EFFECTS OF A MULTI-STRAIN *BACILLUS SUBTILIS*-BASED DIRECT-FED MICROBIAL ON WEANLING PIG GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY, IMMUNITY MARKERS, INTESTINAL MORPHOLOGY, AND MICROBIAL COMMUNITIES

By

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ABSTRACT

EFFECTS OF A MULTI-STRAIN *BACILLUS SUBTILIS*-BASED DIRECT-FED MICROBIAL ON WEANLING PIG GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY, IMMUNITY MARKERS, INTESTINAL MORPHOLOGY, AND MICROBIAL COMMUNITIES

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The objective of this experiment was to evaluate the effects of a multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM) on weanling pig growth performance, nutrient digestibility, immunity markers, intestinal morphology, and microbial communities. A study was conducted to test the hypothesis that DFM supplemented pigs would have greater nutrient digestibility and improvements in specific indicators of health status. Eighty pigs, of equal number of barrows and gilts (initial BW: 7.0 ± 0.60 kg), weaned at 21 ± 1 days of age were randomly allotted to sixteen pens, with five pigs per pen. Two dietary treatments were implemented, a basal control (CON) and a control plus DFM (DFM). Both diets were corn, soybean meal, and distillers dried grains based and were formulated to meet or exceed all nutrient requirements and manufactured on site. Diets were fed for 42 days. Growth performance was recorded on a weekly basis. On d 21 and 42 of the experiment, one pig per pen was randomly selected and euthanized, with equal number of males and females represented. Blood samples were collected prior to euthanasia for assessment of plasma concentrations of immunoglobin A (IgA) and intestinal fatty acid binding protein. Segments of the gastrointestinal tract including duodenum, jejunum, ileum, ascending and distal colon were removed for analysis of nutrient digestibility, intestinal morphology, microbial communities, and concentrations of interleukin 6, interleukin 10 (IL-10), and tumor necrosis factor alpha. Overall growth

performance did not differ between DFM and CON. Overall means ± SD were 0.51 ± 0.05 kg/d, 0.79 ± 0.05 kg/d and 0.66 ± 0.05 for ADG, ADFI, and G:F, respectively. Compared to pigs fed CON, overall digestibility of AA within the jejunum tended to be greater for tryptophan (P = 0.06), methionine (P = 0.10), and cysteine (P = 0.12) for pigs fed DFM. The pH of contents in ascending colon, a possible indicator of varied fiber digestion, did not differ. Apparent total tract nitrogen and energy digestibility did not differ between DFM and CON on d 21 or 42.

Compared to CON, overall jejunal villus height was greater (P = 0.02) (422 vs. 385 ± 10 µm, respectively) and ascending colon crypt depth tended to be greater (P = 0.10) on d 21 (373 vs. 337 ± 14 µm, respectively). Compared to CON, DFM tended to increase IgA (P = 0.06) on d 21 (0.34 *vs.* 0.54 ± 0.07 mg/mL, respectively) and tended to increase IL-10 (P = 0.12) on d 42 (133 *vs.* 237 ± 49 pg/mL, respectively). Addition of a multi-strain *Bacillus subtilis*-based DFM appears to impact select amino acid digestibility within the jejunum. Improvements in digestibility may be related to the DFMs benefit on weanling pig health status, observed via differences in intestinal morphology and specific immunity markers.

This thesis is dedicated to my wife, Caitlin Lewton. Thank you for always believing in me and patiently encouraging me. Thank you for all the sacrifices you have made to be with me through this journey.

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KEY TO ABBREVIATIONS

AA = Amino acid

ADFI = Average daily feed intake

ADG = Average daily gain

CP = Crude protein

DFM = Direct-fed microbial

GE = Gross energy

G:F = Gain to feed ratio, feed efficiency

GIT = Gastrointestinal tract

iFABP = Intestinal fatty acid binding protein

IgA = Immunoglobin A

IL-6 = Interleukin 6

IL-10 = Interleukin 10

N = Nitrogen

Ti = Titanium

TNFα = Tumor necrosis factor alpha

VFA = Volatile fatty acid

INTRODUCTION

Weaning may be the most impactful event of the young pig's life, involving many stressors that when combined, lead to a negative impact on the pig's health and growth performance (Pluske, 2013). Until recent years, the swine industry has helped weanling or nursery pigs cope with this transition by using feed-grade antibiotics, however, due to increased recognition of antibiotic resistant bacteria growth, antibiotic use has become increasingly regulated (Aarestrup et al., 2010; Schultz and Rademacher, 2017). Moving forward, finding antibiotic alternatives has become a major focal point of research within the swine industry. Of the many alternatives available today, direct-fed microbials (DFM) have shown great potential (Lee et al., 2014; Blavi et al., 2018; Tang et al., 2019). Of the many researched and available DFM, *Bacillus subtilis* stands out as one of the species of interest (Larsen et al., 2014). There are many strains of *Bacillus subtilis* available and generally, they appear to share a beneficial effect on growth performance. Augspurger et al. (2016) have conducted three separate experiments, each showing positive impacts of a multi-strain *Bacillus subtilis*-based DFM on nursery pig growth performance. It is unclear how this multi-strain *Bacillus subtilis*-based DFM improves growth performance, and such information is needed to continue developing and improving DFM utilization is nursery pig diets. The overarching hypothesis of this thesis is that the multi-strain *Bacillus subtilis*-based DFM improves growth performance in part by sustaining the gastrointestinal health of piglets in response to weaning. The overall goal was to assess the impact of multi-strain *Bacillus subtilis*-based DFM on markers of small and large intestinal functionality and health. The study that forms the essence of this thesis was conducted to evaluate for following specific objectives: 1) to determine the effects of a multi-strain *Bacillus*

subtilis-based DFM on nutrient digestibility 2) to determine the effects of a multi-strain *Bacillus subtilis*-based DFM on immunological markers, and intestinal morphology and microbiota communities.

In chapter one, the background and relevant research related to this study are provided, focusing on the impact of weaning on the gastrointestinal tract (GIT) of weanling pigs and the use of DFM as an antibiotic alternative to help negate the negative responses associated with weaning. In chapter two, objective 1 addresses the specific hypothesis that that pigs supplemented with *Bacillus subtilis* have greater digestibility of nitrogen, amino acids (AA), gross energy (GE), and increased production of volatile fatty acids (VFA) in different segments of the GIT. In chapter three, objective 2 addresses the specific hypothesis that pigs supplemented with *Bacillus subtilis* would have improved health status indicated by positive changes in immunological markers, greater morphological development of individual gastrointestinal segments, and marked changes in microbial communities. These chapters are then followed by overall conclusions and implications.

CHAPTER 1

LITERATURE REVIEW

1. Introduction

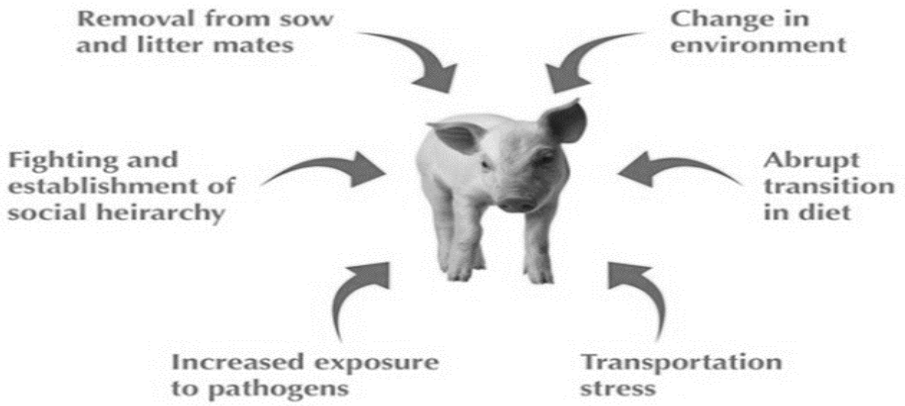
Within the swine industry, the nursery or weanling pig is one of the most researched of all age classes because of the performance challenges that ensue. Indeed, weaning may be the most negatively impacting event of the pig's life, significantly impacting health and growth performance (Pluske, 2013). Of the many weaning factors affecting the nursery pig's performance and health, the most important include: environmental and social stressors, age at weaning, dietary changes, and negative impacts on the gut (Hedemann et al., 2003; Moeser et al., 2007; Lewis, 2008; Campbell et al., 2013; Fels et al. 2014). The use of antibiotics has proven effective for many years in mitigating the impact of these stressors (Cromwell, 2002). With phasing out of antibiotics, antibiotic alternatives have become of great importance to help weanling pigs transition into the nursery. Of the many alternatives available, direct-fed microbials (DFM) have great potential. At the gut level, weaning causes major changes in the microbiome, the community of microorganisms residing in the gastrointestinal tract (GIT) (Issacson and Kim, 2012). The microbiome plays several roles in the body and is constantly changing with shifts in the pig's age and diet (Isaacson and Kim, 2012). One of DFM classes, *Bacillus subtilis*, a gram-positive bacterium (Mingmongkolchai and Panbangred, 2017), appears promising. Several studies have shown that supplementing *Bacillus subtilis* increases growth performance (Augspurger et al. 2016;) and nutrient digestibility (Blavi et al., 2018), and improved intestinal morphology (Lee et al., 2014; Kim et al., 2019), immunological markers (Zhang et al., 2017), and microbiota communities (Lee et al., 2014). The most understood

mechanisms by which *Bacillus subtilis* improved the GIT functionality included increased enzyme (Blavi et al., 2018; Tang et al., 2019) and antimicrobial secretions (Hong et al., 2005), competitive exclusion (Cai et al., 2015), and immune stimulation (Upadhaya et al., 2017; Kim et al., 2019). In this thesis, the effect of a multi-strain *Bacillus subtilis* DFM on parameters of intestinal functionality and health were investigated and presented in Chapters 2 and 3.

2. Impact of Weaning

Weaning piglets from their mother is arguably the most impactful moment of the pig's lifetime (Pluske, 2013). Weaning results in a short-term lag in growth relative to pre-weaning growth and the magnitude to this impact varies depending on several factors shown in Figure 1.1. These factors, or stressors come in many forms including transportation (Lewis, 2008), exposure to new pathogens (Moxley and Duhamel, 1999), and establishment of hierarchy (Fels et al. 2014), which are briefly discussed below. Weaning also involves dietary transitions and may result in post-weaning diarrhea, both impacting the pig's wellbeing and ability to cope during this period (Pluske, 2013). Weaning is associated with changes in in intestinal cell morphology, cell production, and immunological marker concentrations, which in turn appear to be associated with changes in nutrient digestibility (Hedemann et al., 2003; Lee et al., 2014). Furthermore, post-weaning dietary and environmental changes are associated with alteration of the intestinal microbiome (Frese et al., 2015). Minimizing these impacts through the use of DFM may play an important role in helping the weanling pig transition during this critical part of its life.

Figure 1.1. Diagram depicting factors or stressors involved at the time of weaning (Crenshaw et al., 2014)



2.1 Environmental and Social Stressors

Upon weaning, piglets are often transported for up to 24 hr (Lewis, 2008) to a different location. Prolonged transport in the summer can cause heat stress but may also increase fighting during transport, while winter transport increases thermoregulatory behaviors, resulting in increasing piglet fatigue and dehydration (Lewis, 2008). In addition to transport, exposure to a new nursery facility create different environmental challenges, including novel pathogens. A few common bacterial diseases faced by weanling pigs include *Escherichia coli, Clostridium perfringens* and *Salmonella enterica* (Moxley and Duhamel, 1999). The extent of pathogen infection is affected by weaning age, management protocols, and facility cleanliness (Smith et al., 2007; Lewis, 2008). Finally, weanling pigs also face the additional challenge of reestablishing hierarchy between pen mates. Fighting is common and may last as long as 48 to 72 hr until hierarchy is within the pen is established (Fels et al., 2014), significantly depleting bodily stored energy and increasing the piglet's susceptibility to disease and dehydration.

2.2 Weaning Age

One of the more important factors dictating post-weaning success is lactation length or weaning age (Moeser et al., 2007). In natural setting, weaning of piglets occurs around 10 to 12 weeks of age, which is in sharp contrast to weaning under commercial settings. For instance, in 2019, the national average age at weaning in the U.S. was 20.8 ± 2.4 d (Moeser et al., 2017; PigCHAMP, 2019). Weaning pigs as early as 10 to 21 days of age is beneficial as it decreases pathogen transmission from sows to piglets and increase the number of pigs produced each year (Fangman and Tubbs, 1997; Smith et al., 2007). However, it has also led to many challenges, as early weaned pigs have greater difficulty transitioning to plant-based diets, lacking essential enzyme activity (Lindemann et al., 1986). Additionally, they are much more prone to disease outbreak and impaired gut barrier function, as a result of compromised immune system (Campbell et al., 2013). Increasing wean age from 15 to 20 days increased post weaning average daily gain (ADG) and feed efficiency (G:F) (Smith et al., 2007). Further increasing weaning age from 21 to 28 days was shown to decrease intestinal permeability at 24 hr post weaning, which may lead to a positive long-term effect on gut health and integrity (Moeser et al., 2017).

2.3 Dietary Changes and Post Weaning Diarrhea

The dietary transition from milk to a ground, grain-based, feeds may be one of the greatest challenges placed on the weanling pig. Switching pigs to solid food results in a decrease in feed intake, as grain-based diets are less palatable than milk produced by the sow (Campbell et al., 2013). From birth until weaning, a milk-based diet is compatible or matches the production

of specific enzymes, however the transition to solid feed requires production of enzymes that the weanling pig has not yet fully developed, including amylase, lipase, chymotrypsin, and trypsin (Lindemann et al., 1986). This creates a brief time frame in which dietary nutrients may not be adequately absorbed by the body. Creep feeding is a common practice that many farms use in order to reduce the impact of the transition from milk-based to grain-based diets upon weaning. However, the data to support the benefits of creep feeding varies with some research showing positive impacts on weanling pig growth performance (Bruininx et al., 2002b; Cabrera et al., 2013) and gut health (Pluske et al., 1996; Kuller et al., 2007; Jayaraman and Nyachoti, 2017); while suggest the costs may outweigh the benefits, observing limited changes in either growth performance (Barnet et al., 1989; Fraser et al., 1994) or microbial populations and fermentation products (Mathew et al., 1994). Creep feeding benefits may vary from farm to farm or pig to pig and may greatly depend on its dietary composition, as is also the case with post-weaning diet formulation.

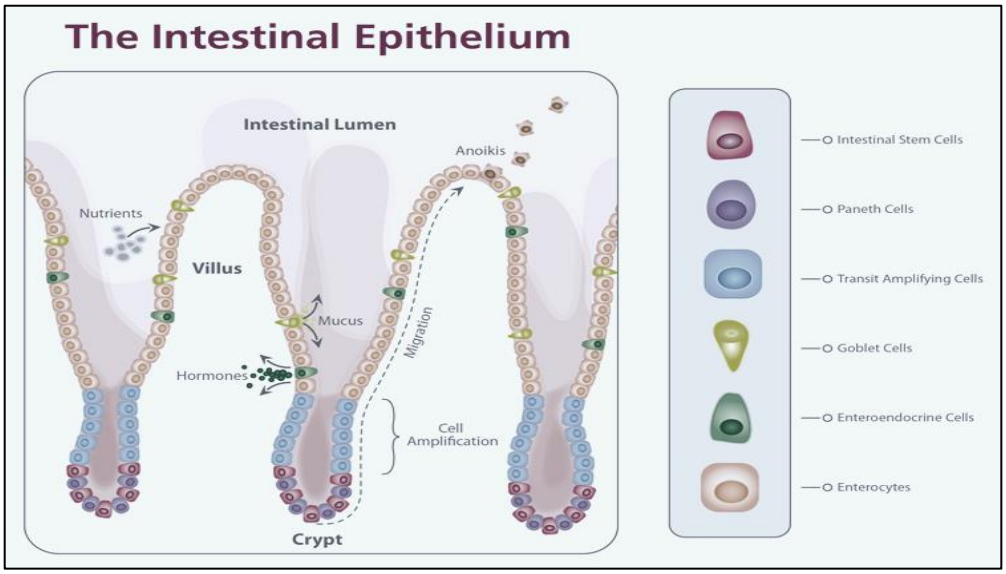
Proper dietary formulation is critical in order to provide adequate, digestible nutrients to low intake, stressed or immunocompromised pigs. Improper dietary formulation may exacerbate the impacts of weaning, leading to post-weaning diarrhea outbreaks. Studies have shown that the amount and type of protein and fiber in the diet can either prevent or cause diarrhea (Pluske, 2013). One strategy of reducing nursery pig diarrhea is by lowering the dietary crude protein (CP) concentrations, limiting the available substrates that microbes are able to restructure into toxic compounds such as ammonia and hydrogen sulfide (Bhandari et al., 2010; Payling et al., 2017). Along with improper formulation, diarrhea is also a shared symptom of several diseases commonly occurring in the nursery. Of the known diarrhea related diseases, many are associated

with decreased growth performance, lowered appetite, and thin appearances (Carpenter and Burlatschenko, 2005). Internally, these diseases are associated with breaking down intestinal cells, morphological structure, and protein stores, as well as destroying red blood cells and decreasing feed efficiency by as much as 25% in some cases (Lecce et al. 1982; Curry et al., 2018).

2.4 Morphological and Immunological Impacts

Weaning greatly alters the normal function of the GIT, damaging the structural components within the gut lumen (Hedemann et al., 2003). The morphology of the intestine, or physical structure undergoes negative changes (Aronoff and Fudeman, 2011). As depicted in Figure 1.2, the intestinal lumen is structurally surrounded by villi and crypts, consisting on several different cell types with unique functions. Weaning is often associated with decreased villus height and increased crypt depth (Hampson, 1986; Cera et al., 1988; Hedemann et al., 2003), both signs of stress, increased cell turnover and tissue demand, and decreased health status (Xu et al., 2003; Awad et al., 2009). On the contrary, greater villus height increases the intestinal surface area and allows for greater nutrient absorption (Caspary, 1992; Awad et al., 2009). Longer villi lead to greater division of epithelial cells while increased crypt depth, within the small intestine, is related to lower performance and decreases the total absorption of available nutrients (Awad et al., 2009). In the colon, crypts may also serve to protect stem cells from harmful metabolites generated by the microbiota primarily located within the colon (Kaiko et al., 2016). Thus, increase in colon crypt depth may indicate the cells responding to greater concentrations of beneficial metabolites being produced in the colon.

Figure 1.2. The intestinal epithelium, showing the intestinal lumen, villus, crypt, and location of specific cells (STEMCELL Technologies Inc., CC)



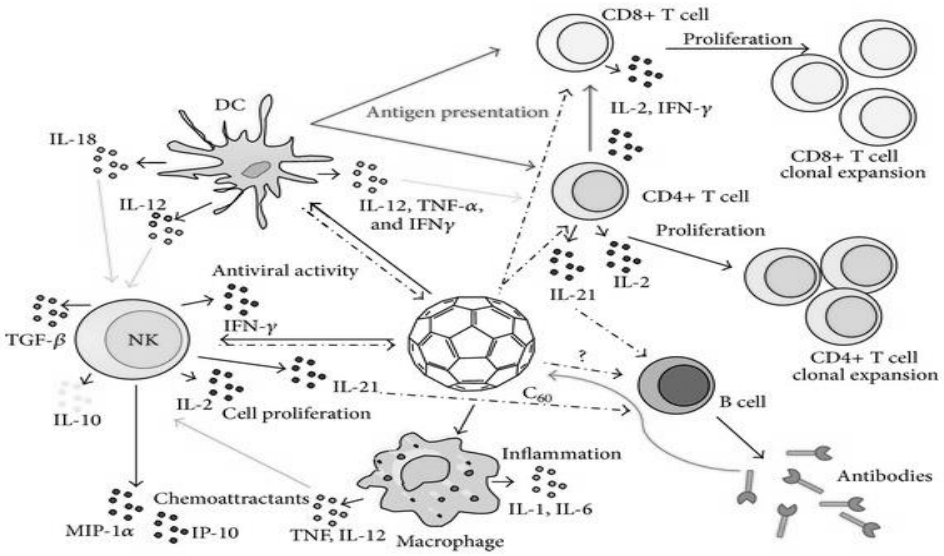
There are many different types of cells located in the intestine, all with a unique purpose. A few examples include: enterocytes that are responsible for nutrient absorption, goblet cells which secrete protective mucus, paneth cells that produce antimicrobial peptides and enteroendocrine cells that produce different hormones (Gerbe et al., 2012). Changes in cell concentrations may be closely related and help explain observed differences in villus height or crypt depth. For examples, an observed increase in villi height combined with shallow crypt depth and greater concentrations of goblet cells are all considered indicators of improved cell health and greater nutrient absorption.

The intestinal epithelial cell integrity serves as the major selective barrier of the gut, protecting the rest of the body from pathogenic invasion (Jacobi and Odle, 2012). All the stressors of weaning lead to a compromised gut barrier function and inability to properly protect the body from invading pathogens (Moeser et al., 2007). Disease challenge studies have looked

at specific markers as indicators of inflammation, antigen presence, and overall health (Bhandari et al., 2010; Kim et al., 2019). These markers are often recovered from blood samples or intestinal mucosal scrapes (Moeser et al., 2007; Jaworski et al., 2017). Evaluating specific markers in both the blood and intestine provides indications of both systemic and localized immunity, relating to gut barrier function and overall immune status of the pig. Each marker is unique and is thought to reflect specific changes in immunity or health status, yet they all function in unison relative to their role in immunity and inflammatory response, as depicted in Figure 1.3. Some may be an indicator of a recovering, healthy pig when in low concentrations, others at increased concentrations. A few commonly evaluated markers are interleukin 6, (IL-6), 10 (IL-10), and 12 (IL-12), in addition to immunoglobulin A (IgA), tumor necrosis factor alpha (TNFα), and intestinal fatty acid binding protein (iFABP) (Lee et al., 2014; Jaworski et al., 2017; Zhang et al., 2017). While IL-12 is a pro-inflammatory marker, IL-10 is an anti-inflammatory marker and IL-6 has both pro and anti-inflammatory contributions (Giudice and Gangestad, 2018). These three markers are involved in acute and chronic immune response, and interact with several other markers in the body, suppressing some, while inducing others. For example, IL-6 stimulates the production of acute phase proteins such as serum amyloid A, while inhibiting the release of TNFα (Giudice and Gangestad, 2018). Tumor necrosis factor alpha plays a similar role in acute phase response as it is involved in inducing fever and ultimately leading to insulin resistance (Wong et al., 2001). Immunoglobin A is an antibody that helps protect the body from harmful microbes and serves as an indicator of mucosal immunity and improved immune function (Lee et al., 2014). On the other hand, iFABP is recognized as an indicator of damaged mucosa, with greater iFABP concentrations correlated to greater mucosal damage (Berkeveld et al., 2008). Because of weaning, this complex of immunity markers are responding as a necessary

to all the stressors being experienced by the pig. These responses may lead to short- or long-term impacts on the pig's health and performance throughout its lifetime (Moeser et al., 2017).

Figure 1.3. Diagram depiction of acute phase inflammatory response, showing various roles of specific cytokines (Petrovic et al., 2015).



Intestinal morphology and immunological markers are addressed in chapter 3 of this thesis under objective 2, regarding the impact of *Bacillus subtilis* on these health-related indicators.

2.5 Altered Digestibility of Dietary Nutrients

Negative changes to both the intestinal morphology and gut barrier function are directly or indirectly related to the pig's ability to digest and absorb available nutrients. Upon weaning, decreased villus height, increased crypt depth, and altered gut barrier function have been linked to decreased utilization of dietary nutrients including gross energy (GE), nitrogen (N), and specific amino acids (AA) (Sohn et al., 1994; Lee et al., 2014). Greater digestibility of GE is

associated with more energy being utilized by the body for both maintenance and growth. During weaning, a decrease in GE digestibility may occur as the result of shorter villi in the small intestine, decreasing total surface area available in the gut (Awad et al., 2009). In the same way, N digestibility may be reduced, which is used as an indication of relative CP digestibility (NRC, 2012).

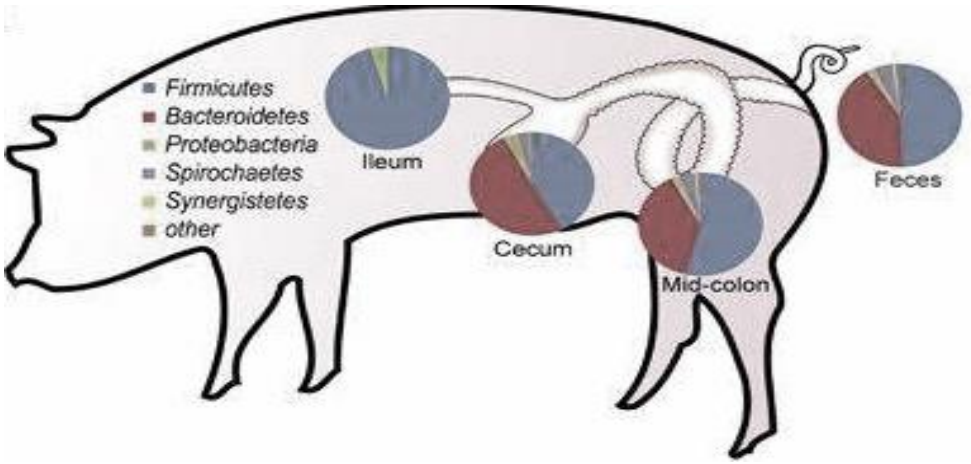
Additionally, weaning stressors may lower the production of volatile fatty acids (VFA) (Giang et al., 2012). Short chain fatty acids, also referred to as VFA represent fatty acids containing one to six carbons (Wong et al., 2006). In the nonruminant, VFA are produced primarily by the microbiota in the large intestine via microbial fermentation of fiber and other undegraded nutrients (Wong et al., 2006). Several studies have evaluated VFA in the feces, or within specific sections of the large intestine of pigs (Kim et al., 2013; Kanengoni et al., 2015). Three major VFA include acetate, butyrate, and propionate, all playing important roles in nonruminant nutrition and metabolism, including their roles as secondary energy sources (Wong et al., 2006). Therefore, weaning can significantly impact the digestibility of specific nutrients, leading to nutritional deficiencies, and requiring expensive, easily digestible diets to be fed during the first few weeks post-weaning. In this thesis, the impact of *Bacillus subtilis* on specific nutrient digestibility will be addressed in chapter 2, under objective 1.

2.6 Intestinal Microbiome

During the pig's lifetime, changes occur in the growth and development of the intestinal microbiome. The intestinal microbiome is simply the community of microorganisms that reside within the GIT, which in mammals has been estimated to be composed of nearly 10^{14} bacteria, ten times greater than the number of eukaryotic cells present in the body (Isaacson and Kim,

2012). The numerous bacteria forming the microbiome help provide necessary functions that the pig cannot perform on its own including production of vitamin K, recycling of bile salts, and fatty acid production in the colon (Isaacson and Kim, 2012). Two separate studies have identified the changes that occur in the microbiome of different GIT segments, as depicted in Figure 1.4 (Looft et al., 2014; Isaacson and Kim, 2012). Nearly 90% of bacteria examined in the fecal material collected from growing pigs belonged to two different phyla, *Firmicutes* and *Bacteroidetes* (Isaacson and Kim, 2012). Within the GIT, unique clusters of bacteria were found in specific regions. The cecum and colon populations contained mostly bacteria of those two phyla, the jejunum was almost entirely of the *Firmicutes* phyla and the ileum consisted of a third phyla, *Proteobacteria* (Looft et al., 2014; Isaacson and Kim, 2012). Differences in species found within sections of the small intestine may be correlated to unique purposes and functions each bacterial species serves within the GIT, as well as how they interact with the rest of the microbiome.

Figure 1.4. Phylum level differences within specific segments of the gastrointestinal tract of 3-month-old growing pigs (Looft et al., 2014).



The above studies suggest that specific microbes dominate different segments of the GIT, suggesting that a particular balance in the microbiome population is preferred or of greater benefit to the pig (Looft et al., 2014; Isaacson and Kim, 2012). Microbial diversity is also of great interest in today's research related to the intestinal microbiome. Greater diversity is often associated with a healthier pig, due to a more stable GIT environment (Davis et al., 2007).

When interpreting microbial diversity, there are two types of diversity often analyzed, alpha and beta diversity. Alpha diversity is a measure of the microbial diversity within a given community, and beta diversity measures diversity between communities (Lozupone and Knight, 2008). Bhandari et al. (2008) studied the ileal microbiota of DFM supplemented pigs, challenged with *E. coli*. They observed changes in alpha diversity at the phylum level, with challenged pigs having a decrease in *Firmicutes* and increase in *Bacteroidetes* bacteria, relative to the negative control. Another study analyzed beta diversity of microbiota within the ileum and cecum when supplementing different xylanases to either corn or wheat-based diets of growing pigs. Differences in beta diversity between diets were found, with wheat-based differing from corn-based, as well as between segments, with the cecum having a greater diversity than the ileum (Zhang et al., 2018). Both types of diversity may be measured either qualitatively, by determining the presence or absence of specific bacteria, or quantitatively in which the number of times each bacteria was found is also recorded in addition to presence/absence (Lozupone and Knight, 2008). For example, based on the results of Looft et al. (2014), a qualitative or quantitative increase in bacteria belonging to the *Firmicutes* phyla, would be beneficial and help restore the preferred balance of the ileal microbiome.

Among the studies evaluating the microbiome, populations or communities are often described quantitatively and qualitatively. Excreta or digesta may be collected to confirm the presence of the DFM species within specific segments of the GIT, analyze differences occurring in the microbiome, or help determine how well the DFM colonizes within the GIT (Leser et al., 2008). Depending on the aim of the study, microbiota may be measured solely in the feces (Lee et al., 2014; Upadhaya et al., 2017), or within specific GIT segments upon euthanasia (Leser et al., 2008). Challenge studies may evaluate fecal microbiota to confirm a decrease in relative concentrations of a specific pathogen, such as *Salmonella* or *E. coli*, over the course of time (Upadhaya et al., 2017; Kim et al., 2019). Analyzing the microbiota can be very difficult and expensive depending on the desired outcomes, and analyzing microbiota at the species level can be challenging, as unique strains of bacteria of the same species may be difficult to distinguish within the pigs GIT (Davis et al., 2007).

While the microbiome of a grown pig is very diverse, the newborn pig has little to no microbiome at birth. However, as they encounter beneficial bacteria, their microbiome will develop and serve greater purposes including greater immunity and disease resistance. Recent research demonstrated how a milk-based diet in young pigs selectively gives advantage to certain microbial populations, leading to a small number of dominant bacterial groups in the GIT (Frese et al., 2015). As pig's age and transition from weaning into the nursery, changes in diet and other stressors alter the microbiome (Issacson and Kim, 2012). These changes often result in a time period of vulnerability and negative impact on the GIT.

Changes within the microbiome upon supplementation of a multi-strain *Bacillus subtilis*-based DFM, will be addressed in chapter 3 of this thesis, under objective 2.

2.7 Antibiotics and Alternatives

To help pigs cope with the stress of weaning, the swine industry has depended on the use of feed-grade antibiotics, providing benefits to both growth performance and health status (Cromwell, 2002). Antibiotics have been estimated to improve growth rate by as much as 25 to 30% and feed efficiency by 12 to 15%, while reducing morbidity and mortality by over 50% in some cases (Cromwell, 2002). However, in more recent years, negative effects of overusing antibiotics have become a major concern to the general public. As a result, the European Union and the United States have recently banned the use of antibiotic as growth promoters in 2006 and 2017, respectively (Aarestrup et al., 2010; Schultz and Rademacher, 2017). Moving forward, weanling pig research has evaluated multiple alternative ways to cope with weaning stress, while avoiding the use of antibiotics. Among the antibiotic alternatives available today, the most common include DFM (Augspurger et al., 2016), dietary acidifiers (Kil et al., 2011), dietary enzymes (Thacker, 2000), fatty acid supplements (Rossi et a., 2010), and nutraceuticals such as copper and zinc (Thacker, 2013). While each class of antibiotic alternatives may be beneficial, their outcomes have been variable, with some showing more effective results than others (Walsh et al., 2007; Zhang et al., 2014; Augspurger et al., 2016).

3. Direct-Fed Microbials

The names DFM and probiotic are used interchangeably. They can both be defined as live microbials supplemented in the diet that are beneficial to the host (Zimmermann et al., 2001). As opposed to antibiotics which directly targets and eliminate specific microorganisms, DFM act more broadly via several mechanisms (Buntyn et al., 2016). In contrast to antibiotics,

DFM are live microorganisms that adapt to their environment. While antibiotics have clear and effective results, the effectiveness and specificity of DFM on health is still unclear (Walsh et al., 2007). Probiotics were suggested to potentially replace dietary short chain fatty acid supplementation (Jacobi and Odle, 2012). With this notion in mind, DFM may share similar mechanisms as fatty acids in the colon including reducing diarrhea via electrolyte uptake, increasing cell proliferation and intestinal surface area, and upregulating mucosal transporters (Jacobi and Odle, 2012).

Feeding a *Bacillus subtilis*-based DFM led to an increase in the diversity of microflora relative to a control diet in one study (Davis et al., 2007). In the same study, strains of uncultivable gram-positive bacterium were identified in the jejunum, suggesting that supplementing DFM may create new stains of bacterium upon interaction with the microbiome. In addition, lower concentrations of disease fighting agents such as sulfated mucins and T lymphocytes were found. It was suggested that these changes in the gut could lead to an increase in immunity, physiological development, and nutritional efficiency. These results give a slight glimpse of the potential impact of DFM on a pig's microbiome. Some DFM appear more effective than others, with specific species standing out as possessing desired qualities (Larsen et al., 2014).

In this thesis, the objective is to test the general hypothesis that pigs supplemented with a multi-strain *Bacillus subtilis*-based DFM will have greater digestibility of specific nutrients, and positive changes in health-related indicators.

3.1 Bacillus subtilis

Bacillus subtilis, one of the many bacterial species within the *Firmicutes* phyla and *Bacillus* genus, has been used as a feed additive in livestock industry for several years and its efficacy and mechanism of action remain unclear. This unique, gram-positive bacteria species is known as saprophytes because it is found in nature and is commonly associated with soil, water, dust, and air (Mingmongkolchai and Panbangred, 2017). According to Stein (2005) there are several hundred *Bacillus subtilis* strains of bacteria. *Bacillus subtilis* has a unique structure, containing both hydrophobic and cyclic structures, in addition to containing unusual constituents including D-amino acids and making it resistant to hydrolysis by peptidases and proteases (Stein, 2005).

Bacillus subtilis is one of a few types of bacteria able to form endospores. Endospore formation is a unique function in which the bacteria are able to form a small, highly resistant form containing the bacterium's whole genome (Higgins and Dworkin, 2012). *Bacillus subtilis* forms endospores when experiencing extreme environments in which it would otherwise not survive. Endospore formation is triggered by changes in the environment and initiated by phosphorylation of different kinases, leading to a cascade of structural changes within the cell (Higgins and Dworkin, 2012). Endospores are able to survive UV radiation, extreme heat and pH, and damaging chemicals for extensive lengths of time and transform back into vegetative cells when exposed to an environment in which they are able to grow and reproduce (McKenney et al. 2013). Therefore, endospores are viable in a gastric environment whereby the pH can be as low as 2 (DeRouchey et al., 2009). Endospore formation could be one of the key explanations as to how *Bacillus subtilis* is able to bypass the stomach and function in the small and large

intestine of weanling pigs. While each strain may differ, according to Isaacson and Kim (2012) as a member of the *Firmicutes* phyla, *Bacillus subtilis* is mainly located and active within the jejunum, cecum, and colon.

Several species of *Bacillus subtilis* have been shown to display varied effects (Larsen et al., 2014), including pathogenic inhibition, cellulase and xylanase production, biofilm formation, sporulation ability, and heat resistance of spores (Larsen et al., 2014). In comparison to the other species, *Bacillus subtilis* had one of the greatest inhibition abilities on *Escherichia coli*, *Staphylococcus aureus, Salmonella typhimurium* and *Clostridium perfringens*. Additionally, *Bacillus subtilis* was one of the few species having greater amounts of cellulose degrading enzymes. Spore formation in *Bacillus subtilis* is consistently above 10% and occasionally as high as 95%. It is less heat resistance and exhibits less biofilm formation than other species (Larsen et al., 2014). When combining all the factors, *Bacillus subtilis* emerged as one of the top candidates for use as a DFM in livestock diets.

In this thesis, *Bacillus subtilis* is the species of interest as the bacteria contained in the multi-strain *Bacillus subtilis*-based DFM researched by Augspurger et al. (2016) that is known to provide benefits to weanling pig growth performance.

3.2 Proven Benefits of Bacillus subtilis

Single specific strain of *Bacillus subtilis*, multiple strains or with mixed cocktails made of several different species of phyla of bacteria have been studied in trials focusing on evaluating a combination of growth performance, nutrient digestibility, intestinal morphology, and/or immunological markers.

Lee et al. (2014) looked at a single strain *Bacillus subtilis* supplement for 21-d old pigs. The DFM was fermented in a cultured broth with citrus juice waste product and fed at different concentration of 0, 1.5, 3.0, 4.5 g/kg diet over a 28-d period. Pigs supplemented with *Bacillus subtilis* at 4.5 g/kg had greater ADG and G:F, greater digestibility of dry matter (DM), CP, and GE, as well as increased villus height and serum immunoglobin G (IgG) and IgA, with no change in mineral digestibility. Upadhaya et al. (2017) conducted a study in which weanling pigs were challenged with *Salmonella*. Sampling the blood at 12 hr post infection they observed increases in red blood cells and lymphocytes, in addition to greater concentrations of serum immunoglobins IgG when supplementing with *Bacillus subtilis*. Tang et al. (2019) used 25-d old pigs during a 42-d trial with two different concentrations of CP, with or without *Bacillus subtilis* supplementation. Apparent total tract digestibility of ether extract and CP were greater when pigs were fed *Bacillus subtilis* and lower dietary CP, with no change in calcium and phosphorus digestibility. In that same study, the acetic acid concentration in ileal digesta was greater for pigs fed lower protein, combined with *Bacillus subtilis*, with no change in propionate and butyrate concentrations.

Two other groups have studied single strain *Bacillus subtilis* DFM in different aged pigs (Hu et al., 2016; Blavi et al., 2018). Hu et al. (2016) evaluated nursing piglets at seven days of age. When supplementing milk replacer, piglets suffering from intrauterine growth restriction provided with *Bacillus subtilis* had improved G:F and greater enzyme activities of maltase and sucrase in the jejunal contents at 21 d of age. Blavi et al. (2018) evaluated *Bacillus subtilis* fed to growing pigs weighing an average of 23 kg. After the trial, pigs supplemented with *Bacillus*

subtilis had improved GE digestibility, but no indication of changes to apparent ileal and hindgut digestibility of CP or individual AA.

Together, these studies suggest significant changes in growth performance, greater utilization or digestibility of specific nutrients including GE and CP, as well as increased villi height, greater enzyme activity, and positive changes in serum or plasma markers of pigs supplemented with a single strain of *Bacillus subtilis*.

Combinations of multiple strains of *Bacillus subtilis* or combinations of various species, as DFM mixtures or 'cocktails,' have been studied. Cai et al. (2015) and Jaworski et al. (2017) both fed three-week-old pigs a three-strain *Bacillus*-based DFM consisting of one strain of *Bacillus subtilis* and two strains of *Bacillus amyloliquefaciens*. Cai et al. (2015) reported improved apparent total tract digestibility of N and reduced blood urea nitrogen, suggesting improved protein utilization. Jaworski et al. (2017) observed that the DFM had no impact on short chain or branched chain fatty acids in cecal or rectal contents. Giang et al. (2012) assessed digestibility in 26 to 28 d-old weaned piglets fed a diet supplemented with a mixture of lactic acid bacterium alone or in combination with *Bacillus subtilis* and *Saccharomyces boulardii*. The complex supplement contained *Enterococcus faecium, Lactobacillus fermentum, Lactobacillus acidophillus,* and *Pediococcus pentosaceus*. Apparent ileal and total tract digestibility of organic matter and CP were improved with no difference seen in crude fiber digestibility. All DFM mixtures increased intestinal lactic acid and total VFA concentrations (Giang et al., 2012). Lan et al. (2017) observed that a multiple-microbe probiotic (*Bacillus coagulans, Bacillus licheniformis, Bacillus subtilis and Clostridium butyricum*) increased digestibility of GE, but not DM or nitrogen in weanling pigs fed from 28 to 70 d of age.

In order to gain a better understanding of the mode of action of *Bacillus subtilis* within the GIT of a young pig, Leser et al. (2008) labeled *Bacillus subtilis* with short pieces of dialysis membrane to track their location and physical structure, being either vegetative cell or endospore, within the GIT. The premises for their work was first, that as *Bacillus subtilis* is a soil microbe, it is also an anaerobe and performs anaerobic respiration and fermentation, which may give it the capabilities of surviving and germinating into a vegetative cell within a pig's GIT; second, that vegetative cells can secrete enzymes and antimicrobials to aid the host in digestion and other processes; and third, that endospores would have the capabilities of competing against pathogenic bacteria and making changes to the pig's immune system. To test their hypothesis, several experiments were conducted in which pigs were separated by treatment and sampled to compare endospores or vegetative cells in each part of the GIT over the course of time. It was reported that endospores were able to germinate within the stomach. While endospores can survive for an extended length of time in harsh environments, their vegetative cells are much less viable; thus decreasing the potential impact of *Bacillus subtilis* in the small and large intestines. These results also suggested that the bacteria were short lived, with decreasing quantities found in the feces as supplementation of the DFM was terminated (Leser et al., 2008). *Bacillus subtilis* appeared to function in endospore form in the GIT but is not able to permanently reside within the GIT, requiring continual supplementation in order to reap the benefits it may provide. Leser et al. (2008) labeled *Bacillus subtilis* as an allochthonous population, one that is a nonresident and simply passes through or serves more as an opportunistic colonizer than a permanent resident of the GIT. However, this may differ between unique strains of *Bacillus subtilis*.

3.3 Bacillus subtilis Mode of Action

Several mechanisms, or modes of action within the host have been proposed for *Bacillus subtilis* supplemented as a DFM. As there are many different strains, each strain may act slightly different, expressing one or multiple mechanisms.

Many researchers agree that *Bacillus subtilis* may act through enzyme secretion. Some of the enzymes that *Bacillus subtilis* is known to secrete include: α-amylase, arabinase, cellulase, dextranase, levansucrase, maltase, alkaline protease, neutral protease, and β-glucanase (Priest, 1977). Additional enzymes have been postulated including specific pectinases and xylanases (Blavi et al., 2018). Increase in apparent total tract digestibility of energy due to the ability of *Bacillus subtilis* to secrete α-amylase, which helps breaks the glycosidic bonds in starch was reported (Blavi et al., 2018). Alternatively, increases in energy utilization may be the result of increased digestion of fiber due to other enzymes secreted by *Bacillus subtilis* including pectinase and xylanase (Blavi et al., 2018). Other researchers attribute changes in growth performance and CP digestibility to the proteases produced by *Bacillus subtilis* (Tang et al., 2019). Other enzymes may aid in the breakdown of non-starch polysaccharides present in soybean meal and other common swine feed ingredients (Upadhaya et al., 2015; Zhang et al., 2017). Increased apparent whole tract digestibility and apparent ileal digestibility of organic matter and CP may be due to production of another unknown enzyme by *Bacillus subtilis* (Giang et al., 2012).

Synthesis of antimicrobials is another potential *Bacillus subtilis* mode of action (Hong et al., 2005). *Bacillus subtilis* is known to synthesize several antimicrobials including different lantibiotics, bacilysocin, and amicoumacins (Stein, 2005). The effectiveness however of each

strain to produce these antimicrobials within the host varies greatly and cannot be accurately replicated *in vitro*.

Another mechanism considered by researchers is that *Bacillus subtilis* may function by competitively excluding pathogenic bacteria (Cai et al., 2015). For instance, many studies observe decreases in ammonia concentrations (Samanya and Yamauchi, 2002; Cai et al., 2015; Upadhaya et al., 2015; Prenafeta-Boldu, et al., 2017), which is potentially due to *Bacillus subtilis* limiting growth of pathogenic populations in the hindgut that metabolize nitrogenous compounds. Samanya and Yamauchi (2002), reported increased villus height in the duodenum and ileum when supplementing *Bacillus subtilis* and suggested that the observed changes was due partly due to a decrease in the population of urease producing bacteria and by doing so, decrease ammonia content in the GIT. Because ammonia is toxic to intestinal cells, it was postulated that decreased production would lead to increased cell mitosis and thereby, increased villus height (Samanya and Yamauchi, 2002). By competitively excluding harmful pathogens, *Bacillus subtilis* helps in balancing the microbiome and increasing the animal's overall health.

Greater production of VFA in one study resulted from increased fermentation in the hindgut, leading to greater utilization of CP and fiber (Prenafeta-Boldú et al., 2017). Volatile fatty acids have significant effects on health and nutrient utilization, serving as substrates for different peripheral tissues, helping decrease pathogenic load, and leading to increased cell proliferation (Tang et al., 2019; Jaworski et al., 2017). These increases in fermentation are likely the result of *Bacillus subtilis* acting to alter the microbial population (Jaworski et al., 2017). Bhandari et al. (2008), conducted a challenge study looking at the effects of supplementation of *Bacillus subtilis*, with *E. coli* infection, and observed changes in microbe populations,

specifically gram-positive bacteria. *Bacillus subtilis* being a gram-positive bacterium acts mostly on other gram-positive bacterium (Bhandari et al., 2008). As they function very similarly, *Bacillus* bacteria may outperform *Lactobacillus* bacteria in the GIT, limiting substrate availability and growth of *Lactobacillus* and other similar gram-positive bacteria (Bhandari et al., 2008).

One final mechanism often considered is that of immune stimulation. *Bacillus subtilis* is often evaluated in combination with an intentional immunological challenge studies in which different measures are taken to determine the effects of *Bacillus subtilis* on pigs recovery rate (Upadhaya et al., 2017; Kim et al., 2019). Several serum markers are associated with immune function and are potential indicators of reduced stress and improved growth performance (Lee et al., 2014). One study reported decreased β-conglycinin in weanling pigs when supplementing *Bacillus subtilis* (Zhang et al., 2017). Pigs with decreased β-conglycinin had greater average daily feed intake (ADFI) and growth, and a reduction of serum proinflammatory cytokines interleukin 4 and 6 (IL-6) and increase in anti-inflammatory cytokine interleukin 10 (IL-10) (Zhang et al., 2017). Changes in morphology, such as greater villus height, indicate improved gut health and nutrient absorption (Lee et al., 2014). On the other hand, greater crypt depth may be the body's response to an infection, attempting to repair the mucosal cell damage caused by the infection (Walsh et al., 2007). Increased ileal villus height may be an indicator of *Bacillus subtilis* stimulating the immune system, but also be related to other previously mentioned mechanisms including synthesis of enzyme or antimicrobials and competitive exclusion of other bacteria. Studies looking at responses to direct immune challenges or pathogenic challenges such as *Escherichia coli*, have observed significant decrease in diarrhea and shortened recovery

windows, when supplementing *Bacillus subtilis* (Bhandari et al., 2008; Buntyn et al., 2016; Kim et al., 2019).

As previously stated, these mechanisms are possibly intricately tied together, leading to common outcomes such as increased villus height, greater feed conversion, and improved nutrient utilization. For this reason, defining the specific mechanism by which *Bacillus subtilis* is working can be quite challenging, and requires a well-designed study and collection of various samples. Of all the different designs and approaches, no one study is completely conclusive. Many studies conclude that more research is needed or the mechanism should be examined more closely (Feng et al., 2007; Walsh et al., 2007; Cai et al., 2015; Upadhaya et al., 2017). As each product has a unique combination of different bacteria species and strains, multiple, varied designs may be required to fully elucidate mechanisms of that specific DFM. In this thesis objectives presented in chapters 2 and 3 focus on evaluating nutrient digestibility and health related indicators, in order to discover and define the specific mechanism(s) by which a multi-strain *Bacillus subtilis*-based DFM functions within the GIT of weanling pigs.

4. Conclusion

There are many factors associated with weaning that all lead to disease susceptibility and decreased growth performance within the first few weeks of moving pigs to the nursery (Pluske, 2013). Direct-fed microbials have become an important area of research as the industry shifts away from using antibiotics. *Bacillus subtilis* has shown promising use as a DFM, because of the reported benefits to weanling pig health and performance (Augspurger et al., 2016; Kim et al., 2019). Of the potential mechanisms by which DFM function within the GIT, each strain or combination may differ. Intestinal digestibility of nutrients, morphology, immunological

markers, and the microbiome were highlighted and discussed to prepare the reader in understanding the study presented in this thesis.

CHAPTER 2

EFFECTS OF A MULTI-STRAIN *BACILLUS SUBTILIS*-BASED DIRECT-FED MICROBIAL ON WEANLING PIG GROWTH PERFORMANCE AND NUTRIENT DIGESTIBILITY

Abstract

A study was conducted to evaluate the effects of a multi-strain *Bacillus subtilis* based direct-fed microbial (DFM) on apparent nutrient digestibility of nursery pigs. Eighty pigs, of equal number of barrows and gilts (initial BW: 7.0 ± 0.60 kg), were weaned at 21 ± 1 d and randomly allotted to sixteen pens, with five pigs per pen. Two dietary treatments were implemented, a basal control (CON) and a control plus DFM (DFM). Both diets were corn, soybean meal, and distillers dried grains based and were formulated to meet or exceed requirements for all nutrients and manufactured on site. Diets were fed for 42 d and growth performance measures were recorded weekly. On d 21 and 42 of the experiment, one pig per pen, with equal number of males and females represented, was randomly selected and euthanized. Digestibility of specific nutrients was evaluated within the duodenum, jejunum, ileum, ascending and distal colon. There were no differences in growth performance between DFM and CON. Overall means ± SD were 0.51 ± 0.05 kg/d, 0.79 ± 0.05 kg/d and 0.66 ± 0.05 for ADG, ADFI, and G:F, respectively. Digestibility of tryptophan within the jejunum tended (P = 0.06) to increase with addition of DFM, as did cysteine (P = 0.12) and methionine (P = 0.10). The analysis also suggested that the impact of the DFM on the digestibility of amino acids may be early in the nursery phase. The pH of contents in ascending colon, a possible indicator of varied fiber digestion, did not differ. Likewise, no differences were observed between treatment in apparent total tract nitrogen and energy digestibility (analysis of distal colon contents). The

addition of a multi-strain *Bacillus subtilis*-based DFM appears to impact select amino acid digestibility depending upon location in the gastrointestinal tract.

Introduction

With the increasing concerns regarding antibiotic resistant bacteria decreasing antibiotic effectiveness in humans, the European Union and the United States have recently banned the use of antibiotic as growth promoters (Aarestrup et al., 2010; Schultz and Rademacher, 2017). With these new restrictions, the industry is investigating alternative ways to achieve the health and performance benefits associated with antibiotics. One alternative is direct-fed microbials (DFM) (Chen et al., 2005). A four-strain *Bacillus subtilis*-based DFM was developed and shows to increase nursery pig performance with of 5-10% greater gain and up to 5% lower feed/gain ratio (Augspurger et al. 2016). While there are several previous studies involving either a single-strain *Bacillus subtilis* or multiple strains within the *Bacillus* genus, there are no published studies involving multiple strains of this specific species. Other research involving feeding of *Bacillus subtilis*-based DFM has been shown to positively impact pig growth performance (Kim et al., 2019) and nutrient digestibility (Lee et al., 2014; Blavi et al., 2018). The specific objective of this study was to evaluate the effect of a multi-strain *Bacillus subtilis*-based DFM on specific nutrient digestibility of the 21-d old weanling pig. The hypothesis was that pigs supplemented with *Bacillus subtilis* have greater digestibility of nitrogen (N), amino acids (AA), gross energy (GE), and increased production of volatile fatty acids (VFA) in different segments of the GIT.

Materials and Methods

Animals, Housing, and Experimental Design

The Institutional Animal Care and Use Committee at Michigan State University reviewed and approved the protocol for this experiment. The animal study was structured as a complete randomized design based on litter, weight, and sex and conducted between the months of August and September 2019 at the Michigan State University Swine Teaching and Research Center. Eighty crossbred pigs (PIC 359 x Yorkshire) equally balanced by sex were weaned at 21 ± 1 d (7.0 ± 0.6 kg, initial BW) and randomly allotted into sixteen (1.22 × 1.83 m) cohorts with five pigs per cohort. Cohorts were randomly allotted to pens located in one of four mechanically-ventilated identical nursery rooms. Cohort allotment was based on litter (dam), weight, and sex; maintaining a varied distribution of weights of pigs in each pen. Lastly, one of the two treatments was assigned to each pen. Each pen held five pigs, half of the pens within each treatment contained three gilts and two barrows, and half contained three barrows and two gilts. Pens were equipped with round-rod steel flooring, vertical-rod, fiberglass fencing and gates, single-sided two-hole feeders, and one nipple drinker. Rooms were operated on an all-in/all-out system and were disinfected using bleach (15.6 mL/L) two to five days before pigs were placed in the pens. All dams were vaccinated pre-breeding for parvovirus, leptospirosis, and erysipelas. Processing of newborn pigs on days one and two included: ear notching, tail docking, and 1.0 mL iron dextran (200 mg/mL). All pigs received an additional 1.0 mL iron dextran (200 mg/mL) between d 7 and 10 and males were castrated. Upon weaning, pigs were vaccinated for the prevention of circovirus and erysipelas. All water nipples had a flow rate of 25 ± 1 mL/sec.

Diets and Feeding

Two dietary treatments were used: a control diet with no DFM supplementation (CON) and diet with supplementation of a multi-strain *Bacillus subtilis*-based DFM (DFM) at an inclusion rate of 0.5 g/kg of feed. Treatments were imposed over three dietary phases (d 0-7, 7-14, and 14-28) with d 0 representing the day of weaning (21 ± 1 d of age). All diets were formulated using recommendations made available by Kansas State University and based on values published by the NRC (2012) (Menegat et al., 2019) and were mixed at Michigan State University swine farm using a 113-kg paddle ribbon mixer (Table 2.1). All micro ingredients were pre-mixed in the Michigan State University non-ruminant nutrition laboratory, prior to mixing with macro ingredients. Dietary Cu and Zn were set at or slightly above levels suggested by NRC (2012).

Analysis of Feed

Feed analysis was completed for each dietary phase (Table 2.2). Samples were prepared as described below, and then shipped to the University of Missouri Experimental Station Chemical Laboratory (Columbia, MO) for analysis. Feed samples shipped included one sample per treatment, per phase, both a composite sample from the mixer rations as well as a composite of the complementary diets taken from individual feeders. Phases two and three had additional samples including titanium dioxide (0.1%), making a total of twenty analyzed feed samples. The following analyses were performed: Dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose, cellulose, lignin, and total dietary fiber (TDF), crude fiber, N, ether extract, individual AA Ca and P, and ash. Analyses were performed by the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO) in accordance

to the standard methods of AOAC (2007). Neutral detergent fiber was determined by use of neutral detergent and heat stable amylase according to the methods of Van Soest et al. (1991). Hemicellulose was calculated as NDF-ADF. Titanium (Ti) in the feed was calculated according to Myers et al. (2004). Individual AAs were determined in accordance with the standard methods of AOAC (2007).

Data Recording and Sample Collection

Weekly performance data was collected by weighing each pig individually each wk and averaging weights within each pen to measure for average daily gain (ADG). Feed disappearance was measured by vacuuming out remaining feed of pens feeder and subtracting that from total weekly feed additions to calculate average daily feed intake (ADFI). Feed efficiency (G:F) was calculated by dividing ADG by its corresponding ADFI.

At the end of wk 3 and 6, one pig per pen, with consideration of sex, was euthanized for analysis of nutrient digestibility and pH of ascending colon content. One pig was sacrificed at a time alternating between CON and DFM. Prior to euthanasia, pigs were sedated using Telazol-T (2.5 mg/kg); Ketamine-K (1.25 mg/kg); and Xylazine-X (1.25 mg/kg); used as TKX (0.025 mL/kg) in a single intramuscular injection with a 22 G needle. Pigs were then euthanized via sodium pentobarbital (1 mL/4.5 kg) in a single intra-cardiac injection with an 18 G needle.

Upon euthanasia pigs were opened lengthwise and the cecum was located. Immediately anterior to the cecum, the ileum was tied with string and cut along the mesentery for approximately 50 cm. Digesta anterior to this section, yet within the ileum was manually pushed into the 50 cm section before tying off the other end and removing that section of the ileum. The ascending colon was then removed in a similar manner, tying off immediately posterior to the

cecum and measuring a 50 cm section, tying off the other end and removing the whole ascending colon, stretching it lengthwise by cutting along the mesentery. The distal colon segment was collected by tying off both ends and removing the entire distal colon, from the rectum to the beginning of the descending colon. This section was about 20 cm in length. The jejunum was located by beginning immediately anterior to the section of removed ileum and cutting along the mesentery for approximately 8 m to be certain the mid-jejunum had been located. Jejunum tissue was tied off in this location and a second tie was placed 30 cm anterior. Jejunal digesta anterior to this section was moved manually into this 30 cm section similar to that performed in the ileum. The duodenum was located by first locating the distal end of the stomach and tying off the start of the duodenum. A second tie was placed approximately 20 cm posterior to the first and this section of the duodenum was removed.

Upon removal of each individual segment, digesta was collected from each of the five sections (duodenum, jejunum, ileum, ascending and distal colon) for the analysis of GE, N, VFA, complete AA profile, and Ti. All digesta samples were collected into labeled, plastic 50 mL tubes. Ileal, jejunal, and ascending colon digesta were collected into three separate tubes. One tube was designated for AA and N analysis, another for Ti and GE, and a third for VFA. Digesta was removed by cutting off one of the tied ends and gently stripping the digesta lengthwise from the tissue into each tube proportionally. Both the duodenum and distal colon were limited in available digesta. For this reason, little to no digesta was collected from the duodenum. Contents of the distal colon were collected using the same technique as described above with only small amounts collected for some pigs. For those, the small amount was placed into a single tube for

GE, nitrogen, and Ti analysis. Following collection, each tubes cap was wrapped in paraffin paper and placed on ice, and then frozen at -20º C until further analysis.

The pH was collected from the ascending colon content. After removal of digesta, the pH was recorded from one of the 50 mL tubes containing digesta from the ascending colon by placing the pH probe directly into the tube. Following each measurement, the pH probe was cleaned with distilled water.

Analysis of Digesta

Samples of digesta were prepared for specific analysis of GE, nitrogen, Ti, individual AA, and VFA. All samples were freeze dried (HarvestRight 115V, 3/4HP Salt Lake City, UT) except for those used to evaluate VFA. Immediately after being removed from the freezer, whole tube weights were recorded and tubes were thawed placing digesta in appropriately labeled weigh boats or whirl pack baggies to increase surface area when in the freeze drier. Samples were then refrozen before placing in the freeze drier. After complete drying of individual samples, they were finely ground using a Willey mill micro grinder (Swedesboro, NJ) with a one mm sized screen.

Titanium in the digesta was calculated on site using an adjusted protocol based on that of Myers et al. (2004). Samples were weighed (150 mg) into a 100 mL Digesdahl flasks in guidance with the expected protein values being between 21-25%. Four mL of concentrated H_2SO_4 was added to each flask, swirled to cover all digesta and kept over-night to digest. Flasks were then placed on a Digesdahl burner (Model 23130-20, Loveland, CO) and vacuum system to boil the acid for six minutes, followed by the addition of 10 mL of 50% H_2O_2. After completely burning off H_2O_2, flasks were cooled and then diluted to the 100 mL mark with distilled water. Upon

water dilution to the 100 mL mark, 160 µL of standards and individual samples were transferred to micro plates in duplicate. Standard concentrations used for Ti analysis were 0, 0.5, 1.0, 1.5, and 2.0 mg/dL. Plates were read at an absorbance of 460 nm on a well plate reader (Molecular Devices SpectraMax Plus 384, San Jose, CA). All sample duplicates having a CV < 5 and were averaged for a final Ti concentration.

As it utilized the same sample used to analyze Ti, N was also analyzed on site according to the Hach total nitrogen method (Hach et al., 1987). Following the same steps described for Ti, samples were weighed into a 100 mL Digesdahl flask, followed by overnight digestion using concentrated H_2SO_4. Flasks were then placed on a Digesdahl burner (Model 23130-20, Loveland, CO) and vacuum system to boil the acid for six minutes, followed by the addition of 10 mL of 50% H_2O_2. After completely burning off H_2O_2, flasks were cooled and then diluted to the 100 mL mark with distilled water. 800 µL aliquot of each digested sample was transferred to centrifuge tubes and combined with 20 mL of 0.1 g/L solution of polyvinyl alcohol. The standard curve consisted of dilutions of 0-0.08 mg/dL. Once prepared, 160 µL of standards and samples were transferred to micro plates in duplicate after which 40 µL of PVA and Nessler's reagent solution was transferred to each well. Plates were read at an absorbance of 460 nm on a well plate reader (Molecular Devices SpectraMax Plus 384, San Jose, CA), well plate reader. All sample duplicates with a CV < 5 were averaged for a final nitrogen concentration. Upon confirmation with results received by the, digesta values for N, collected on site, were used in the final analysis when calculating apparent digestibility.

Gross energy was analyzed using an Adiabatic Bomb Calorimeter (115V) Parr model 12141 with 11108 oxygen combustion vessel (Parr Instrument Co., Moline, IL). Approximately

0.5 g of dried samples was pelleted and weighed before prior to bomb assembly, pressurizing with 35 psi of oxygen, and combusting in the bomb upon reaching a steady state temperature. Gross energy presented as Kcal/g was calculated by correcting for sample weight, change in temperature, and individual bomb correction factors.

Individual AA were determined for digesta samples by the University of Missouri Experimental Station Chemical Laboratory (Columbia, MO) as described above. As sample quantity was limited, proximate analysis of digesta samples was determined as secondary, for each sample containing enough remaining digesta. Total nitrogen, or crude protein was analyzed prior to other proximate analyses, following the same procedures as described above for feed sample analysis.

Analysis and results related to VFA are still pending.

Calculations and Statistical Analysis

Apparent digestibility was calculated for each nutrient analyzed from the collected digesta samples including GE, N, and AA. The following equation derived from Stein et al. (2007), was used to estimate % digestibility using analyzed Ti values as a correction factor:

% digestibility = [1 - (Nutrient digesta / Nutrient feed) * (Marker feed / Marker digesta)] * 100

where marker represents analyzed Ti values in both the feed and digesta, and nutrient represents the analyzed value of individual nutrients in both the feed and digesta (Stein et al., 2007).

Data from the experiment was analyzed using the PROC GLIMMIX procedure in SAS (SAS Institute, Inc., Cary, NC) with pen as the experimental unit, nested within treatment. Data was analyzed as a repeated measure over time both for performance data, and collection day

data. Digestibility data was analyzed as a repeated measure over both time (week) and space (GIT segment). The model included dietary treatments and week as fixed effects and pen nested within treatment as the random effect. Individual treatment means were separate using the Tukey-Kramer multiple comparison test. Differences were considered significant at $P < 0.05$ and tendencies at $P \geq 0.05$ and < 0.15.

While typical statistical analysis recognizes tendencies as being set to a $P < 0.10$, the analysis of our results evaluated statistical tendencies at a $P < 0.15$. Results were evaluated in this manner in order to more accurately depict the specific mechanisms by which the DFM used in this study many be functioning within the GIT. The growth performance of the pigs used in this study was well beyond what is observed on an average commercial farm, and also exceeded that of Augspurger et al. (2016). This was in part due to a clean facility and superior health status of the pig herd, as well as lower stocking density than what is observed in a commercial setting. Therefore, in order to provide the best understanding of the mechanisms by which this multi-strain *Bacillus subtilis*-based DFM was functioning within the GIT of weanling pigs, statistical tendencies were set to a $P < 0.15$ in the present study.

Results and Discussion

Morbidity, Mortality, and Growth Performance

This study was designed to evaluate the effects of a multi-strain *Bacillus subtilis*-based DFM on nutrient digestibility. There was no mortality and two pigs were treated, both belonging to the DFM treatment, one due to lameness and the other due to weight loss, for a total morbidity

of 2.5%. There were no overall differences in growth performance (Table 2.3). Although some differences were observed within given weeks, as ADG tended to be greater when feeding DFM, relative to CON, during wk 2 (P = 0.08), and ADFI was significantly increased relative to CON during wk 2 (P = 0.04) and 3 (P = 0.02). Feed efficiency was decreased with addition of DFM, relative to CON during wk 6 (P = 0.04) (Table 2.3). The study, however, was not designed to confirm performance results previously obtained by Augspurger et al. (2016) who observed a 5-10% greater gain and 1.4-5% greater feed conversion with addition of DFM. In the current study, with pen being the experimental unit, eight pens were assigned to each treatment, as this was considered enough statistical power to detect differences in nutrient digestibility (Lee et al., 2014) this however was not expected to provide enough power to determine differences in growth performance. Furthermore, pen density, with five pigs per pen, favored maximum performance and was not representative of commercial farm stocking densities. Overall means for this study were 0.51 kg/d, 0.79 kg/d and 0.66 for ADG, ADFI, and G:F, respectively, hence pigs in this study performed exceptionally well. Compared to Augspurger et al. (2016) containing a minimum of 10 pigs per pen, ADG was 0.36 and 0.29 kg/d for experiments two and three, respectively. Average daily feed intake and G:F for experiment three were 0.42 kg/d, and 0.69, respectively (Augspurger et al., 2016). The pigs in the current study had a 30% greater ADG and consumed nearly 47% more feed than that of pigs used by Augspurger et al. (2016), while maintaining a similar G:F. When compared to other studies with similar pig numbers and pen sizes, pigs in this study performed numerically better (Guo et al., 2006; Walsh et al., 2007; Lee et al., 2014; Tang et al., 2019).

Amino Acid Digestibility

In the current study, overall AA absorption appeared to numerically increase when moving from the jejunum to the ileum (Table 2.5). Yet, differences between treatments occurred strictly within the jejunum (Table 2.4). In our experiment, we were unable to collect sufficient amounts of digesta from the duodenum and distal colon segments, and therefore unable to compare changes in AA digestibility of those two segments to the rest of the GIT. In the present study, pigs supplemented with DFM appeared to have a slight increase in specific AA absorption in the jejunum, compared to CON. Relative to CON, overall digestibility tended to be greater for tryptophan ($P = 0.06$), cysteine ($P = 0.12$), and methionine ($P = 0.10$) with addition of DFM (Table 2.4). Expressed as a percent, DFM increased digestibility by 5, 11, and 15% for methionine, tryptophan, and cysteine, respectively. However, no differences were observed in AA digestibility within the ileum or ascending colon (Table 2.5). These results appear to indicate significant activity of *Bacillus subtilis* by the middle of the small intestine, specifically the jejunum. The site of primary AA absorption has been debated, with some research providing evidence that the mid-intestine, or jejunum, is where most AA absorption occurs (Johansson, 1974; Silk et al. 1985). This may depend on the species of interest, as research has shown that most laboratory animals absorb AA primarily in the jejunum (Webb, 1990).

When adjusting the model to evaluate wk 3 alone as a repeated measure over space (GIT segment), but not time (week), additional tendencies were observed in arginine ($P = 0.11$), histidine ($P = 0.11$), and threonine ($P = 0.14$). While not presented in a table, LSmeans for CON and DFM digestibility's were (47.8 vs. 59.1% ± 4.8), for arginine, (33.6 vs. 47.0% ± 5.6), for

histidine, and (37.7 vs. 50.1% ± 6.2) for threonine, respectively. When analyzed this way, the results may indicate an early impact of the DFM, within 3 wk of initial supplementation.

In 2012, Isaccson and Kim conducted a pilot study and observed unique differences in the microbiota within different GIT segments, with the jejunum being composed of primarily bacteria belonging to the *Firmicutes* phyla (> 90%), while the ileum consisted of a broader mix of *Firmicutes* and *Proteobacteria*. Their findings agree with the effects of *Bacillus subtilis* on digestibility within the jejunum of pigs fed in the current study. As a member of the *Firmicutes* phyla, *Bacillus subtilis* may have helped restore the proper balance of the jejunum, being a naturally *Firmicutes* dominate environment. There are limited studies evaluating the effects of *Bacillus subtilis* or other similar DFM on AA digestibility. Kaewtapee et al. (2017) recently evaluated AA digestibility of growing pigs with and without a mixed *Bacillus* spp. DFM. This DFM included one strain of both *Bacillus subtilis* and *Bacillus licheniformis*. When fed with a wheat, barley, and soybean meal-based diet, the DFM led to no differences in digestibility of any AA by the terminal end of the ileum. In the present study, no differences were observed in ileal digestibility of AA, however DFM led to an improved digestion of essential AA in the jejunum.

Bacillus subtilis may alter digestibility through enzyme secretion. Some of the enzymes it has been known to secrete include: α-amylase, arbinase, cellulase, dextranase, lavansucrrase, maltase, alkaline protease, neutral protease, and β-glucanase (Priest, 1977). Blavi et al. (2018) reported that increases in apparent total tract digestibility of energy may be due to the ability of *Bacillus subtilis* to secrete α-amylase, which helps breaks the glyosidic bonds in starch. They suggested that increases in energy utilization may alternatively be the result of increased digestion of fiber due to other enzymes secreted by *Bacillus subtilis* including pectinase and

xylanase. However, differences in GE digestibility were not observed in this study, lessening support of this as a primary mechanism. Other research attributes changes in growth performance and digestibility to the proteases produced by *Bacillus subtilis* (Tang et al., 2019), or other unidentified enzymes aiding in the breakdown of CP, non-starch polysaccharides, or other substrates present in soybean meal and other common swine feed ingredients (Giang et al., 2012; Upadhaya et al., 2015). Each single strain or mixed cocktail seems to act differently, as the DFM used in the current study appears to improve digestibility of specific AA, rather than directly impacting energy digestibility. This may be an indication that the *Bacillus subtilis* strains involved in this study secrete unique proteases, specifically improving absorption of cysteine, methionine, and tryptophan. Recent research has been conducted to evaluate dietary supplementation of proteases in nursery pig diets. Two separate studies conducted in 2016 (Pan et al., 2016; Yu et al., 2016) found improved AID of several AA both indispensable (arginine, histidine, isoleucine, leucine, lysine, methionine, and threonine), and dispensable (alanine, cysteine, and tyrosine) with protease supplementation. However, neither protease improved digestibility of tryptophan, the AA most greatly affected by the DFM used in the current study.

In addition to being involved in protein synthesis, tryptophan is used to synthesize serotonin (Waclawiková and Aidy, 2018). However, serotonin production must compete with various other pathways that utilize tryptophan, one being that of the kynurenine biosynthesis pathways, which metabolizes nearly 90% of digested tryptophan (Waclawiková and Aidy, 2018). As it plays an important role in immune homeostasis, decreased levels of serotonin may lead to an increased inflammatory response at the gut level. Serotonin is also involved in regulating mood and appetite, with greater serotonin concentrations leading to an increase in voluntary feed

intake (Henry et al., 1992; Kwon et al., 2019), which may explain the increase in ADFI associated with the addition of DFM in the present study, previously mentioned above. In the present study, tryptophan digestibility was increased in the jejunum with the addition of DFM, relative to CON (Table 2.4). Once again, DFM supplementation led to an increase in ADFI during wk 2 ($P = 0.04$) and 3 ($P = 0.02$) (Table 2.3) , during which pigs were overcoming late responses to weaning stress, along with undergoing a dietary change from phase one to two. A significant increase in tryptophan digestibility may have led to an increase in available tryptophan for synthesis of serotonin, decreasing inflammation in the GIT, which we have observed via greater production of anti-inflammatory markers in the jejunum (Chapter 3, Lewton et al., 2020b).

Cysteine is a key component in the production of glutathione, an antioxidant involved in the removal of reactive oxygen species, along with enzyme regulation and formation of DNA precursors (Meister and Anderson, 1983). An increase in cysteine digestibility may lead to increased production and recycling of glutathione in the liver, however this is tightly regulated (Stipanuk et al., 2006). Excess cysteine may also lead to an increase in taurine synthesis, which plays a key role in proinflammatory cell regulation and bile salt formation (Redmond et al., 1998). An increase in glutathione and/or taurine production resulting from addition of *Bacillus subtilis*, would improve antioxidant capacity and lead to positive changes in inflammatory cytokines and related immunity markers, as has been observed in our study (Chapter 3, Lewton et al., 2020b).

Direct-fed microbial supplementation increasing digestibility of methionine in the jejunum may also indicate the changes discussed above. Methionine is a cysteine sparing AA,

although the use of this pathway in the methionine metabolism cycle differs depending on several factors, including digestibility of methionine presence in the diet. It has been shown that at greater available methionine concentrations, production of cysteine via the transulfuration pathway may substantially increase (Finkelstein and Martin, 1984). The increased absorption of methionine observed in the current study, may have led to greater production of cysteine end products, upon meeting the pig's methionine requirement.

Digestibility of Gross Energy and Nitrogen

Limited studies have evaluated multiple segments of the GIT for apparent digestibility, with most evaluating for AID or apparent total tract digestibility (ATTD). In the present study, digestibility of N and GE appeared to numerically increase at each consecutive GIT segment from the jejunum to distal colon, with CON digestibility values of 52.0, 62.1, 70.2, and 77.8% for GE in the jejunum, ileum, ascending colon, and distal colon, respectively (Table 2.6). Similarly, N digestibility values for CON were 43.3, 55.8, 57.4, and 71.5% for the jejunum, ileum, ascending colon, and distal colon, respectively (Table 2.6). However, no treatment differences were observed within the ileum and ascending colon for any response variables (Table 2.6). The values obtained from our control group, appear to be small, yet similar to those obtained from other weanling pig studies. Giang et al. (2012) fed weaned pigs a lactic acid bacteria supplement in addition to *Bacillus subtilis* and observed greater digestibility values for nitrogen than the current study, with an AID of N approaching 80% and ATTD close to 85-90%. A second study evaluating a *Bacillus subtilis* fermentation biomass on nutrient digestibility, found an ATTD close to 70% for nitrogen and 80% for GE, when obtained 4 wk into the nursery phase (Lee et al., 2014). A third study evaluating a mixed *Bacillus* spp. DFM containing one

strain of *Bacillus subtilis* found the ATTD of both nitrogen and GE fell between 70 and 80%, with a slight decrease in both measures from d 14 to 42 post weaning (Cai et al., 2015). Agreeing with the above, Tang et al. (2019) evaluated the effects of a *Bacillus subtilis*-based DFM (DSM32315) on nursery pig ATTD in a 2 × 2 factorial involving two amounts of CP and with or without DFM supplementation, suggesting that the *Bacillus subtilis* may have a greater impact on digestibility in low protein diets. Low protein combined with *Bacillus subtilis* led to a 5% increase in ATTD of N (75% vs 80%). Contrary to the results of the present study, these studies appear to observe a slight increase in either AID or ATTD with addition of a *Bacillus subtilis*-based DFM. While not statistically significant, ATTD of both N and GE appear to decrease with addition of DFM, in the current study. Also differing from earlier studies, Blavi et al. (2018) saw no differences in ATTD of N, while AID was slightly decreased with the addition of *Bacillus subtilis*. These variations could be the result of differences in the studies strains of bacteria, pig age and growth performance, dietary composition, inclusion rate, and/or interaction with other feed additives (Chesson, 1994; Chen et al., 2005). The diets fed in the current study were made with corn, soybean meal, and distillers dried grains with solubles (DDGS). Inclusion of DDGS has been shown to significantly decrease AID of GE and ATTD of GE, N, and several essential AA (Agyekum et al., 2016). This may explain the relatively low digestibility values observed in the current study.

In the current study, relative to CON, overall N digestibility tended to decrease (P = 0.08) in the distal colon with the addition of DFM (71.5 vs. 58.8 ± 5.3 %) (Table 2.6). In contrast, others reported greater fecal or ATTD of N when supplementing a *Bacillus subtilis*-based DFM (Lee et al., 2014; Cai et al., 2015; Tang et al., 2019). The decrease in N digestibility

observed in the present study may have been related to changes in the richness or diversity of the microbiome of the distal colon upon addition of DFM, leading to increased N recycling and endogenous secretions. Bacteria utilize and synthesize amino acids and other N containing metabolites (Davila et al., 2013). Up to 25% of the urea produced in the liver enters the intestinal lumen, mostly within the small intestine but also in the colon in growing pigs (Bergen and Wu, 2009). Microbes are essential for the hydrolysis of urea into ammonia and carbon dioxide, and subsequent conversion of ammonia to form glutamate and glutamine (Bergen and Wu, 2009). In the current study, glutamate digestibility was not measured within the distal colon due to limited sample volume. If it had been evaluated, differences in glutamate digestibility would have been of great benefit in helping interpret the change relating to N digestibility in the distal colon in association with DFM supplementation in the current study.

Colonic Contents pH

Agyekum et al. (2016) measured colonic pH as a secondary indication of changes in the microbiome or hindgut fermentation and production of VFA. As the primary site of bacterial fermentation and microbial communities in nonruminants, we expected to see the greatest impact of *Bacillus subtilis* on N digestibility, VFA production, and pH within the colon (Tajima and Aminov, 2015). However, no differences were observed in the pH or nutrient digestibility of ascending colon content in this study. While not significant ($P = 0.18$), at the end of wk 6, DFM had a numerically greater pH than CON, 5.8 vs. 5.6, respectively. However, this is the opposite of what occurred at the end of wk 3 with pH values of 5.8 and 5.7, for CON and DFM, respectively, which was also not significant. While difficult to interpret, this may be related to the type of diets fed in this study. Over the course of the 42-d study, both treatments were fed

increasing amounts of DDGS, 5, 10, and 20% for phases one, two, and three, respectively. Other studies have demonstrated the effects of DDGS on the microbiota of nursery pigs, finding that inclusion of 30% DDGS led to a significant decrease in the *Firmicutes*:*Bacteroidetes* ratio, mostly due to a decrease in many *Lactobacillus* species (Burrough et al., 2015). As they are known for producing lactic acid and reducing digest pH, a decrease in *Lactobacillus* within the ascending colon, could result in an increase in digesta pH. However, this was not supported in the current study, as pH was numerically decreased 5.8 vs 5.6 in the control diet when increasing DDGS inclusion from 10 to 20% for pigs euthanized on wk 3 and 6, respectively. Between wk 3 and 6, colonic pH of CON and DFM appear to move in opposite directions, possibly indicating changes occurring within the microbiome with prolonged addition of *Bacillus subtilis*. While *Lactobacillus* experiences optimal growth at a lower pH, *Bacillus subtilis* has been shown to prefer an environment with a higher pH. Some studies suggest an optimal pH of 5.5, while others suggest a pH much closer to 6.5, depending on the specific strains and activity of interest (Chantawannakul et al., 2002; Koni et al., 2017).

Conclusions

In conclusion, supplementation of a multi-strain *Bacillus subtilis*-based DFM appeared to have some beneficial effects on the nutrient digestibility of nursery pigs, specifically within the jejunum, tending to increase digestibility of cysteine, methionine, and tryptophan. These improvements in specific AA digestibility could help explain the improvements in ADG and G:F observed in the study by Augspurger et al. (2016). *Bacillus subtilis* also appears to lead to changes in hindgut digestibility and fermentation, observed through a tendency for decreased

digestibility of N within the distal colon, relative to CON. The current study evaluated nutrient digestibility across multiple segments of the GIT. No previous DFM studies have evaluated digestibility in the jejunum and ascending colon, with this being the first to do so. While enzyme secretions related to protein digestion were not evaluated in this study, we speculate that the DFM may be secreting enzymes that improve digestibility of specific amino acids. Further research is required to confirm this. Additional studies are needed to identify specific mechanisms by which multi-strain *Bacillus subtilis*-based DFM may be improving AA digestibility in the jejunum and other GIT segments.

APPENDIX

APPENDIX

Table 2.1. Composition of diets, control and diet containing multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM), across all three dietary phases, as-fed basis

	Phase 1, d 0-14		Phase 2, d 14-28		Phase 3, d 28-42	
Ingredient %	Control	DFM	Control	DFM	Control	DFM
Corn	40.18	40.13	48.36	48.31	50.99	50.95
Soybean meal, 47.5% CP	17.30	17.30	21.15	21.15	22.65	22.65
Corn DDGS, 7.5% oil	5.00	5.00	10.00	10.00	20.00	20.00
Dried whey, 72% lactose	25.00	25.00	10.00	10.00	-	-
Fish meal	3.00	3.00	4.50	4.50	-	-
Spray-dried bovine plasma	4.00	4.00	-	-	-	-
Corn oil	3.00	3.00	3.00	3.00	3.00	3.00
Calcium carbonate, 38.5% Ca	0.65	0.65	0.60	0.60	0.85	0.85
Monocalcium phosphate, 21.5% P	0.55	0.55	0.55	0.55	0.45	0.45
Salt	0.30	0.30	0.55	0.55	0.60	0.60
L-Lysine HCl	0.35	0.35	0.50	0.50	0.65	0.65
DL-Methionine	0.15	0.15	0.15	0.15	0.14	0.14
L-Threonine	0.13	0.13	0.19	0.19	0.21	0.21
L-Tryptophan	0.03	0.03	0.06	0.06	0.06	0.06
L-Valine	0.07	0.07	0.10	0.10	0.10	0.10
VTM premix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Phytase	0.05	0.05	0.05	0.05	0.05	0.05
DFM[2]	-	0.05	-	0.05	-	0.05
Total, 100%	100	100	100	100	100	100

[1]VTM premix provided the following vitamins and microminerals in the following concentrations: Zinc 83.4 g/kg, iron 66.7 g/kg, manganese 33.4 g/kg, copper 10 g/kg, iodine 0.3 g/kg, selenium 0.2 g/kg, vitamin A 7,363 KIU, vitamin D 1,177 KIU, vitamin E 44,112 IU, menadione 1.5 g, vitamin B12 0.02 g, riboflavin 4.7 g, pantothenic acid 14.7 g, niacin 29.4 g, thiamine 0.7 g, pyridoxine 2.9 g, folic acid 1.1 g, and biotin 0.1 g.
[2]Direct-fed microbial was mixed at an inclusion rate of 0.5 g/kg of diet

Table 2.2. Analyzed composition of control diet and diet containing multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM), across all three dietary phases

	Phase 1, d 0-14		Phase 2, d 14-28		Phase 3, d 28-42	
Item	Control	DFM	Control	DFM	Control	DFM
DM, %	91.45	91.40	90.31	90.34	90.61	90.91
GE, kcal/kg	4,608	4,611	4,678	4,597	4,762	4,736
CP, %	21.70	21.40	21.70	22.00	21.90	22.60
Crude Fat, %	4.50	4.30	5.20	5.10	6.60	6.70
Crude Fiber, %	1.60	1.70	2.10	2.20	3.30	3.00
NDF, %	6.20	6.20	8.30	8.10	11.10	12.00
ADF, %	2.90	2.50	4.00	4.00	5.50	5.80
Titanium, ppm	-	-	684	671	661	692
Indispensable AA, %						
Arg	1.10	1.10	1.10	1.10	1.10	1.10
His	0.51	0.51	0.50	0.50	0.52	0.53
Ile	0.91	0.92	0.90	0.91	0.87	0.88
Leu	1.85	1.89	1.83	1.84	1.95	2.02
Lys	1.58	1.53	1.56	1.46	1.49	1.71
Met	0.50	0.42	0.45	0.48	0.42	0.46
Met + Cys	0.91	0.81	0.76	0.81	0.76	0.79
Phe	0.98	0.97	0.98	0.99	1.05	1.07
Thr	1.00	1.03	0.97	0.96	0.91	0.94
Trp	0.30	0.30	0.28	0.34	0.26	0.28
Val	1.13	1.15	1.08	1.10	1.05	1.07
Dispensable AA, %						
Ala	1.06	1.07	1.11	1.09	1.13	1.16
Asp	1.94	1.93	1.86	1.87	1.77	1.73
Cys	0.41	0.39	0.31	0.33	0.34	0.33
Glu	3.28	3.27	3.32	3.33	3.37	3.32
Gly	0.80	0.80	0.88	0.86	0.79	0.79
Pro	1.17	1.19	1.23	1.20	1.29	1.32
Ser	0.84	0.84	0.80	0.80	0.82	0.82
Tyr	0.66	0.68	0.65	0.63	0.68	0.68

Table 2.3. Weekly growth performance of control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)[1]

Item	Control	DFM	SEM	*P*-value
ADG, kg				
Wk 1	0.170	0.160	0.01	0.31
Wk 2	0.295[y]	0.341[x]	0.02	0.08
Wk 3	0.428	0.446	0.02	0.54
Wk 4	0.581	0.573	0.03	0.82
Wk 5	0.722	0.751	0.02	0.35
Wk 6	0.832	0.809	0.03	0.59
Overall	0.507	0.513	0.01	0.67
ADFI, kg				
Wk 1	0.274	0.260	0.01	0.25
Wk 2	0.399[b]	0.436[a]	0.01	0.04
Wk 3	0.630[b]	0.693[a]	0.02	0.02
Wk 4	0.854	0.877	0.02	0.49
Wk 5	1.146	1.165	0.03	0.63
Wk 6	1.359	1.401	0.04	0.41
Overall	0.777	0.805	0.02	0.23
G:F				
Wk 1	0.619	0.618	0.02	0.98
Wk 2	0.735	0.779	0.03	0.25
Wk 3	0.680	0.642	0.02	0.28
Wk 4	0.679	0.653	0.02	0.28
Wk 5	0.630	0.646	0.01	0.44
Wk 6	0.613[a]	0.577[b]	0.01	0.04
Overall	0.659	0.653	0.01	0.58

[1] Performance data taken from n=8 pens per treatment, 5 pigs per pen from d 0-21, 4 pigs per pen from d 21-42
[a,b]Values in a common row lacking a common superscript differ ($P \leq 0.05$)
[x,y]Values in a common row lacking a common superscript tend to differ ($P \leq 0.15$)

Table 2.4. Apparent jejunal digestibility of indispensable and dispensable amino acids, for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)[1]

Item	Control	DFM	SEM	*P*-value
Indispensable AA, %				
Arg	59.53	64.91	3.72	0.29
His	49.53	55.41	4.17	0.30
Ile	53.04	57.54	3.67	0.37
Leu	56.86	58.43	4.01	0.77
Lys	63.96	70.59	3.92	0.22
Met	67.83[y]	73.35[x]	2.46	0.10
Met+Cys	46.20[y]	55.31[x]	3.70	0.07
Phe	56.77	59.69	4.04	0.59
Thr	50.85	58.36	4.73	0.24
Trp	56.33[y]	67.27[x]	4.20	0.06
Val	51.95	57.49	4.73	0.39
Dispensable AA, %				
Ala	49.63	53.04	3.95	0.52
Asp	46.34	52.01	4.68	0.37
Cys	14.44[y]	29.20[x]	7.02	0.12
Glu	52.01	54.75	4.36	0.64
Gly	-2.79	-1.56	8.27	0.91
Pro	47.46	49.56	4.37	0.72
Ser	46.69	51.80	4.44	0.40
Tyr	54.68	58.00	4.29	0.57
All indispensable AA, %	57.89	62.31	3.17	0.31
All dispensable AA, %	45.00	48.14	3.93	0.55
All AA, %	49.88	54.34	4.37	0.45

[1]Digestibility coefficients within jejunum, n=6-8 representative pigs per treatment at both d 21 and 42, n=3-4 for Trp d 21 due to lack of sufficient sample collection

[a,b]Values in a common row lacking a common superscript differ ($P \leq 0.05$)

[x,y]Values in a common row lacking a common superscript tend to differ ($P \leq 0.15$)

Table 2.5. Apparent digestibility of indispensable amino acids, across segments of the gastrointestinal tract, for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)[1]

Item %	Control	DFM	SEM	*P*-value
Arg				
Jejunum	59.53	64.91	3.72	0.29
Ileum	75.86	76.56	3.29	0.88
Ascending colon	76.82	78.05	3.83	0.81
His				
Jejunum	49.53	55.41	4.17	0.30
Ileum	68.34	66.21	3.62	0.68
Ascending colon	72.43	73.43	4.30	0.86
Ile				
Jejunum	53.04	57.54	3.67	0.37
Ileum	69.85	68.53	3.39	0.78
Ascending colon	62.01	65.39	5.93	0.67
Leu				
Jejunum	56.86	58.43	4.01	0.77
Ileum	70.75	68.36	3.40	0.62
Ascending colon	69.09	71.45	4.14	0.67
Lys				
Jejunum	63.96	70.59	3.92	0.22
Ileum	74.68	76.18	3.41	0.76
Ascending colon	72.49	75.48	4.06	0.58
Met				
Jejunum	67.83[y]	73.35[x]	2.46	0.10
Ileum	77.71	78.57	2.38	0.80
Ascending colon	67.13	71.50	4.97	0.51
Phe				
Jejunum	56.77	59.69	4.04	0.59
Ileum	70.20	68.67	3.47	0.76
Ascending colon	68.35	70.71	4.17	0.67
Thr				
Jejunum	50.85	58.36	4.73	0.24
Ileum	64.75	62.47	4.12	0.70
Ascending colon	66.33	67.41	4.87	0.87
Trp				
Jejunum	56.33[y]	67.27[x]	4.20	0.06
Ileum	74.13	73.19	3.67	0.85
Ascending colon	79.35	82.35	3.67	0.54
Val				
Jejunum	51.95	57.49	4.73	0.39
Ileum	67.81	66.26	4.05	0.79
Ascending colon	61.74	65.27	4.87	0.58

[1]All values are overall LSmeans of d 21 and 42 combined data, total amino acids were not reported in distal colon due to insufficient sample size, n=12 to16 representative pigs per treatment, n=8 to 11 for Trp due to insufficient sample collection

[x,y]Values in a common row lacking a common superscript tend to differ ($P \leq 0.15$)

Table 2.6. Apparent digestibility of gross energy, nitrogen, and total amino acids across segments of the gastrointestinal tract, for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)[1]

Item, %	Control	DFM	SEM	*P*-value
Gross Energy				
Jejunum	52.04	53.58	3.86	0.78
Ileum	62.13	60.00	3.58	0.67
Ascending colon	70.18	65.15	3.97	0.35
Distal colon	77.77	69.15	3.97	0.17
Nitrogen				
Jejunum	43.29	45.19	5.20	0.79
Ileum	55.77	59.21	4.61	0.60
Ascending colon	57.39	53.64	4.90	0.58
Distal colon	71.50[x]	58.85[y]	5.33	0.08
Total amino acids				
Jejunum	49.88	54.34	4.37	0.45
Ileum	66.96	65.18	3.78	0.74
Ascending colon	74.62	71.71	6.06	0.99

[1]All values are overall LSmeans of d 21 and 42 combined data, total amino acids were not reported in distal colon due to insufficient sample collection, n=6 to 8 representative pigs per treatment for both d 21 and 42

[a,b]Values in a common row lacking a common superscript differ ($P \leq 0.05$)

[x,y]Values in a common row lacking a common superscript tend to differ ($P \leq 0.15$)

CHAPTER 3

EFFECTS OF A MULTI-STRAIN *BACILLUS SUBTILIS*-BASED DIRECT-FED MICROBIAL ON IMMUNITY MARKERS, INTESTINAL MORPHOLOGY, AND MICROBIAL COMMUNITIES IN DIETS FED TO WEANLING PIGS

Abstract

The objective of this experiment was to evaluate the effects of a multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM) on nursery pig health as indicated by intestinal mucosal and blood plasma immunological markers, intestinal morphology, and microbiota. Eighty pigs, of equal number of barrows and gilts (initial BW: 7.0 ± 0.60 kg), weaned at 21 ± 1 days of age were randomly allotted to sixteen pens, with five pigs per pen. Two dietary treatments were implemented, a basal control (CON) and a basal control plus DFM (DFM). Both diets were corn, soybean meal, and distillers dried grains based and were formulated to meet or exceed all nutritional requirements (NRC, 2012) and manufactured on site. Diets were fed for 42 d. On d 21 and 42 of the experiment, one pig per pen was randomly selected and euthanized, with equal number of males and females represented. Blood samples were collected prior to euthanasia for assessment of plasma concentrations of immunoglobin A (IgA) and intestinal fatty acid binding protein. Segments of the gastrointestinal tract including duodenum, jejunum, ileum, ascending and distal colon were removed for analysis of intestinal morphology, microbial communities, and levels of interleukin 6, interleukin 10 (IL-10), and tumor necrosis factor alpha. Jejunal villus height was greater (P = 0.02) in the DFM pigs as compared with CON pigs (422 vs. 385 ± 10 µm, respectively) and ascending colon crypt depth tended to be greater (P = 0.10) on d 21 (373 vs. 337 ± 14 µm, respectively). Compared to CON, DFM treatment tended to

increase IgA (P = 0.06) on d 21 (0.34 *vs.* 0.54 ± 0.07 mg/mL, respectively) and tended to increase IL-10 (P = 0.12) on d 42 (133 *vs.* 237 ± 49 pg/mL, respectively). Addition of a multi-strain *Bacillus subtilis*-based DFM may have a potential early benefit to nursery pig health status.

Introduction

The nursery phase of production is arguably the most critical time point of the market pig's life. The many transitions associated with weaning lead to a great deal of stress, impacting the pig's gut health, immune status, and growth rate (Pluske, 2013). As the industry moves away from the use of antibiotic growth promoters, finding new ways to decrease the negative impact of weaning has become of great importance. Among the many researched antibiotic alternatives, direct-fed microbials (DFM), or probiotics, are one of great promise. Augspurger et al. (2016) evaluated a multi-strain *Bacillus subtilis*-based DFM on 21-d-old weanling pig performance, finding impacts of 5-10% greater gain and up to 5% lower feed/gain ratio. However, the mode of action of this unique DFM has yet to be defined. To date, the few published single-strain studies suggest an ability of *Bacillus subtilis* to impact health status through specific changes in immunological markers, intestinal morphology, or microbiota populations (Bhandari et al., 2008; Lee et al., 2014; Zhang et al., 2017). Therefore, the objective of this research was to evaluate the effect of a multi-strain *Bacillus subtilis*-based DFM on intestinal mucosal and blood plasma immunological markers, intestinal morphology, and microbiota as indicators of health status of the 21-d-old weanling pig. A study was conducted to test the hypothesis that pigs supplemented with *Bacillus subtilis* would have improved health status indicated by positive changes in

immunological markers, greater morphological development of individual segments of the gastrointestinal tract (GIT), and marked changes in microbial communities.

Materials and Methods

Animals, Housing, and Experimental Design

The Institutional Animal Care and Use Committee at Michigan State University reviewed and approved the protocol for this experiment. The study was structured as a complete randomized design based on litter, weight, and sex. Eighty crossbred pigs (PIC 359 x Yorkshire) from the MSU Swine Farm were weaned at 21 ± 1 days old (7.0 ± 0.60 kg, initial BW) and randomly allotted into sixteen (1.22 x 1.83 m) mixed sex cohorts. Cohorts were then randomly allotted to pens located in one of four mechanically-ventilated nursery rooms at Michigan State University Swine Teaching and Research Center. Cohort allotment was based litter (dam), weight, and sex; maintaining a varied distribution of weights of pigs in each pen. All housing, vaccinating, and processing of piglets and dams was conducted as explained in chapter 2 by Lewton et al. (2020a). All pig work was completed between the months of August and September 2019.

Diets and Feeding

Two treatments were used in the study (1) a basal control diet with no DFM supplementation (CON) and (2) basal control plus supplementation of a multi-strain *Bacillus subtilis* based DFM (DFM) at an inclusion rate of 0.5 g/kg of feed. The nursery study was split up into three dietary phases (d 0-7, d 7-14, and d 14-28), with d 0 representing the day of

weaning (21 ± 1 d of age). All diets were formulated using recommendations made available by Kansas State University (Menegat et al., 2019) based on values published by the NRC (2012), and were mixed at Michigan State University swine farm using a 113 kg paddle-ribbon mixer (Table 3.1). All micro ingredients were pre-mixed in the Michigan State University nonruminant-nutrition laboratory, prior to mixing with macro ingredients. Dietary Cu and Zn were set at or slightly above levels suggested by NRC (2012). All dietary sampling and analyses were conducted following the methods described in chapter 2 by Lewton et al. (2020a). Analyzed composition of the diets are shown in Table 3.2.

Data Recording and Sample Collection

At the end of wk 3 and 6, one pig per pen, with consideration of sex, was euthanized for analysis of systemic and localized immunity, intestinal morphology, and intestinal microbiota. One pig was sacrificed at a time alternating between CON and DFM. Prior to euthanasia, pigs were sedated using Telazol-T (2.5 mg/kg); Ketamine-K (1.25 mg/kg); and Xylazine-X (1.25 mg/kg), used as TKX (0.025 mL/kg) in a single intramuscular injection with a 22 G needle. After sedation and prior to euthanasia, blood samples were collected from each pig using a 10 mL BD Vacutainer via jugular venipuncture. Blood tubes were temporarily placed on ice. Pigs were then euthanasia via sodium pentobarbital (1 mL/4.5 kg) in a single intracardiac injection with an 18 G needle.

After euthanasia carcasses were opened lengthwise and the cecum was located. Collection of the duodenum, jejunum, ileum, ascending colon, and distal colon was conducted according to the procedures detailed in chapter 2 by Lewton et al. (2020a). Upon removal, sections of intestine were placed on a metal pan and a portion of tissue was collected for

histology, opened lengthwise and immediately placed in chilled 1x PBS. Once cleaned, these tissues were flattened and stapled, mucosa side up, to appropriately labeled cardboard paper and stored in Carnoy's 2000 (MasterTech Scientific, Inc.) for 24 hr to prep for histological viewing.

For analysis of microbiota, FTA card samples were collected from each intestinal section (duodenum, jejunum, ileum, ascending colon, distal colon). Upon opening lengthwise, tissues were swabbed with a plastic cotton swab by gently rubbing the intestinal lining for three to five seconds. Swabs were then transferred to FTA cards by rubbing directly onto the card for three to five seconds. Cards were then placed in a clean, dry area and lightly covered with paper towel for a minimum of three hours before being packaged in plastic bags with a small desiccate pack and shipped to Microbial Discovery Group (Franklin, WI) for microbiome assessment. Analyses and results of FTA cards are still pending.

After removal of digesta and swab collection, a portion of each section, excluding the distal colon (ileum, jejunum, duodenum, and ascending colon) was placed in 1x PBS until clean, and laid flat on a metal pan to collect the mucosal layer. Mucosal scrapes were collected using two glass slides and gently removing the mucosa from the muscular layer of the tissue. Scrapings were then flash frozen with liquid nitrogen, wrapped in whirl pack bags, rolled up, and placed in liquid nitrogen. These were then transferred to a -80º C for storage until analysis of localized immunity markers.

Chemical Analysis

Blood tubes were placed directly on ice and then aliquoted to collect the blood plasma. Tubes were centrifuged for 30 min at 2,300 rpms and 4º C. Plasma was then separated into labeled sample tubes in a covered sample container and stored at -80º C until analysis of specific

cytokines and immunoglobins. Plasma samples were analyzed for intestinal fatty acid binding protein (iFABP) and immunoglobulin A (IgA). Fatty acid binding protein was analyzed using a human FABP2/I-FABP immunoassay from R&D Systems, Inc (Minneapolis, MN). Samples and all materials were allowed two hr to thaw, prior to dilution of samples (25 µL sample per 300 µL calibrator diluent), and preparation of eight standards (0 - 1,000 pg/mL). Upon completion of the immunoassay, optical density of each well was determined using a microplate reader at 450 nm within 30 min of adding stop solution. Immunoglobin A was analyzed in a similar manner using a pig IgA ELISA kit from Bethyl Laboratories, Inc. (Montgomery, TX). Samples were thawed for 2 hr prior to diluting with a 1:15,000 dilution scheme of sample to diluent. A standard curve was established using a series of eight dilutions (0-1,000 ng/ml). Plates were measured for optical density at an absorbance of 450 nm within 30 min of adding stop solution.

To analyze localized immunity, three markers were selected: interleukin 6 (IL-6), interleukin 10 (IL-10), and tumor necrosis factor alpha (TNFα). Markers were analyzed using ELISA kits produced by RayBiotech, Inc. (Norcross, GA) for IL-6, and R&D Systems, Inc. (Minneapolis, MN) for IL-10 and TNFα. All ELISAs followed the same principles as discussed above for analysis of systemic immunity markers. Protein concentration was diluted two-fold prior to running ELISAs for IL-6 and IL-10. Standard curves were prepared for IL-6 (0 – 10,000pg/ml), IL-10 (0 – 2,000 pg/ml) and TNFα (0 -1,500 pg/ml) using a series of eight dilutions. Upon completion of assay and addition of stop solution, optical density was measured at an absorbance of 450 nm.

Twenty-four hr post collection, tissues for analysis of intestinal morphology were moved to a new container with 1x PBS + 0.1% sodium azide (1.50 L 1x PBS + 1.5 g sodium azide),

where they remained for an additional 24 hr. Following this time period, sections of each tissue was cut and moved into a separate plastic cassette, placed in 70% ethanol, and delivered to the Michigan State University Investigative Histopathology Laboratory (East Lansing, MI) for slide preparation and staining. According to their procedures, tissue samples previously fixed in 10% Neutral Buffered Formalin were processed and vacuum infiltrated with paraffin on the Sakura VIP 2000 tissue processor, followed by embedding with the ThermoFisher HistoCentre III embedding station. Once blocks are cooled, excess paraffin was removed from the edges; placed on a Reichert Jung 2030 rotary microtome and faced to expose tissue samples. Once the block was faced it was cooled and finely sectioned at four to five microns. Sections were dried at 56º C slide incubator to ensure adherence to the slides for 2 to 24 hr not exceeding this temperature which would potentially destroy antigenic components. Slides were removed from the incubator and stained with a routine hematoxylin and eosin method, followed by coverslipping with synthetic mounting media for permanent retention and visualization with light microscopy.

Slides were then viewed microscopically using a Leica DM750 brightfield microscope fit with Leica LAS EZ software (Buffalo Grove, IL). Within the ascending colon, crypt depth and muscle thickness were measured using a minimum of 20 representative crypts and measurements of muscle thickness. Within the duodenum, jejunum, and ileum villi height, and crypt depth were evaluated by selecting a minimum of 20 representative villi and crypt. Villi to crypt ratio was calculated using a minimum of eight adjacent villi and crypt. Photos taken and used for analysis of villi and crypt, were also used to evaluate goblet cell concentrations within the duodenum and ascending colon. Using 8 to 15 representative crypt, total goblet cells per crypt and goblet cells

per µm of crypt depth. The goblet cell concentrations in the jejunum and ileum were not evaluated, because of poor quality photos of those two segments.

Statistical Analysis

Data from the experiment was analyzed using the PROC GLIMMIX procedure in SAS (SAS Institute, Inc., Cary, NC) with pen as the experimental unit, nested within treatment. Data was analyzed as a repeated measure over time, accounting for the two separate collection periods. Dietary treatment and week were set as fixed effects and pen within treatment as the random effect in the analysis. Individual treatment means were separate using the Tukey-Kramer multiple comparison test. Differences from this experiment were considered significant at $P < 0.05$ and tendencies at $P \geq 0.05$ and < 0.15.

As mentioned in chapter 2 by Lewton et al. (2020a), in the present study results were evaluated by setting statistical tendencies at a $P < 0.15$ in order to better depict the mechanisms by which this DFM may be functioning within the GIT of weanling pigs. The growth performance of the pigs used in this study was well beyond what is observed on an average commercial farm, and also exceeded that of Augspurger et al. (2016). This was in part due to a clean facility and superior health status of the pig herd, as well as lower stocking density than what is observed in a commercial setting. Therefore, in order to provide the best understanding of the mechanisms by which this multi-strain *Bacillus subtilis*-based DFM was functioning within the GIT of weanling pigs, statistical tendencies were set to a $P < 0.15$ in the present study (Chapter 2, Lewton et al., 2020a).

Results and Discussion

Morbidity and Mortality

The overall health of pigs was excellent, resulting in no mortality and only two pigs being treated due to lameness and/or weight loss, for a total morbidity of 2.5%, as mentioned in chapter 2 by Lewton et al. (2020a). As discussed by Lewton et al. (2020a), this study involved pigs originating from a very healthy herd and were maintained in a very clean research facility. Differences in performance observed between the current group of pigs and those of Augspurger et al. (2016), may be related to differences in overall herd health status. Furthermore, pen density, with five pigs per pen, favored maximum performance and was not representative of commercial farm stocking densities (Chapter 2, Lewton et al., 2020a).

Intestinal Morphology

In the current study, minimal differences were observed between treatments in intestinal morphology. However, difference observed appeared to be correlated with our results presented elsewhere, with the DFM appearing to have the greatest impact within the jejunum (Chapter 2, Lewton et al., 2020a). Relative to CON, overall jejunal villus height was increased by 9% (P = 0.02) in pigs supplemented with DFM (385 vs. 422 ± 10 µm, respectively; Table 3). While not statistically significant, similar degree of improvement was observed for both d 21 and 42, suggesting an early, yet lasting impact of the DFM (Table 3). Other groups have evaluated *Bacillus subtilis*-based products for morphological changes, with varied results. Similar to the current study, Walsh et al. (2007) fed weanling pigs a mixed *Bacillus*-based DFM and observed an increase in ileal villus height on d 34 of the trial. These improvements were limited to pigs

given a single bolus dose in addition to the DFM being supplied in the feed. Another group of researchers observed greater differences when feeding a *Bacillus subtilis* fermented biomass to 21-d-old weanling pigs (Lee et al., 2014). A linear improvement was observed on d 28 of the study, with the highest inclusion of the biomass (4.5 g/kg) leading to increased villus height and villus height to crypt depth ratio in the duodenum, jejunum, and ileum (Lee et al., 2014). Still other research has shown improvements in ileal villus height of 7-d-old piglets receiving formula milk supplemented with *Bacillus subtilis* PB6, appearing to have the greatest impact on pigs suffering from intrauterine growth restriction (Hu et al., 2016). The same variation in response occurs across species, with poultry studies observing variable responses depending on the specific product (Samanya and Yamauchi, 2002; Sikander et al., 2017). In agreeance with the current study, these studies all appear to suggest that *Bacillus subtilis* has a greater impact on villus height than crypt depth within the small intestine.

In the current study, crypt depth within the ascending colon on d 21, tended to increase with addition of DFM ($P = 0.10$), relative to CON (337 vs. 373 ± 14 μm), respectively (Table 3.3). Jin et al. (1994) have shown high fiber diets increase colon crypt depth of 13 kg pigs, being significantly altered, within a 14-d time span. More recent studies have confirmed this, observing increased colon crypt depth when feeding high fiber or resistant starch diets to nursery pigs (Hedemann et al., 2006; Hedemann and Knudsen, 2007). A study evaluating a different *Bacillus* species found no differences in crypt depth between treatments, in either the small intestine or colon (Reiter et al., 2006). However, limited studies have evaluated the effects of DFMs on colonic morphology. Much is known about the role of the villi role in enzyme production and absorption of dietary nutrients, however, less is known about the crypt (Pluske et al., 1996;

Hedemann et al., 2003). Deeper crypts in the small intestine have been associated with greater cell production and turnover and are often linked with negative responses in growth performance (Hedemann et al., 2003; Awad et al., 2009). However, a study in mice suggested that the crypt may also serve to protect stem cells from harmful metabolites generated by the microbiota primarily located within the colon (Kaiko et al., 2016). An observed increase in colon crypt depth may indicate the cells responding to greater concentrations of metabolites produced in the colon. As some of these metabolites, including butyrate and other volatile fatty acids are beneficial to the host, serving as secondary energy sources, increased colonic crypt depth with the addition of *Bacillus subtilis* may be related to positive changes in the microbiota, increasing metabolite production (Wong et al., 2006; Kaiko et al., 2016). Still other researchers have demonstrated the importance of increased crypt size for the initiation of crypt fission, a necessary part of intestinal growth and regeneration (Park et al., 1997).

Goblet Cell Concentrations

In the current study, no differences were observed in goblet cell concentration within the duodenum or ascending colon, when expressed either as goblet cells per crypt or goblet cells per µm of crypt depth. Concentrations of goblet cells per crypt d 21 were (14.1 vs. 15.3 ± 1.6 cells; $P = 0.59$) in the duodenum, and (20.3 vs. 20.6 ± 2.6 cells; $P = 0.93$) in the ascending colon for CON and DFM, respectively. On d 42, goblet cell concentrations were (21.3 vs. 19.2 ± 1.5 cells; $P = 0.35$) in the duodenum, and (26.7 vs. 28.7 ± 2.6 cells; $P = 0.60$) in the ascending colon for CON and DFM, respectively. When expressed as goblet cells per µm of crypt depth, overall concentrations for CON and DFM were (0.049 vs. 0.047 ± 0.002 cells/µm; $P = 0.66$) in the duodenum, and (0.083 vs. 0.081 ± 0.007 cells/µm; $P = 0.84$) in the ascending colon,

respectively. While there are multiple classes of goblet cells, being neutral or acidic, and within the acidic class existing as either sulfated and non-sulfated (Croix et al., 2011), they were not distinguished in the current study. Davis et al. (2007) found that administering *Lactobacillus brevis* to piglets throughout lactation and the nursery phase, led to greater production of non-sulfated goblet cells in the duodenum, while decreasing the number of sulfated goblet cells in the duodenum and jejunum. Similar decreases in the non-sulfated goblet cells were observed in the pigs supplemented *Lactobacillus brevis* during lactation and followed with either an antibiotic or *Bacillus* DFM in the nursery (Davis et al., 2007). Another group, Brown et al. (2006) found no differences in goblet cells when comparing two different management strategies, conventional and segregated early weaning, observing no effects of management on goblet cell concentration. However, neutral goblet cells significantly decreased upon weaning, returning to above pre-weaning concentrations by 25 d post-weaning (Brown et al., 2006). Another study agreed with these results, observing effects of weaning on goblet cell concentration, with pigs evaluated at weaning having greater goblet cell concentration than those evaluated 5 d post-weaning (Bruininx et al., 2002a). However, in the current study pigs were not evaluated until wk 3 and 6 post-weaning, likely beyond the minimal time required for full recovery of the effects of weaning on goblet cell concentrations. Goblet cells primarily function as protective cells, secreting mucins that form a layer of protection across the lumen of the small and large intestine (Specian and Oliver, 1991). A greater concentration of goblet cells in a specific segment of the GIT, may be an indication of greater protection of the mucosal layer. Additionally, goblet cell mucin production has been shown to increase in response to microbes, as colonization of mucus provides a great platform for bacterial growth (Deplancke and Gaskins, 2001). However, that did not appear to be the case in the current study. In the present study, goblet cell concentration was

not increased to coincide with the tendency of deeper crypts within the ascending colon, in pigs fed DFM (Table 3.3). Results of this analysis may have been affected by the use of photos collected for analysis of villi height and crypt depth. These photos were not originally taken for the purpose of analyzing goblet cells, and therefore goblet cell count may have been slightly affected by differences in magnification among photos, and measurement bar, which covered part of each crypt.

Immunological Markers

In the current study, DFM led to an increase in plasma levels of IgA (P = 0.03), by more than 20% relative to CON (0.58 vs. 0.37 ± 0.05 mg/ml), respectively (Table 3.4). This antibody plays an important role in clearing antigens present in the gut lumen, preventing them from entering the epithelium (Corthësy, 2009). The current results are in agreeance with other *Bacillus subtilis* studies involving nursery pigs. Lee et al. (2014) observed an increase in plasma IgA, with a linear increase as more *Bacillus subtilis* was added to the diet, leading up to a 13% increase relative to pigs on the control diet. Dong et al. (2014) observed a similar increase in plasma IgA in 70-d-old pigs, when supplemented with *Bacillus subtilis* alone, but observed no differences when *Bacillus subtilis* was combined with *Lactobacillus plantarum*. Another study supplementing *Bacillus subtilis* as part of a mixed cocktail DFM did not observed any differences in plasma IgA concentrations (Kim et al., 2014). These observed differences may be due to differences in the mechanism between mixed DFM and those composed of *Bacillus subtilis* alone. Poultry studies also appear to indicate an immune enhancement by *Bacillus subtilis*, observed through significant increases in plasma or mucosal IgA concentrations

(Amerah et al., 2013; Rajput et al., 2013; Bai et al., 2018). *Bacillus subtilis* appears to play an important role in stimulating a systemic immune response, inducting IgA production.

Intestinal fatty acid binding protein has been recognized as a biomarker of mucosal damage, as the two are highly correlated (Lau et al., 2016; Pietro et al., 2019). In the current study, no differences were observed in plasma iFABP (Table 3.4). There are no other known studies evaluating *Bacillus*-based products on iFABP concentrations as a marker for tissue injury. Research comparing plasma iFABP in weaned or nursing pigs indicated that differences in plasma iFABP may involve factors beyond those associated with weaning, observing a greater within-litter correlation to iFABP levels of their mother (Berkeveld et al., 2008). A study using a rodent model evaluated the ability of *Bacillus subtilis* to minimize the adverse effects associated with excessive exercise. Supplemented rats were able to maintain a similar concentration of plasma iFABP to that of sedentary rats, while control exercised rats had significantly greater concentrations, indicating a protective role of *Bacillus subtilis* to environmental stressors (Ducray et al., 2019).

Gastrointestinal functionality biomarkers have been evaluated in many studies involving a *Bacillus subtilis*-based DFM, however there does not appear to be a single, representative biomarker for capturing its local effects within the GIT, but rather requires analysis of several markers for an accurate interpretation (Pietro et al., 2019). In the current study, DFM tended to increase jejunal IL-10 concentrations on d 42 ($P = 0.12$; 133 *vs.* 237 ± 49 pg/mL, respectively) and overall ($P = 0.10$; 113 vs. 194 ± 35 pg/mL), No other differences were observed in either the jejunum or ileum (Table 4). This increase in anti-inflammatory cytokine IL-10 has been observed previously (Zhang et al. 2017). In contrast, Walsh et al. (2012) found that *Salmonella*

typhimurium challenged pigs supplemented with *Bacillus subtilis* did not have greater mucosal TNFα concentrations, relative to a negative control group. Others observed no changes in plasma TNFα with *Bacillus subtilis* and two different concentrations of fiber (Jaworski et al., 2017). Zhang et al. (2017) found when feeding *Bacillus subtilis* fermented soybean meal, relative to a control diet, fermented soybean meal led to decreases in IL-6 and increases in IL-10 in both the jejunum and ileum. However, other researchers feeding *Bacillus subtilis* to pre-weaned dairy calves found no changes in either IL-6 or IL-10 (Sun et al., 2010). Variations in these results may involve several factors such as dietary composition and the health status of the animals, in addition to differences in the bacterial strains used in the study. Rodent macrophages cultured with *Bacillus subtilis* displayed a stimulated immune response with greater concentrations of nitric oxide and various cytokines, including IL-10 and IL-6 (Xu et al., 2012). These results appear to indicate that *Bacillus subtilis* may support the simulation of a local intestinal immune response by altering concentrations of specific cytokines and other immunity markers.

Rotavirus

Within the first week of the study, routine diarrhea cases occurred throughout the nursery. Pooled fecal samples collected on d 9 of the study were sent to Iowa State University Veterinary Diagnostic Lab (Ames, IA) for analysis. Laboratory analysis of these samples indicated the presence of rotavirus in the feces of sampled pigs, from both treatments. However, PCR results indicated a numerically lower concentration of rotavirus A and C in pigs supplemented with DFM (Figure 3.1). Statistical analysis of these samples was not conducted as it was not a planned comparison and analysis was limited to two pooled samples per treatment. These differences may help explain other observed results, as an indicator of the *Bacillus subtilis*

competitively excluding specific pathogens present in the GIT, helping to restore the balance of the microbiome (Zimmerman et al., 2001). The role of *Bacillus subtilis* may be two part, directly decreasing the presence of pathogens, and increasing the production of IgA. In monogastric, including pigs, secretory IgA is the primary immunoglobin present in milk (Saif and Fernandez, 1996). While in lactation, piglets receive needed immunoglobins in the form of milk, however the transition to grain-based diets upon weaning eliminates the passive immunity received from the mother. In the current study, *Bacillus subtilis* led to a significant increase in plasma IgA within the first 21 d post weaning. This increase in IgA, an antibody important for clearing antigens to protect the epithelial layer (Corthësy, 2009), possibly decreased the presence of specific pathogens, including rotavirus A and C, in the GIT of DFM supplemented pigs.

Conclusions

In conclusion, addition of a multi-strain *Bacillus subtilis*-based DFM in nursery diets appeared to lead to beneficial impacts on intestinal morphology, observed through greater villus height in the jejunum, and deeper crypts in the ascending colon. In agreeance with other results of our research (Chapter 2, Lewton et al., 2020a), DFM appears to provide benefits specific to the mid-jejunum. These changes appear to occur as soon as three weeks into supplementation and are sustained as long as six weeks. *Bacillus subtilis* appears to improve the immune function of nursery pigs, observed in 20% higher plasma IgA concentrations and increased expression of jejunal anti-inflammatory cytokine IL-10. The current study was limited by the overall superior health of the pigs involved. Compared to other similar studies, this group of pigs performed

exceptionally well (Chapter 2, Lewton et al., 2020a), which may have limited the effects of DFM on intestinal morphology and immunological markers.

APPENDIX

APPENDIX

Table 3.1. Composition of diets, control and diet containing multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM), across all three dietary phases, as-fed basis

	Phase 1, d0-14		Phase 2, d14-28		Phase 3, d28-42	
Ingredient %	Control	DFM	Control	DFM	Control	DFM
Corn	40.18	40.13	48.36	48.31	50.99	50.95
Soybean meal, 47.5% CP	17.30	17.30	21.15	21.15	22.65	22.65
Corn DDGS, 7.5% oil	5.00	5.00	10.00	10.00	20.00	20.00
Dried whey, 72% lactose	25.00	25.00	10.00	10.00	-	-
Fish meal	3.00	3.00	4.50	4.50	-	-
Spray-dried bovine plasma	4.00	4.00	-	-	-	-
Corn oil	3.00	3.00	3.00	3.00	3.00	3.00
Calcium carbonate, 38.5% Ca	0.65	0.65	0.60	0.60	0.85	0.85
Monocalcium phosphate, 21.5% P	0.55	0.55	0.55	0.55	0.45	0.45
Salt	0.30	0.30	0.55	0.55	0.60	0.60
L-Lysine HCl	0.35	0.35	0.50	0.50	0.65	0.65
DL-Methionine	0.15	0.15	0.15	0.15	0.14	0.14
L-Threonine	0.13	0.13	0.19	0.19	0.21	0.21
L-Tryptophan	0.03	0.03	0.06	0.06	0.06	0.06
L-Valine	0.07	0.07	0.10	0.10	0.10	0.10
VTM premix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Phytase	0.05	0.05	0.05	0.05	0.05	0.05
DFM[2]	-	0.05	-	0.05	-	0.05
Total, 100%	100	100	100	100	100	100

[1]VTM premix provided the following vitamins and microminerals in the following concentrations: zinc 83.4 g/kg, iron 66.7 g/kg, manganese 33.4 g/kg, copper 10 g/kg, iodine 0.3 g/kg, selenium 0.2 g/kg, vitamin A 7,363 KIU, vitamin D 1,177 KIU, vitamin E 44,112 IU, menadione 1.5 g, vitamin B12 0.02 g, riboflavin 4.7 g, pantothenic acid 14.7 g, niacin 29.4 g, thiamine 0.7 g, pyridoxine 2.9 g, folic acid 1.1 g, and biotin 0.1 g.
[2]Direct-fed microbial was mixed at an inclusion rate of 0.5 g/kg of diet

Table 3.2. Analyzed composition of control diet and diet containing multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM), across all three dietary phases

	Phase 1, d0-14		Phase 2, d14-28		Phase 3, d28-42	
Item	Control	DFM	Control	DFM	Control	DFM
DM, %	91.45	91.40	90.31	90.34	90.61	90.91
GE, kcal/kg	4,608	4,611	4,678	4,597	4,762	4,736
CP, %	21.70	21.40	21.70	22.00	21.90	22.60
Crude Fat, %	4.50	4.30	5.20	5.10	6.60	6.70
Crude Fiber,%	1.60	1.70	2.10	2.20	3.30	3.00
NDF, %	6.20	6.20	8.30	8.10	11.10	12.00
ADF, %	2.90	2.50	4.00	4.00	5.50	5.80
Titanium, ppm	-	-	684	671	661	692
Indispensable AA, %						
Arg	1.10	1.10	1.10	1.10	1.10	1.10
His	0.51	0.51	0.50	0.50	0.52	0.53
Ile	0.91	0.92	0.90	0.91	0.87	0.88
Leu	1.85	1.89	1.83	1.84	1.95	2.02
Lys	1.58	1.53	1.56	1.46	1.49	1.71
Met	0.50	0.42	0.45	0.48	0.42	0.46
Met + Cys	0.91	0.81	0.76	0.81	0.76	0.79
Phe	0.98	0.97	0.98	0.99	1.05	1.07
Thr	1.00	1.03	0.97	0.96	0.91	0.94
Trp	0.30	0.30	0.28	0.34	0.26	0.28
Val	1.13	1.15	1.08	1.10	1.05	1.07
Dispensable AA, %						
Ala	1.06	1.07	1.11	1.09	1.13	1.16
Asp	1.94	1.93	1.86	1.87	1.77	1.73
Cys	0.41	0.39	0.31	0.33	0.34	0.33
Glu	3.28	3.27	3.32	3.33	3.37	3.32
Gly	0.80	0.80	0.88	0.86	0.79	0.79
Pro	1.17	1.19	1.23	1.20	1.29	1.32
Ser	0.84	0.84	0.80	0.80	0.82	0.82
Tyr	0.66	0.68	0.65	0.63	0.68	0.68

Table 3.3. Intestinal morphology across gastrointestinal segments, measured at two time points for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)[1]

Day	Segment	Control	DFM	SEM	*P*-value
Villi height, µm					
21	Duodenum	432	451	22	0.55
	Jejunum	370	406	21	0.24
	Ileum	370	381	12	0.51
42	Duodenum	512	486	20	0.38
	Jejunum	399	439	21	0.20
	Ileum	407	404	12	0.89
Overall[2]	Duodenum	472	468	17	0.87
	Jejunum	385	422	10	0.02
	Ileum	388	393	8	0.67
Crypt depth, µm					
21	Duodenum	340	359	11	0.25
	Jejunum	297	319	13	0.24
	Ileum	238	233	7	0.67
	Ascending colon	337	373	14	0.10
42	Duodenum	354	359	11	0.72
	Jejunum	316	321	13	0.81
	Ileum	252	248	7	0.73
	Ascending colon	410	424	14	0.52
Overall	Duodenum	347	359	8	0.29
	Jejunum	307	320	8	0.24
	Ileum	245	241	5	0.60
	Ascending colon	374	398	11	0.15

[1]Data representing intestinal morphology, n=6 to 8 observations per treatment, 1 to 2 missing values in duodenum and ileum due to damaged tissue upon collection

[2]Overall values are based on average of d 21 and 42 LSmeans

[a,b]Values in a common row lacking a common superscript differ ($P \leq 0.05$)

[x,y]Values in a common row lacking a common superscript tend to differ ($P \leq 0.15$)

Table 3.4. Immunological markers in both plasma and intestinal mucosa, measured at two time points for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)[1]

Location/Item	Day	Control	DFM	SEM	*P*-value
Plasma					
IgA, mg/mL					
	21	0.34[y]	0.54[x]	0.07	0.06
	42	0.82	0.92	0.07	0.29
	Overall[2]	0.58[b]	0.73[a]	0.05	0.03
iFABP, pg/mL					
	21	467	410	83	0.63
	42	496	347	88	0.24
	Overall	481	379	61	0.25
Ileum					
IL-6, pg/mL					
	21	363	331	41	0.57
	42	437	480	39	0.44
	Overall	400	405	29	0.90
IL-10, pg/mL					
	21	147	168	48	0.77
	42	225	258	56	0.69
	Overall	186	213	36	0.61
TNFa, pg/mL					
	21	110	85	16	0.27
	42	110	101	15	0.68
	Overall	110	93	11	0.30
Jejunum					
IL-6, pg/mL					
	21	328	382	38	0.33
	42	403	384	40	0.72
	Overall	366	382	30	0.69
IL-10, pg/mL					
	21	93	152	35	0.26
	42	133[y]	237[x]	49	0.13
	Overall	113[y]	195[x]	35	0.10
TNFa, pg/mL					
	21	79	76	15	0.89
	42	78	62	15	0.46
	Overall	78	69	11	0.54

[1]Data of immunological markers, n=6 to 8 observations per treatment, deemed outliers when greater than ± 2 SD from mean

[2]Overall values are based on average of d 21 and 42 LSmeans

[a,b]Values in a common row lacking a common superscript differ ($P \leq 0.05$)

[x,y]Values in a common row lacking a common superscript tend to differ ($P \leq 0.15$)

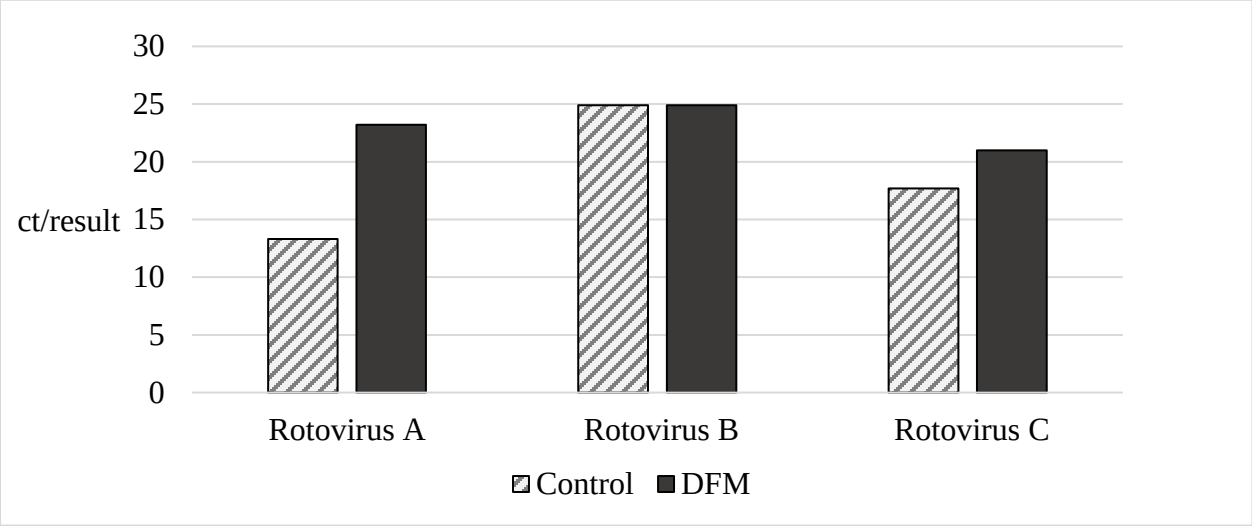


Figure 3.1. PCR of pooled fecal sample analysis, a total of two representative pooled samples from a total of four pigs per treatment, represented as cycle threshold per test (ct/test), for control diet and multi-strain *Bacillus subtilis*-based direct-fed microbial (DFM)

CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

The event of weaning is extremely stressful on a young pig's body, involving many associated factors that all add up to a very susceptible, compromised weanling pig (Pluske, 2013; Moeser et al., 2007). As the industry continues to seek potential antibiotic alternatives, *Bacillus subtilis*-based direct-fed microbial (DFM) may be one of the most reasonable solutions. While much research is still needed, and each strain may slightly differ in its mechanics and characteristics, *Bacillus subtilis* has proven itself as an effective microbial on many occasions (Lee et al., 2014; Blavi et al., 2018; Tang et al., 2019; Kim et al., 2019). Within the gastrointestinal tract (GIT), the specific multi-strain *Bacillus subtilis*-based DFM utilized by Augspurger et al. (2016) appears to have a multi-part mechanism, effecting intestinal morphology and immunological marker concentrations, in addition to specific nutrient digestibility. Increases in immunoglobulin A (IgA) with the addition of *Bacillus subtilis*, may be related to changes in the intestinal microbiota and decreases in specific pathogens present in the GIT, however, this has yet to be confirmed. As an antibody important for protecting the mucosal barrier (Corthësy, 2009), greater IgA concentrations likely led to less mucosal damage and increased cell proliferation within specific segments of the GIT. This was confirmed by increased villus height and interleukin 10 (IL-10) concentrations in the jejunum. Related to these structural changes, *Bacillus subtilis* appears to impact digestibility of specific amino acids (AA) within the jejunum, including cysteine, methionine, and tryptophan. Observed improvements in growth performance may be related to greater absorption of AA and improved environment of the GIT. *Bacillus subtilis* also appears to lead to greater hindgut fermentation observed through

tendencies of decreased nitrogen (N) digestibility in the distal colon. These changes may be related to tendencies for deeper crypts in the ascending colon, an indication of greater metabolite production (Kaiko et al., 2016), including volatile fatty acids (VFA), however VFA analysis of digesta is yet to be completed in this study.

Addition of this multi-strain *Bacillus subtilis*-based DFM may be beneficial in a variety of commercial settings, with pig health being one of the major factors determining overall effectiveness. Additionally, dietary formulation may alter the impact of this DFM on weanling pig growth performance and health status. Diets used in this study included corn distillers dried grains with solubles (DDGS), up to 20% in phase three (Tables 2.1 and 3.1), which presents varied nutrient digestibility and maybe a source of unknown microbes, may have impacted the effects of *Bacillus subtilis* in other subtle ways. This could also be the case for other common alternative feed ingredients added to nursery diets such as blood plasma, prebiotics, dietary enzymes, or pharmacological inclusions of copper or zinc, as each of these ingredients are known to impact pig health and performance (Bhandari et al., 2008; Lee et al., 2009; Perez et al., 2011; Zhang et al., 2014). As we continue to learn more about the benefits of DFM supplementation and those normally present in feed and the environment, the focus may shift toward feeding specific bacterial strains at different times throughout the growing pig's lifetime; prior to birth, pre-weaning, post-weaning, in the nursery, and during the grower/finishing phase of production. There also may be sex specific strains or strain combinations that are more beneficial to barrows than gilts and vice versa. These are all questions that should be addressed as we continue to learn more about the microbiome and DFM supplementation.

During the presentation of these results, a question was asked regarding differences in some measures that may have been related to sex of the pig. The present research was conducted using pen as the experimental model, a comparison of sex differences was not planned as the experiment was not designed to do so. However, research has demonstrated sex differences in inflammatory response in the post-weaning period (Medland et al., 2016; Pohl et al., 2017). Out of the interest in continuing to learn more about this unique DFM, sex was added to a second analysis of the immunological markers analyzed in this study. Intestinal fatty acid binding protein was the sole marker to indicate any sex differences, with females fed CON tended to have a greater intestinal fatty acid binding protein (iFABP) plasma concentration ($P = 0.12$) than females fed DFM (580 vs 342 ± 90 pg/mL), respectively. As females are known to have a greater inflammatory response and heightened intestinal permeability in the post-weaning period (Medland et al., 2016; Pohl et al., 2017), the DFM may have helped reduce intestinal permeability and provide greater protection to the females hypersensitive response. While not part of our original analysis, this may provide a slight indication that specific DFM supplementation may provide sex-specific benefits.

Based on the results of this research, this unique multi-strain *Bacillus subtilis*-based DFM performs in the GIT of weanling pigs by way of stimulation of the immune system. Moving forward, additional research is needed focusing on other mechanisms including competitive exclusion (Cai et al., 2015) and specific enzyme (Priest, 1977) or antimicrobial secretions (Hong et al., 2005). The present study was the first to evaluate a time by GIT segment interaction on nutrient digestibility bringing to light many interesting results. Our research also indicated that this DFM has an early impact in the nursery period. We believe continued study of mechanisms

within specific segments of the weanling pig GIT, across several time points, would be of great value.

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