ABSTRACT

BROOKS, KAYLA LEANNE. The Use of *Bacillus*-based Direct-fed Microbials to Improve Growth Performance and Gut Health of Nursery Pigs. (Under the direction of Dr. Sung Woo Kim.)

Concerns about the use of antibiotics as growth promotors in livestock production have led to the removal of many antimicrobial growth promotors from livestock production. The use of direct-fed microbials (DFMs) as an antibiotic alternative has generated significant interest over the last decade. However, research findings in pigs have been extremely variable and the novel strain *Bacillus subtilis* 29784 has yet to be evaluated in pigs. Therefore, to address the current issue, three experiments were conducted.

The objective of experiment 1 was to determine the effects of *Bacillus*-based DFMs on growth performance, gut health, immune status, and oxidative stress status in nursery pigs. Three-hundred and sixty nursery pigs were randomly assigned to 1 of 3 treatments based on a randomized complete block design, with sex and initial body weight as blocks: 1) Basal diet (CON); 2) Basal diet + DFM *Bacillus subtilis* 29784 (DFM-1); 3) Basal diet + DFM based on *Bacillus subtilis* and *Bacillus licheniformis* (DFM-2). Both DFM treatments improved ($P < 0.05$) average daily gain (ADG) compared to the CON from d 15 to 42 and during the overall experimental period. Both DFM treatments also improved ($P < 0.05$) average daily feed intake (ADFI) compared to the CON during the overall experimental period. Both DFM treatments tended to ($P = 0.090$) lower the pH in colon digesta. In addition to improvements in growth, DFM-1 increased ($P < 0.05$) concentrations of tight junction protein zonula occludens-1 (ZO-1) compared to the CON and DFM-2 treatments.

The objective of experiment 2 was to determine the optimal dose of *Bacillus subtilis* 29784 to improve growth performance of nursery pigs. Fifty-four newly weaned piglets were

randomly allotted to 1 of 3 treatments: 1) T1: Basal diet; 2) T2: T1 + *Bacillus subtilis* (1.0 x 10^8 CFU/kg feed); 3) T3: T1 + *Bacillus subtilis* (2.5 x 10^8 CFU/kg feed). Days 1 to 14 were considered phase 1 and days 15 to 42 were considered phase 2. Increasing concentrations of *Bacillus subtilis* had a quadratic effect ($P < 0.05$) on d 14 BW with the greatest d 14 BW (10.1 kg) achieved with T2. From d 1 to 14, the concentrations of *Bacillus subtilis* tended to quadratically affect both ADG ($P = 0.064$) and ADFI ($P = 0.097$) with the highest phase 1 ADG (242 g/d) and ADFI (259 g/d) achieved with T2. Concentration of *Bacillus subtilis* did not affect G:F from d 1 to 14, d 15 to 42, or the overall experimental period. No effects of *Bacillus subtilis* concentrations on growth performance were observed from d 15 to 42 or the overall experimental period. The concentration of *Bacillus subtilis* did not affect fecal scores.

The objective of experiment 3 was to determine the effects of *Bacillus subtilis* 29784 on gut health and prevention of post-weaning diarrhea caused by $F18^+$ *Escherichia coli*. Forty nursery pigs were randomly allotted to 1 of 4 treatments with initial BW as a blocking factor: 1) NC: a diet with no additive and no $F18^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and $F18^+$ *Escherichia coli* challenge; 3) AGP: PC + antibiotics (CTC 100 and Denagard for phase 1 and Mecadox for phase 2) and ZnO; 4) PRO: PC + *Bacillus subtilis* 29784. The $F18^+$ *Escherichia coli* challenge was administered on d 7. Body weights were not affected by treatment during wk 1, 2 or 3. During wk 4, both AGP and PRO had higher body weights than PC ($P < 0.05$) but were not different from NC (Table 2). No effects of treatment were observed on ADG during the entire experimental period. During wk 2, AGP and PRO improved ($P < 0.05$) ADFI compared to PC but not compared to NC (Table 2). There were no differences in ADFI among treatments during wk 3. However, during wk 4, all challenged treatments (PC, AGP, and PRO) had decreased ($P < 0.05$) ADFI compared to NC (Table 2). No effects of treatment were observed on G:F during wk 1, 2, or 4. However, during wk 3, the challenged treatments (PC, AGP, PRO) tended to have higher ($P = 0.095$) G:F than NC (Table 2). The PRO and AGP tended to ($P = 0.097$) have lower protein carbonyl than PC which was an indicator of improved oxidative stress status. Contrary to the hypothesis, The PRO did not prevent postweaning diarrhea caused by F18$^+$ *Escherichia coli*.

In conclusion, experiments 1 and 3 demonstrated that *Bacillus*-based DFMs can improve growth performance and gut health of nursery pigs in natural exposure conditions and may reduce the relative abundance of pathogenic bacteria during a pathogen challenge. Experiment 2 demonstrated that in order to achieve optimal growth performance, *Bacillus subtilis* 29784 should be supplemented at 1.0 x 10^8 CFU/kg feed. Overall, these experiments show that *Bacillus*-based DFMs could be a potential alternative to antibiotics although more research is needed.

The Use of *Bacillus*-based Direct-fed Microbials to Improve Growth Performance and Gut Health of Nursery Pigs

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BIOGRAPHY

Kayla Leanne Brooks was born on September 13th, 1991 in South Boston, Virginia. Her interest in livestock animals and agriculture began during her childhood in rural Virginia. She received her B.S. degree in Animal and Poultry Sciences in 2012 from Virginia Polytechnic Institute and State University. After completing her B.S., Leanne decided to pursue a M.S. degree in Animal and Poultry Sciences under the direction of Dr. Robert Rhoads where she studied the safety and adequacy of galactooligosaccharides and fructooligosaccharides in neonatal pig formula. She received her M.S. degree in 2014 from Virginia Polytechnic Institute and State University. In 2014, she spent three months as an intern at the Novozymes location in Salem, Virginia where she worked as part of the direct-fed microbials group. In the fall of 2014, Leanne began her Ph.D. in Animal Science under the direction of Dr. Sung Woo Kim. Her research explored the use of *Bacillus*-based direct-fed microbials in nursery pigs.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADG: average daily gain

ADFI: average daily feed intake

BW: body weight

CFU: colony forming unit

CP: crude protein

d: day

DM: dry matter

DFM: direct-fed microbial

ETEC: enterotoxigenic *Escherichia coli*

FDA: Food and Drug Administration

g: gram

G:F: gain to feed ratio

h: hour

IgA: immunoglobulin A

IgG: immunoglobulin G

MDA: malondialdehyde

min: minute

NRC: National Research Council

PC: protein carbonyl

SEM: standard error of the mean

SID: standardized ileal digestibility

SOD: superoxide dismutase

STEC: Shiga toxin producing *Escherichia coli*

TLR: toll-like receptor

TNFα: Tumor necrosis factor α

USDA: U.S. Department of Agriculture

VH:CD: villus height to crypt depth ratio

VFD: Veterinary Feed Directive

wk: week

ZO-1: zonula occludens-1

CHAPTER 1

LITERATURE REVIEW

Introduction

Direct-fed microbials (DFMs) have been used in livestock production for many years. However, interest in the supplementation of DFMs as a method of improving growth performance and animal health has increased in the last few decades due to increased concerns about the use of antibiotics in livestock production. Growing concerns about the development of antibiotic resistance as well as consumer demands for antibiotic-free products led to the implementation of the Veterinary Feed Directive (VFD) which restricts the use of any “medically important” antimicrobial agent in, or on animal feed. These factors combined have made the reduction or elimination of antibiotic usage in livestock production imperative. This poses a challenge for pig producers as the removal of antibiotics often leads to compromised growth performance and increased incidents of disease (Wierup, 2001; Stein, 2002) in certain stages of production.

Antibiotics can often be removed from the diets of growing-finishing pigs without causing any evident disease problems, whereas occurrence of disease and performance problems may be exacerbated during the post weaning period (Wierup, 2001). In order to minimize the adverse effects of weaning, especially in non-medicated swine production, new strategies to improve nutrition and health are crucial. One strategy of interest is the use of antibiotic alternatives to improve animal health and performance. The use of antibiotic alternatives such as DFMs to improve animal health and performance is one such strategy.

The increased interest in DFMs has led to an extensive body of research. However, the results from this research have been conflicting. Studies conducted in this area have been extremely variable, both in the type and strain of DFM used as well as in the experimental design. Several experiments have evaluated DFMs in natural exposure conditions whereas others have incorporated an immune challenge. DFMs can enhance animal performance and health by various modes of action. However, individual DFMs may differ in the modes of action used to benefit animal performance and health. This review will focus on the use of DFMs in swine production with a specific focus on research conducted on the supplementation of *Bacillus*-based DFMs and their potential modes of action.

Direct-fed Microbials

The use of DFMs in livestock production has occurred for many years with increased interest during the last 15 years due to concerns about the use of antibiotics in livestock production. Formerly referred to as probiotics, the U.S. Food and Drug Administration (FDA) now defines DFMs as “products that are purported to contain live, viable microorganisms” (FDA, 2015). The FDA and American Association of Feed Control Officials regulate DFMs as feed ingredients and recognize a list of microorganisms that have been approved for use in animal feed. There are several categories of DFMs including *Bacillus*, lactic acid bacteria, and yeasts. Numerous DFMs within these categories have been studied with the majority of the studies focusing on bacteria.

Several criteria are used in order to determine which microorganisms are suitable for use as DFMs. Probiotic bacteria must be safe for consumption by animals or humans, prepared on a large scale and in a viable manner, they must remain stable and viable in storage and during use, they must be able to survive in the gastrointestinal tract, and they should confer direct and indirect beneficial effects on their host (Fuller, 1989; Ohashi and Ushida, 2009). In order for DFMs to survive in the gastrointestinal tract, they must be resistant to gastric acid, bile acid, and digestive enzymes (Dunne, 2001; Larsen et al., 2014).

Furthermore, certain effects of a DFM on its host depends on the number of viable cells that are present in the gastrointestinal tract (Charteris et al., 1998; Alexopoulos et al., 2004) and therefore, the actual level of bacteria present and their viability must be assessed.

Sources of variability in DFM studies

It is often concluded that the effects of DFMs are more inconsistent than other feed additives, such as antibiotics. It is important to note that when comparing results from DFM studies, there are many sources of variability. Studies vary in experimental design, DFM species, DFM dose, and the type of challenge used, if any (Buntyn et al., 2016). Part of the inconsistency could be due to the fact that there are several experimental models that have been used to investigate the effects of DFMs in livestock including natural exposure conditions, a pathogen challenge, or direct immune challenge. The use of these experimental models depends on the specific mode of action being investigated.

The efficacy of DFMs depends on numerous factors such as the species composition, administration level, viability, method of application, and supplementation frequency. In addition to the variability in DFMs, other aspects of experimental studies must also be evaluated as potential sources of variability. Environmental conditions, stage of production (age), herd health status, and farm cleanliness can also influence the effectiveness of DFMs and contribute to the inconsistent results often associated with DFM research (Buntyn et al., 2016).

Another crucial aspect of DFM studies to consider is the variation in individual response. Regardless of species, numerous studies have reported benefits of DFM supplementation whereas little to no effects have been observed in other studies. Moreover, in studies reporting beneficial effects, statistical significance is rarely achieved even with a difference of 5% or greater (Simon et al., 2001). This indicates large variability in the reactions of the individual animals to a DFM. Therefore, when evaluating the literature in this area and making comparisons between DFM studies, it is important to account for all of these factors before drawing any conclusions.

Bacillus species

Bacteria from the genus *Bacillus* have been researched extensively due to their ability to form highly resistant endospores, produce antimicrobial compounds, and synthesize a variety of exogenous enzymes. Gram-positive *Bacillus* species are well known for their production of enzymes and it has been discovered that a large fraction of the genetic capacity is dedicated to the ability to utilize a variety of carbon sources including several molecules derived from plants (Kunst et al., 1997). Given all of these characteristics, *Bacillus* species are primarily distinguished from other types of DFMs, such as lactic acid bacteria, by their unique ability to form spores.

When environmental conditions become unfavorable, bacteria from the *Bacillus* genus have the ability to form spores. These spores protect the bacterial genome until favorable environmental conditions are reestablished and the spore can germinate into a vegetative state (McKenney et al., 2012). In order to survive in a harsh environment with fewer nutrients, spores are partially dehydrated and metabolically dormant (McKenney et al., 2012). Spores are extremely resistant to a variety of conditions, including extreme heat, a broad range of pH, chemicals such as peroxide and hypochlorite, and ultraviolet radiation (Nicholson et al., 2000; McKenney et al., 2012). These characteristics, make *Bacillus* species ideal DFM candidates because spores can survive the feed manufacturing process and therefore, can be incorporated in not only mash diets but pelleted diets as well. The resistant properties of spores also allow them to survive the low pH in the stomach, and pass to the small intestine where they can germinate and begin exerting their effects (Casula and Cutting, 2002; Leser et al., 2008). Spores have both a unique structure and components that are responsible for resistance to environmental stress and dormancy (Setlow, 2006). Spores contain several layers that are not present in vegetative cells. All endospores formed by *Bacillus* are enclosed in a coat, which in some species, may be the outermost layer of the spore, whereas other species may contain an additional layer known as the exosporium (Henriques and Moran, 2007). Both the coat and exosporium protect the spore and have important roles in the reaction of the spore with its environment (Henriques and Moran, 2007; Mckenney et al., 2013). Spores can actively interact with their environment, despite the metabolic inactivity of the core of the spore (Henriques and Moran, 2007). The layers under the coat consist of the outer membrane, the peptidoglycan cortex, the peptidoglycan germ cell wall, and the inner spore membrane (Setlow, 2014).

Bacillus spores are considered dormant due to the fact that they have little to no metabolic activity. However, when provided with favorable conditions, germination can occur followed by outgrowth, which results in a vegetative cell. Germination can be germination can be triggered by a variety of agents including nutrients, calcium dipicolinate (CaDPA), dodecylamine, fragments of peptidoglycan, and high pressure (Setlow, 2014). In nature, it is probable that nutrients such as single amino acids, sugars or purine nucleosidases initiate germination by binding to spore-specific protein complexes known as germinant receptors in the inner membrane (Setlow, 2003; Setlow, 2014). Specifically, spores of

Bacillus subtilis will germinate in response to the free amino acids, L-Ala, L-Val, and L-Apn, whereas D-amino acids will not initiate germination (Atluri et al., 2006; Setlow, 2014). Spores may germinate as quickly as a few minutes, however some spores may take longer than 24 h to germinate following exposure to a germinant. Once a spore has committed to germination, ions including H+, K+, and Na+, followed by CaDPA are released due to a change in the permeability of the inner membrane (Setlow, 2014). Following the release of CaDPA, the peptidoglycan cortex is degraded by cortex-lytic enzymes which allows the germ cell wall to expand so that the core of the spore can expand and take up water, which allows metabolism to begin (Setlow, 2014).

Given that spores will germinate in response to nutrients, when incorporated into livestock feed, *Bacillus* spores will germinate in the gastrointestinal tract and become vegetative cells; however, little to no outgrowth of the species occurs (Casula and Cutting, 2002; Leser et al., 2008). Therefore, *Bacillus subtilis* and *Bacillus licheniformis* are unable to permanently colonize the gut and thus require continuous administration in both pigs and poultry (Cartman et al., 2008; Leser et al., 2008; Latorre et al., 2014).

The species of *Bacillus* most commonly used in livestock feed are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, and *Bacillus coagulans* (Ohashi and Ushida, 2009). Although many species of *Bacillus* have been investigated, *Bacillus subtilis* is by far the most widely used. *Bacillus subtilis* has been studied for many years and therefore it is currently the best-characterized Gram-positive bacterium (Earl et al., 2008). *Bacillus subtilis* is known for its adaptability to environmental changes, high product yields, exceptional fermentation properties and lack of toxic secondary metabolites (van Dijl and Hecker, 2013). The genome of *Bacillus subtilis* also contains several genes that are involved in the production of secondary metabolites, such as antibiotics (Kunst et al., 1997). These qualities make *Bacillus subtilis* a model species for use as a DFM in livestock feeds.

Modes of action

There have been several proposed mechanisms by which DFMs are able to exert their beneficial effects on animal performance and health. DFMs differ from antibiotics in that they exert their effects through indirect mechanisms. Proposed mechanisms include but are not limited to: enzyme activity (van Dijl and Hecker, 2013), lowering pH by acid production (Ohara and Yahata, 1996), antimicrobial production (Katz and Demain, 1977; Cladera-Olivera et al., 2004; Stein, 2005), improvement of nutrient digestibility (Lei et al., 2015; Jorgensen et al., 2016), competitive exclusion of pathogens (La Ragione, 2001; La Ragione and Woodward, 2003), modulation of the gut microbiome (Bhandari et al., 2008; Cui et al., 2013a; Yang et al., 2016; Dubreuil, 2017) and modification of the immune response (Lessard et al., 2008; Klaenhammer et al., 2012; Salim et al., 2013; Zhang et al., 2017). The majority of DFM studies have evaluated one or more of these potential modes of action. This review will further explore these potential modes of action in pigs.

Bacillus-based Direct-fed Microbial Research in Swine

The effects of DFM supplementation have been evaluated in all stages of swine production. However, given that weaning is one of the most stressful events in a pig's life, the majority of the studies that incorporated a pathogen challenge have focused on the post-weaning period. At weaning, pigs are exposed to a multitude of challenges including environmental, physiological and social stressors. In addition to being separated from the sow and transported to a new environment with new pathogens, pigs concurrently experience a shift in diet as they transition from the sow's milk to a dry, less digestible feed. As a result, weaning can result in reduced pig growth, health, and feed intake as well as increased morbidity and mortality due to intestinal and immune system dysfunctions (Campbell et al., 2013). Therefore, it is important to evaluate the use of DFMs as a strategy to assuage the effects of weaning on pig health and growth performance.

Despite the reported benefits of *Bacillus*-based DFMs, there is still some debate about the consistency of results from the use of DFM products in pigs. *Bacillus* research in pigs has yielded variable results. Several studies show improved growth performance, lower morbidity and mortality, improved gastrointestinal health of piglets, and decreased pathogen colonization (Alexopoulos et al., 2004; Cui et al., 2013; Hu et al., 2014; Lee et al., 2014). Conversely, other studies have not detected effects on growth performance (Walsh et al., 2007; Bhandari et al., 2008) or nutrient digestibility (Kaewtapee et al., 2017). It is important to note that studies conducted with *Bacillus subtilis* vary in design and strains of bacteria used which may explain the inconsistency.

Effects on growth performance and nutrient digestibility

The effects of *Bacillus*-based DFMs on growth performance of pigs have been evaluated in many different experiments. The ability of Bacillus-based DFMs to improve all aspects of growth performance (ADG, ADFI, G:F) either individually, or simultaneously, has been well documented in the literature (Alexopoulos et al., 2004; Cui et al., 2013; Upadhaya et al., 2015; Jorgensen et al., 2016; Lan et al., 2016). Due to the ability of *Bacillus* species to produce various exogenous enzymes (Kunst et al., 1997), improvements in growth performance, particularly feed efficiency, are naturally attributed to improved nutrient digestibility (Choi et al., 2011; Upadhaya et al., 2015; Jorgensen et al., 2016; Lan et al., 2016). As expected, reported improvements in feed efficiency are usually accompanied by improvements in average daily gain. However, it has also been demonstrated that *Bacillus*-based DFMs may improve ADG by increasing feed intake (Choi et al., 2011; Cui et al., 2013). These results suggest that the primary mode of action may differ among DFMs.

The beneficial effects of *Bacillus*-based DFMs on pigs may begin as early as gestation and lactation. Baker et al. (2013) reported that sows fed diets supplemented with *Bacillus subtilis* at a dose of 3.75 x 10^8 CFU/kg feed weaned more pigs, had litters with greater weaning weights and tended to have increased litter ADG compared to sows fed a control diet. These results are in accordance with a study conducted by Kritas et al. (2015) in which sows fed a diet supplemented with *Bacillus subtilis* C-3102 at 3.0 x 10^8 CFU/kg feed had litters with improved birth weights, higher weaning weights, and more piglets weaned during the second cycle compared to sows fed a control diet. Sows fed the DFM supplemented diet also had improved ADG of piglets from birth to weaning during the first cycle (Kritas et al., 2015). These studies are unique in that the majority of DFM research in pigs has focused on the production phases following weaning.

To investigate the efficacy of a DFM product containing both *Bacillus subtilis* and *Bacillus licheniformis* (Chr. Hansen BioPlus 2B) on the productivity and health of pigs from wean to finish, Alexopoulos et al. (2004) fed pigs 5 different diets with no DFM (double control) during any stage, BioPlus 2B (1.28 x 10^9 CFU/kg of feed) during weaning only, BioPlus 2B during weaning followed be either a low (0.64 x 10^9 CFU/kg of feed), medium (1.28 x 10^9 CFU/kg of feed), or high dose (1.92 x 10^9 CFU/kg of feed) of BioPlus2B during the growing and finishing phases. As expected, the pigs treated with the DFM had lower morbidity and mortality (14.8% and 3.7%, respectively) compared to the double controls (25.9% and 11.1%, respectively) (Alexopoulos et al., 2004). This reduction in morbidity and mortality was believed to be a result of a reduction in post-weaning diarrhea caused primarily by *Escherichia coli*. The BioPlus 2B improved ADG and feed conversion ratio during the weaning and growing periods with no effects on growth performance observed during the finishing stage. There were also no effects on ADFI observed during any stage (Alexopoulos et al., 2004). During the growing stage, the beneficial effects of BioPlus 2B were more apparent with the medium and high doses which could have been due to the presence of more vegetative cells.

Another BioPlus 2B® study conducted by Wang et al. (2009) investigated the effects on growing pigs. In a 35 d feeding trial, pigs were fed either a basal diet (CON) or a basal diet with one of three different levels of BioPlus 2B (0.05%, 0.10%, and 0.20%). During the experiment, increasing levels of BioPlus 2B tended to linearly increase ($P = 0.08$) ADG and also linearly increase ($P < 0.05$) ADFI (Wang et al., 2009). In contrast to Alexopoulos et al. (2004), no effects of BioPlus 2B on feed efficiency were observed. Wang et al. (2009) also reported no linear or quadratic effects of the DFM on ATTD on DM and N which contradicts results reported in other studies (Choi et al., 2011; Jorgensen et al., 2016; Lan et al., 2016). The variation in the results of these studies could be attributed to the difference in dose, or probiotic administration strategy.

The results described by Alexopoulos et al. (2004) are analogous to results from a study conducted by Jorgensen et al. (2016), in which 28 d old pigs were allotted to treatments based on a 2 x 2 factorial arrangement with 2 levels of energy (standard or reduced) and 2 levels of the *Bacillus*-based DFM (0 or 400 mg/kg) of a commercial product (BioPlus YC®) containing a mixture of *Bacillus subtilis* (DSM 5750) and *Bacillus licheniformis* (DSM 5749) at a minimum concentration of 3.2 x 10^{12} viable spores/kg of product. This experiment followed piglets from weaning through finishing.

Although no effects on growth performance were observed during the prestarter period (28 to 42 d of age), during the starter period (42 to 70 d of age), BioPlus YC® improved ADG and F:G in both the standard and reduced energy diets compared to their control equivalents (Jorgensen et al., 2016). BioPlus YC improved growth rate by 11.2% and F:G by 8.6% during the grower period (70 to 120 d of age), however it negatively affected F:G during the finisher period (120 to 182 d of age) (Jorgensen et al., 2016). No effects of BioPlus YC were observed on ADFI during any experimental period. Data from the entire experimental period (28 to 182 d of age) confirmed that the improvements in growth and F:G by BioPlus YC remained during the whole period. The authors partly attributed the improvements in growth performance during the grower phase to coinciding improvements in ATTD of fat and phosphorus (Jorgensen et al., 2016) which supports the proposed DFM mechanism of improving nutrient digestibility.

Based on the observed negative effect of the DFM on feed efficiency of finisher pigs, Jorgensen et al. (2016) also speculated that the age of the pig may influence the efficacy of DFMs and that older pigs may be more capable of resisting intestinal disorders. This theory is further supported by observations reported by Meng et al. (2010) in which a combination of *Bacillus subtilis* and *Clostridium butyricum* improved ADG and G:F during the grower phase but did not improve G:F during the finisher phase. These studies suggest that DFMs may be more effective during the weaning and growing periods than the finishing period.

The effects of *Bacillus*-based DFMs reported by Alexopoulus et al. (2004) and Jorgensen et al. (2016) are in partial agreement with results reported by Cui et al. (2013) in which *Bacillus subtilis* (2.0 x 10^{13} CFU/kg) fed to pigs weighing 10 to 110 kg BW improved ADG (597 g/d) compared to the control (577 g/d) and also improved feed to gain ratio (3.06) compared to the control (3.13). In contrast to the studies conducted by Alexopoulos et al. (2004), and Jorgensen et al. (2016), Cui et al. (2013) also demonstrated that *Bacillus subtilis* supplementation improved ADFI (1,826 g/d) compared to the control (1,806 g/d). The results reported by Cui et al. (2013) and Jorgensen et al. (2016) are comparable to results from a study conducted by Choi et al. (2011) in which weaned piglets were fed either a control diet (NC), a diet with antibiotics (PC), or one of two DFM diets prepared by either low (LT) or high (HT) drying temperatures.

Similarly to Jorgensen et al. (2016), no effects on growth performance were observed during phase 1 (d 1 to 14 post-weaning) (Choi et al., 2011). Conversely, during phase 2 (d 15 to 28 post-weaning), both LT and HT improved ADG (437 g/d and 431 g/d respectively), compared to NC (379 g/d) but were not superior to PC (453 g/d). The LT and HT also improved ADFI (621 g/d and 627 g/d, respectively), and G:F (703 g/kg and 688 g/kg, respectively), compared to NC (581 g/d and 653 g/kg) during phase 2 but were not superior to PC (638 g/d and 711 g/kg). Given that there were no differences between LT and HT, the authors concluded that the beneficial effects of DFMs used may reach a maximum level beyond which, no additional effects would be observed (Choi et al., 2011). The improvements in growth performance in this study were mainly attributed to improved nutrient digestibility given the enhancement of ATTD of DM by LT and ATTD of GE by HT compared to PC and NC (Choi et al., 2011) and these results are comparable to Jorgensen et al. (2016). Whereas these results further support the beneficial effects of *Bacillus subtilis*, it is important to note that the DFM used by Choi et al. (2011) was a combination of *Bacillus subtilis*, *Lactobacillus acidophilus*, *Saccharomyces cervisiae*, and *Aspergillus oryzae*.

Another study using a multi-strain DFM was conducted by Lan et al. (2016). In this study, 125 weaned pigs at 28 d of age were fed 1 of 5 diets including a basal diet (control), or diets with 4 different doses of a multi-strain DFM containing a mixture of spores of *Bacillis subtilis*, *Bacillus licheniformis*, *Bacillus coagulance*, and *Clostridium butyricum* (0.01%, 0.03%, 0.06%, and 0.10%). This study reported that the DFM treatments linearly improved ADG during d 1 to 14, however no effects on ADFI or G:F were observed. However, during d 15 to 42, the linear improvement in ADG by the DFM treatment was accompanied by linear improvements in G:F although no differences in ADFI were observed. The improvements in ADG and G:F in this study could have been attributed to improvements in nutrient digestibility, as the DFM treatments linearly improved the ATTD of DM, N, and GE compared to the control (Lan et al., 2016).

In contrast to studies exhibiting an improvement in ADG accompanied by either an improvement in ADFI or G:F, Upadhaya et al. (2015) reported improvements in only ADG during wks 0 to 6 of a study in growing pigs fed either a control diet or a diet containing 1.47 x 10^{11} CFU/kg of *Bacillus* organisms. Although, at wk 6, the DFM treatment improved ATTD of DM and N compared to the control and also decreased fecal ammonia emissions at the end of wk 6 and 15 (Upadhaya et al., 2015). Therefore, the authors concluded that *Bacillus* organisms could improve growth performance and DM digestibility whilst also reducing fecal ammonia content.

Collectively, these results demonstrate that *Bacillus*-based DFMs can have beneficial effects on growth when used either alone or in combination with other species. These studies also indicate that the age of pigs, preparation of the DFM, composition of the diet, and the DFM inclusion level may affect the potential benefits of DFMs on growth performance and nutrient digestibility. Consequently, when producers are choosing a DFM for potential use in their production system, there are a lot of factors to consider. Whereas improvements in growth performance and nutrient digestibility are extremely beneficial, DFMs may also be capable of improving other aspects of animal performance such as gut health that could further benefit animal production and welfare.

Effects on gut health

Several of the previously proposed modes of action of *Bacillus*-based DFMs pertain to improvements in gut health. Numerous DFMs have been shown to affect gut-associated immunity in livestock (Mack and Lebel, 2004; Scharek et al., 2007; Schierack et al., 2007). However, other studies have shown little to no benefit of DFMs on immune response (Walsh et al., 2012; Jaworski et al., 2017). Studies that have investigated the effects of *Bacillus*-based DFMs on gut health have varied in the incorporation of an immune challenge, the type of immune challenge and the gut health parameters evaluated.

Bacillus-based DFMs may improve gut health via improvements in several aspects of gut health such as intestinal morphology (Baum et al., 2002), kinetics of nutrient transport through the enterocytes (Walsh et al., 2012), enterocyte proliferation (Babińska et al., 2005; Walsh et al., 2007), as well as modulation of the intestinal immune system (Scharek et al., 2007; Schierack et al., 2007). *Bacillus*-based DFMs also have the ability to alter the gut microbiota (Baker et al., 2013; Hu et al., 2014; Kritas et al., 2015; Lan et al., 2016) at both the phylum and genus levels.

Effects on pH

The effects of BioPlus 2B® on growth performance and pH of slurry in growing pigs were evaluated by Wang et al. (2009). Crossbred barrows were fed either a basal diet (CON), or a basal diet including either 0.05%, 0.10%, or 0.20% BioPlus 2B® (B0.05, B0.1, and B0.2, respectively) and pH of slurry waste was measured daily for the 5 d after feeding. The slurry pH was lower in the B0.1 and B0.2 groups compared to the CON and B0.05 groups at 72, 96, and 120 h (Wang et al., 2009). No differences in slurry pH were observed among treatments at 24 or 48 h or between CON and B0.05 throughout the experimental period (Wang et al., 2009). These results support the notion that DFMs can alter pH and also that the effects of BioPlus 2B® on pH may be dose dependent.

A study conducted by He et al. (2017) compared two different methods of *Bacillus subtilis* supplementation. Piglets were fed either a *Bacillus subtilis* fermented liquid feed (1.28×10^{12} CFU/kg) (BFLD) or a *Bacillus subtilis*- supplemented commercial pelleted diet (2.80×10^{11} CFU/kg) (BCPD) from 7 to 31 d of age. At 32 d of age, the BFLD treatment lowered the pH of the colonic luminal content of pigs compared to the BCPD treatment (He et al., 2017). No effects on pH of the jejunal luminal content were reported. The decreased pH in the colonic luminal content of the BFLD treatment could have been due to the higher concentrations of lactic, chenodeoxycholic and lithocholic acids that were also observed (He et al., 2017). The higher concentrations of lactic acid produced by the BFLD were in accordance with the authors expectations given that more active components for the growth of lactic acid bacteria were present in the BFLD (He et al., 2017). This study supports the concept that DFMs are able to influence gastrointestinal pH by the production of organic acids.

Effects on immune response

The effects of 2 DFMs on the humoral immune system in sows and their piglets were investigated by Scharek et al. (2007). In the sows given feed supplemented with *Bacillus cereus* var. *toyoi*, there was an increase in fecal IgA compared to the sows supplemented with *Enterococcus faecium* although there were no differences in serum IgG by either DFM (Scharek et al., 2007). Just prior to weaning, supplementation of *Bacillus cereus* resulted in higher fecal IgA whereas, 1 wk post-weaning, the *Enterococcus faecium* caused a decrease in levels of IgA. Levels of IgG were decreased by both DFMs post-weaning compared to the controls. This study demonstrated the ability of *Bacillus cereus* to increase IgA secretion in the gastrointestinal tract in both sows and piglets. The authors attributed this increase in IgA to an improved mucosal immune response which subsequently led to reduced systemic IgG levels in piglets post-weaning (Scharek et al., 2007). These results have also been observed in broiler chickens fed a combination of *Bacillus subtilis, Lactobacillus reuteri,* and *Saccharomyces cerevisiae* (Salim et al., 2013). The DFM combination caused increases in plasma IgG, IgM, and IgA compared to the control (Salim et al., 2013).

In a study conducted by Schierack et al. (2007), 70 pigs were fed either an untreated control diet or a diet containing *Bacillus cereus* var. *toyoi*. Several of the pigs fed each of these diets were further divided into an influenza vaccination group to obtain data on cytokine production, or a mycoplasma vaccination group to obtain data on antibody titers

(Schierack et al., 2007). Peripheral blood mononuclear cells in the DFM group produced higher concentrations of IL-4 and IFN-γ cytokines and had a higher CD4+/CD8+ ratio which, according to Appleyard et al. (2002) is indicative of the effectiveness of a secondary immune response (Schierack et al., 2007). Therefore, Schierack et al. (2007) concluded that *Bacillus cereus* var. *toyoi* had the ability to alter the immune system of piglets.

The effects of DFMs on growth performance and gut health were also evaluated by Walsh et al. (2007) by feeding 188 weanling pigs either a control diet (Trt 1), 1 of 3 DFM diets containing either a DFM bolus (*Lactobacillus acidophilus* and *enterococcus faecium*) fed at weaning (Trt 2), a DFM (BioPlus 2B® containing *Bacillus subtilis* and *Bacillus licheniformis*) in the feed only for d 1 to 34 (Trt 3), or a combination of a DFM bolus at weaning and DFM in the feed for d 1 to 34 (Trt 4), or a diet with in-feed antibiotics (Trt 5) (Walsh et al., 2007). On d 20, Trt 4 increased the migration rate of duodenal and ileal enterocytes compared to Trt 1 (negative control) and Trt 4 (Walsh et al., 2007). On d 34, DFM Trt 3 increased enterocyte proliferation in ileal crypts compared to Trt 1 and Trt 4 which is in agreement with results reported by Babińska et al. (2005) in which piglets fed *Lactobacillus acidophilus* and *Bifidobacterium breve* had enhanced crypt cell proliferation.

Another study conducted by Walsh et al. (2012) evaluated the ability of DFMs or organic acids to control a *Salmonella* infection in newly weaned pigs. Pigs weaned at 19 ± 2 d of age were fed either a control diet, a diet containing DFMs (*Bacillus subtilis*, *Bacillus licheniformis*, and *Enterococcus faecium*) in drinking water, a diet containing an organic acid-based blend, or a diet with an antibiotic (55 mg carbadox/kg feed) for 14 d. Water delivery of DFMs did not affect TNFα concentrations in either serum or mucosa and no effect on the prevalence of *Salmonella* in the feces, gastrointestinal tract, or lymph nodes was observed (Walsh et al., 2012). On d 4 post infection, the DFM and organic acid treatments resulted in deeper crypts in the jejunum compared to the other treatments and on d 2 post infection, caused greater basal short-circuit current, which is indicative of active ion transport, than the antibiotic treatment (Walsh et al., 2012). Based on the findings from these studies, the authors concluded that water delivery of DFMs provided little to no benefit to pigs during a *Salmonella* infection.

Similarly to results reported by Walsh et al. (2012), a study conducted by Jaworski et al. (2017) described no DFM effects on plasma TNFα concentrations when pigs were fed diets containing a combination of either a low or high fiber diet with either 0 or 60 g of a multi-strain *Bacillus*-based DFM (1.5 x 10^8 CFU/kg feed) containing equal proportions of 1 strain of *Bacillus subtilis* and 2 strains of *Bacillus amyloliquefaciens*. He et al. (2017) also reported no differences in serum TNFα, or IL-1β levels between pigs fed a *Bacillus subtilis* fermented liquid diet and pigs fed a *Bacillus subtilis* supplemented commercial pelleted diet. Although, the fermented liquid diet increased levels of IL-6 in the serum compared to the commercial pelleted diet. Results from these studies indicate that DFM supplementation has little to no effect on the pro-inflammatory immune response at the systemic level (Walsh et al., 2012; He et al., 2017; Jaworski et al., 2017).

Effects on gut barrier function

In pigs, the largest and most critical barrier against the external environment is the intestinal epithelium which is composed of a single cell layer. The intestinal epithelium has two major functions: 1) preventing the passage of harmful microorganisms, toxins, or other antigens (Podolsky, 1999; Groschwitz and Hogan, 2009) and 2) serving as a filter in order to allow important dietary nutrients, electrolytes, and water to translocate from the lumen of the intestine into circulation (Kunzelmann and Mall, 2002; Blikslager et al., 2007; Broer, 2008). Therefore, improved gut integrity is an extremely important aspect of gut health.

The ability of *Bacillus*-based DFMs to modulate the immune response and improve gut barrier function during a pathogen challenge was further demonstrated by Yang et al. (2016). Weaned pigs were given either a low (3.9×10^8 CFU/d) or high (7.8×10^8 CFU/d) oral dose of a mixture of *Bacillus subtilis* and *Bacillus licheniformis* spores for 1 wk before an *Escherichia coli* challenge. In the pigs challenged with *Escherichia coli*, the mRNA expression of TLR4, NOD2, iNOS, IL-8, and IL-22 in the small intestine were upregulated by the *Bacillus*-based DFMs (Yang et al., 2016). The low-dose of the DFM elevated ZO-1 expression and increased the ratio of $CD4^{-}CD8^{-}$ T-cell subpopulations in peripheral blood whereas the high dose resulted in the extension of $CD4^{-}CD8^{-}$ T-cells in the inflamed intestine (Yang et al., 2016). The data from this study suggest that enteritis caused by *Escherichia coli* can be somewhat alleviated by *Bacillus*-based DFMs by preserving the integrity of the intestinal epithelial barrier by increasing ZO-1 expression and upregulating intestinal IL-22 expression (Yang et al., 2016). However, it is important to note that during an enteropathogenic bacterial infection, IL-22 can also trigger an inflammatory response. In conjunction with other tight junction proteins including claudin, occludin, and actin, ZO-1 plays an essential role in the structural arrangement of tight junctions (Stevenson et al., 1986) and therefore improvements in ZO-1 concentrations would be valuable to maintain gut integrity.

The effect of *Bacillus*-based DFMs on the intestinal epithelial barrier described by Yang et al. (2016) is further supported by an *in vitro* study conducted by Gu et al. (2014).

This study found that treating a porcine epithelial cell line (IPEC-J2) with *Bacillus subtilis* improved the concentrations of occludin and ZO-1 during the process of tight junction formation and therefore permeability of the tight junctions was decreased (Gu et al., 2014). When the IPEC-J2 cells were treated with deoxynivalenol (DON), the cells treated with Bacillus subtilis had increased expression of ZO-1 (Gu et al., 2014). Therefore, it was concluded that dysfunction of the intestinal epithelial barrier induced by DON could be prevented via improvement in the expression of tight junction proteins by *Bacillus subtilis*.

Effects on gut histology

The effects of *Bacillus cereus* on intestinal morphology and mucins of the intestinal mucosa in weaned pigs were evaluated by Baum et al. (2002). In the small intestine, the supplementation of *Bacillus cereus* led to an increased villus length, whereas there were no differences observed in morphology of the crypts, total number of goblet cells or the number of Ki67-positive cells (Baum et al., 2002). These results are comparable to results observed in broiler chickens fed a combination of *Bacillus subtilis*, *Lactobacillus reuteri*, and *Saccharomyces cerevisiae* (Salim et al., 2013). The broiler chickens fed the DFM combination had increased villus height and width in the ileum in addition to increased thickness of the muscularis externa which is indicative of a strong gut integrity (Salim et al., 2013).

Modulation of gut microbiota

The ability to modulate the intestinal microbiota is another proposed mechanism by which DFMs are able to exert their beneficial effects on their host. As previously discussed, DFMs have been shown to alter the intestinal immune response in pigs. In order for animals to maintain a healthy gut environment, homeostasis between the host and the microbial communities in the gut must be sustained. The intestinal immune response is a crucial factor in maintaining the mutually beneficial host-microbial relationship and therefore, resident gut bacteria largely influence mammalian immunity (Hooper et al., 2012). Consequently, it is speculated that DFMs may affect the immune response primarily via modulation of the gut microbiota.

Modulation of the gut microbiota by *Bacillus*-based DFMs may begin as early as gestation. In a study conducted by Baker et al. (2013), sows were fed either a control diet or a diet supplemented with *Bacillus subtilis* at a dose of 3.75 x 10^8 CFU/kg feed and the effects of the DFM on their piglets were observed at d 3 and d 10 of lactation. On d 3 of lactation, the pigs nursing the sows fed the DFM had a greater quantity of *Lactobacillus gasseri* and *Lactobacillus johnsonii* in the ileum and a lower quantity of *Escherichia coli* in the colon than the pigs on the control sows (Baker et al., 2013). On d 10 of lactation, pigs nursing the DFM sows had a greater quantity of *Lactobacillus* species in the colon compared to pigs nursing the control sows, which also tended to have *Clostridium perfringens* present in the ileum (Baker et al., 2013). This study is in agreement with a study conducted by Kritas et al. (2015) in which sows fed a diet supplemented with 3.0 x 10^8 CFU/kg feed of *Bacillus subtilis* C-3102 had piglets with decreased quantities of *Escherichia coli* and *Clostridum* spp. in the feces. Jointly, these studies indicate that *Bacillus*-based DFM supplementation can be beneficial for sows and their piglets prior to weaning by altering the developing intestinal microbiota.

In addition to the neonatal period, *Bacillus*-based DFMs have also been shown to modulate the gut microbiota in the phases following weaning. In a study conducted by Hu et al. (2014), supplementation of *Bacillus subtilis* KN-42 in was shown to alter bacterial communities in weaned pigs, with a dose of 4.0 x 10^9 CFU/kg feed achieving the highest bacterial diversity. Supplementation of *Bacillus subtilis* KN-42 reduced the relative number of *Escherichia coli* and when supplemented at 20.0 x 10^9 CFU/kg feed, increased the relative number of *Lactobacillus* (Hu et al., 2014). These results are in accordance with Lan et al. (2016) which reported higher fecal *Lactobacillus* counts and lower *Escherichia coli* counts when pigs were fed a basal diet supplemented with 0.1% of a multi-strain probiotic containing a mixture of 1 x 10^{12} CFU/kg of *Bacillus coagulance*, 5 x 10^{11} CFU/kg of *Bacillus lichenformis*, 1 x 10^{12} CFU/kg of *Bacillus subtilis* and 1 x 10^{11} CFU/kg of *Clostridium butyricum* respectively.

In addition to impacting the gut microbiota at the genus level, *Bacillus*-based DFMs have also shown the ability to alter the gut microbiota at the phylum level. When *Bacillus subtilis* was fed to 8-wk old pigs until they reached 110 kg BW, the copy numbers and percentage of *Bacteroidetes* was decreased while the percentage of *Firmicutes* was increased in the cecal digesta (Cui et al., 2013). Concurrently, lipid metabolism was up-regulated through increased gene expression of fatty acid synthase and acetyl CoA carboxylase in adipose tissue which supported the authors conclusion that changes in lipid metabolism could be due to the altered proportion of *Bacteroidetes* and *Firmicutes* in the gut (Cui et al., 2013).

The method of *Bacillus subtilis* supplementation may also impact the effects on the gut microbiota as demonstrated by He et al. (2017). Pigs fed a *Bacillus subtilis* fermented liquid diet had higher counts of *Firmicutes*, and lower intestinal bacterial diversity, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* than pigs fed a commercial pelleted diet supplemented with *Bacillus subtilis* (He et al., 2017). Given that *Bacillus* species belong to the *Firmicutes* phylum, it is no surprise that the supplementation of *Bacillus* DFMs may increase proliferation of *Firmicutes* bacteria in the gut although the level of increase may depend on the source of the *Bacillus subtilis* and the method of supplementation.

Competitive exclusion of pathogens

The ability to competitively exclude pathogens from adhering to the intestinal epithelium is an important mode of action by which DFMs may benefit livestock. In pigs and poultry, several studies have demonstrated that *Bacillus*-based DFMs can competitively exclude pathogens including *Escherichia coli*, *Salmonella enterica*, and *Clostridium perfringens* (Maruta et al. 1995; La Ragione et al., 2001; La Ragione and Woodward, 2003; Tsukahara et al., 2013). In livestock production, pathogenic bacteria can negatively impact production via losses in growth performance and poor animal health.

A study conducted in weaning pigs demonstrated the capability of *Bacillus subtilis* to competitively exclude Shiga toxin 2e-producing *Escherichia coli* (STEC), which leads to porcine edema disease (ED) (Tsukahara et al., 2013). The numbers of STEC in the ileal digesta and feces were reduced by *Bacillus subtilis* DB9011 and therefore, the symptoms of ED were improved (Tsukahara et al., 2013). These results are in agreement with results reported from a previous feeding trial by Guo et al. (2006). Piglets were fed diets supplemented with different levels of *Bacillus subtilis* MA139 (2.2 x 10^5, 2.2 x 10^6, or 2.2 x 10^{10} CFU/kg of feed) and compared to a negative control and a positive control containing an antibiotic. The pigs fed *Bacillus subtilis* MA139 at 2.2 x 10^8 CFU/kg feed had a numerical decrease in fecal *Escherichia coli* counts and a significant increase in *Lactobacilli* counts (Guo et al., 2006).

These results were similar to outcomes discovered in poultry in a study by Ragione et al. (2001) investigated the ability of *Bacillus subtilis* to exclude *Escherichia coli* O78:K80 in poultry. All symptoms of *Escherichia coli* infection were suppressed by one inoculum of *Bacillus subtilis* spores (2.5 x 10^8 CFU). Throughout the entire experiment, *Bacillus subtilis* remained in the intestine and concomitantly, intestinal colonization and *Escherichia coli* shedding were reduced during the entire experiment (Ragione et al., 2001).

A study in broilers investigated whether or not *Bacillus subtilis* C-3102 (3.0 x 10^8 CFU/kg feed) could competitively exclude *Salmonella* and *Campylobacter* in the gastrointestinal tract (Maruta et al., 1995). In a field trial experiment, the detection rate of *Campylobacter* and *Salmonella*-positive chickens was lower in the *Bacillus subtilis* supplemented group compared to the control as well as significant decreases in *Enterobacteriaceae* and *Clostridium perfringens* (Maruta et al., 1995). These results are comparable to results reported by Ragione and Woodward (2003), in which a single inoculum of *Bacillus subtilis* (1.0 x 10^9 spores) inhibited colonization and persistence of *Clostridium perfringens* and *Salmonella enterica*. For the duration of the experiment *Bacillus subtilis* remained in the intestine and concurrently, the shedding of *Salmonella enterica* in the feces was significantly reduced (Ragione and Woodward, 2003).

Collectively, these studies in both pigs and poultry demonstrate the ability of *Bacillus*-based DFMs to competitively exclude the growth of certain pathogenic bacteria via colonization inhibition, and subsequently reduced fecal shedding. Although the specific details of the mechanisms by which *Bacillus*-based DFMs are able to suppress the growth of pathogens have yet to be elucidated, it is possible that the production of antimicrobial compounds by *Bacillus* species (Katz and Demain, 1977; Cladera-Olivera et al., 2004; Stein, 2005) could play an important role.

Scope of the Present Dissertation

The United States is a major player in the global production of pork. Currently, the U.S. is the third-largest producer and consumer of pork and pork products and in recent years, the U.S. has consistently been among the top exporters of pork and pork products (USDA, 2017). However, due to consumer demands for antibiotic-free meat and concerns about the development of antibiotic resistant microorganisms, the FDA has made important progress in recent years regarding the use of medically important antibiotics in water or feed of animals used for human consumption through the establishment of the VFD.

In order to guarantee that antibiotics are used appropriately for certain reasons pertaining to animal health, the FDA is now focused on the elimination of the use of medically important antibiotics for production purposes, such as growth promotion and improved feed efficiency. In addition, any other therapeutic uses in animal water or feed will now be supervised by a licensed veterinarian (FDA, 2017). The removal of these antibiotics by the VFD has paved the way for more feed additive research to find proven antibiotic alternatives and DFMs are one such additive that have generated substantial interest in the last few decades.

Many different types of DFMs have been evaluated in pigs and a good portion of the research has investigated the effects of *Bacillus*-based DFMs. Various aspects of animal growth performance and health have been evaluated. However, studies are extremely variable in many areas including the DFM strain used, DFM dose, and the incorporation of a pathogen challenge or not. Few studies have examined the effects of the same *Bacillus*-based DFM in both challenged and non-challenged experiments.

The genetic makeup of *Bacillus subtilis* 29784 is significantly different from *Bacillus subtilis* strains currently used in the market and therefore, this strain is unique. This strain is a naturally-occurring bacterial strain that does not contain any resistance genes to antibiotics commonly used in human medicine (Adisseo, 2017). Additionally, unlike some DFM products, *Bacillus subtilis* 29874 does not cause hemolysis and is not cytotoxic to animal cells (Adisseo, 2017) In poultry, this strain has proven to be consistent, extremely stable, and has the ability to germinate in a timely manner which allows it to exert effects at the intestinal level. To date, the effects of *Bacillus subtilis* 29784 have only been investigated in poultry. Therefore, the first study (Chapter II) aims to evaluate the effects of *Bacillus subtilis* 29784, compared to a commercially available product containing both *Bacillus subtilis* and *Bacillus licheniformis*, on growth performance and gut health of nursery pigs.

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of post-weaning diarrhea in pigs. Infections caused by ETEC can lead to reduced growth performance and impaired gut health in pigs, especially during the neonatal and post-weaning period. These effects of ETEC can result in production losses and consequently, effective mitigation strategies are crucial. Therefore, the second study (Chapter III) aims to evaluate the effects of *Bacillus subtilis* 29784 on gut health and prevention of post-weaning diarrhea caused by F18^{+} *Escherichia coli* in comparison to antibiotics.

Previous research in pigs and other livestock has indicated that the effects of DFMs may be dose-dependent. The dose of a DFM will affect the number of viable cells that may be present in the gastrointestinal tract. However, depending on which DFM is being used, the dose levels reported in the literature have been extremely variable and may also depend on the source of the DFM. Therefore, the third study (Chapter IV) aims to determine the optimal dose of *Bacillus subtilis* 29784 to improve growth performance of nursery pigs. Collectively, these studies aim to further investigate the effects of *Bacillus*-based DFMs on growth performance and gut health of nursery pigs in both a natural exposure study and an immune challenge study and to compare the effects of *Bacillus subtilis* to antibiotics, with a specific focus on the ideal dosage and effects of a novel strain of *Bacillus subtilis* 29784.

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CHAPTER II

THE EFFECTS OF *BACILLUS*-BASED DIRECT FED MICROBIALS ON GROWTH PERFORMANCE AND GUT HEALTH OF NURSERY PIGS

SUMMARY

Bacillus-based DFMs have commonly been fed to pigs as a means to improve growth performance and gut health. The objective of this study was to determine the effects of *Bacillus*-based DFMs on growth performance, gut health, immune status, and oxidative stress status in nursery pigs. Pigs (180 barrows and 180 gilts) newly weaned at 21 d of age (6.3 ± 1.0 kg BW) were randomly assigned to 1 of 3 treatments based on a randomized complete block design with sex and initial BW as blocks: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* (1.28 x 10^9 CFU/kg feed) and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed). Pigs were distributed into 30 pens per treatment with 4 pigs per pen and given *ad libitum* access to feed and water for 42 d with d 1 to 14 representing phase 1 and d 15 to 42 representing phase 2. Feed intake and BW were measured at the beginning and end of each phase to calculate ADG, ADFI, and G:F. At d 42, 8 pigs (1 per pen) per treatment (4 barrows and 4 gilts) were euthanized for sample collection. Serum and jejunal mucosal tissues were obtained to measure concentrations of tumor necrosis factor α (TNF-α), immunoglobulins G and A (IgG and IgA), protein carbonyl (PC), and malondialdehyde (MDA). Jejunal tissues were obtained to measure concentrations of tight junction proteins (claudin, occludin, and ZO-1). Pen was the experimental unit and proc MIXED of SAS was used for statistical analysis using treatment and sex as fixed effects and BW block as a random effect. The ADG was greater ($P < 0.05$) in the DFM-1 (550 and 427 g/d) and DFM-2 (546 and 424 g/d) compared to the CON (503 and 392 g/d) for both phase 2 and the overall experimental period, respectively. Overall ADFI was increased ($P < 0.05$) in the DFM-1 (552 g/d) and DFM-2 (555 g/d) compared to the CON (516 g/d), although there were no effects on G:F. The DFM-1 had higher ($P < 0.05$)

ZO-1 than the CON and higher (*P* < 0.05) claudin and ZO-1 than the DFM-2. DFM -1 (6.27) and DFM- 2 (6.21) tended to (*P* = 0.090) decrease pH in the colon compared to the CON digesta pH of 6.51. There were no differences in TNF-α, IgG, IgA, PC, or MDA concentrations in serum and jejunal mucosa among treatments. In conclusion, both *Bacillus*-based DFMs improved growth of nursery pigs primarily by increasing feed intake, whereas the *Bacillus subtilis* 29784 additionally improved gut integrity without affecting immune or oxidative stress status in the small intestine.

INTRODUCTION

Bacillus-based DFMs have been fed to livestock for many years as a means to improve growth performance and gut health. However, research on *Bacillus*-based DFMs in pigs has yielded inconsistent results. Most research has been conducted on nursery pigs using either a single *Bacillus* strain or a combination of *Bacillus* strains. Several studies have reported improved growth performance including improved ADG and G:F in both nursery and grower phases (Alexopoulos et al., 2004; Guo et al., 2006; Davis et al., 2008; Cui et al., 2013; Hu et al., 2014; Cai et al., 2015; Jorgensen et al., 2016) as well as reduced morbidity and mortality (Alexopoulos et al., 2004; Davis et al., 2008). Conversely, other studies showed no effect on growth performance (Walsh et al., 2007; Bhandari et al., 2008). Studies have also demonstrated the ability of *Bacillus*-based DFMs to improve gastrointestinal health of pigs and broilers through improvements in intestinal morphology and intestinal immune status (Scharek et al., 2007; Deng et al., 2013; Lee et al., 2013; Salim et al., 2013; Cai et al., 2015). It has also been demonstrated that *Bacillus subtilis* can modulate the composition of the intestinal microbiota (Cui et al., 2013; Kaewtapee et al., 2017).

Recently, a new *Bacillus subtilis* strain that has been proven to be a novel subspecies, has received increased attention in poultry. *Bacillus subtilis* 29784 has been shown to improve body weight and feed conversion ratio in broilers in a variety of rearing conditions (Rhayat et al., 2017). In 3 broiler studies, animal performance and villus height in the small intestine were improved compared to the negative controls (Devillard et al., 2016). *Bacillus subtilis* 29784 also altered the cecal microbiota which resulted in increased butyrate producing species (Devillard et al., 2016). Although these data have demonstrated the potential beneficial effects of *Bacillus subtilis* 29784 in broilers, this strain has yet to be evaluated in pigs.

Based on previous research, it was hypothesized that feeding nursery pigs with *Bacillus*-based DFMs would improve growth performance by creating a healthy gut environment unfavorable to the growth of pathogens. Therefore, the objective of this study was to determine the effects of *Bacillus*-based DFMs on growth performance, gut health, and immune and oxidative stress status of nursery pigs in natural exposure conditions.

MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University (Raleigh, NC, USA).

Animals and experimental design

Three hundred and sixty crossbred (PIC 337 x Camborough 22) barrows and gilts (6.3 ± 1.0 kg BW) newly weaned at approximately 21 d of age were utilized for this experiment. Pigs were grouped according to BW (5 blocks) and randomly assigned to 3 treatments within a BW group. Treatments were: 1) CON: a diet with no feed additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed); and 3) DFM-2: CON + *Bacillus subtilis* (DSM 5750) (1.28 x 10^9 CFU/kg feed) and *Bacillus licheniformis* (DSM 5749) (1.28 x 10^9 CFU/kg feed). Each treatment had 30 replicates (15 barrow pens and 15 gilt pens) with 4 pigs per pen. Pigs were housed in pens (1.42 x 3.86 m) with solid concrete floors at the Swine Evaluation Station (Clayton, NC) of North Carolina State University. Temperatures in the building were between 79^{o}F and 92^{o}F for the duration of the study. Pigs were given *ad* *libitum* access to feed and water for the 42 d experimental period. Pigs were fed 2 nursery phase diets (Table 1) with d 1 to 14 representing phase 1 and d 15 to 42 representing phase 2. Phase 1 targeted 5 to 7 kg BW and phase 2 targeted 7 to 11 kg BW. All diets were formulated to meet nutrient requirements suggested by NRC (2012).

Growth performance

Initial BWs were taken upon arrival at the Swine Evaluation Station of North Carolina State University and prior to allotment. BW measurements were taken at d 14 and d 42 for phase 1 and phase 2 respectively to determine ADG. The amount of feed given was recorded and feeder weights were measured on d 14 and d 42 to estimate ADFI and G:F. Fecal scores were recorded on d 2, 4, 6, 8, 10, and 14 based on a 1 to 3 scale with 1 being solid and 3 being watery diarrhea. Mortality was also recorded.

Sample Collection

On d 42 at the conclusion of the study, 8 pigs per treatment (4 barrows and 4 gilts) that represented the average BW from each BW group were euthanized for sample collection. Blood samples were collected in vacutainer tubes (BD Vacutainer®; Becton, Dickinson and Company, NJ, USA) immediately before euthanasia and then centrifuged at 3,000 x g for 15 min to obtain serum for immune and oxidative stress status analyses. Digesta samples were collected from the terminal jejunum, ileum, and colon and immediately placed on ice for pH measurements or snap frozen in liquid nitrogen and stored at -80^{o}C for later gut microbiome sequencing. Jejunal mucosa samples were collected from the terminal jejunum and snap frozen in liquid N and also stored at -80^{o}C until microbiome sequencing. Tissue from the jejunum was also collected to measure concentrations of tight junction proteins (claudin, occludin, and zonula occludens-1). Additional tissue samples were taken from the duodenum and jejunum, flushed with 0.9% saline and placed in formalin for histology measurements.

pH measurements

Immediately after collection, digesta samples from the jejunum, ileum and colon were placed on ice until pH was measured. Measurements were recorded immediately upon arrival at the laboratory using a pH meter (Thermo Fisher Scientific, MA, USA).

Histology measurements and immunohistochemistry

After collection, tissue samples from the duodenum and jejunum remained in 10% formalin for 3 wk. A 1 cm thick cross section of jejunal tissue was sliced using a scalpel, placed in a cassette, placed in ethanol and sent to the Histology Laboratory at the College of Veterinary Medicine of North Carolina State University for hematoxylin and eosin staining. The Infinity Analyze and Capture software (Lumenera Corporation, Ottawa, Canada) was used to measure villus height, villus width (width measurements were taken at the base of a villus, at one-half of the villus height, and the tip of a villus) and crypt depth in both tissues. Measurements of 20 intact villi and their associated crypt were measured from each slide. The same person executed all intestinal morphology analyses. Cell proliferation was measured in jejunal crypts by using a Ki67 antibody stain (Histology Laboratory) and subsequent analysis using the browser-based ImageJS application (Almeida et al., 2012). Ki67 is a protein expressed in the nucleus of proliferating cells and is not expressed in quiescent cells. Ki67 antibody stains are performed using a primary antibody and peroxidase blocking, followed by an incubation with a secondary antibody. After the secondary antibody incubation, a peroxidase solution is administered followed by a peroxidase substrate solution and lastly, a counterstain solution.

Immune and oxidative stress measurements

Porcine specific ELISAs were used to quantify tumor necrosis factor α (TNFα) (Cat. PTA00, R&D Systems, MN, USA), Immunoglobulin G (IgG) (Cat. E100-104, Bethyl Laboratories, TX, USA), protein carbonyl (Cat. STA-310, Cell Biolabs, Inc., CA, USA), and malondialdehyde (Cat. STA-330, Cell Biolabs, Inc.) in both serum and jejunal mucosal tissue. Immunoglobulin A (IgA) was measured in jejunal mucosal tissue by a porcine specific ELISA (Cat. E101-102, Bethyl Laboratories).

Protein isolation and western blotting

Jejunal tissue from each treatment were used to measure tight junction proteins as described by Yang et al. (2015). Tissue samples (50 mg) of jejunum were weighed and suspended into 0.5 mL RIPA lysis and extraction buffer containing 5 µL protease inhibitor cocktail. Tissue samples were then homogenized and kept on ice. The homogenate was centrifuged at 10,000 x g at 4^{o}C for 10 min and supernatant was collected. A bicinchoninic acid assay (BCA) protein assay (#23225, ThermoFisher Scientific) was used to measure total protein concentration in the supernatant. The concentration was then adjusted to 4 µg/µL and denatured in a 100^{o}C water bath for 5 min. Samples were loaded into individual wells for SDS-PAGE. The gel was then moved onto polyvinylidene difluoride (PVDF) membrane and the target proteins were transferred to the membrane. Proteins were transferred electrophoretically for 1 h at 90 mV. After blocking with 5% skim milk, overnight incubation with primary antibodies against claudin, occludin, zonula occludens-1 (ZO-1), and β-actin occurred at 4^{o}C. After incubation, the membrane was washed and incubated with horseradish-conjugated secondary antibodies for 1 h at room temperature. The DAB substrate kit (34002; Pierce, Rockford, IL, USA) was used to develop the immunoblot and band density was determined by image analyzer software (LI-COR Biosciences, Lincoln, NE, USA).

Microbiome sequencing

Jejunal digesta and mucosal samples were sent to the Microbiome Core Facility at the University of North Carolina (Chapel Hill, North Carolina) for qPCR analysis of 16S rDNA sequences from gut bacteria using the QuantStudio 3D Digital PCR System (ThermoFisher Scientific, MA, USA) and Ion PGM System for Next-Generation Sequencing (ThermoFisher Scientific). The method used is based on the conserved 16S rDNA gene that is present in all bacteria. Quantitative PCR can indirectly convey the number of bacteria present by calculating the number of conserved 16S rDNA genes (Woo et al., 2008; Barghouthi, 2011).

Microbial diversity

Microbial diversity was measured in jejunal digesta and jejunal (Edgar, 2017). The number of observed species was calculated based on the number of unique OTUs in each sample. Alpha diversity was calculated using rarefaction analyses (Edgar, 2017). The Shannon index was used to characterize species diversity within a community (Morris et al., 2014). The Shannon index accounts for both abundance and evenness of the species present and is calculated by using the proportion of unique species relative to the total number of species multiplied by the natural logarithm of this proportion. The resulting calculation is summed across all species and multiplied by -1. The Chao 1 index calculates the estimated true species diversity of a sample by dividing the number of singletons squared by twice the number of doubletons and then adding the result to the observed number of species (Edgar, 2017).

Statistical analysis

Data were analyzed using Proc Mixed of SAS 9.3 (Cary, NC, USA). Pen was the experimental unit. Treatment and sex were fixed effects and BW block was a random effect. Main effects of treatment and sex were evaluated as well as their interaction. Fecal score data were analyzed with the Kruskal Wallis Test by using the NPAR1WAY procedure in SAS 9.3. Microbiome raw sequencing data were organized using the Ion Reporter Metagenomics 16S algorithm (ThermoFisher Scientific). Data were collected, filtered by primers and length, adaptor sequences were trimmed, and bin barcodes were assigned. Data was then compared against the 16S database (GreenGenes and MicroSeq) using BLAST (Basic Local Alignment Search Tool). The abundance was then generated based on the number of sequences for variable regions and statistical analysis was performed on relative abundance data using Proc Mixed of SAS 9.3. P values of less than 0.05 were considered significant and $0.05 \leq P < 0.10$ were considered a tendency.

RESULTS

Growth performance

Growth performance was not affected during phase 1 of the experiment. However, during phase 2, ADG was improved ($P < 0.05$) in both DFM-1 and DFM-2 (550 and 546 g/d) compared to the CON (503 g/d) (Table 2). Both DFM-1 and DFM-2 tended to increase ($P = 0.100$) ADFI during phase 2 (721 and 712 g/d) compared to the CON (676 g/d) (Table 2). During the overall experimental period, ADG was increased ($P < 0.05$) in DFM-1 (427 g/d) and DFM-2 (424 g/d) compared to the CON (392 g/d) (Table 2). Both DFM-1 and DFM-2 increased ADFI ($P < 0.05$) during the overall experimental period (552 and 555 g/d respectively), compared to the CON (516 g/d) (Table 2). Gain to feed ratio was not affected at any point during the experiment.

Fecal scores and mortality

Fecal scores on d 6 were improved ($P < 0.05$) by DFM-1 (1.20) and DFM-2 (1.23) compared to CON (1.47) (Table 3). There were no differences in fecal scores among treatments on d 2, 4, 8, 10, or 14. There was also no effect of treatment on mortality (Table 3).

Immune status, oxidative stress status and pH

Immune status was not affected by treatment. No differences were observed in IgG in either serum or jejunal mucosa (Table 4). IgA in jejunal mucosa was also not affected by treatment (Table 4). There were no differences in TNFα in the serum or jejunal mucosa (Table 4). Oxidative stress status was also not affected by treatment. There were no differences in MDA or protein carbonyl in either serum or jejunum mucosa (Table 5). No differences in pH were observed in either jejunal or ileal digesta. Both DFM-1 and DFM-2 treatments tended to decrease (*P* = 0.090) pH in the colon (6.3 and 6.2 respectively) compared to the CON (6.5) (Table 7).

Gut histology and immunohistochemistry

Villus height, crypt depth and villus height to crypt depth ratio were not affected in either duodenal or jejunal tissues. DFM-2 tended to increase (*P* = 0.061) villus width in the duodenum (178 µm) compared to DFM-1 (154 µm) and CON (161 µm) (Table 6). Villus width in the jejunum was not affected by treatment. There were no differences in enterocyte proliferation among treatments.

Tight junction proteins

The DFM-1 increased (*P* < 0.05) claudin concentration (0.23 fold) compared to DFM-2 (0.10 fold) but was not different than the CON (Table 8). DFM-1 also tended to increase (*P* = 0.078) occludin concentration compared to DFM-2 (0.19 fold vs. 0.12 fold) but was not different than the CON (Table 8). The DFM-1 improved (*P* < 0.05) ZO-1 concentration (0.53 fold) compared to the CON (0.26 fold) and DFM-2 (0.20 fold) (Table 8).

Gut microbiota

No effects of treatment were observed on the alpha diversity of bacterial species in either the mucosa or digesta. There were also no effects of treatment on the number of observed species in either mucosa or digesta (Table 9). In the mucosa, both DFM treatments tended to decrease (*P* = 0.064) the relative abundance of *Cyanobacteria* (0.231% and 0.113%) for DFM-1 and DFM -2 respectively, compared to the CON (0.321%) (Table 10). There were no treatment effects observed on the microbiome at the phylum level in the digesta (Table 11). The relative abundance of certain bacterial phyla differed between the jejunal mucosa and jejunal digesta. The relative abundance of *Bacteroidetes* was greater ($P < 0.05$) in the jejunal mucosa compared to the jejunal digesta whereas the relative abundance of *Firmicutes* was greater ($P < 0.05$) in the jejunal digesta compared to the jejunal mucosa (Table 12). The relative abundance of *Proteobacteria* was greater ($P < 0.05$) in the jejunal mucosa compared to the jejunal digesta. The *Firmicutes* to *Bacteroidetes* ratio was higher ($P < 0.05$) in the jejunal digesta compared to the jejunal mucosa (Table 12). Both measures of alpha diversity, Chao1 and Shannon index, were greater ($P < 0.05$) in the jejunal mucosa compared to the jejunal digesta (Table. 13). The observed number of species was also higher ($P < 0.05$) in the jejunal mucosa compared to the digesta (Table 13).

DISCUSSION

The improvements in ADG by the DFM treatments are comparable to other publications (Alexopoulos et al., 2004; Chen et al., 2006; Guo et al., 2006; Davis et al., 2008; Choi et al., 2011; Cui et al., 2013; Hu et al., 2014; Jorgensen et al., 2016; Balasubramanian et al., 2017; Jaworski et al., 2017). However, while others have also reported improvements in feed efficiency (Alexopoulos et al., 2004; Guo et al., 2006; Davis et al., 2008; Choi et al., 2011; Cui et al., 2013; Hu et al., 2014; Jorgensen et al., 2016; Balasubramanian et al., 2017; Jaworski et al., 2017) this was not observed in the present study. Similar to our results, Wang et al. (2017) did not observe differences in feed efficiency in piglets fed diets containing

Bacillus amyloliquefaciens (2 x 10^8 CFU/kg) for 28 d. Similarly, when nursery pigs were fed a probiotic containing a combination of *Lactobacillus* and *Streptococcus* for 3 wk, no differences in feed efficiency were reported (Estienne and Hartsock, 2005). Another study also found no improvements in feed efficiency in pigs fed a diet containing *Bacillus subtilis* (1.0 x 10^7 CFU/g), however, this study was conducted in finishing pigs (Chen et al., 2006).

In the present study, the improvements in ADG by *Bacillus subtilis* and a combination of *Bacillus subtilis* and *Bacillus licheniformis* were primarily due to improvements in ADFI. This is similar to results reported by Cui et al. (2013) and Wang et al. (2017). Another study in agreement with the present study reported improved ADFI when weaned pigs were fed a multi-species DFM containing *Bacillus subtilis, Lactobacillus acidophilus, Sacchromyces cerevisiae,* and *Aspergillus oryzae* for 28 d (Choi et al., 2011). However, feed efficiency was also improved and therefore it is difficult to determine whether the improvement in ADG was primarily due to improvements in ADFI or feed efficiency (Choi et al., 2011). It has been previously reported that the manipulation of gut microbial communities can alter body fat percentage (Ley et al., 2005). Previously, an increase in the *Firmicutes* to *Bacteroidetes* ratio has been associated with increased body mass index (Koliada et al., 2017) and pigs with increased *Firmicutes* and decreased *Bacteroidetes* were more obese than their lean counterparts (Ley et al., 2005). Cui et al. (2013) noted an increase in ADFI and a concurrent increase in *Firmicutes* and decrease in *Bacteroidetes*. The proportion of Bacteroidetes has also been shown to have a negative correlation with body weight (Guo et al., 2008). However, in the current study, the relative abundances of *Firmicutes* and *Bacteroidetes* were not affected by treatment, although, the ratio of *Firmicutes* to *Bacteroidetes* was higher in the jejunal digesta than in the jejunal mucosa.

Prior studies in which improvements in feed intake were reported, did not provide an explanation of the potential mode of action by which DFMs may lead to an increase in feed consumption. Therefore, the relationship of the *Firmicutes* to *Bacteroidetes* ratio in relation to feed consumption, as well as the potential mechanisms by which DFMs may increase feed consumption remain to be elucidated and therefore more research in this area is needed. In the current study, it is speculated that improvements in ADG and ADFI could be partially due to improvements in gut health.

In the present study, there are several potential mechanisms by which the Bacillus-based DFMs enhanced gut health. In the current study, both *Bacillus*-based DFMs tended to lower the pH in the colon. This finding is comparable to other studies (Wang et al., 2009; He et al., 2017) conducted in pigs. He et al. (2017) reported that *Bacillus subtilis* fermented liquid feed lowered the pH of colonic luminal content and the pH of slurry in growing pigs was lowered by combined supplementation of *Bacillus subtilis* and *Bacillus licheniformis* (Wang et al., 2009). The lowered pH in the colon could be a result of organic acid production by the vegetative cells of *Bacillus subtilis* and *Bacillus licheniformis*. It has been demonstrated that both *Bacillus subtilis* and *Bacillus licheniformis* produce a wide array of organic acids including malic acid, lactic acid, citric acid, succinic acid, and butyric acids (Ohara and Yahata, 1996; Kunst et al., 1997; Yan et al., 2013). In addition, to the aforementioned acids, *Bacillus licheniformis* can produce propionic acid (Yan et al., 2013) and upon germination, *Bacillus* spores will release dipicolinic acid (Simon et al., 2001). He et al. (2017) also attributed the lowered pH observed in their study to higher concentrations of lactic, chenodeoxycholic and lithocholic acids that were observed simultaneously. It has also been demonstrated that *Bacillus subtilis* maintains internal pH homeostasis via the pH dependent expression of various components of catabolism and respiration that can result in *Bacillus subtilis* consuming acids at a low pH or producing acids at a high pH (Wilks et al., 2009). Therefore, it is speculated that in the current study, the fluctuating pH of the gastrointestinal tract may have elicited more acid production by *Bacillus* species and thus affected the pH of the colon digesta. This reduction in pH could have improved gut health by suppressing the growth of harmful bacteria (Bajagai et al., 2016).

This concept is further supported by the concomitant reduction in the relative abundance of *Cyanobacteria* exhibited by the supplementation of DFM-1 and DFM-2. This reduction of *Cyanobacteria* in the gastrointestinal tract is beneficial because *Cyanobacteria* produce numerous toxins that can negatively impact humans and livestock (Mello et al., 2017). This finding is comparable to other studies (Cui et al., 2013; Hu et al., 2014; Kritas et al., 2015; Lan et al., 2016; He et al., 2017) that demonstrated the ability of *Bacillus*-based DFMs to modulate the gut microbiota at both the phylum and genus level.

In the current study, the supplementation of one *Bacillus* strain was more effective at improving gut barrier integrity than a combination of the 2 *Bacillus* strains. The supplementation of *Bacillus subtilis* 29784 improved the integrity of the gut barrier by increasing the concentrations of tight junction proteins claudin, occludin, and ZO-1. The largest improvement in the tight junction proteins was in ZO-1 which is in agreement with other studies (Gu et al., 2016; Yang et al., 2016) that reported that *Bacillus*-based DFMs are capable of increasing ZO-1 concentration during the formation of tight junctions as well as during an intestinal challenge with either pathogenic bacteria or mycotoxins. Tight junction proteins claudin, occludin, and ZO-1 play an important role in the structural arrangement of tight junctions between enterocytes in the gastrointestinal tract (Stevenson et al., 1986) and therefore, improvements in the concentrations of tight junction proteins would be favorable in order to maintain the integrity of the gut barrier.

Toll like receptors (TLR) are a class of transmembrane pattern-recognition receptors that play an important role in microbial recognition and are present on porcine intestinal epithelial cells (Cao et al., 2015; Gu et al., 2016). A member of the toll-like receptor family, TLR2 recognizes conserved molecular patterns associated with both Gram-positive and Gram-negative bacteria (Modlin, 2002; Akira, 2003; Cario, 2008). It has been reported that TLR2 may play an important role in the regulation of tight junction associated intestinal epithelial barrier integrity (Cario et al., 2004; Cario et al., 2007) specifically by enhancing ZO-1 (Cario et al., 2004). The role of TLR2 in regulating tight junctions is further supported by a study exhibiting enhancements in the expression of tight junction proteins in porcine intestinal epithelial cells through signaling by TLR2 ligands (Gu et al., 2016). Another study also reported increased TLR2 expression when pigs were fed a combination of *Bacillus subtilis* and *Lactobacillus salivarius* (Deng et al., 2013). Therefore, based on previous literature, it is speculated that in the current study, *Bacillus subtilis* may have enhanced the expression of tight junction proteins, particularly ZO-1 through TLR2 ligand signaling. However, further research is needed to conclusively demonstrate that *Bacillus subtilis* 29784 enhances gut barrier integrity via toll-like receptors.

The supplementation of *Bacillus*-based DFMs in the present study did not affect the concentrations of serum TNFα, IgG, or mucosal IgA. Similarly, other studies reported no effects of *Bacillus*-based DFMs on TNFα concentrations in the serum, plasma, or mucosa (Walsh et al., 2012; He et al., 2017; Jaworski et al., 2017). Given that the current study did not incorporate a specific immune challenge, it is not surprising that TNFα concentrations were not affected. In contrast to our results, Scharek et al. (2007) reported a decrease in serum IgG and an increase in IgA secretion in the gastrointestinal tract when pigs were fed *Bacillus cereus* post-weaning. A study in broiler chickens also reported increases in plasma IgG, IgM, and IgA when chickens were fed a combination of *Bacillus subtilis*, *Lactobacillus reuteri*, and *Saccharomyces cerevisiae* (Salim et al., 2013). However, it is important to note that when comparing DFM studies, there are many sources of variation and therefore, differences in results could be due to factors such as type and strain of DFM used, the preparation and dose of the DFM, and the conditions in which the DFM is tested.

In conclusion, this study demonstrated the ability of *Bacillus*-based DFMs to enhance growth performance, modulate the gut microbiota, improve gut integrity, and somewhat improve gut health in nursery pigs. The enhancements in growth performance were exhibited by increases in ADG and ADFI throughout the experiment. Both DFM treatments lowered the pH of colonic digesta which may have led to the decrease in *Cyanobacteria* abundance in the jejunal mucosa. The supplementation of *Bacillus subtilis* 29784 potentially improved the integrity of the intestinal gut barrier by increasing the concentrations of ZO-1. This study shows that *Bacillus*-based DFMs could potentially be used to improved growth performance in nursery pigs.

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Table 1: Composition of experimental diets

Item	Age of pigs	Phase 1 d 21 to 35	Phase 2 d 35 to 63
Ingredient, %			
Yellow corn, ground		39.94	61.79
Whey permeate		19.00	0.00
Poultry fat		2.00	2.00
Soybean meal		21.00	30.00
Poultry meal		5.00	0.00
Blood plasma		6.00	2.00
Fishmeal		4.00	0.00
L-Lys HCl		0.32	0.30
DL-Met		0.15	0.09
L-Thr		0.08	0.06
Limestone		0.85	1.00
Dicalcium phosphate		0.00	1.10
Salt		0.22	0.22
Trace mineral premix[1]		0.15	0.15
ZnO		0.25	0.25
Vitamin premix[1]		0.03	0.03
Phytase[2]		0.01	0.01
Supplement[3]		1.00	1.00
Total		100.00	100.00
Calculated composition[5]			
ME, kcal/kg		3,437	3,385
SID[4] Lys, %		1.50	1.26
SID[4] Met + Cys, %		0.82	0.69
SID[4] Thr, %		0.88	0.74
SID[4] Trp, %		0.27	0.24
Ca, %		0.85	0.71
P total, %		0.66	0.61
STTD[6] P, %		0.45	0.35
Analyzed composition			
DM, %		91.86	90.58
CP, %		24.03	20.28
Ca, %		0.86	0.74
Total P, %		0.71	0.59
Mg, %		0.17	0.15
Na, %		0.42	0.15
K, %		1.14	0.88
S, %		0.31	0.23

Table 1. Continued

[1] Premix is added to provide the following nutrients per kg of complete diet: 6,613.8 IU of vitamin A; 992.0 IU of vitamin D3; 19.8 IU of vitamin E; 2.64 mg of vitamin K; 0.03 mg of vitamin B12; 4.63 mg of riboflavin; 18.52 mg of pantothenic acid; 24.96 mg of niacin; 0.07 mg of biotin; 4.0 mg of Mn as manganous oxide; 165 mg of Fe as ferrous sulfate; 165 mg of Zn as zinc sulfate; 16.5 mg of Cu as copper sulfate; 0.30 mg of I as ethylenediamine dihydroiodide; and 0.30 mg of Se as sodium selenite.

[2] Phytase: Quantumblue 5G to provide 500 FTU/kg feed.

[3] Supplement: corn (1%) for CON; corn (0.9666%) + *Bacillus subtilis* 29784 (0.0334%) for DFM-1, and corn (0.96%) + Bacillus subtilis DSM 5750 and Bacillus licheniformis DSM 5749 (0.04%) for DFM-2.

[4] SID: standardized ileal digestible.

[5] Calculated composition was based on NRC (2012)

[6] STTD: standardized total tract digestible

Table 2. Growth performance of pigs fed experimental diets from 21 to 63 d of age

Item	Treatment[1]				
	CON	DFM-1	DFM-2	SEM	*P* value
Body weight, kg					
Initial	6.3	6.3	6.3	0.4	1.000
d 14	8.6	8.8	8.8	0.5	0.623
d 42	22.7[a]	24.2[b]	24.1[b]	1.3	0.028
ADG, kg/d					
Phase 1[2]	0.169	0.181	0.182	0.013	0.468
Phase 2[2]	0.503[a]	0.550[b]	0.546[b]	0.029	0.019
Overall[2]	0.392[a]	0.427[b]	0.424[b]	0.022	0.019
ADFI, kg/d					
Phase 1[1]	0.210	0.215	0.213	0.012	0.849
Phase 2[1]	0.676[A]	0.721[B]	0.712[B]	0.051	0.100
Overall[1]	0.516[a]	0.552[b]	0.555[b]	0.035	0.026
G:F					
Phase 1[1]	0.809	0.843	0.846	0.028	0.580
Phase 2[1]	0.759	0.771	0.784	0.040	0.545
Overall[1]	0.769	0.779	0.773	0.035	0.683

[ab] Means within a row without a common superscript are significantly different ($P < 0.05$)

[AB] Means within a row without a common superscript tended to be different ($P < 0.10$)

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34×10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28×10^9 CFU/kg feed).

[2] Phase 1 represents d 1 to 14, phase 2 represents d 15 to 42, and overall represents d 1 to 42.

Table 3. Fecal score of pigs fed experimental diets in phase 1[1]

	Treatment[2]				P value
Item	CON	DFM-1	DFM-2	SEM	
Mortality, %	98.3	98.3	97.6	1.5	0.664
Fecal score[3]					
d 2	1.17	1.11	1.13	0.04	0.511
d 4	2.06	1.92	1.94	0.09	0.491
d 6	1.47[a]	1.20[b]	1.23[b]	0.05	< 0.001
d 8	1.28	1.2	1.18	0.05	0.370
d 10	1.15	1.13	1.06	0.04	0.108
d 14	1.23	1.22	1.18	0.05	0.293

[ab] Means within a row without a common superscript are significantly different ($P < 0.05$)

[1] Fecal score was based on a 1 to 3 scale with solid feces given a score of 1 and diarrhea given a score of 3.

[2]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed).

[3] Fecal score data was analyzed with Kruskal Wallis Test by using procedure of NPAR1WAY in SAS 9.3.

Table 4. Immune status measurements from pigs fed experimental diets from 21 to 63 d of age

Item	Treatment[2]			SEM	*P* value
	CON	DFM-1	DFM-2		
TNFα[2]					
Serum, pg/mL	101.3	75.0	87.2	13.4	0.430
Jejunum, pg/mg protein	2.59	1.43	2.08	0.66	0.408
IgG[2]					
Serum, mg/mL	1.88	1.86	1.89	0.06	0.673
Jejunum, ug/mg protein	0.48	0.39	0.5	0.06	0.314
IgA[2]					
Jejunum, ug/mg protein	4.39	4.88	5.76	1.36	0.775

[1] Tumor necrosis factor α: TNF α; immunoglobulin G: IgG; and immunoglobulin A: IgA.
[2]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed).

Table 5. Oxidative stress status of pigs fed experimental diets from 21 to 63 d of age

	Treatment[2]				*P* value
Item	CON	DFM-1	DFM-2	SEM	
MDA[1]					
Serum, umol/L	13.8	11.7	12.1	3.1	0.272
Jejunum, umol/g	0.27	0.17	0.24	0.05	0.451
Protein carbonyl					
Serum, nmol/mL	4.99	4.64	5.36	0.29	0.242
Jejunum, nmol/mg	5.08	5.25	5.23	0.24	0.842

[1]Malondialdehyde

[2]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed).

Table 6. Histology measurements of pigs fed experimental diets from 21 to 63 d of age

Item	Treatment[1]			SEM	*P* value
	CON	DFM-1	DFM-2		
Villus height, μm					
Duodenum	487	422	472	48	0.527
Jejunum	349	385	389	28	0.513
Villus width, μm					
Duodenum	161^{A}	154^{A}	178^{B}	8	0.061
Jejunum	150	151	138	9	0.125
Crypt depth, μm					
Duodenum	224	199	219	11	0.165
Jejunum	187	191	202	9	0.504
VH:CD					
Duodenum	2.38	2.28	2.37	0.18	0.869
Jejunum	2.08	2.20	2.13	0.15	0.835
Enterocyte proliferation[2]					
Jejunum, %	34	36	39	4	0.501

[AB] Means within a row without a common superscript tended to be different ($P < 0.10$)

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34×10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28×10^9 CFU/kg feed).

[2]Enterocyte proliferation was measured in jejunal crypts using a Ki67 antibody stain. Ki67 is a protein expressed in the nucleus of proliferating cells.

Table 7. Digesta pH of pigs fed experimental diets from 21 to 63 d of age

Item	Treatment[1]			SEM	*P* value
	CON	DFM-1	DFM-2		
Digesta pH					
Jejunum	6.73	6.82	6.73	0.08	0.581
Ileum	6.82	6.95	6.55	0.17	0.261
Colon	6.51^{A}	6.27^{B}	6.21^{B}	0.11	0.090

^{AB} Means within a row without a common superscript tended to be different ($P <$ 0.10)

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed).

Table 8. Tight junction protein concentrations of pigs fed experimental diets from 21 to 63 d of age.

	Treatment[1]				*P* value
Item	CON	DFM-1	DFM-2	SEM	
Band intensity normalized with β-actin, % of control					
Claudin	0.17[ab]	0.23[a]	0.10[b]	0.03	0.013
Occludin	0.14[AB]	0.19[A]	0.12[B]	0.04	0.078
Zonula occludens-1	0.26[a]	0.53[b]	0.20[a]	0.03	<0.001

[ab] Means within a row without a common superscript are significantly different ($P < 0.05$)
[AB]Means within a row without a common superscript tended to be different ($P < 0.10$)
[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34×10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28×10^9 CFU/kg feed).

Table 9. Microbial diversity and species richness measured in jejunal digesta and mucosa of pigs fed experimental diets from 21 to 63 d of age

	Treatment[1]				*P* value
Item	CON	DFM-1	DFM-2	SEM	
Jejunal digesta					
Chao1[2]	260.9	228.5	215.7	132.6	0.953
Shannon Index[3]	2.9	2.6	2.5	0.8	0.944
Observed species[4]	111	101	92	55	0.956
Observed OTUs[5]	18,402	23,178	21,279	5,080	0.735
Jejunal mucosa					
Chao1[2]	852.2	959.4	958.4	57.8	0.347
Shannon Index[3]	6.5	7.2	7.2	0.4	0.444
Observed species[4]	350	391	390	25	0.436
Observed OTUs[5]	7,627	6128	4893	1476	0.443

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed).

[2] The Chao 1 index calculates the estimated true species diversity of a sample by dividing the number of singletons squared by twice the number of doubletons and then adding the result to the observed number of species (Edgar, 2017).

[3] The Shannon index accounts for both abundance and evenness of the species present and is calculated by using the proportion of unique species relative to the total number of species multiplied by the natural logarithm of this proportion. The resulting calculation is summed across all species and multiplied by -1 (Morris et al., 2014)

[4] The number of observed species was calculated based on the number of unique OTUs in each sample (Edgar, 2017).

[5]Operational taxonomic unit

Table 10. Microbial phyla quantified in jejunal mucosa of pigs fed experimental diets from 21 to 63 d of age

Item	Treatment[1]			SEM	*P* value
	CON	DFM-1	DFM-2		
Relative abundance of bacterial phylum, %					
Actinobacteria	0.253	0.210	0.270	0.101	0.913
Bacteroidetes	18.610	19.900	16.920	5.429	0.928
Cyanobacteria	0.321[A]	0.231[AB]	0.113[B]	0.059	0.064
Deferribacteres	0.017	0.005	0.006	0.008	0.423
Fibrobacteres	0.025	0.182	0.013	0.074	0.214
Firmicutes	43.080	35.120	28.830	7.271	0.297
Proteobacteria	1.360	2.558	3.329	0.710	0.176
Spirochaetes	0.346	0.618	0.254	0.211	0.289
TM7	0.028	0.005	0.106	0.041	0.224
Tenericutes	0.282	0.688	4.672	2.406	0.380

[AB]Means within a row without a common superscript tended to be different ($P < 0.10$)

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34×10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28×10^9 CFU/kg feed).

Table 11. Microbial phyla quantified in jejunal digesta of pigs fed experimental diets from 21 to 63 d of age

Item	Treatment[1]			SEM	*P* value
	CON	DFM-1	DFM-2		
Relative abundance of bacterial phylum, %					
Actinobacteria	0.406	0.504	0.423	0.237	0.903
Bacteroidetes	3.672	0.827	1.853	2.009	0.432
Cyanobacteria	1.444	5.516	3.013	2.552	0.372
Firmicutes	90.660	81.830	90.270	7.975	0.597
Fusobacteria	0.004	0.085	0.023	0.048	0.474
Proteobacteria	0.380	0.856	0.786	0.338	0.573
Spirochaetes	0.405	0.017	0.156	0.236	0.397
TM7	0.014	0.012	0.030	0.008	0.242
Tenericutes	0.306	0.114	0.178	0.148	0.629
Verrucomicrobia	0.017	0.007	0.011	0.009	0.728

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34×10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28×10^9 CFU/kg feed).

Table 12. Microbial phyla relative abundance in jejunal digesta and mucosa of pigs fed experimental diets from 21 to 63 d of age

	Treatment[1]			*P* value
Item	Digesta	Mucosa	SEM	
Relative abundance of bacterial phylum, %				
Actinobacteria	0.44	0.25	0.15	0.101
Bacteroidetes	2.29	18.47	2.83	<.0001
Cyanobacteria	3.32	0.31	1.31	0.016
Firmicutes	87.59	35.68	5.63	<.0001
Proteobacteria	0.67	2.42	0.41	0.001
Spirochaetes	1.00	0.46	0.29	0.170
TM7	0.03	0.13	0.04	0.065
Tenericutes	0.21	1.89	1.30	0.271
Firmicutes: Bacteroidetes	56.09	0.07	18.41	0.011

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed).

Table 13. Microbial diversity in jejunal digesta and mucosa

	Treatment[1]			*P* value
Item	Digesta	Mucosa	SEM	
Chao1[2]	235	923	68	<.0001
Shannon Index[3]	2.7	6.9	0.4	<.0001
Observed Species[4]	102	377	26	<.0001
Observed OTUs[5]	20953	6216	2361	<.0001

[1]Treatments were: 1) CON: A diet with no additives; 2) DFM-1: CON + *Bacillus subtilis* 29784 (3.34 x 10^9 CFU/kg feed) and 3) DFM-2: CON + *Bacillus subtilis* and *Bacillus licheniformis* (1.28 x 10^9 CFU/kg feed).

[2] The Chao 1 index calculates the estimated true species diversity of a sample by dividing the number of singletons squared by twice the number of doubletons and then adding the result to the observed number of species (Edgar, 2017).

[3] The Shannon index accounts for both abundance and evenness of the species present and is calculated by using the proportion of unique species relative to the total number of species multiplied by the natural logarithm of this proportion. The resulting calculation is summed across all species and multiplied by -1 (Morris et al., 2014)

[4] The number of observed species was calculated based on the number of unique OTUs in each sample (Edgar, 2017).

[5]Operational taxonomic unit

CHAPTER III

DOSE EFFECTS OF *BACILLUS SUBTILIS* ON GROWTH PERFORMANCE OF NURSERY PIGS

SUMMARY

The dose of DFMs can impact the purported health benefits. Therefore, the objective of this study was to determine the most effective dose of *Bacillus subtilis* 29784 needed to enhance growth performance of newly weaned piglets fed a standard diet from 21 to 63 d of age. Pigs (27 barrows and 27 gilts) newly weaned at 21 d of age (6.57 ± 0.91 kg BW) were randomly assigned to 3 treatments based on a randomized complete block design with sex and initial BW as blocks: Treatments were 1) T1: Basal diet 2) T2: T1 + *Bacillus subtilis* (1.0 x 10^8 CFU/kg feed) 3) T3: T1 + *Bacillus subtilis* (2.5 x 10^8 CFU/kg feed). Diets were formulated to meet requirements suggested by NRC (2012). The experiment lasted for 42 d with d 1 to 14 being considered phase 1 and d 15 to 42 considered phase 2. BW and feeder weights were recorded at the beginning and end of each phase to determine ADG, ADFI and G:F. Fecal scores were recorded on d 2, 4, 7, 10, 14, 21, and 35 based on a 1 to 3 scale with 1 being solid and 3 being diarrhea. Data were analyzed using Proc Mixed of SAS with pig as the experimental unit and polynomial contrasts were used to detect treatment effects. The increasing concentrations of *Bacillus subtilis* quadratically affected ($P < 0.05$) d 14 BW with the greatest d 14 BW achieved with T2 (10.01 kg). In phase 1, the concentrations of *Bacillus subtilis* tended to have a quadratic effect on both ADG ($P = 0.064$) and ADFI ($P = 0.097$) with the highest phase 1 ADG (242 g/d) and ADFI (259 g/d) achieved when *Bacillus subtilis* was supplemented at 1.0 x 10^8 CFU/kg feed (T2). Concentration of *Bacillus subtilis* did not affect G:F during phase 1, phase 2, or the overall experimental period. No effects of *Bacillus subtilis* concentrations on growth performance were observed in phase 2 or the overall experimental period. The concentration of *Bacillus subtilis* did not affect fecal scores on d 2, 4, 7, 10, 14, 21, or 35. In conclusion, optimal growth performance was achieved when

Bacillus subtilis 29784 was supplemented at 1.0 x 10^8 CFU/kg feed from d 1 to 14 post weaning.

INTRODUCTION

Although DFMs have been shown to benefit growth performance and animal health, it is often concluded that the effects of DFMs are more inconsistent than other feed additives. These inconsistent results could be due to several factors such as variations in experimental design, DFM species, DFM dose, feed formulation and the type of challenge used, if any (Vondruskova et al., 2010; Buntyn et al., 2016). Several studies have investigated the effects of DFM dose on growth performance, nutrient digestibility, and immune status (Alexopoulos et al., 2004; Wang et al., 2009; Li et al., 2012; Hu et al., 2014a; Zhu et al., 2014; Lan et al., 2016; Yang et al., 2016) in both natural exposure conditions and with the inclusion of a pathogen challenge.

In studies that incorporated a pathogen challenge, the results indicated that high doses of particular DFMs may negate any positive effects by modifying the established microbiota and altering the response of the mucosal immune system to enteric pathogens (Li et al., 2012; Zhu et al., 2014; Yang et al., 2016). Studies conducted in natural exposure conditions reported beneficial effects of doses ranging from 1.2×10^{9} CFU/kg feed to 2.0×10^{13} CFU kg/feed (Alexopoulos et al., 2004; Wang et al., 2009; Cui et al., 2013; Hu et al., 2014; Lan et al., 2016) that could potentially be attributed to a higher number of vegetative cells present. Collectively, these studies have demonstrated that beneficial effects of DFMs may be dose dependent and therefore proper dosage level must be determined for each DFM strain.

Currently, the appropriate dose of *Bacillus subtilis* 29784 in pigs has yet to be determined. Consequently, it is hypothesized that there is an optimal level at which *Bacillus subtilis* 29784 exerts the most benefit on growth performance and fecal scores of nursery pigs. Therefore, the objective of the current study was to determine the most effective dose of

Bacillus subtilis 29784 needed to enhance growth performance of newly weaned piglets fed a standard diet from 21 to 63 d of age.

MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Animals and experimental design

Fifty-four newly weaned pigs, PIC 337 x Camborough 22, half barrows, half gilts (6.6 ± 0.9 kg BW) at approximately 21 d of age were utilized for this experiment. Pigs were grouped according to BW and randomly assigned to 3 treatments within each BW group. Treatments were 1) T1: Basal diet 2) T2: T1 + *Bacillus subtilis* (1.0 x 10^8 CFU/kg feed) 3) T3: T1 + (2.5 x 10^8 CFU/kg feed). Each treatment had 18 replicates, 9 BW blocks, and pigs were housed individually. Pigs were penned in indoor pens (1.50 x 0.74 m) with slatted floors at the North Carolina State University Metabolism Education Unit. Pigs were given ad libitum access to feed and water for the duration of the experiment. The experiment lasted for 42 d with d 1 to 14 being considered phase 1 and d 15 to 42 considered phase 2. All diets were formulated to meet nutrient requirements suggested by NRC (2012).

Growth performance and fecal scoring

Initial BWs were taken upon arrival at the Metabolism Education Unit and prior to allotment. BW measurements were taken at d 14 and d 42 to determine ADG for each phase.

The amount of feed given was recorded and feeder weights were also measured on d 14 and d 42 to estimate ADFI and G:F for each phase. Fecal scores were recorded throughout the experiment on d 2, 4, 7, 10, 14, 21, and 35. Scores were based on a 1 to 3 scale with 1 being solid and 3 being diarrhea. Mortality was also recorded.

Statistical analysis

The effect of treatment was evaluated by ANOVA using Proc Mixed of SAS 9.3 (SAS Institute Cary, NC). The statistical model included treatment, gender, and the treatment by gender interaction as fixed effects and block as a random effect. Pen was the experimental unit. Polynomial contrasts were used to determine linear and quadratic effects of dose level. Fecal score data were analyzed with the Kruskal Wallis Test by using the NPAR1WAY procedure in SAS 9.3. P values of less than 0.05 were considered significant and $0.05 \leq P < 0.10$ were considered a tendency.

RESULTS

Growth performance and fecal scores

Body weights on d 14 were quadratically affected ($P < 0.05$) by the increasing concentrations of *Bacillus subtilis* 29784 with the greatest d 14 BW achieved with T2 (10.01 kg) (Table 2). In phase 1, the concentrations of *Bacillus subtilis* tended to have a quadratic effect on both ADG ($P = 0.064$) and ADFI ($P = 0.097$). The highest phase 1 ADG (242 g/d) and ADFI (259 g/d) were achieved when *Bacillus subtilis* was supplemented at 1.0×10^8 CFU/kg feed (T2) and ADG (218g/d) and ADFI (232 g/d) were decreased when supplementation reached 2.5×10^8 CFU/kg feed (Table 2). Gain to feed ratio was not affected by *Bacillus subtilis* concentrations in either phase 1, phase 2, or the overall experimental period. No effects of *Bacillus subtilis* concentrations on growth performance were observed in phase 2 or the overall experimental period (Table 2). The concentration of *Bacillus subtilis* did not affect fecal scores on d 2, 4, 7, 10, 14, 21, or 35.

DISCUSSION

Previous research has shown that the beneficial effects of DFMs may be dose-dependent (Alexopoulos et al., 2004; Wang et al., 2009; Li et al., 2012; Zhu et al., 2014; Hu et al., 2014; Lan et al., 2016; Yang et al., 2016) and therefore the optimal supplementation level of each DFM must be evaluated for variables of interest. The supplementation level of a DFM is an important factor that can lead to variation between studies and therefore, when making comparisons between studies, it is important to consider the dose of the DFM used in each study. In challenge studies, results have indicated that high doses of certain DFMs may negate any positive effects by altering the established microbiota and modifying the response of the mucosal immune system to enteric pathogens (Li et al., 2012; Zhu et al., 2014; Yang et al., 2016). Studies conducted in natural exposure conditions reported that medium to high doses provided more beneficial effects than lower doses (Alexopoulos et al., 2004; Wang et al., 2009; Hu et al., 2014; Lan et al., 2016). The dose effect of DFMs may be primarily due to the number of vegetative cells that are present in the gastrointestinal tract.

The improvements in ADG during phase 1 of this study were comparable to results reported by other publications (Alexopoulos et al., 2004; Wang et al., 2009; Hu et al., 2014). In the current study, the greatest improvements in phase 1 BW, ADG and ADFI were observed when *Bacillus subtilis* 29784 was supplemented at 1.0 x 10^8 CFU/kg feed. These improvements were reduced when supplementation reached 2.5 x 10^8 CFU/kg of feed. The ideal dose determined in the present study is slightly lower than the optimal doses of 1.28 x 10^9 spores/kg of feed and 1.92 x 10^9 spores/kg of feed needed to achieve the best ADG and G:F of pigs compared to the low dose of 0.64 x 10^9 CFU/kg feed as reported by Alexopoulos et al. (2004). However, Alexopoulus et al. (2004) used a combination of *Bacillus subtilis* and *Bacillus licheniformis,* whereas in the present study, only *Bacillus subtilis* was used. The improvements in ADG and ADFI observed during the present study are somewhat in agreement with results that indicated linear improvements of ADG and ADFI reported with increasing levels of *Bacillus subtilis* and *Bacillus licheniformis* (0.5, 0.1, and 0.2% of a product containing 3.2 x 10^{12} CFU/kg feed) (Wang et al., 2009). Similar to our results, no linear or quadratic effects of supplementation on G:F were observed (Wang et al., 2009). However, in contrast to our results, Wang et al. (2009) did not discover a point at which further supplementation no longer improved growth performance and the supplementation levels used were higher than those in the present study. In agreement with Wang et al. (2009), a multi-strain study also exhibited a linear improvement in ADG and G:F with increasing supplementation levels of a product containing a mixture of 1 x 10^{12} CFU/kg of *Bacillus coagulance*, 5 x 10^{11} CFU/kg of *Bacillus lichenformis*, 1 x 10^{12} CFU/kg of *Bacillus subtilis* and 1 x 10^{11} CFU/kg of *Clostridium butyricum* respectively (Lan et al., 2016). The supplementation levels used by Lan et al. (2016) were much higher than the dose of 1.0 x 10^8 CFU/kg feed that was demonstrated to be the most effective dose in the current study. Collectively, these results indicate that the number of DFM strains and the strains used may influence the required supplementation level.

In addition to the number of DFM strains used, the specific strain that is used may influence the administration level required in order to achieve the greatest results. In the current study, *Bacillus subtilis* 29784 was the strain evaluated and it was determined that doses greater than 1.0 x 10^8 CFU/kg feed diminished the improvements in growth performance. Conversely, another study that investigated the effects of different doses of *Bacillus subtilis* KN-42 reported that the best ADG and feed efficiency were achieved when *Bacillus subtilis* was supplemented at 4 x 10^9 and 20 x 10^9 CFU/kg feed (Hu et al., 2014) which are higher than the ideal dose observed in the current study. Although Hu et al. (2014) evaluated the same DFM species as the current study, the differences in the perceived ideal dose level could have been due to the fact that the strain of *Bacillus subtilis* was not consistent in both studies. Hu et al. (2014) also reported that despite supplementation level, *Bacillus subtilis* KN-42 resulted in a lower diarrhea index in pigs than the negative control. This finding is in contrast to the current study which observed no effects of *Bacillus subtilis* 29784 on fecal scores of nursery pigs.

In conclusion, the ideal supplementation level of DFMs is influenced by numerous factors including DFM species, DFM strain, and the number of strains used. The beneficial effects of *Bacillus subtilis* 29784 on growth performance appear to be dose-dependent and therefore it is concluded that there is a maximum supplementation level beyond which, no additional improvements would be observed. Fecal scores were not affected by the dose of *Bacillus subtilis* 29784. The dose of *Bacillus subtilis* 29784 needed to achieve optimal growth performance of nursery pigs from 21 to 35 d of age was 1.0 x 10^8 CFU/kg feed.

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Table 1. Composition of experimental diets

Item	Age of pig	Phase 1 d 21 to 35	Phase 1 d 35 to 63
Ingredient, %			
Yellow corn, ground		39.94	61.79
Whey permeate		19.00	0.00
Poultry fat		2.00	2.00
Soybean meal		21.00	30.00
Poultry meal		5.00	0.00
Blood plasma		6.00	2.00
Fishmeal		4.00	0.00
L-Lys HCl		0.32	0.30
DL-Met		0.15	0.09
L-Thr		0.08	0.06
Limestone		0.85	1.00
Dicalcium phosphate		0.00	1.10
Salt		0.22	0.22
Trace mineral premix[1]		0.15	0.15
ZnO		0.25	0.25
Vitamin premix[1]		0.03	0.03
Phytase[2]		0.01	0.01
Supplement[3]		1.00	1.00
Total		100.00	100.00
Calculated composition[5]			
ME, kcal/kg		3,437	3,385
SID[4] Lys, %		1.50	1.26
SID[4] Met + Cys, %		0.82	0.69
SID[4] Thr, %		0.88	0.74
SID[4] Trp, %		0.27	0.24
Ca, %		0.85	0.71
Total P, %		0.66	0.61
STTD[6] P, %		0.45	0.35
Analyzed composition			
DM, %		91.61	90.73
CP,%		24.09	21.03
CF, %		4.99	5.31
Ash		6.44	5.11

Table 1. continued

[1]Premix is added to provide the following nutrients per kg of complete diet: 6,613.8 IU of vitamin A; 992.0 IU of vitamin D3; 19.8 IU of vitamin E; 2.64 mg of vitamin K; 0.03 mg of vitamin B12; 4.63 mg of riboflavin; 18.52 mg of pantothenic acid; 24.96 mg of niacin; 0.07 mg of biotin; 4.0 mg of Mn as manganous oxide; 165 mg of Fe as ferrous sulfate; 165 mg of Zn as zinc sulfate; 16.5 mg of Cu as copper sulfate; 0.30 mg of I as ethylenediamine dihydroiodide; and 0.30 mg of Se as sodium selenite.

[2]Phytase: Quantumblue 5G to provide 500 FTU/kg feed

[3]Supplement: *Bacillus subtilis* 29784 supplemented at (0, 10, 25 and 500 g/ton) for T1, T2, T3, and T4

[4]SID = standardized ileal digestible

[5]Calculated composition was based on NRC (2012)

[6] STTD: Standardized total tract digestible

Table 2. Growth performance of pigs fed experimental diets from 21 to 63 d of age

	Bacillus subtilis 29784, 10^8 CFU/kg feed				*P* value	
Item	0	1.0	2.5	SEM	Linear	Quadratic
BW, kg						
Initial	6.6	6.6	6.6	0.3	0.828	0.498
d 14	9.4^{a}	10.0^{b}	9.6^{ab}	0.4	0.713	0.047
d 42	28.3	29.7	29.1	0.9	0.349	0.105
ADG, g/d						
Phase 1[1]	205^{A}	242^{B}	218^{AB}	13.10	0.658	0.064
Phase 2[1]	672	704	696	20.23	0.339	0.281
Overall[1]	516	550	537	16.19	0.336	0.116
ADFI, g/d						
Phase 1	232^{A}	259^{B}	232^{A}	13.41	0.838	0.097
Phase 2	927	949	957	34.70	0.441	0.780
Overall	696	719	715	25.89	0.505	0.529
G:F						
Phase 1	0.89	0.93	0.94	0.02	0.125	0.473
Phase 2	0.73	0.75	0.73	0.01	0.937	0.373
Overall	0.75	0.77	0.75	0.01	0.887	0.224

^{ab} Means within a row without a common superscript are significantly different (*P* <0.05)

^{AB}Means within a row without a common superscript tended to be different (*P* < 0.10)

[1]Phase 1 represents d 1 to 14, phase 2 represents d 15 to 42, and overall represents d 1 to 42.

Table 3. Fecal scores of pigs fed experimental diets from 21 to 63 d of age

	Bacillus subtilis 29784, 10^8 CFU/kg feed				*P* value	
Item	0	1.0	2.5	SEM	Linear	Quadratic
Fecal score[1]						
d 2	2.39	2.25	2.22	0.20	0.541	0.739
d 4	2.39	2.65	2.50	0.17	0.716	0.293
d 7	2.17	2.41	2.33	0.21	0.623	0.495
d 10	1.50	1.88	1.56	0.20	0.974	0.137
d 14	1.50	1.65	1.78	0.18	0.289	0.875
d 21	1.22	1.29	1.17	0.10	0.634	0.462
d 35	1.22	1.47	1.39	0.13	0.394	0.235

[1]Fecal scores were based on a scale of 1 to 3 with solid feces given a score of 1 and diarrhea given a score of 3.

CHAPTER IV

THE EFFECTS OF *BACILLUS SUBTILIS* ON GUT HEALTH AND PREVENTION OF POSTWEANING DIARRHEA CAUSED BY F18^{+} *ESCHERICHIA COLI*

SUMMARY

Enterotoxigenic *Escherichia coli* (ETEC) is a major problem in the swine industry and is known to cause post weaning diarrhea and edema disease in piglets. An ETEC infection can lead to poor growth performance, scouring, and increased mortality in newly weaned piglets. The objective of this study was to determine if *Bacillus subtilis* 29784 could improve growth performance, gut health, and prevent post-weaning diarrhea caused by $F18^+$ *Escherichia coli*. Forty newly weaned barrows (6.3 ± 1.0 kg BW) at approximately 21 d of age were randomly assigned to 4 treatments with initial BW as a blocking factor. Treatments were: 1) NC: a diet with no additive and no $F18^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and $F18^+$ *Escherichia coli* challenge (2×10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1×10^8 CFU/kg). Diets were formulated according to requirements suggested by NRC (2012). The experiment lasted for 27 d with d 1 to 14 being considered phase 1 and d 15 to 27 considered phase 2. BW and FW were recorded weekly to determine ADG, ADFI, and G:F. The $F18^+$ *Escherichia coli* was inoculated on d 7. Fecal scores were recorded on d 3, 5, 7, 8, 9, 10, 11, 12, 13, 14, 16, 18, 20, 23, and 26 based on a 1 to 3 scale with 1 being solid and 3 being diarrhea. Data were analyzed using Proc Mixed of SAS 9.3 with pig as the experimental unit, and treatment as a fixed effect, and BW block as a random effect. During wk 4, both AGP and PRO had higher body weights than PC ($P < 0.05$) but were not different from NC. No effects of treatment were observed on ADG during the entire experimental period. During wk 2, AGP and PRO improved ($P < 0.05$) ADFI compared to PC but not compared to NC. During wk 4, all challenged treatments (PC, AGP, and PRO) had decreased ($P < 0.05$) ADFI compared to NC.

No effects of treatment were observed on G:F during wk 1, 2, or 4. Although, during wk 3, the challenged treatments (PC, AGP, PRO) tended to have higher ($P < 0.10$) G:F than NC. On both d 11 and d 12, the challenged treatments (PC, AGP, and PRO) had higher ($P < 0.05$) fecal scores than NC. Both supplemented treatments (AGP and PRO) tended to ($P < 0.10$) have a higher pH in ileal digesta compared to PC. There were no differences in TNFα detected among treatments in either d 14 or d 26 serum samples. Both AGP and PRO tended to ($P < 0.10$) have lower serum protein carbonyl on d 14 compared to PC and PRO had lower ($P < 0.05$) serum MDA on d 26 compared to AGP. Claudin concentrations in jejunal tissue were higher ($P < 0.05$) in the supplemented treatments (AGP and PRO) compared to PC (Table 8). Occludin concentrations in jejunal tissue were higher ($P < 0.05$) in AGP (0.383) than PRO (0.236) (Table 8). Concentrations of ZO-1 in jejunal tissue were also higher ($P < 0.05$) in AGP (0.349) compared to PRO (0.214) (Table 8). On d 14, the relative abundance of *Bacteroidetes* was increased ($P < 0.05$) in PRO (36.6%) fecal samples compared to AGP (23.6%) fecal samples (Table 11). In d 14 fecal samples, the relative abundance of *Proteobacteria* was decreased ($P < 0.05$) by AGP and PRO compared to PC (Table 11). PRO had a lower ($P < 0.05$) relative abundance of *Proteobacteria* (14.88%) compared to AGP (27.33%) (Table 11). In conclusion, *Bacillus subtilis* 29784 offered little to no benefit to nursery pigs challenged with $F18^+$ *Escherichia coli* and did not prevent postweaning diarrhea.

INTRODUCTION

Postweaning diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is a concern in the swine industry and is responsible for economic losses due to poor growth performance, diarrhea and increased morbidity and mortality in newly weaned pigs. The most common types of *Escherichia coli* that cause post weaning diarrhea carry the F4 (K88) or F18 adhesion (Fairbrother et al., 2005). In the past, this problem has been controlled by the supplementation of antibiotics in the feed. However, due to the increased consumer demand for antibiotic free pork production and concerns about antibiotic resistance, antibiotic alternatives are needed more than ever. The use of DFMs as potential alternatives to antibiotics has generated significant interest in the last fifteen years (Buntyn et al., 2016).

DFMs are purported to competitively exclude pathogenic bacteria. Numerous studies in pigs and poultry have shown that DFMs can competitively exclude and prevent adherence of gram-negative bacteria such as *Escherichia coli* and *Salmonella* both *in vitro* and *in vivo* (Jin et al., 2000; Forestier et al., 2001; La Ragione, 2001; La Ragione and Woodward, 2003; Roselli et al., 2006; Tsukahara et al., 2013). Nevertheless, the mechanisms by which DFMs exert beneficial effects on their host during an ETEC infection may vary depending on the species and strain being investigated. In the previous study (Chapter II), *Bacillus subtilis* 29784 improved growth performance and gut health of nursery pigs in natural exposure conditions. However, the potential role of *Bacillus subtilis* 29784 in alleviating the symptoms of an ETEC infection in pigs has yet to be elucidated.

Therefore, it is hypothesized that supplementing *Bacillus subtilis* 29784 would improve pig performance, gut health and reduce post weaning diarrhea caused by $F18^{+}$ *Escherichia coli*. The objective of this study was to determine the effects of supplementation of *Bacillus subtilis* 29784 compared to the effects of antimicrobial growth promotors on growth performance, gut health and prevention of post weaning diarrhea caused by F18$^+$ *Escherichia coli*.

MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University (Raleigh, NC, USA).

Animals and experimental design

Forty newly weaned Chester White barrows (6.3 ± 1.0 kg BW) at approximately 21 d of age were utilized for this experiment. Pigs were grouped according to BW and randomly assigned to 4 treatments within each BW group. Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Each treatment had 10 replicates, 5 BW blocks, and pigs were house individually. Pigs were housed in indoor pens (1.50 x 0.74 m) with slatted floors with a waterer and self-feeder in each pen at the Metabolism Education Unit (MEU) of North Carolina State University. Pigs were given *ad libitum* access to feed and water for the duration of the experiment. Non-infected and infected pigs were housed at opposite ends of the room with 10 empty pens between the groups. Airflow in the building flowed from the direction of the non-infected pigs to the infected pigs and infected pigs had nose to nose contact through their pens. The experiment lasted for 27 d with d 1 to 10 being considered phase 1 and d 11 to 27 considered phase 2. Phase 1 targeted 5 to 7 kg BW and phase 2 targeted 7 to 11 kg BW. All diets were formulated to meet nutrient requirements suggested by NRC (2012).

Growth performance

Initial BW were taken upon arrival at the MEU and prior to allotment. BW measurements were taken at d 7, 14, 21, and 27 to determine ADG. The amount of feed given was recorded and feeder weights were also measured on d 7, 14, 21, and 27 to estimate ADFI and G:F. Fecal scores were recorded throughout the experiment on d 3, 5, 7, 8, 9, 10, 11, 12, 13, 14, 16, 18, 20, 23, and 26 based on a 1 to 3 scale with 1 being solid and 3 being watery diarrhea. Mortality was also recorded.

Escherichia coli challenge

Strain preparation

The strain of *Escherichia coli* used was selected for resistance to nalidixic acid according to Cutler et al. (2007). In order to prepare this strain, 1 individual colony from the original tube of F18^{+} *Escherichia coli* was selected and then inoculated into 500 μL of lysogeny broth (LB) and mixed well. This mixture was then used to inoculate 100 μL tubes each containing 1 mL of LB with increasing doses of nalidixic acid (10, 25, 50, and 100 mg/L). The tubes were incubated overnight with shaking. Following the overnight incubation, the tube with the highest amount of nalidixic acid was chosen to determine if bacteria growth was still present. The bacteria concentration was determined by spectrophotometry at a wavelength of 600 nm with an optical density value greater than 0.75

considered adequate. An amount of 100 μL was taken from this tube and used to inoculate tubes with 1 mL of new media at 1.5X, 2.0X, 2.5X and 3.0X the original nalidixic acid concentration. These tubes were incubated overnight with shaking. This process was repeated until the nalidixic acid concentration reached 150 mg/L. The strain was then cultured in media containing nalidixic acid and tested on plates with 25 and 50 mg/L nalidixic acid to ensure survival.

Inoculation preparation

The seed culture was prepared by using 10 mL of LB broth (BD; 743-29238) with 1.5 mg of nalidixic acid in a 50 mL Erlenmeyer flask. This broth was autoclaved and 1 colony from the last step of preparation was inoculated into the broth and then incubated at 37^{o}C shaking at 150 rpm overnight. The main culture was then prepared from this seed culture. After the overnight incubation, 1 mL of the cultured media was inoculated into 500 mL of autoclaved LB broth with 75 mg of nalidixic acid in a 2 L Erlenmeyer flask. This mixture was allowed to grow overnight at 37^{o}C with shaking at 150 rpm. After overnight growth, 100 μL of the cultured media was spread on LB agar plates with 50 mg/L of nalidixic acid according to a serial dilution. These plates were incubated at 37^{o}C overnight. After plating, the remaining 500 mL of cultured media was stored at 4^{o}C. Following the overnight incubation, the colonies on the agar plate were counted and 50 mL of the remaining media was transferred to a new tube. The tube was centrifuged at 3,000 x g for 10 min at 4^{o}C. After centrifugation, the supernatant was discarded and the remaining pellet was resuspended in 20% dextrose and 5% nonfat dry milk. This solution was kept on ice until inoculation. On d 7 of the experiment, pigs were given an oral dose of (2 x 10^9 CFU/0.5 mL) of F18$^+$ *Escherichia coli* in the morning and a second time in the evening.

Blood and fecal sample collection

On d 14 and 26 of the study, 10 mL of blood was collected from the jugular vein in vacutainer tubes (BD Vacutainer®; Becton, Dickinson and Company, New Jersey, USA). Blood samples were centrifuged at 3,000 x g for 15 min to obtain serum. Serum was aliquoted and stored at -80ºC until further analyses. Fecal samples were also obtained on d 14 and d 26 and stored at -20ºC until later microbiome sequencing.

Sample collection

On d 27 at the conclusion of the study, pigs were euthanized via captive bolt followed by exsanguination for sample collection. Digesta samples were collected from the terminal jejunum, ileum, cecum and colon for pH measurements. A separate jejunal digesta sample was collected and snap frozen in liquid nitrogen and stored at -20ºC for later gut microbiome sequencing. Jejunal mucosa samples were collected and snap frozen in liquid nitrogen and then stored at -80ºC until further analyses. Tissue from the jejunum was also collected to measure tight junction proteins (claudin, occludin, and zonula occludens-1). An additional tissue samples was taken from the jejunum, flushed with 0.9% saline and placed in 10% formalin for histology measurements.

pH measurements

Immediately after collection, pH of digesta samples from the jejunum, ileum cecum and colon were measured using a pH meter (Thermo Fisher Scientific, MA, USA.) after standardization with buffers at a pH of 4.0 and 7.0.

Histology measurements

After collection, tissue samples from the jejunum remained in 10% formalin for 3 wk. Cross sections of 1 cm thickness were sliced from jejuncal tissue, placed in cassettes, covered in ethanol, and sent to the Histology Laboratory at the College of Veterinary Medicine of North Carolina State University for hematoxylin and eosin staining. The Infinity Analyze and Capture software (Lumenera Corporation, Ottawa, Canada) was used to measure villus height, villus width (Figure 1) and crypt depth in both tissues. Measurements of 20 intact villi and their associated crypt were measured from each slide. The same person executed all intestinal morphology analyses.

Immune and oxidative stress measurements

Porcine specific ELISAs were used to quantify TNFα (Cat. PTA00, R&D Systems, MN, USA), protein carbonyl (Cat. STA-310, Cell Biolabs, Inc., CA, USA), and malondialdehyde (Cat. STA-330, Cell Biolabs, Inc.) in both d 14 and d 26 serum and jejunal mucosal tissue.

Protein isolation and western blotting

Jejunal tissue from each treatment was used to measure tight junction proteins as described by Yang et al. (2015). Tissue samples (50 mg) of jejunum were weighed and suspended into 0.5 mL RIPA lysis and extraction buffer containing 5 µL protease inhibitor cocktail. Tissue samples were then homogenized and kept on ice. The homogenate was centrifuged at 10,000 x g at 4^{o}C for 10 min and supernatant was collected. A BCA protein assay (#23225, ThermoFisher Scientific) was used to measure total protein concentration in the supernatant. The concentration was then adjusted to 4 μg/μL and denatured in a 100ºC water bath for 5 min. Samples were loaded into individual wells for SDS-PAGE. The gel was then moved onto polyvinylidene difluoride (PVDF) membrane and the target proteins were transferred to the membrane. Proteins were transferred electrophoretically for 1 h at 90 mV. After blocking with 5% skim milk, overnight incubation with primary antibodies against claudin, occludin, ZO-1 and β-actin occurred at 4ºC. After incubation, the membrane was washed and incubated with horseradish-conjugated secondary antibodies for 1 h at room temperature. The DAB substrate kit (34002; Pierce, IL, USA) was used to develop the immunoblot and band density was determined by image analyzer software (LI-COR Biosciences, NE, USA).

DNA extraction and microbiota analysis

The QIAmp® DNA stool Mini Kit (Cat No. 51504, Qiagen, Germany) was used to extract bacterial DNA from mucosa and fecal samples prior to microbiota analysis. After DNA extraction, d 14 and d 26 fecal samples and jejunal mucosal samples were sent to Mako Medical Laboratories in Raleigh, North Carolina for qPCR analysis of 16S rDNA sequences from gut bacteria and counting of F18^{+} *Escherichia coli*. Samples were prepared for template preparation on the Ion Chef TM instrument and sequencing on the Ion S5 TM system. Variable regions V2, V3, V4, V6, V7, V8, and V9 of the 16S rRNA gene were amplified with the Ion 16S TM Metagenomics Kit (Cat. A26216, ThermoFisher Scientific). Libraries were prepared from the amplified target regions with the Ion Xpress TM Plus Fragment Library Kit (Cat. 4471269, ThermoFisher Scientific) and the IonCode TM Barcode Adapters 1-384 Kit (Cat. A29751, ThermoFisher Scientific) was used for barcoding and multiplexing of the prepared libraries. The libraries were quantified with the Ion Universal Library Quantitation Kit (Cat. A26217, Thermofisher Scientific) and samples were diluted to equal concentration and pooled into multiplexed libraries for template preparation. Template preparation and chip loading were performed using the Ion Chef TM instrument and sequencing was performed on the Ion S5 TM system with the Ion 520 TM & Ion 530 TM Kit-Chef (Cat. A30010, ThermoFisher Scientific) and the Ion 530 TM Chip Kit-4 Reactions (Cat. A27763, ThermoFisher Scientific). Sequences were processed using the Torrent Suite TM Software (version 5.2.2) to produce unaligned. bam files for further analysis. Sequence data analysis, alignment to GreenGenes and MicroSeq databases, alpha and beta diversity plot generation, and OTU table generation were performed by the Ion Reporter TM Software Suite (version 5.2) of bioinformatics analysis tools. Samples were analyzed using Ion Reporter's Metagenomics 16S workflow (version w1.1). All samples had a depth of sequencing coverage > 1000X. Sample preparation and analysis settings were performed to manufacturer's recommended protocols and analysis settings. Sequencing was performed by the Genomics Department of Mako Medical Laboratories (8461 Garvey Drive, Raleigh, N.C. 27616).

F18 plasmid quantitation of F18^{+} Escherichia coli strains

For quantitation of F18^{+} *Escherichia coli* strains, a synthetic plasmid containing the fedA major subunit fimbrial adhesin gene sequence (Accession no. M61713, NCBI GeneBank) was constructed by GeneArt (ThermoFisher Scientific). The synthetic gene F18 was assembled from synthetic oligonucleotides and/or PCR products. The fragment was inserted into pMK-RQ-Bs. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by Sanger sequencing. The sequence congruence within the insertion sites was 100%. ThermoFisher Scientific also provided a TaqMan probe specific for the fedA gene. The plasmid was used as a standard for quantitation of $F18^+$ plasmids within samples. Before sequencing using qPCR the standard plasmid was linearized by SmaI digestion (Cat. FD0664, ThermoFisher Scientific). Based on the plasmid size (914 bp) and concentration the count of the stock standard was calculated. The standard was serially diluted to 2.86×10^7, 2.86×10^6, 2.86×10^5, 2.86×10^4, and 2.86×10^3 plasmid counts. Quantitative PCR of samples and standards was performed with Taqpath qPCR Master Mix CG (Cat. A15297, ThermoFisher Scientific) on the QuantStudio 12K Flex (ThermoFisher Scientific) per manufacturer's instructions. Sample F18 plasmid count was calculated based on the standard dilution using linear regression. Sequencing was performed by the Genomics Department of Mako Medical Laboratories (8461 Garvey Drive, Raleigh, N.C. 27616).

Statistical analysis

Data were analyzed using Proc Mixed of SAS 9.3 (Cary, NC). Pig was the experimental unit. Treatment was a fixed effect and BW block was a random effect. Orthogonal contrasts were also made between 'NC vs. PC + AGP + PRO', 'PC vs. AGP + PRO', and 'AGP vs. PRO'. Fecal score data were analyzed with the Kruskal Wallis Test by using the NPAR1WAY procedure in SAS 9.3. Microbiome raw sequencing data were organized using the Ion Reporter Metagenomics 16S algorithm (ThermoFisher Scientific). Data were collected, filtered by primers and length, adaptor sequences were trimmed, and bin barcodes were assigned. Data was then compared against the 16S database (GreenGenes and

MicroSeq) using BLAST (Basic Local Alignment Search Tool). The abundance was then generated based on the number of sequences for variable regions and statistical analysis was performed on relative abundance data using Proc Mixed of SAS 9.3. P values of less than 0.05 were considered significant and $0.05 \leq P < 0.10$ were considered a tendency.

RESULTS

Growth performance

Body weights were not affected by treatment during wk 1, 2 or 3. During wk 4, both AGP and PRO had higher body weights than PC ($P < 0.05$) but were not different from NC (Table 2). No effects of treatment were observed on ADG during the entire experimental period. During wk 2, AGP and PRO improved ($P < 0.05$) ADFI compared to PC but not compared to NC (Table 2). There were no differences in ADFI among treatments during wk 3. However, during wk 4, all challenged treatments (PC, AGP, and PRO) had decreased ($P < 0.05$) ADFI compared to NC (Table 2). No effects of treatment were observed on G:F during wk 1, 2, or 4. Although, during wk 3, the challenged treatments (PC, AGP, PRO) tended to have higher ($P < 0.10$) G:F than NC (Table 2).

Fecal scores and mortality

On d 3, both AGP and PRO tended to ($P = 0.069$) have higher fecal scores (1.4) than the PC and NC treatments (1.0) (Table 3). On d 7, PRO tended to ($P = 0.057$) have lower fecal scores (1.0) than AGP (1.4) and PRO also had lower ($P < 0.05$) fecal scores on d 8 (1.0) compared to AGP (1.0) (Table 3). On both d 11 and d 12, the challenged treatments (PC, AGP, and PRO) had higher ($P < 0.05$) fecal scores than NC (Table 3). The challenged treatments tended to have (P = 0.068) higher fecal scores on d 14 compared to NC (Table 3). On d 26, AGP had lower (P < 0.05) fecal scores (1.0) than PRO (1.3) (Table 3). No effects of treatment were observed on d 5, 9, 10, 13, 16, 18, 20, or 23 (Table 3). All pigs remained alive until the end of the study and therefore no differences in mortality were observed.

Gut histology and pH

Both supplemented treatments (AGP and PRO) tended to (P < 0.10) have a higher pH in ileal digesta compared to PC (Table 4). The pH of digesta from the jejunum, colon, and cecum was not affected by treatment (Table 4). Villus height, villus width, villus base, crypt depth and villus height to crypt depth ratio were not affected by treatment. The width of the villus tip was greater (P < 0.05) in the challenged treatments compared to NC (Table 5).

Immune status and oxidative stress status

There were no differences in TNFα detected among treatments in either d 14 or d 26 serum samples (Table 6). Both AGP and PRO tended to (P < 0.10) have lower serum protein carbonyl on d 14 compared to PC (Table 7) and PRO had lower (P < 0.05) serum MDA on d 26 compared to AGP (Table 7). Serum protein carbonyl concentrations were not affected by treatment on d 26 or in jejunal mucosa (Table 7). No differences in serum MDA on d 14 or jejunal mucosa were observed among treatments (Table 7).

Tight junction proteins

Claudin concentrations in jejunal tissue were higher (P < 0.05) in the supplemented treatments (AGP and PRO) compared to PC (Table 8). Occludin concentrations in jejunal tissue were higher ($P < 0.05$) in AGP (0.383) than PRO (0.236) (Table 8). Concentrations of ZO-1 in jejunal tissue were also higher ($P < 0.05$) in AGP (0.349) compared to PRO (0.214) (Table 8).

Gut microbiota and F18$^+$ Escherichia coli counts

Although no differences in F18$^+$ *Escherichia coli* counts were detected in fecal samples from d 14 or d 26, on d 14, PC had the highest count of F18$^+$ *Escherichia coli* (120,909) and NC had the lowest (1,028) (Table 9). By d 26, counts of F18$^+$ *Escherichia coli* had decreased in all treatments while numerically, PC still had the highest (123) and NC had the lowest (4) with AGP (29) and PRO (5) having counts close to that of NC (Table 9). The counts of F18$^+$ *Escherichia coli* did not differ between d 14 and d 26 (Table 10).

On d 14, the relative abundance of *Bacteroidetes* was increased ($P < 0.05$) in PRO (36.6%) fecal samples compared to AGP (23.6%) fecal samples (Table 11). In d 14 fecal samples, the relative abundance of *Proteobacteria* was decreased ($P < 0.05$) by AGP and PRO compared to PC (Table 11). PRO had a lower ($P < 0.05$) relative abundance of *Proteobacteria* (14.9%) compared to AGP (27.3%) (Table 11). No phylum differences were detected among treatments in d 26 fecal samples (Table 11).

DISCUSSION

Enterotoxigenic Escherichia coli (ETEC) causes postweaning diarrhea in the swine industry which can lead to decreased growth performance, and increased mortality and morbidity which results in economic losses for the producer. The majority of *Escherichia coli* that results in postweaning diarrhea carry either the F4 or F18 fimbrial adhesion. Newly weaned piglets are extremely susceptible to *Escherichia coli* infections due to the stress of weaning, environmental stress, and dietary stress. As a result of the development of antibiotic resistance and the current reduction in the use of antibiotics, other mitigation strategies to alleviate the symptoms of *Escherichia coli* infections must be evaluated. In recent years, the use of DFMs has gained more interest as a potential alternative to antibiotics.

The ability of *Bacillus*-based DFMs to improve growth performance in natural exposure conditions has been well documented in numerous studies; however, this was not observed in the current study (Alexopoulos et al., 2004; Chen et al., 2006; Guo et al., 2006; Davis et al., 2008; Choi et al., 2011; Cui et al., 2013; Hu et al., 2014; Jorgensen et al., 2016; Balasubramanian et al., 2017; Jaworski et al., 2017). Both the antibiotics and *Bacillus subtilis* 29784 improved ADFI during wk 2 of the post-challenge period. In contrast to this finding, other studies reported no improvements in growth performance by *Bacillus subtilis* during an *Escherichia coli* challenge in weaning pigs (Bhandari et al., 2008; Tsukahara et al., 2013).

The *Escherichia coli* challenge increased the occurrence of diarrhea on d 4 and 5 post-challenge compared to the negative control indicating that the *Escherichia coli* challenge was successful. However, in the present study, *Bacillus subtilis* did not reduce postweaning diarrhea caused by $F18^{+}$ *Escherichia coli*. This finding differs from results discussed by Bhandari et al. (2008) which reported that *Bacillus subtilis* decreased scours at 24 h post infection with *Escherichia coli*, and Scharek-Tedin et al. (2013), which demonstrated that *Bacillus cereus* supplementation reduced the occurrence of diarrhea in nursery pigs challenged with *Salmonella typhimurium*. However, similar to the results from the current study, the supplementation of lactic acid bacteria, *Lactobacillus paracasei* and

Pediococcus pentosaceus did not prevent diarrhea caused by F18^{+} *Escherichia coli* in neonatal pigs (Andersen et al., 2017).

Although the *Bacillus subtilis* did not reduce diarrhea caused by F18^{+} *Escherichia coli*, other results suggested that the DFM was able to alleviate some aspects of the *Escherichia coli* infection. In order for an ETEC infection to progress to a symptomatic disease, the *Escherichia coli* must be able to colonize the small intestine in sufficient numbers following the attachment of fimbrial adhesions to the intestinal epithelium (Francis, 2002; John M Fairbrother et al., 2005; Dubreuil, 2017). It has been previously demonstrated that *Bacillus*-based DFMs can competitively exclude a number of pathogenic bacteria including *Escherichia coli*, *Salmonella enterica*, and *Clostridium perfringens* (Maruta et al., 1995; La Ragione et al., 2001; La Ragione and Woodward, 2003; Scharek-Tedin et al., 2013; Tsukahara et al., 2013). Specifically, *Bacillus*-based DFMs can assuage the symptoms of an *Escherichia coli* infection in pigs and poultry by competitively excluding the pathogen in the gastrointestinal tract (La Ragione et al., 2001; Guo et al., 2006; Tsukahara et al., 2013).

The findings from the current study indicated that *Bacillus subtilis* 29784 was able to suppress the growth of F18+ *Escherichia coli* in the gastrointestinal tract at 7 d post challenge and by 19 d post challenge, the *Bacillus subtilis* had lowered the fecal F18^{+} *Escherichia coli* counts to the level of the negative control whereas, the positive control still had relatively higher fecal counts of F18^{+} *Escherichia coli*. This suppression of *Escherichia coli* growth is in agreement with other studies in which *Bacillus subtilis* supplementation was shown to reduce *Escherichia coli* counts in the feces and digesta of pigs (Guo et al., 2006; Tsukahara et al., 2013; Yang et al., 2016) and in poultry (La Ragione et al., 2001). In addition to reduced F18^{+} *Escherichia coli* fecal counts, *Bacillus subtilis* 29784 also modulated the gut microbiota at the phylum level by reducing the relative abundance of fecal *Proteobacteria* 7 d post challenge. Due to the fact that *Proteobacteria* is a phylum containing a variety of gram-negative pathogenic bacteria including *Escherichia coli*, this result is of great importance. Concurrently, *Bacillus subtilis* supplementation increased the relative abundance of fecal *Bacteroidetes* 7 d post challenge which is comparable to results reported by Bhandari et al. (2008) in which pigs fed *Bacillus subtilis* also had a greater incidence of *Bacteroidetes* in ileal digesta 7 d post challenge. The ability of *Bacillus*-based DFMs to alter the gut microbiota at the phylum level has previously been established (Cui et al., 2013; He et al., 2017). The results from the present study further support the concept that *Bacillus subtilis* can suppress the growth of pathogenic bacteria in the gastrointestinal tract and modulate the gut microbiota.

Despite reductions in the growth of pathogenic bacteria, *Bacillus subtilis* did not affect TNFα levels, digesta pH, jejunal histology or the concentrations of tight junction proteins. These results are comparable to other studies that reported no effects of *Bacillus*-based DFMs on pH, TNFα levels, or tight junction protein expression in pigs challenged with *Escherichia coli* (Bhandari et al., 2008; Yang et al., 2016). The pH results from the current study are in contrast to results reported in experiment 1. However, supplementation of *Bacillus subtilis* and AGP positively affected the oxidative stress status 7 d post-challenge by decreasing levels of serum protein carbonyl. The reduction of protein carbonyl is beneficial to the animal because reactive oxygen species can damage all types of biological molecules and the redox environment of the intestinal epithelium is crucial to maintain proper organ function and efficient nutrient digestion and absorption (Circu and Aw, 2012). Proteins can be oxidized to form radical species including superoxide, hydroxyl, and peroxyl groups (Dalle-Donne et al., 2003). The oxidation of proteins leads to the formation of carbonyl groups on the protein side chains and the stability of these compounds is useful for their detection and makes them an ideal biomarker of oxidative stress (Dalle-Donne et al., 2003). It has been established that *Bacillus subtilis* produces a superoxide dismutase (SOD) which is an important antioxidant enzyme that protects organisms against oxidative damage by catalyzing the reduction of the superoxide radical to dioxygen and hydrogen peroxide (Liu et al., 2007). Specifically, the *sodA* gene in *Bacillus subtilis* encodes a manganese-containing SOD that is vital for oxidative stress resistance in sporulating and growing cells (Inaoka et al., 1999). A previous study exhibited the ability of a *Bacillus*-based DFM to enhance the antioxidant capacity of pigs by increasing the activities of antioxidant enzymes including SOD, and concurrently enhancing antioxidant gene expressions, including *SOD* mRNA levels in the jejunum (Wang et al., 2017). Therefore, it is speculated that the reduction of serum protein carbonyl by *Bacillus subtilis* 29784 observed in the current study could have been due to amplified antioxidant gene expression or the increased production or activity of SOD. Further research is needed to determine the specific antioxidant genes or enzymes that may be affected or produced by *Bacillus subtilis* 29784.

In conclusion, *Bacillus subtilis* 29784 supplementation provided minimal improvements in growth performance of nursery pigs. Although, *Bacillus subtilis* 29784 improved the oxidative stress status by reducing levels of serum protein carbonyl 7 d post-challenge. The supplementation of *Bacillus subtilis* 29784 also lowered the relative abundance of fecal *Proteobacteria* and F18$^+$ *Escherichia coli* counts and increased fecal *Bacteroidetes* abundance. However, *Bacillus subtilis* was not superior to the antibiotics and did not prevent postweaning diarrhea caused by F18$^+$ *Escherichia coli*. Overall, supplementing *Bacillus subtilis* 29784 can confer limited benefits to pigs during an F18$^+$ *Escherichia coli* infection and was not superior to antibiotics.

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Table 1. Composition of experimental diets

Item	Phase 1	Phase 2
Ingredient, %		
Corn, yellow dent (4-02-861)	40.00	54.00
Whey permeate (MB2014)	20.00	10.00
Poultry fat (4-09-319)	3.50	1.80
Soybean meal, dehulled (5-04-612)	22.00	23.50
Blood plasma (no IFN, NRC 2012)	6.00	3.00
Fish meal (5-01-977)	0.00	0.00
L-Lys HCl	0.48	0.47
DL-Met	0.22	0.18
L-Thr	0.15	0.13
L-Trp	0.00	0.00
Dical (NCSU2014)	0.60	0.85
Limestone, ground (6-02-632)	0.95	0.95
Vitamin premix	0.03	0.03
Mineral premix	0.15	0.15
Salt	0.22	0.22
Zinc oxide	0.25	0.25
Poultry meal (MB2014)	5.00	4.00
Additive[2] (AGP or PRO)	0.45	0.47
Total	100.00	100.00
Calculated composition[4]		
DM, %	90.8	90.1
ME, kcal/kg	3481	3388
CP, %	23.0	21.6
SID[3] Lys, %	1.50	1.35
SID[3] Met + Cys, %	0.82	0.74
SID[3] Trp, %	0.25	0.22
SID[3] Thr, %	0.88	0.79
Ca, %	0.86	0.81
STTD[5] P, %		
Analyzed composition		
DM, %	90.16	90.34
CP, %	22.74	20.98
Ca, %	0.88	0.89
Total P, %	0.71	0.66
Mg, %	0.14	0.15
Na, %	0.34	0.27
K, %	1.13	1.02

Table 1. Continued

[1] Premix is added to provide the following nutrients per kg of complete diet: 6,613.8 IU of vitamin A; 992.0 IU of vitamin D3; 19.8 IU of vitamin E; 2.64 mg of vitamin K; 0.03 mg of vitamin B12; 4.63 mg of riboflavin; 18.52 mg of pantothenic acid; 24.96 mg of niacin; 0.07 mg of biotin; 4.0 mg of Mn as manganous oxide65 mg of Fe as ferrous sulfate; 165 mg of Zn as zinc sulfate; 16.5 mg of Cu as copper sulfate; 0.30 mg of I as ethylenediamine dihydroiodide; and 0.30 mg of Se as sodium selenite.

[2] Additive: 0.375% AGP (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2); 0.001% PRO to provide 1 x 10^8 CFU/kg feed.

[3] SID = standardized ileal digestible

[4] Calculated composition was based on NRC (2012).

[5] STTD: Standardized total tract digestible

Table 2. Growth performance of pigs fed experimental diets from 21 to 48 d of age

	Treatment[1]					*P* value		
Item	NC	PC	AGP	PRO	SEM	NC vs. PC +AGP+PRO	PC vs. AGP+PRO	AGP vs. PRO
BW, kg								
Initial	6.2	6.2	6.2	6.2	0.5	0.812	0.828	0.987
Wk 1	6.6	6.4	6.4	6.8	0.6	0.594	0.247	0.180
Wk 2	8.9	8.3	8.3	9	0.7	0.341	0.375	0.221
Wk 3	12.5	12	12.1	12.8	0.9	0.601	0.430	0.371
Wk 4	16.3	15.4	16.6	16.4	1.1	0.463	0.024	0.719
ADG, g/d								
Wk 1	51	38	57	80	18	0.768	0.111	0.349
Wk 2	325	269	289	319	29	0.289	0.386	0.351
Wk 3	518	523	579	544	33	0.492	0.190	0.421
Wk 4	630	614	599	616	37	0.367	0.924	0.695
Pre-challenge[2]	51	38	57	80	18	0.768	0.111	0.349
Post-challenge[2]	484	456	501	479	27	0.830	0.159	0.457
ADFI, g/d								
Wk 1	92	97	126	119	15	0.249	0.186	0.696
Wk 2	414	355	385	429	40	0.329	0.039	0.227
Wk 3	730	674	753	656	50	0.426	0.414	0.147
Wk 4	981	849	855	808	86	0.037	0.510	0.473
Pre-challenge[2]	92	97	126	119	15	0.249	0.186	0.696
Post-challenge[2]	694	610	685	615	48	0.091	0.264	0.114
G:F								
Wk 1	0.63	0.48	0.47	0.57	0.12	0.326	0.474	0.492
Wk 2	0.78	0.78	0.77	0.75	0.04	0.879	0.760	0.449
Wk 3	0.71	0.8	0.78	0.88	0.05	0.095	0.657	0.157
Wk 4	0.66	0.78	0.75	0.89	0.09	0.168	0.397	0.341
Pre-challenge[2]	0.63	0.48	0.47	0.57	0.12	0.326	0.474	0.492
Post-challenge[2]	0.70	0.76	0.75	0.79	0.03	0.071	0.678	0.358

[AB] Means within a row without a common superscript tended to be different ($P < 0.10$)
[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2×10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1×10^8 CFU/kg).
[2]Challenge was initiated on d 7 with an oral dose of 2×10^9 CFU of F18$^+$ *Escherichia coli*

Table 3. Fecal scores of pigs fed experimental diets and challenged with ETEC on d 7

	Treatment[1]					*P* value		
Item	NC	PC	AGP	PRO	SEM	NC vs PC + AGP + PRO	PC vs AGP + PRO	AGP vs. PRO
Fecal score[2]								
d 3	1.0	1.0	1.4	1.4	0.2	0.192	0.069	1.000
d 5	1.1	1.5	1.6	1.3	0.2	0.150	0.836	0.287
d 7	1.3	1.1	1.4	1.0	0.1	0.427	0.573	0.057
d 8	1.2	1.2	1.5	1.0	0.1	0.845	0.782	0.022
d 9	1.2	1.3	1.4	1.3	0.2	0.491	0.807	0.673
d 10	1.3	1.4	1.5	1.3	0.2	0.741	0.641	0.420
d 11	1.2	1.6	1.5	1.9	0.2	0.027	0.643	0.115
d 12	1.1	1.9	1.4	1.9	0.2	0.010	0.318	0.088
d 13	1.3	1.2	1.5	1.4	0.2	0.739	0.243	0.683
d 14	1.0	1.4	1.2	1.4	0.2	0.068	0.597	0.362
d 16	1.2	1.2	1.2	1.2	0.1	1.000	1.000	1.000
d 18	1.3	1.2	1.2	1.2	0.1	0.536	1.000	1.000
d 20	1.3	1.2	1.2	1.2	0.1	0.525	1.000	1.000
d 23	1.2	1.3	1.3	1.1	0.1	0.829	0.542	0.293
d 26	1.2	1.0	1.0	1.3	0.1	0.374	0.211	0.034

[ab] Means within a row without a common superscript are significantly different ($P < 0.05$)
[AB] Means within a row without a common superscript tended to be different ($P < 0.10$)
[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).
[2]Fecal scores were measured based on a 1 to 3 scale with 1 being solid feces and 3 being diarrhea.

Table 4. Digesta pH of pigs fed experimental diets and challenged with ETEC on d 7

	Treatment[1]					*P* value		
Item	NC	PC	AGP	PRO	SEM	NC vs PC + AGP + PRO	PC vs AGP + PRO	AGP vs PRO
pH								
Jejunum	6.73	6.70	6.83	6.96	0.11	0.543	0.442	0.718
Ileum	6.74	6.48	6.66	6.92	0.14	0.651	0.082	0.187
Colon	6.21	6.15	6.23	6.25	0.09	0.927	0.594	0.865
Cecum	6.01	6.02	5.99	6.01	0.07	0.916	0.809	0.818

[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 5. Gut histology of pigs fed experimental diets and challenged with ETEC on d 7

Item	Treatment[1]				SEM	*P* value		
	NC	PC	AGP	PRO		NC vs. PC +AGP + PRO	PC vs. AGP + PRO	AGP + PRO
Jejunum histology								
Villus height, mm	431	438	454	431	19	0.663	0.851	0.415
Villus width, mm	112	116	115	114	4	0.572	0.785	0.873
Villus base, mm	116	116	112	110	5	0.529	0.390	0.742
Villus tip, mm	68	79	78	83	4	0.008	0.724	0.327
Crypt depth, mm	155	164	154	138	11	0.804	0.189	0.327
Villus height: crypt depth	3	3	3	3	0	0.784	0.328	0.711

[ab] Means within a row without a common superscript are significantly different (P <0.05)

[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 6. Immune status measurements of pigs fed experimental diets and challenged with ETEC on d 7

Item	Treatment[1]				SEM	*P* value		
	NC	PC	AGP	PRO		NC vs. PC + AGP + PRO	PC vs. AGP + PRO	AGP vs. PRO
Tumor necrosis factor α								
Serum d 14, pg/mL	75	106	97	131	26	0.229	0.810	0.373
Serum d 26, pg/mL	59	63	78	59	9	0.423	0.603	0.128
Jejunum, pg/mg protein	1.19	0.57	0.72	0.49	0.23	0.428	0.629	0.370

[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a adiet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 7. Oxidative stress status measurements of pigs fed experimental diets and challenged with ETEC on d 7

Item	Treatment[1]				SEM	*P* value		
	NC	PC	AGP	PRO		NC vs. PC + AGP + PRO	PC vs. AGP + PRO	AGP vs. PRO
Protein carbonyl, nmol/mg								
Serum d 14	0.95	1.97	1.36	1.12	0.38	0.188	0.097	0.636
Serum d 26	1.14	1.17	0.93	1.01	0.22	0.654	0.433	0.770
Jejunum	2.55	2.79	2.39	2.5	0.32	0.987	0.372	0.807
MDA, uM								
Serum d 14	12.47	11.02	9.04	10.72	3.04	0.499	0.742	0.674
Serum d 26	10.35	7.07	13.78	8.32	1.83	0.768	0.085	0.042
Jejunum, uM/g protein	0.51	0.41	0.48	0.48	0.09	0.552	0.402	0.977

[1]Treatments were: 1) NC: a diet with no additive and no F18^{+} *Escherichia coli* challenge; 2) PC: a diet with no additive and F18^{+} *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 8. Tight junction protein concentrations of pigs fed experimental diets and challenged with ETEC on d 7

Item	Treatment[1]				SEM	P value		
	NC	PC	AGP	PRO		NC vs. PC + AGP + PRO	PC vs. AGP + PRO	AGP vs. PRO
Band intensity normalized with β-actin, % of control								
Claudin	0.095	0.094	0.154	0.117	0.015	0.154	0.041	0.109
Occludin	0.276	0.269	0.383	0.236	0.042	0.684	0.445	0.022
ZO-1[2]	0.204	0.152	0.349	0.214	0.021	0.187	<.01	<0.01

[ab] Means within a row without a common superscript are significantly different (P <0.05)
[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).
[2]Zonula occludens-1

Table 9. F18$^+$ *Escherichia coli* counts in fecal samples of pigs fed experimental diets and challenged with ETEC on d 7

Item	Treatment[1]				SEM	*P* value		
	NC	PC	AGP	PRO		NC vs. PC + AGP + PRO	PC vs. AGP + PRO	AGP vs. PRO
F18$^+$ *Escherichia coli* count								
d 14	1,028	120,909	5,440	9,877	40,880	0.356	0.032	0.941
d 26	4	123	29	5	46	0.391	0.067	0.722

[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 10. F18+ *Escherichia coli* counts in d 14 and d 26 fecal samples of pigs challenged with ETEC on d 7

	Day			*P* value		
Item	d 14	d 26	SEM	Trt	Day	Trt x Day
F18+ *Escherichia coli* count	53758	18286	23499	0.150	0.116	0.155

[1]Treatments were: 1) NC: a diet with no additive and no F18+ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18+ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 11. Phylum relative abundance in day 14 fecal samples from pigs fed experimental diets and challenged with ETEC on d 7

	Treatment					*P* value		
Item	NC	PC	AGP	PRO	SEM	NC vs. PC + AGP +PRO	PC vs. AGP + PRO	AGP vs. PRO
Fecal, d 14 phylum, relative %								
Actinobacteria	2.11	1.01	0.42	0.67	0.98	0.215	0.728	0.888
Bacteroidetes	28.72	25.16	23.59	36.59	2.59	0.926	0.128	0.001
Firmicutes	44.38	38.54	46.87	44.64	3.83	0.818	0.138	0.691
Proteobacteria	19.72	34.77	27.33	14.88	4.30	0.209	0.012	0.039
Spirochaetes	1.87	2.06	0.64	1.40	0.80	0.575	0.247	0.452
Tenericutes	3.73	3.30	2.24	3.32	0.91	0.440	0.652	0.434
Firmicutes: Bacteroidetes	1.65	1.61	2.10	1.34	0.22	0.894	0.703	0.023

[ab] Means within a row without a common superscript are significantly different (*P* <0.05)

[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 12. Phylum relative abundance in d 26 fecal samples from pigs fed experimental diets and challenged with ETEC on d 7

	Treatment					*P* value		
Item	NC	PC	AGP	PRO	SEM	NC vs. PC + AGP +PRO	PC vs. AGP + PRO	AGP vs. PRO
Fecal, d 26 phylum, relative %								
Actinobacteria	3.48	0.99	0.88	1.59	1.72	0.199	0.906	0.815
Bacteroidetes	26.12	27.02	28.60	28.78	3.60	0.631	0.707	0.971
Firmicutes	44.65	42.46	47.44	45.88	5.29	0.903	0.431	0.799
Proteobacteria	23.10	28.23	20.47	22.00	6.18	0.949	0.358	0.861
Spirochaetes	2.20	0.64	0.91	1.03	0.43	0.031	0.566	0.873
Tenericutes	2.58	1.07	3.17	1.75	0.76	0.530	0.138	0.177
Firmicutes: Bacteroidetes	1.98	1.70	2.06	1.84	0.37	0.784	0.549	0.652

[1]Treatments were: 1) NC: a diet with no additive and no F18+ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18+ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

Table 13. Phylum relative abundance in fecal samples samples from pigs challenged with ETEC on d 7

	Treatment[1]			*P* value		
Item	d 14	d 26	SEM	Trt	Day	Trt x Day
Fecal, phylum, relative %						
Actinobacteria	1.37	1.98	0.86	0.524	0.521	0.933
Bacteroidetes	28.54	27.63	1.87	0.110	0.678	0.194
Firmicutes	43.74	45.11	3.06	0.401	0.636	0.963
Proteobacteria	24.08	23.45	3.25	0.081	0.862	0.435
Spirochaetes	1.51	1.18	0.33	0.237	0.456	0.461
Tenericutes	3.15	2.14	0.42	0.706	0.094	0.318
Firmicutes: Bacteroidetes	1.68	1.90	0.19	0.327	0.283	0.770

[1]Treatments were: 1) NC: a diet with no additive and no F18$^+$ *Escherichia coli* challenge; 2) PC: a diet with no additive and F18$^+$ *Escherichia coli* challenge (2 x 10^9 CFU); 3) AGP: PC + antibiotics (CTC 100 and Denagard for Phase 1 and Mecadox for Phase 2) and ZnO (2,500 mg/kg); 4) PRO: PC + *Bacillus subtilis* 29784 (1 x 10^8 CFU/kg).

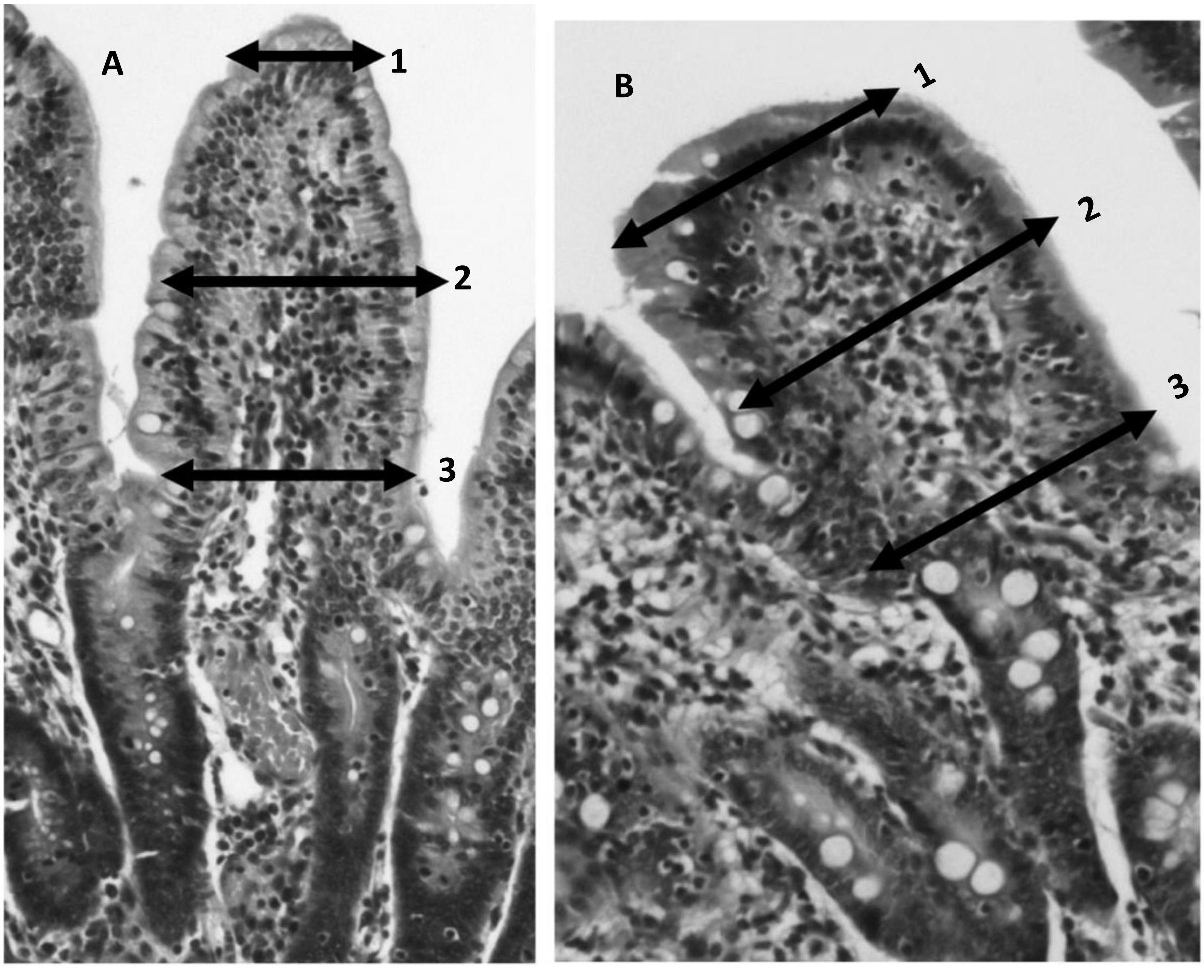

Figure 1. Jejunal villus measurements from negative and positive controls

A) NC: a diet with no additive and no F18^{+} *Escherichia coli* challenge; B) PC: a diet with no additive and F18^{+} *Escherichia coli* challenge (2 x 10^{9} CFU). Villus measurements were taken at the tip of the villus (1), at half the height of the villus (2), and the base of the villus (3).

CHAPTER V

GENERAL CONCLUSIONS

The swine industry currently faces the challenge of finding alternatives to antibiotics that can maintain consistent performance while also reducing feed costs and enhancing production. Many feed additives have been investigated including acidifiers, plant extracts, prebiotics, probiotics, and nutraceuticals such as copper and zinc. DFMs are one such alternative that may offer economic and health benefits. DFMs have demonstrated potential in their ability to improve growth performance, gut health, immune status, oxidative stress status, and their capacity to module the gut microbiota. DFMs have been evaluated in various conditions ranging from natural exposure conditions in which no specific challenge is incorporated, to studies involving an immune challenge such as pathogenic bacteria or mycotoxins.

However, despite promising results, there are still inconsistencies in the literature. A wide range of factors including DFM type, strain, source, supplementation level, administration method, farm sanitation and experimental design can contribute to the variation among DFM studies and lead to misperception about the purported benefits of DFMs. Additionally, there are many proposed modes of action by which DFMs may exert benefits on their hosts, including improvement of nutrient digestibility, lowering intestinal pH, enzyme activity, modification of the immune response, competitive exclusion of pathogenic bacteria and modulation of the gut microbiota. In contrast to antibiotics, DFMs benefit animal performance and health through indirect mechanisms and therefore, in order to fully understand how DFMs work, these modes of action require further investigation. Given that individual DFMs may not utilize all of the proposed modes of action and therefore, previously conducted studies have typically limited their investigation to only a few modes of action for a DFM of interest in certain conditions. Therefore, this research investigated the effects of a novel strain of *Bacillus subtilis* 29784 on growth performance and gut health in nursery pigs in both natural exposure and challenged conditions and the potential modes of action utilized by this strain. The first study demonstrated the ability of *Bacillus*-based DFMs to improve growth performance and gut health, enhance gut barrier integrity, and modulate the gut microbiota of nursery pigs in natural exposure conditions. The improvements in ADG were primarily due to increased ADFI which could have been due to improvements in gut health. Both *Bacillus subtilis* and a combination of *Bacillus subtilis* and Bacillus *licheniformis* lowered the pH of colonic digesta which subsequently could have led to the decrease in a phylum of toxin-producing bacteria, *Cyanobacteria* in the jejunal mucosa. The supplementation of *Bacillus subtilis* 29784 alone additionally improved the integrity of the intestinal gut barrier by increasing the concentration of ZO-1. This study provides support for the ability of Bacillus-based DFMs to improve growth performance in nursery pigs.

The ideal supplementation level of a DFM is influenced by numerous factors including DFM species, DFM strain, and the number of strains used. The second study established that the beneficial effects of *Bacillus subtilis* 29784 on growth performance appear to be dose-dependent and therefore it is concluded that there is a maximum supplementation level beyond which improvements begin to diminish. The dose of *Bacillus subtilis* 29784 needed to attain optimal growth performance of nursery pigs from 21 to 35 d of age was approximately 1.0 x 10^8 CFU/kg feed. Nevertheless, it is recommended that the correct supplementation level of each DFM strain should be evaluated before further use.

The third study demonstrated that *Bacillus subtilis* 29784 supplementation offers little to benefit on growth performance during an F18$^+$ *Escherichia coli* challenge. Although, this study exhibited the antioxidant capacity of *Bacillus subtilis* 29784 to improve systemic oxidative stress status by reducing levels of serum protein carbonyl. This study also showed that *Bacillus subtilis* 29784 can competitively exclude pathogenic bacteria. The supplementation of *Bacillus subtilis* 29784 lowered the relative abundance of fecal *Proteobacteria* and $F18^+$ *Escherichia coli* counts and increased fecal *Bacteroidetes* abundance. However, *Bacillus subtilis* was not superior to the antibiotics and did not prevent postweaning diarrhea caused by $F18^+$ *Escherichia coli*. Overall, the supplementation of *Bacillus subtilis* 29784 was not superior to antibiotics, did not prevent postweaning diarrhea and conferred few benefits to pigs during an *Escherichia coli* infection.

Collectively, this research confirms the benefits of the supplementation of a novel strain of *Bacillus subtilis* that had previously only been investigated in poultry. Overall, these studies exhibited the numerous modes of action by which *Bacillus*-based DFMs may subsequently improve growth performance and gut health in nursery pigs. This research indicates that *Bacillus*-based DFMs could be a feasible option for swine producers to use to enhance production and animal health in certain conditions.