa. The inclusion of *Bacillus*-based DFMs altered the mucosa-associated microbiota to preserve or reestablish homeostasis in the GIT. Greater

abundance of *Prevotella_9* and *Veillonella* in the colon mucosa was observed in pigs fed DFM1 than those fed PC. This indicates DFM1 is effective in elevating the abundance of potentially beneficial bacteria in the intestine, though DFM1 also increased *Helicobacter* (OTU 11) compared with PC. Supplementation of DFM2 increased *Streptococcus* and reduced *Escherichia-Shigella, Chlamydia, Helicobacter* (OTU 140), and *Fusobacterium* in the intestinal mucosa compared with PC. This demonstrates the ability of DFM2 to reduced pathogenic bacteria load. Taken together with results from Becker et al. (2019), these findings suggest that the ETEC challenge negatively altered gastrointestinal function by inducing microbial dysbiosis through the increase in potentially pathogenic bacteria and the reduction of beneficial bacteria in the mucosa. The supplementation of a DFM containing 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis* may be a beneficial feeding strategy used to alleviate the effects of an ETEC challenge in weaned pigs.

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Table 4.1. Ingredient and nutrient composition of the experimental diets (as-fed basis, %)

Item	Control	Control+DFM1[1]	Control+DFM2[2]
Ingredient			
Corn	58.07	58.04	58.04
Soybean meal	15.00	15.00	15.00
Whey powder	9.00	9.00	9.00
HP300[3]	8.50	8.50	8.50
Fishmeal	4.00	4.00	4.00
Soybean oil	2.00	2.00	2.00
Limestone	1.27	1.27	1.27
Monocalcium phosphate	0.10	0.10	0.10
Salt	0.68	0.67	0.67
Vitamin premix[4]	0.20	0.20	0.20
Trace mineral premix[5]	0.20	0.20	0.20
L-Lysine HCl	0.52	0.52	0.52
DL-Methionine	0.19	0.19	0.19
L-Threonine	0.16	0.16	0.16
L-Valine	0.06	0.06	0.06
L-Tryptophan	0.03	0.03	0.03
Phytase[6]	0.02	0.02	0.02
DFM1	--	0.03	--
DFM2	--	--	0.03
Calculated nutrient levels[7]			
ME, kcal/kg	3,407	3,407	3,407
NE, kcal/kg	2,559	2,559	2,559
Crude protein	20.34	20.34	20.34
Ether extract	4.92	4.92	4.92
Total P	0.53	0.53	0.53
STTD P	0.43	0.43	0.43
Calcium	0.85	0.85	0.85
SID Lys	1.40	1.40	1.40
SID Met + Cys	0.77	0.77	0.77
SID Thr	0.83	0.83	0.83
SID Trp	0.24	0.24	0.24
Analyzed nutrient levels			
Dry matter	85.52	85.63	85.45
GE, kcal/kg	4,329	4,338	4,321
Crude protein	21.51	22.32	21.68
aEE[8]	5.69	5.56	5.82

[1]Direct-fed microbial 1 (DFM1) = Three strains of *Bacillus amyloliquefaciens;* 7.5×10^5 cfu/g of feed, Danisco Animal Nutrition, Marlborough, UK.

[2]Direct-fed microbial 2 (DFM2) = Two strains of *Bacillus amyloliquefaciens* and one strain of *Bacillus subtilis;* 1.5×10^5 cfu/g of feed, Danisco Animal Nutrition, Marlborough, UK.

[3]Enzymatically-treated soybean meal; Hamlet Protein, Findlay, OH
[4]Provided per kg of diet: 7,656 IU vitamin A, 875 IU vitamin D, 63 IU vitamin E, 4 mg vitamin K, 70 mg niacin, 34 mg pantothenic acid, 14 mg riboflavin, and 0.06 mg vitamin B_{12}.
[5]Provided per kg of diet: 165 mg Zn (zinc sulfate), 165 mg Fe (iron sulfate), 39 mg Mn (manganese sulfate), 17 mg Cu (copper sulfate), 0.3 mg I (calcium iodate), and 0.3 mg Se (sodium selenite).
[6]2,000 FTU/kg of feed provided 0.109% available P; AxtraPhy, Danisco Animal Nutrition, Marlborough, UK.
[7]STTD = standardized total tract digestible; SID = standardized ileal digestible.
[8]aEE: acid-hydrolyzed ether extract.

Table 4.2. Top 25 most abundant operational taxonomic units (OTU) across all intestinal mucosal sites.

OTU	Number of sequences	Phylum	Family	Genus	Relative abundance (%)
1	948550	*Epsilonbacteraeota*	*Helicobacteraceae*	*Helicobacter*	17.49
2	396193	*Firmicutes*	*Lactobacillaceae*	*Lactobacillus*	7.31
3	286215	*Proteobacteria*	*Enterobacteriaceae*	*Escherichia-Shigella*	5.28
4	122143	*Firmicutes*	*Clostridiaceae_1*	*Clostridium_sensu_stricto_1*	2.25
5	108787	*Firmicutes*	*Streptococcaceae*	*Streptococcus*	2.01
6	65624	*Firmicutes*	*Lactobacillaceae*	*Lactobacillus*	1.21
7	61776	*Firmicutes*	*Lactobacillaceae*	*Lactobacillus*	1.14
8	59116	*Firmicutes*	*Veillonellaceae*	*Megasphaera*	1.09
9	57376	*Epsilonbacteraeota*	*Campylobacteraceae*	*Campylobacter*	1.06
10	57250	*Chlamydiae*	*Chlamydiaceae*	*Chlamydia*	1.06
11	54946	*Epsilonbacteraeota*	*Helicobacteraceae*	*Helicobacter*	1.01
12	52144	*Actinobacteria*	*Bifidobacteriaceae*	*Bifidobacterium*	0.96
13	51472	*Firmicutes*	*Ruminococcaceae*	*Ruminiclostridium_5*	0.95
14	47779	*Bacteroidetes*	*Prevotellaceae*	*Prevotella_9*	0.88
15	46607	*Proteobacteria*	*Pasteurellaceae*	*Actinobacillus*	0.86
16	42735	*Firmicutes*	*Erysipelotrichaceae*	*Turicibacter*	0.79
17	40855	*Firmicutes*	*Lactobacillaceae*	*Lactobacillus*	0.75
18	38051	*Firmicutes*	*Ruminococcaceae*	*Ruminococcaceae_UCG-008*	0.70
19	37300	*Proteobacteria*	*Pasteurellaceae*	*Pasteurellaceae_unclassified*	0.69
20	36357	*Firmicutes*	*Peptostreptococcaceae*	*Terrisporobacter*	0.67
21	34886	*Firmicutes*	*Streptococcaceae*	*Streptococcus*	0.64
22	33389	*Firmicutes*	*Ruminococcaceae*	*Subdoligranulum*	0.62
23	29873	*Firmicutes*	*Lactobacillaceae*	*Lactobacillus*	0.55
24	29449	*Proteobacteria*	*Pasteurellaceae*	*Actinobacillus*	0.54
25	29419	*unknown_unclassified*	*unknown_unclassified*	*unknown_unclassified*	0.54

Table 4.3. Effects of treatment on microbial abundance in the ileal mucosa, %

OTU	Genus	Treatment[1]				SEM	q – value contrasts[2]		
		NC	PC	DFM1	DFM2		1	2	3
3	*Escherichia-Shigella*	0.322	14.781	22.191	0.207	2.881	0.004	0.929	0.004
11	*Helicobacter*	0.001	0.003	0.844	0.003	0.045	0.726	0.004	1.000
16	*Turicibacter*	0.108	1.446	0.642	1.223	0.105	0.036	0.695	1.000
21	*Streptococcus*	1.627	0.017	0.027	0.624	0.126	0.004	0.929	0.029
24	*Actinobacillus*	0.008	3.302	0.270	0.344	0.416	0.007	0.432	0.503
30	*Enterococcus*	0.173	0.902	0.421	0.006	0.079	0.500	0.860	0.007
34	*Lactobacillus*	0.834	1.370	0.058	0.011	0.115	0.911	0.052	0.004
38	*Fusobacterium*	0.000	0.441	1.174	0.001	0.201	1.000	0.883	0.031
82	*Acidaminococcus*	0.030	0.001	0.058	0.006	0.153	0.068	0.034	0.571
88	*Intestinibacter*	0.008	0.000	0.001	0.001	0.003	0.036	0.842	0.982

[1]NC = non-challenged control (n=10); PC = positive challenged control (n=10); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5 $\times 10^5$ cfu/g of feed); DFM2 = PC + DFM2 (n=8; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5 $\times 10^5$ cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK).

[2] Preplanned contrasts: 1 = NC vs. PC; 2 = PC vs. DFM1; 3 = PC vs. DFM2

Table 4.4. Effect of treatment on microbial abundance in the colon mucosa, %

OTU	Genus	Treatment[1]				SEM	*q* – value contrasts[2]		
		NC	PC	DFM1	DFM2		1	2	3
3	*Escherichia-Shigella*	3.337	1.529	0.514	0.009	5.853	0.868	0.800	0.008
10	*Chlamydia*	0.024	0.298	0.316	0.006	0.028	0.035	0.999	0.003
26	*Romboutsia*	0.020	0.418	1.490	0.389	0.338	0.024	0.561	0.999
37	*Prevotella_9*	1.241	0.003	0.202	0.012	0.280	0.003	0.037	0.698
64	*Anaerovibrio*	0.266	0.267	0.009	0.456	0.040	0.999	0.008	0.891
77	*Veillonella*	0.062	0.000	0.639	0.029	0.125	0.066	0.008	0.160
96	*Clostridium_sensu_stricto_1*	0.004	0.087	0.123	0.038	0.008	0.028	0.962	0.800
105	*Methanobrevibacter*	0.145	0.297	0.000	0.111	0.042	0.960	0.017	0.909
134	*Desulfovibrio*	0.044	0.219	0.001	0.183	0.028	0.693	0.017	0.999
140	*Helicobacter*	0.052	0.292	0.074	0.002	0.029	0.517	0.695	0.017
186	*Bacteroides*	0.058	0.168	0.007	0.024	0.012	0.688	0.047	0.325

[1]NC = non-challenged control (n=10); PC = positive challenged control (n=10); DFM1 = PC + DFM1 (n=8; 3 strains of *Bacillus amyloliquefaciens;* 7.5 $\times 10^5$ cfu/g of feed); DFM2 = PC + DFM2 (n=8; 2 strains of *Bacillus amyloliquefaciens* and 1 strain of *Bacillus subtilis;* 1.5 $\times 10^5$ cfu/g of feed). Supplementation rates were based on manufacturer's recommendations (Danisco Animal Nutrition, Marlborough, UK).

[2] Preplanned contrasts: 1 = NC vs. PC; 2 = PC vs. DFM1; 3 = PC vs. DFM2

CHAPTER V

INTEGRATIVE SUMMARY

General discussion

Throughout all production stages, pigs are exposed to several stressors and pathogenic organisms that reduce growth performance and increase mortality and morbidity. Newly weaned pigs are particularly more susceptible to pathogenic infection due to their underdeveloped gastrointestinal tract (GIT) and immune system. Post-weaning diarrhea (PWD) induced by enterotoxigenic *Escherichia coli* (ETEC) has been a long-recognized problem in swine production worldwide that has contributed to significant economic losses for producers. Following colonization and proliferation in the GIT, ETEC produces one or more enterotoxins that alter water and ion flux in the small intestine, resulting in clinical symptoms of diarrhea and dehydration (Sun and Kim, 2017). As pork producers continue to limit or remove antibiotics from production practices, there is an undoubted need for alternative strategies to prevent or mitigate these production losses. In-feed supplementation of direct-fed microbials (DFMs) is one such strategy that has been proposed to improve intestinal function and health of the weaned pig in order to reduce negative impacts on growth performance (Yirga, 2015); however, results in pigs have been inconsistent. The objectives of this thesis were to evaluate the potential beneficial effects of *Bacillus*-based DFMs under normal physiological and ETEC challenge conditions and to characterize the impact of an ETEC challenge in weaned pigs on growth, intestinal function, immune response, and microbiota.

Experiment 1 (Chapter 2) evaluated the effects of two novel *Bacillus*-based direct-fed microbial blends on nursery pig growth performance in a commercial-like environment. The data from this experiment revealed that supplementation of either DFM did not impact the growth performance of nursery pigs with in good health. It is also hypothesized that the efficacy of the DFM could have been altered by dietary ingredients, such as zinc oxide. It is also possible that the response to DFM supplementation would be greater in pigs with a poor health status (McEwen and Fedorka-Cray, 2002). Additionally, a further understanding of how ETEC is physiologically impacting the pig is necessary in order to develop effective feeding strategies. Therefore, the objectives of Experiment 2 (Chapters 3 and 4) were to investigate the impact of an ETEC challenge on growth performance, intestinal barrier function, immune response, and microbial homeostasis of weaned pigs while concurrently evaluating the potential protective effects of DFMs.

In chapter 3, it was found that an ETEC challenge reduced rectal temperature, impaired intestinal barrier integrity through alterations in tight junction proteins, increased systemic and localize IL-8 production, and reduced ileal mucosal sIgA. This resulted in blunted growth performance during the 10-d post-challenge period. Pigs were subjected to an extremely severe health challenge, resulting in acute clinical symptoms and high mortality outcomes (23%), which is not unusual in ETEC challenged pigs if left untreated (Fairbrother and Gyles, 2012). However, pigs appeared to be recovering from infection by the end of the challenge period, as evidenced by decreases in ETEC shedding and a lack of *E. coli* attachment to the intestinal epithelial lining. The lack of febrile response in ETEC challenged pigs is possibly explained by the reduction in feed intake and dehydration observed, and also agrees with the lack of response of other cytokines. Supplementation of

DFM1 did not appear to attenuate the impacts of ETEC; however, DFM2 may provide some protection to nursery pigs challenged with ETEC by improving ETEC shedding and intestinal barrier integrity.

In chapter 4, an ETEC challenge disrupted microbial homeostasis in the intestinal mucosa by increasing the abundance of potentially pathogenic genera in the ileal and colonic mucosa, including *Escherichia-Shigella, Helicobacter, Fusobacterium,* and *Actinobacillus.* This was accompanied by decreases in beneficial bacteria, including *Lactobacillus, Faecalibacterium, Alloprevotella,* and *Ruminococcus*. The supplementation of DFM2 appeared to have altered the mucosa-associated microbiota to preserve or reestablish homeostasis in the GIT by reducing pathogenic bacterial load, which may explain the improved intestinal barrier integrity observed in chapter 3.

Recommendations for future research

When considering nutritional strategies to aid in the prevention and mitigation of post-weaning stressors and infection, *Bacillus*-based DFMs appear to be a promising candidate as a feed additive. Future research evaluating non-antibiotic alternative products and their interactions with each other, as well as dietary ingredients, is necessary in order to understand their mode of action in the pig. This is especially important when characterizing the microbial population in the GIT, as substrate is the major driver of population composition. Future research evaluating products in health-challenged conditions should consider using a non-challenged control group in order to quantify the severity of the challenge. This would also allow differentiation of microbiota changes due to disease challenge or diet differences, thus making comparisons across studies easier and more relevant.

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