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Evaluation and Selection of a Bacillus based Direct-Fed Microbial Candidate for In situ Enzyme Production to Improve Gut Health Integrity, Bone Quality and Growth Performance in Poultry

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Evaluation and Selection of a *Bacillus* based Direct-Fed Microbial Candidate for *In situ* Enzyme Production to Improve Gut Health Integrity, Bone Quality and Growth Performance in Poultry

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ABSTRACT

During the last decade, the increasing interest in renewable energy sources has been changing the distribution of corn utilization from human and animal consumption to biofuel production, leading to a continuous rise in feed costs of livestock diets. Therefore, alternative feed ingredients such as distillers dried grains with solubles (DDGS), as well as cereals like wheat, barley, and sorghum have become part of the feed matrix to maintain or reduce production costs. However, these raw materials often contain a higher concentration of antinutritional factors in comparison to corn, including non-starch polysaccharides (NSP) which increase digesta viscosity and reduce nutrient absorption in monogastric animals. As a result, the addition of exogenous enzymes in poultry feed has steadily increased to maximize nutrient utilization and maintain performance parameters with diets containing less digestible ingredients. On the other hand, the poultry industry is also facing social concerns regarding the use of antibiotic growth promoters (AGP) and the development of antibiotic resistant microorganisms. One alternative among others is the utilization of direct-fed microbials (DFM) as substitutes of AGP and also as a prophylactic practice to reduce the incidence of bacterial gastrointestinal diseases. Therefore, the objectives of the present dissertation were to evaluate and select different *Bacillus spp*. strains as DFM candidates based on enzyme production profiles to improve nutrient absorption and intestinal integrity, as well as, maintain a healthy microflora balance in poultry consuming commercial and alternative diets. Due to *Bacillus* endospores are in a dormant state when delivered into the feed, it was crucial to evaluate the spores' germination rate, distribution and persistence in the gastrointestinal tract (GIT) of chickens to understand the probable mechanism of action of this remarkable beneficial microorganism. It was observed that some full life-cycle development occurred and around 90 % of the spores germinated in the GIT, suggesting that a continuous administration is advisable for consistent improvement. Additionally, in a series of *in vitro*

experiments, three *Bacillus spp*. strains were selected based on their enzyme production activity profile of amylase, cellulase, protease, lipase, xylanase and phytase. Analysis of the 16S rRNA sequence classified two strains as *B. amyloliquefaciens* and one of the strains as *B. subtilis*. The three isolates were combined in an equal ratio (1:1:1) and showed to reduce viscosity and *Clostridium perfringens* proliferation in an *in vitro* digestive model simulating different compartments of the GIT of poultry. For *in vivo* trials in broilers and turkeys a rye-based diet was used as a source of high soluble NSP. Inclusion of the *Bacillus*-DFM candidate significantly reduce digesta viscosity and bacterial translocation to the liver, resulting in an increase of performance and bone quality parameters, along with maintenance of the beneficial microflora in the GIT. Moreover, due to the wide availability of DDGS from the ethanol industry, a different set of experiments including 8% of DDGS in the grower diet were developed. Supplementation with the *Bacillus*-DFM candidate improve growth performance, bone mineralization, and intestinal morphology in comparison to the control group ($P < 0.05$), suggesting that the dietary inclusion of selected *Bacillus spp.* spores is a viable alternative in commercial diets, having a positive impact in gut health and production parameters.

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DEDICATION

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Latorre, J. D., X. Hernandez-Velasco, G. Kallapura, A. Menconi, N. R. Pumford, M. J. Morgan, S. L. Layton, L. R. Bielke, B. M. Hargis and G. Tellez. 2014. Evaluation of germination, distribution, and persistence of *Bacillus subtilis* spores through the gastrointestinal tract of chickens. Poult. Sci. 93:1793-1800. (Chapter I).

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I. INTRODUCTION

Social concern about the development of multi drug resistant pathogens is challenging the poultry industry to find economically viable strategies to the conventional use of antibiotic growth promoters (AGP) in poultry diets without affecting production parameters (Boyle et al., 2007). Continuous and extensive research of suitable alternatives include feed additives such as probiotics and direct-fed microbials (DFM) (Hong et al., 2005), organic acids and essential oils from plant extracts (Ricke, 2003), or bacteriophage therapy (Andreatti Filho et al., 2007). The use of one or a mixture of these potential substitutes of AGP could potentially provide an extraordinary tool to deal with human food-borne pathogens and at the same time maintain customer preferences to avoid a reduction in consumption per capita of poultry meat.

In the case of probiotics, the most common and commercial type is based on lactic acid bacteria (LAB) that include the genus *Lactobacillus* and *Pediococcus* which are normally part of the microflora of different animal species (Tellez et al., 2012). However, LAB probiotics required to be microencapsulated, refrigerated and/or lyophilized to prolong storage shelf-life, and usually are administered in the drinking water because they are not feed-stable. In this regard, among the large number of probiotic products in use today, some are bacterial spore formers mostly of the genus *Bacillus*. Used primarily in their spore form, some *Bacillus* direct-fed microbials (DFM) have been shown to prevent selected gastrointestinal disorders with an astonishing diversity of species and applications (Hong et al., 2008). While not all *Bacillus* spores are highly heat tolerant, some isolates are the toughest life form known on earth (Vreeland et al., 2000) and can be used under extreme heat and pressure conditions (pelletization). Several studies have shown that either live vegetative cells or endospores of some *Bacillus* isolates can prevent colon

carcinogenesis (Park et al., 2007). Moreover, it has been previously investigated that selected *Bacillus* strains can produce antimicrobial compounds against Gram-negative enteropathogens, such as *Salmonella spp.*, *Escherichia coli*, and *Campylobacter spp*. (Tellez et al., 2012). Additionally, there is scientific evidence suggesting that *Bacillus* spores can germinate in the GIT into metabolically active vegetative cells, therefore, being considered as part of the microflora and not just transiently present in the gut (Hoa et al., 2001).

On the other hand, steady increments in corn utilization for production of biofuels and reduction in harvest yield due to drought seasons have increased corn demand and cost. Corn is usually the main source of energy in poultry diets, but at times it is difficult to formulate least cost diets using this cereal and unconventional grains have to be used. When chickens are fed alternative grains such wheat or rye that are high in non-starch polysaccharides (NSP), poor performance and unmanageable litter conditions caused by sticky droppings are reported (Fengler and Marquardt, 1988). NSP in these cereal grains are comprised mainly of highly branched arabinoxylans, increasing digesta viscosity responsible for poor digestibility through interference with the movement of particles and solutes across the intestinal lumen, preventing the access of digestive enzymes to the endosperm contents and reducing intestinal absorption of nutrients. In addition, increased digesta viscosity reduce conjugated bile acids, affecting fat emulsification and fat digestibility (Langhout et al., 1999). Additionally, a elevated digesta viscosity prolonges the feed passage rate, increasing the time available for digesta associated bacteria to multiply prior to evacuation in the feces, and provides more substrate availability in the distal parts of the intestine for microbial fermentation (Kiarie et al., 2013). Alterations in gut permeability are connected to bacterial overgrowth in poorly digested diets causing bacterial translocation into the portal and/or systemic circulation in several types of leaky gut syndromes leading to systemic

bacterial infections (Seki and Schnabl, 2012). Since poultry has little or no intrinsic enzymes capable of hydrolyzing NSP, exogenous carbohydrases as additives are used in an attempt to reduce the effect of these anti-nutritive factors (Bedford and Schulze, 1998). One of the principal sources of exogenous enzymes and antibiotics from bacterial origin are produced by different *Bacillus* strains. However, some but not all *Bacillus* species have the capacity to produce different enzymes including amylase, protease, lipase, cellulase, xylanase, and phytase (Hendricks et al., 1995). The objectives of the present dissertation were to evaluate and select a *Bacillus*-DFM candidate based on different *in vitro* enzyme production profiles to improve gut health integrity, bone quality and growth performance in poultry.

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II. LITERATURE REVIEW

Bacillus direct-fed microbials: Spore germination, distribution in the gastrointestinal tract, and effect on health and performance parameters in poultry

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ABSTRACT

Indiscriminate and inappropriate use of antibiotics has led to the emergence of multidrug resistant pathogens, resulting in the ban of the use of antibiotics in animal diets in several countries. As an alternative, probiotics have been under investigation as feed additives to establish an adequate intestinal microflora that improve productive responses in animals. Bacteria from the genus *Bacillus* are receiving important attention, because of their properties to control enteropathogens and their remarkable attribute to produce endospores. In addition, some *Bacillus* species have the capacity to synthesize different exogenous enzymes, including protease, phytase, xylanase, keratinase, lipase, and cellulase, that have been reported to improve absorption of nutrients due to degradation of anti-nutritional factors such as non-starch polysaccharides (NSP), phytate and low digestible proteins in poultry diets. These benefits make supplementation of *Bacillus* spores an available and applicable alternative for the use of antibiotic growth promoters, reducing the incidence of various gastrointestinal diseases and improving production performance in poultry under commercial conditions. In this review, we summarize the fate, dissemination, and efficacy of *Bacillus* direct-fed microbials candidates in the gastrointestinal tract of poultry and their effect on health and performance parameters.

INTRODUCTION

Due to current intensive management practices in poultry production, animals are susceptible to enteric microflora imbalances leading to diminishment on performance parameters. To mitigate the effect of dysbiosis in the gastrointestinal tract, diets have been commonly supplemented with antibiotics as growth promoters showing effective decrease in the presentation of digestive disorders and increase in performance (Parker and Armstrong, 1987). However, the

indiscriminate and inappropriate use of antibiotics has led to the emergence of multidrug resistant pathogens, leading to the prohibition of antibiotics in animal diets in the European Union in 2006 (Tellez et al., 2012). The worst scenario is the possible contribution of these resistant bacteria from animal production resulting in serious medical implications in humans (Klein, 2003).

As an alternative to the use of antibiotic growth promoters (AGP) in poultry diets, probiotics have been under investigation as feed additives to establish an adequate intestinal microflora, promoting adequate productive responses in animals (Becquet, 2003; La Ragione et al., 2004). Among the species of microorganisms used as probiotics, some facultative anaerobic gram positive bacteria from the genus *Bacillus* are receiving important attention through enhancing digestion and absorption of nutrients, and control of enteropathogens such as *Salmonella spp*., *Clostridium perfringens, Campylobacter spp., and Escherichia coli* in the gastrointestinal tract (GIT) of different animal species (Jadamus et al., 2001; Wolfenden et al., 2011; Tellez et al., 2012). Additionally, the genus *Bacillus* has the extraordinary capacity to produce endospores under stressful environmental conditions; some of these spores have the ability to resist high temperatures used during feed preparation (pelletization), extreme pH, dehydration, high pressures and contact with caustic chemical substances (Menconi et al., 2013). These admirable features make selected *Bacillus* spores a direct-fed microbial (DFM) suitable for commercialization and distribution due to a long-shelf life and stability (Vreeland et al., 2000; Shivaramaiah et al., 2011).

On the other hand, it is really important to understand the factors that affect germination and distribution of *Bacillus* spores throughout the gastrointestinal tract. *Bacillus* spores germinate into vegetative cells depending on nutritional and non-nutritional factors known as germinants

(such as L-alanine, asparagine, glucose, fructose, potassium chloride) and the effect of a non-lethal heat treatment under different pressures (100 – 600 Megapascals) (Setlow, 2003). There is evidence supporting the idea that some *Bacillus* spores germinate in the GIT of chickens, mice, pigs, dogs and humans, therefore, being metabolically active and having responses such as, production of antimicrobial substances, immunomodulatory effects on the intestinal mucosa, and function as competitive exclusion agents interacting with host cells (Hoa et al., 2000; Duc et al., 2004; Tam et al., 2006). Furthermore, some *Bacillus* species have the capacity to produce different exogenous enzymes, including protease, phytase, xylanase, keratinase, lipase, and cellulase (Hendricks et al., 1995; Monisha et al., 2009; Mazzotto et al., 2011; Jani et al., 2012; Mittal et al., 2012; Shah et al., 2012). These enzymes help to degrade complex feed molecules, improve absorption of nutrients, reduce intestinal viscosity in non-starch polysaccharide rich diets (NSP), and decrease the amount of substrates available for growth of pathogenic bacteria. Additionally, it has been shown that the presence of *Bacillus* species such as *Bacillus subtilis*, enhance growth of other beneficial microorganisms such as *Lactobacillus* by production of subtilisin, catalase, and also decreasing intestinal pH (Hosoi et al., 2000).

All the benefits related to the utilization of *Bacillus*-DFM in the diet, make supplementation of *Bacillus* spores an available and applicable alternative instead of the use of antibiotic growth promoters, avoiding an increment in the presentation of different gastrointestinal diseases and maintaining or improving performance parameters in poultry production under commercial conditions.

SPORE GERMINATION AND GERMINANTS

Although *Bacillus* spores are in a dormancy state and are considered one of the most stable and resistant forms of life. Spores have an active sensor system that allows them to undergo the process of germination when environmental and nutritional conditions are favorable. Spores respond to the presence of nutritional or non-nutritional factors known as germinants, which trigger the start of a series of interconnected reactions that will finally have as a result the generation of a metabolically active vegetative cell. Among the nutritional germinants involved in germination of *Bacillus subtilis*, L-alanine is the most common, but also the mixture of asparagine, fructose, glucose and potassium chloride have been demonstrated to stimulate the initiation of germination (Setlow, 2003). In the case of *Bacillus megaterium*, L-proline has been recognized as an important germinant (Foster and Johnstone, 1990).

The first step implicated in germination is the interaction between a nutritional germinant like L-alanine and the receptor of the spore located in the inner membrane. These receptors are composed by different proteins (GerA, GerB and GerK), that in the case of *Bacillus subtilis* are encoded by *gerA*, *gerB* and *gerK* operons (Paidhungat and Setlow, 2000). Following the binding of the germinant with the cell receptor, there is an increase in the spore core permeability, due to the movement of Ca^{++} cations and dipicolinic acid (DPA) from the core accompanied by uptake of water into the core. These steps are considered the first phase of the germination process, however, some researchers mention that a non-lethal heat treatment is important to activate the spore receptors before binding the germinant (Moir et al., 2002). The second phase of the germination process includes the activation of cortex-lytic enzymes that finally allow the complete rehydration of the core and activation of the metabolic enzyme activity of the future vegetative cell (Setlow, 2003).

The mechanism by which the cortex-lytic enzymes are activated is not completely known. *B. subtilis* enzymes CwlJ and SleB are involved in the degradation of the cortex peptidoglycan after previous activation of the cell receptor by a specific germinant (Chirakkal et al., 2002, Makino and Moriyama, 2002). One hypothesis is that CwlJ activity may be induced by the presence of Ca^{++} and DPA released from the inner membrane of the spore, starting in this way the hydrolytic disruption of the cortex and permitting the movement of water inside the spore core. Moreover, the increment of stress in the structure of the dissolving cortex could also induce SleB activity, having as result the expansion of the germ cell wall, which is going to be the cell membrane of the vegetative cell. The substrate of the cortex-lytic enzymes is the muramic-δ-lactam present in the peptidoglycans layer, being the target of enzyme activity, however, other enzymes and other compound of the cortex may be involved in this process in different *Bacillus* species (Atrih and Foster, 2001).

In addition to nutrient germinants, there are also non-nutrient factors that stimuli germination, such as; heat shock, salts, presence of Ca^{++} cations and DPA from other germinating spores, and different amounts of pressure (100 – 600 MPa). Nevertheless, there is contradictory evidence related to low and high pressures and their effect on germination. Wuytack and co-workers observed that low pressures (100 – 200 MPa) may influence the activation of inner membrane receptors promoting germination; however, at high pressures (600 MPa) germination was not completed due to interruption of the final phase steps of the process (Wuytack et al., 2000). In contrast, Paidhungat et al. (2000), reported that higher pressures (500 – 600 MPa) incentive germination even without presence of nutritional germinants, indicating that pressure may affect the release of Ca^{++} and DPA, therefore avoiding the first phase of activation of spore receptors, and acting directly over degradation of cortex peptidoglycans.

Additionally, different aspects such as osmoregulation of the core by the cortex layer, degradation of small acid-soluble proteins (SASPs) as source of amino acids for cell growth, and permeability of the coat and cortex to different nutrient germinants to reach the inner membrane of the core are fundamental to complete germination, together with the chain of reactions mentioned before. Spore germination is still a process that must be investigated in detail, because there are still unknown facts about how the binding of the germinant and the receptor is integrated in a response that finish with the activation of cortex-lytic enzymes, and also how proteins of the inner membrane receptor interact between each other and with different germinants in variable *Bacillus* species (Moir et al., 2002; Setlow, 2003). Have knowledge about the aspects that affect germination and sporulation of the genus *Bacillus* is crucial to understand how these bacteria may act in the GIT of animals when supplemented as direct-fed microbials.

DISTRIBUTION AND GERMINATION OF *BACILLUS* SPORES THROUGHOUT THE GASTROINTESTINAL TRACT IN DIFFERENT ANIMAL MODELS INCLUDING POULTRY

Due to *Bacillus* spores are recognized to be in a dormant state in comparison to other probiotic bacteria such as *Lactobacillus*, determination of germination of *Bacillus* spores in the GIT is of vital importance. The capacity of certain spores to germinate under gastrointestinal conditions is directly related with the possible mechanism of action through which these bacteria will benefit the host (Tellez, 2014). Metabolically active cells are required to secrete antimicrobial substances, stimulate beneficial microbiota, and act as competitive exclusion agents (Ozawa et al., 1981). Additionally, vegetative cells of some, but not all, *Bacillus* isolates have shown to produce exogenous enzymes that may promote an increase in digestibility of different nutrients

from the diet (Leser et al., 2008). On the contrary, according to Tam and co-workers, other beneficial effects of *Bacillus* spores such as competition for attachment sites and immunomodulation do not require germinated spores to have positive effects on the host, therefore both stages (vegetative cells and spores) of the *Bacillus* life cycle could provide a different set of advantages supporing their utilization as functional feed additives (Tam et al., 2006).

Equally essential is to know the distribution of the spores throughout the GIT to realize where the major advantages of the direct-fed microbial supplementation would be expected to occur. Furthermore, it is also crucial to recognize if these bacteria have the capacity to accomplish some full life cycle in the GIT or if they are transient occurring, requiring constant supplementation in the diet to persist in the digestive tract (Cartman et al., 2007).

In the case of poultry, Cartman et al. (2008) demonstrate that *B. subtilis* spores, when provided orally, germinate in the GIT. Identification and quantification of spores and vegetative cells were done using RT-PCR, qRT-PCR and a strain of *B. subtilis* (SC2362) that harbored a fusion gene (*rrnO-lacZ*) and a chloramphenicol acetyltransferase gene (*cat*). After 20 hours of spores administration, the number of vegetative cells was higher in comparison to the number of spores present in different segments of the GIT. This finding suggests that even when *Bacillus spp.*, are considered aerobic bacteria, spores have the ability to germinate into vegetative cells and survive in the anoxic environment of the digestive tract. This could be the result of the use of nitrite or nitrate molecules as terminal electron acceptors in the electron transport chain by some *Bacillus* strains (Nakano and Zuber, 1998). Additionally, Studies conducted in our laboratory have shown that approximately 90% of *B. subtilis* spores germinate within 60 min in the presence of starter broiler feed *in vitro,* this was observed after a heat-shock treatment (75^{o}C for 10 minutes) that

allowed counting of spores only (Table 1). Extended *in vivo* studies confirmed that viable *B. subtilis* spores were recovered during 120 h, from different sections of the GIT of broiler chickens after constantly receiving feed supplemented with spores (10^6 spores/g) or a single oral-gavage dose (10^6 spores/0.25 mL). Approximately a 1 $\log_{10}$ colony forming units (CFU) reduction of spore numbers was observed after 24 hours of administration, which may suggest a germination rate of around 90% in the GIT (Figure 1) (Latorre et al., 2014a). Similarly, Jadamus et al. (2001) observed that spores of *B. cereus* var. *toyoi* germinated fast in the crop of chickens. Moreover, when vegetative cells were orally administered, spores were collected from different segments of the GIT, meaning that both processes were occurring, either germination of spores or sporulation of vegetative cells. Furthermore Cartman et al. (2008), found that *B. subtilis* spores can be detected after six weeks of a single oral administration (10^9 spores/0.1mL), meaning that compared to the passage rate of the digesta in chickens (6 -7 hours), spores tend to persist over time in the GIT (Shires et al., 1987).

This behavior was also supported by examination of the fate of *Bacillus* spores in the GIT of different animal models such as mice and pigs. For instance, Hoa and co-workers evaluate the amount of spores and vegetative cells present in different parts of the GIT of pathogen-free mice, showing that occasionally the amount of spores excreted was higher than the single oral original inoculum administer to the mice (Hoa et al., 2001). This result may imply that spores can germinate, growth and sporulate, completing a full-life cycle under digestive tract of conditons. Furthermore, Leser et al. (2008), evaluate the germination and outgrowth of *B. subtilis* and *B. licheniformis* spores in the GIT of pigs using a flow cytometry technique (FCM) and also plate counting spores after heat-treatment. In the quantitation of spores and vegetative cells using FCM, cells were stained with a dye (Syto-13), and differentiate by the low concentration of the

dye present in the spore cell wall, in contrast to the high concentration observed in the cell wall of vegetative cells. It was revealed that, similar to the GIT of poultry, the number of spores diminished with time, meanwhile the number of vegetative cells tend to increase. Additionally, in this research was also possible to appreciate that the number of vegetative cells slightly increased in comparison with the original inoculum administered, suggesting little outgrowth of *Bacillus* cells in the GIT. Even when conditions of the GIT are not completely suitable for germination of *Bacillus* spores, it has been shown that in different animal species some *Bacillus* isolates are capable to develop a transformation from metabolically dormant spores to metabolically active vegetative cells. Spores are tolerant to the acidic pH of the stomach or proventriculus, additionally it has been suggested that the change of pH can trigger germination in the fore and hind gut (Jadamus et al., 2001; Leser et al., 2008). However, vegetative cells are highly susceptible to the presence of bile salts, probably in this way influencing the beginning of the sporulation process inside the GIT of the host, and promoting competition of some full life cycle development of the *Bacillus* bacteria before being excreted (Guo et al., 2006).

EFFECT OF *BACILLUS* DIRECT-FED MICROBIALS ON HEALTH

In day-old hatch birds, the digestive tract has a reduced bacterial population, making this almost unpopulated environment susceptible for colonization by pathogenic bacteria, therefore, affecting from the beginning the future performance of affected animals. This is one of the reasons for the utilization of probiotics or direct-fed microbials starting from early phases of life in livestock animals. Additionally, the prohibition of inclusion of antibiotics in animal diets since 2006 (Europe Community), increase the necessity to find alternatives to prevent presentation of diseases without diminishing production standards (Cartman et al., 2007). The most common

bacteria used as probiotics are from the genus *Lactobacillus* (LAB), however, they must be administered for example in the drinking water, and maintained under optimal conditions to prolong shelf-life.

Due to this restrictions, use of some spore former bacteria from the genus *Bacillus* have earned interest in the last years. As mentioned before, spores are resilient to harsh environmental conditions, and have a long shelf-life, making them feed-stable and suitable for commercialization (Hong et al., 2005; Hong et al., 2008).

Nevertheless, it is important to understand that not all *Bacillus* species are used as direct-fed microbials; each isolate has different characteristics according to the chemical substances that produce, heat resistance temperatures, rate of growth, rate of sporulation, persistence in the GIT, and probable advantages to the host. Several studies have shown that vegetative cells of certain *Bacillus* isolates prevent colonization of the GIT by enteropathogens such as *Salmonella spp.*, *Clostridium perfringens*, and *Campylobacter jejuni* (Teo and Tan, 2005; Svetoch et al., 2005; Wolfenden et al., 2011). For instance, Shivaramaiah et al. (2011) administered spores of different *Bacillus spp.* strains to evaluate their effect on *Salmonella* Typhimurium exclusion and growth performance, showing at the end a reduction in the recovery of this pathogenic bacteria from the crop and ceca of chicks and poults that consumed *Bacillus*-DFM supplemented diets in comparison to the untreated group ($P<0.01$). Additionally, it was also observed an improvement on performance parameters when broilers and poults were fed with *Bacillus*-supplemented diets compared to the control unsupplemented group ($P<0.05$). Furthermore in a different study, Knap et al. (2010) evaluated the effect of the inclusion of *B. licheniformis* (DSM 17236) in the diet of broiler chickens on the presentation of necrotic enteritis (*C. perfringens*). The study reported that the performance of broilers receiving the direct-fed microbial (10^6 and 10^7 cfu/g) was similar to

the group of birds that consume a medicated diet (Virginiamycin 15 g/ton feed), moreover no significant difference were observed regarding to the necrotic enteritis lesion score or mortality. In addition, multiple published studies support the fact that some isolates of *B. subtilis* have the ability to decrease the persistence of *C. perfringens*, avian pathogenic *Escherichia coli* and *Salmonella* serovar Enteritidis in the GIT of poultry (La Ragione et al., 2001; La Ragione and Woodward, 2003). The mode of action of *Bacillus* vegetative cells to reduce colonization of enteropathogens is not completely known.

Some *Bacillus* isolates have the capacity to produce antimicrobial compounds against different pathogens (Figure 2) or stimulate the immune system of the host. Moreover in the case of *C. perfringens*, due to the ability of some strains of *Bacillus* to produce proteases, could be possible that *Clostridium* toxins (α-toxin, NetB) were degraded by these enzymes (Knap et al., 2010). On the other hand, some *Bacillus* species have also been studied for detoxification or protective effect on cases of mycotoxicosis (Ma et al., 2012; Galarza-Seeber et al., 2015). In addition to the use of *Bacillus* spores as direct-fed microbials, spore forming bacteria have also been studied as a vector for oral vaccines, providing in this way an excellent alternative combining the benefits of a probiotic with the advantages of a possible tool to increase acquire immune responses for different diseases without the presentation of vaccine reactions (Duc et al., 2003).

EFFECT OF *BACILLUS* DIRECT-FED MICROBIALS ON PERFORMANCE PARAMETERS

In the case of poultry performance and feed formulation, one of the principal problems is the continuous utilization of cereal grains such as corn for biofuel production, therefore, affecting feedstuffs availability and feed cost, which represents around a seventy percent of the production

expenses in the poultry industry. Ethanol production and variability in corn prices have led to the use of alternative and less digestible energy sources in poultry diets. Cereals such as wheat and barley, as well as by products of biofuel production (Distiller's dried grains with solubles) have become occasionally feed ingredients used in poultry diets. Unfortunately, these alternative raw materials increased the amount of less digestible non-starch polysaccharides (NSP) in the feed, which as a result generate an increment in digesta viscosity in monogastrics animals (Tellez et al., 2014; Tellez et al., 2015). Utilization of *Bacillus* direct-fed microbials is one of the alternatives to optimize the digestibility of NSP rich diets, because some isolates have been recognized as xylanase, cellulase and β-glucanase producers (Robson and Chambliss, 1987; Hendricks et al., 1995; Monisha et al., 2009). Xylanase is one of the enzymes that have shown reduction of intestinal viscosity, which is one of the factors involved in presentation of *Clostridium*-associated enteritis (Engberg et al., 2004; Wu et al., 2004). More recently, our laboratory have demonstrated that inclusion of certain *Bacillus*-DFM candidates that produce exogenous enzymes such as xylanase in high NSP diets significantly reduced both viscosity and *C. perfringens* proliferation in an *in vitro* digestive model study simulating different compartments of GIT of poultry (Latorre et al., 2015). This results were also observed during *in vivo* experiments conducted with chickens and turkeys fed with a high NSP rye-based diet. When the *Bacillus*-DFM candidate was included in the experimental rye-based diet, significant improvements in intestinal viscosity, performance parameters, bacterial translocation and bone quality were observed in supplemented animals (Tables 2 and 3), suggesting that the consumption of a selected *Bacillus*-DFM producing a variable set of enzymes, could contribute to enhance nutrient digestibility and promote healthy intestinal integrity (Latorre et al., 2014b). Additionally, some *Bacillus* species can also synthesize proteases, which could be used to help in

the degradation of low quality proteins present in the diet, hence, preventing detrimental enteric microflora changes that could result in the proliferation of *C. perfringens*. One qualitative method to screen different *Bacillus* strains for protease activity is the utilization of milk agar medium, followed by the measurement of the zone of clearance present around the evaluated bacterial colony after 24 hours of incubation at 37ºC (Jani et al., 2012).

Furthermore, different studies have supported the hypothesis that incorporation of *B. subtilis* spores in the diet improved production parameters in poultry (Jiraphocakul et al., 1990; Santoso et al., 1995; Wu et al., 2011; Lei et al., 2013; Zhang et al., 2013). Samanya and Yamauchi (2002) reported that the supplementation of *B. subtilis* var. *natto* in the diet of chickens increased villi height and enterocyte proliferation, showing also a decrease in blood ammonia concentration which was correlated with a better intestinal function.

Moreover, Wolfenden et al. (2010) included two different *Bacillus* isolates (PHL-RW35 and PHL-RW41) at 10^7 and 10^5 spores/g of feed respectively, and obtained significant increases in body weight after 11 days of age in broiler chicks. Similarly, in another study done by the same author (Wolfenden et al., 2011), inclusion of a *B. subtilis* (PHL-NP122) in the diet of turkeys under commercial conditions resulted in a similar improvement on body weight at 23 days of age in comparison with a group of chickens consuming a medicated diet (Nitarsone). These results suggest that utilization of some *Bacillus* isolates could be an effective alternative to maintain or increase production parameters without utilization of antibiotics growth promoters in the poultry industry.

CONCLUSION AND FUTURE PERSPECTIVES

Besides control of pathogen colonization of the GIT through production of bacteriocins and stimulation of the immune system, some *Bacillus* direct-fed microbials have the capacity to produce a variable set of enzymes that may contribute to enhance performance through improving digestibility, reducing intestinal viscosity and promoting healthy intestinal integrity in commercial poultry. Additionally, it has been shown that some *Bacillus* isolates are candidates to be used as vectors for oral vaccines, which add one advantage more to the set of benefits obtained by this amazing microorganism. However, there are still a lot of unknowns about physiological aspects involved in the germination and sporulation process, and also the mechanism of action used by these bacteria to control colonization of enteropathogens and improve performance parameters.

Table 1. Evaluation of germination and growth of *Bacillus* PHL-NP122 (log_{10} cfu/g) spores in an *in vitro* crop assay using a corn and soybean feed with or without heat shock[†]. Adapted from Latorre et al., 2014a.

Time (min)	No heat shock (log_{10} cfu /g)	Heat shock (log_{10} cfu /g)
0	6.98 ± 0.1[a]	6.78 ± 0.1[a]
10	6.58 ± 0.2[a]	6.52 ± 0.2[a]
15	6.78 ± 0.2[a]	6.56 ± 0.2[a]
30	7.06 ± 0.1[a]	6.66 ± 0.1[b]
40	7.12 ± 0.1[a]	6.58 ± 0.1[b]
60	7.16 ± 0.1[a]	6.33 ± 0.2[b]

[a–b]Means within a row with different superscripts differ ($P < 0.05$).
[†]Data is expressed as mean ± SE of five replicates per treatment in each timepoint.

Table 2. Evaluation of body weight, digesta viscosity, and bacterial translocation to the liver in neonatal turkey poults fed with a rye-soybean based diet or rye-soybean based diet with *Bacillus* direct-fed microbial (DFM) supplementation. Adapted from Latorre et al., 2014b.

	Experiment 1			Experiment 2		
	Body weight[†] (g)	Digesta viscosity[‡] (cP Log_{10})	Bacterial translocation[£] (cfu Log_{10})	Body weight[†] (g)	Digesta viscosity[‡] (cP Log_{10})	Bacterial translocation[£] (cfu Log_{10})
CON[c]	65.91 ± 3.6[b]	2.03 ± 0.3[a]	3.03 ± 0.5[a]	74.47 ± 1.6[b]	2.80 ± 0.4[a]	2.13 ± 0.7[a]
TRT[d]	82.85 ± 4.2[a]	1.54 ± 0.2[b]	1.24 ± 0.5[b]	95.60 ± 2.2[a]	1.62 ± 0.5[b]	0.35 ± 0.4[b]

[a-b]Superscripts within columns indicate significant difference at $P < 0.05$.
[c]Control rye based diet without DFM.
[d]Control rye based diet with candidate DFM (10^6 spores/g of feed).
[†]Body weight n=25; Data is express as Mean ± SE.
[‡]Digesta viscosity is expressed in Log_{10} (in centipoise, cP = 1/100 dyne s/cm^2), n = 12.
[£]Liver bacterial translocation (expressed in cfu Log_{10} /g of tissue), n = 12.

Table 3. Evaluation of bone strength and bone composition in neonatal turkey poults fed with a rye-soybean based diet without or with *Bacillus* direct-fed microbial (DFM) supplementation[†]. Adapted from Latorre et al., 2014b.

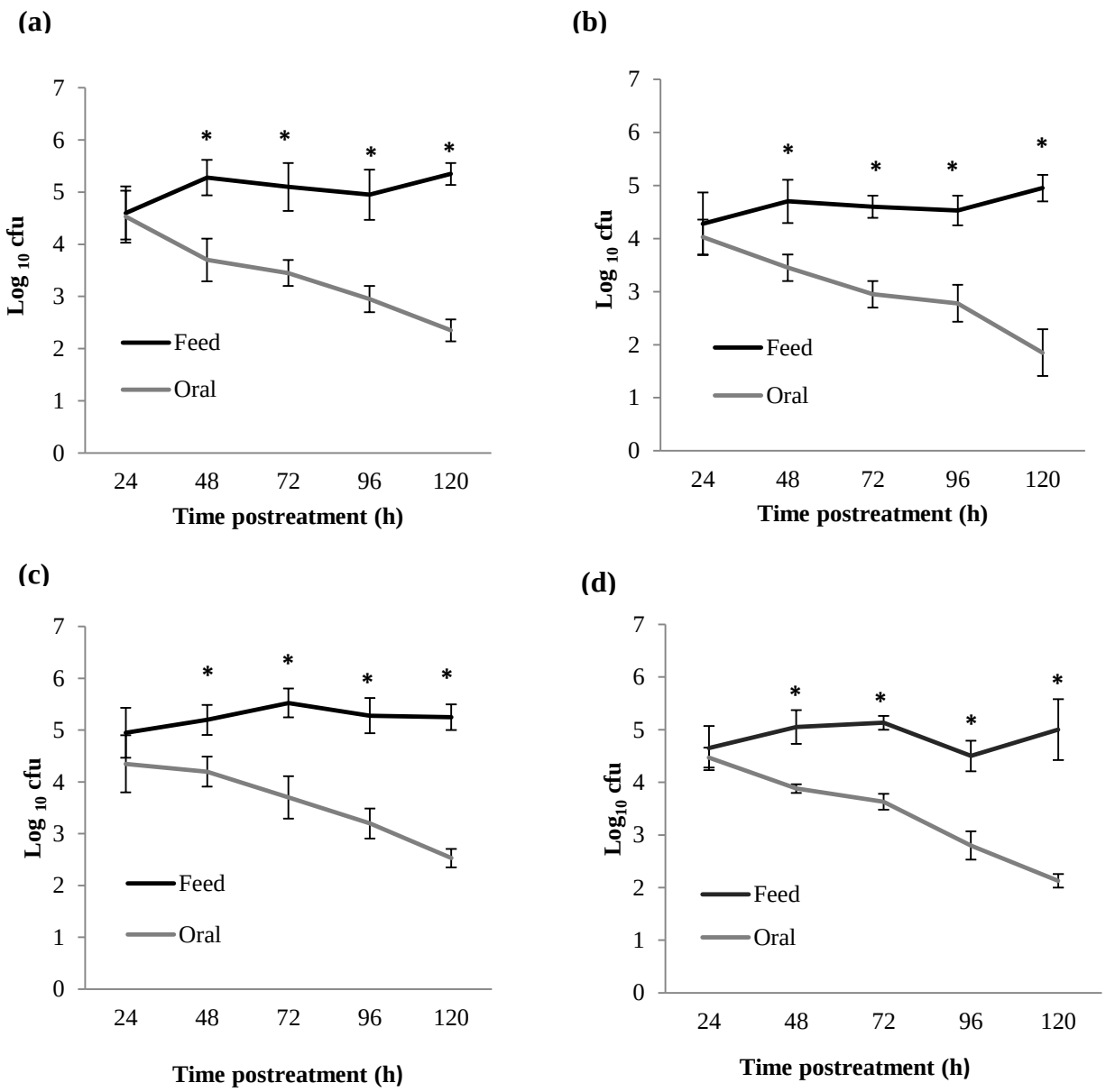
	Tibia strength load at yield (kg/mm)	Tibia diameter (mm)	Total ash from tibia (%)	Calcium (% of ash)	Phosphorus (% of ash)
CON[c]	1.14 ± 0.2[b]	4.45 ± 0.3[b]	35.61 ± 0.8[b]	27.35 ± 0.1[b]	16.35 ± 0.5[b]
TRT[d]	2.55 ± 0.1[a]	5.82 ± 0.8[a]	50.87 ± 0.7[a]	40.31 ± 0.5[a]	22.67 ± 0.3[a]

[a-b] Superscripts within columns indicate significant difference at $P < 0.05$.

[c]Control rye based diet without DFM.

[d]Control rye based diet with candidate DFM (10^6 spores/g of feed).

[†]Tibias from twelve poults were collected to evaluate bone quality. Data is expressed as mean ± SE.



*Data is expressed as mean and SE of 5 replicates in each time point ($P < 0.05$). Comparisons of spore counting were performed between constant supplementation of spores in the feed or a single oral dose. Adapted from Latorre et al., 2014a.

Figure 1. *Bacillus subtilis* (Log_{10} cfu/g) in crop (a), ileum (b), ceca (c) and feces (d) of broiler chickens given a single oral dose or constant administration of spores in the feed.

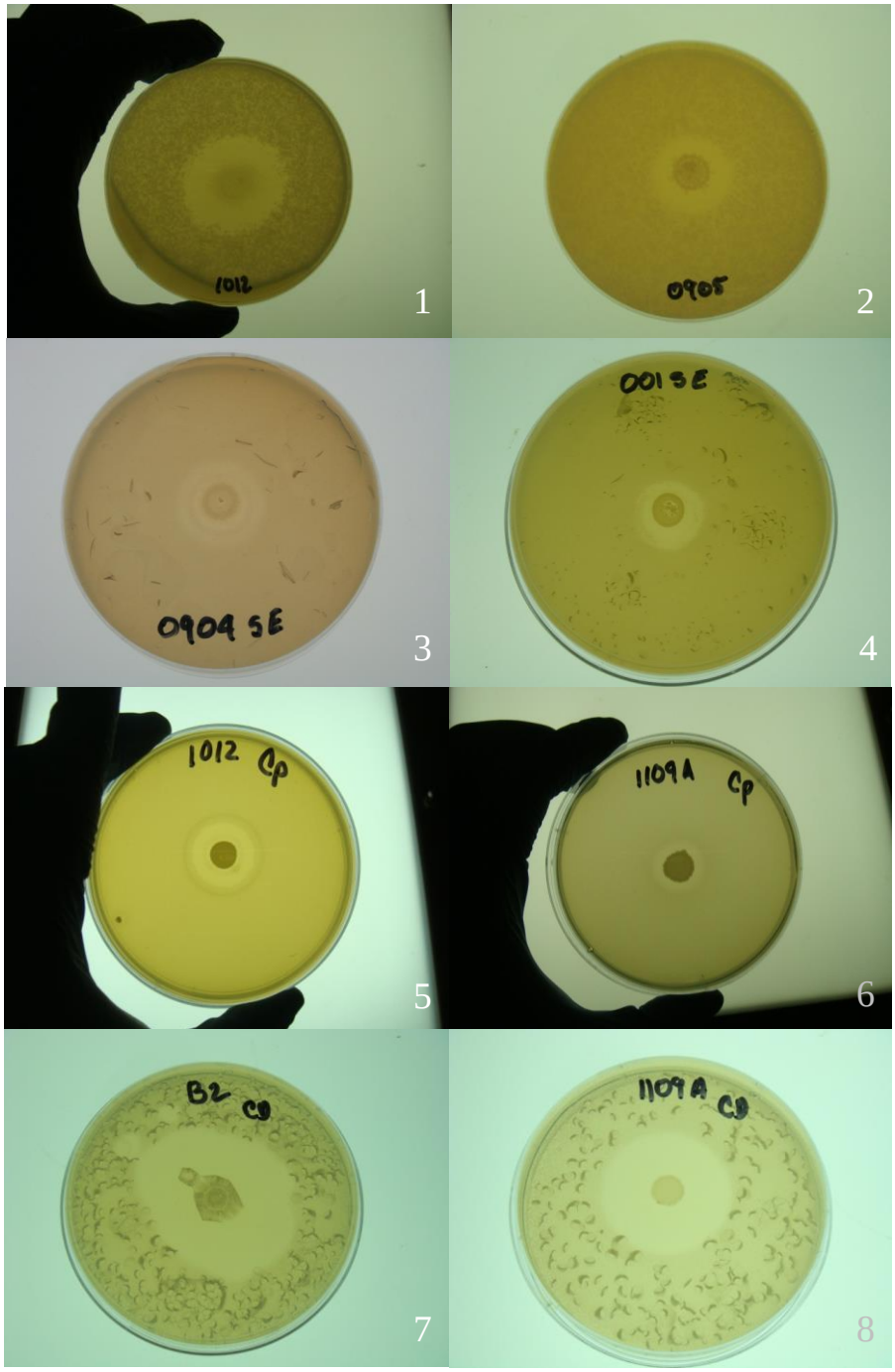


Figure 2. Evaluation of bacteriocin-like compounds synthesis from different *Bacillus spp.* as direct-fed microbial candidates using an overlay methodology. 1 *Escherichia coli* F18 – *Bacillus* 1012 and 0905 showing 20 and 14 mm of inhibition zone respectively; 2 *Salmonella* Enteritidis – *Bacillus* 0904 and 001 showing 16 and 10 mm of inhibition zone respectively; 3 *Clostridium perfringens* – *Bacillus* 1012 and 1109A showing 14 and 6 mm of inhibition zone respectively; 4 *Clostridium difficile* – *Bacillus* B2 and 1109A showing 26 and 24 mm of inhibition zone respectively.

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III. CHAPTER I

Evaluation of germination, distribution, and persistence of *Bacillus subtilis* spores through the gastrointestinal tract of chickens

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ABSTRACT

Spores are popular as direct-fed microbials, though little is known about their mode of action. Hence, the first objective of the present study was to evaluate the in vitro germination and growth rate of *Bacillus subtilis* spores. Approximately 90% of *B. subtilis* spores germinate within 60 min in the presence of feed in vitro. The second objective was to determine the distribution of these spores throughout different anatomical segments of the gastrointestinal tract (GIT) in a chicken model. For *in vivo* evaluation of persistence and dissemination, spores were administered to day-of-hatch broiler chicks either as a single gavage dose or constantly in the feed. During 2 independent experiments, chicks were housed in isolation chambers and fed sterile corn soy-based diets. In these experiments one group of chickens was supplemented with 106 spores/g of feed, whereas a second group was gavaged with a single dose of 10^6 spores per chick on day of hatch. In both experiments, crop, ileum, and cecae were sampled from 5 chicks at 24, 48, 72, 96, and 120 h. Viable *B. subtilis* spores were determined by plate count method after heat treatment (75°C for 10 min). The number of recovered spores was constant through 120 h in each of the enteric regions from chickens receiving spores supplemented in the feed. However, the number of recovered *B. subtilis* spores was consistently about 10^5 spores per gram of digesta, which is about a 1-$\log_{10}$ reduction of the feed inclusion rate, suggesting approximately a 90% germination rate in the GIT when fed. On the other hand, recovered *B. subtilis* spores from chicks that received a single gavage dose decreased with time, with only approximately 10^2 spores per gram of sample by 120 h. This confirms that *B. subtilis* spores are transiently present in the GIT of chickens, but the persistence of vegetative cells is presently unknown. For persistent benefit, continuous administration of effective *B. subtilis* direct-fed microbials as vegetative cells or spores is advisable.

Keywords: *Bacillus subtilis*, spore, germination, direct-fed microbial, probiotic

INTRODUCTION

Concerns regarding development of antibiotic-resistant microorganisms and social pressures, have continued the trend to ban the use of antibiotics as growth promoters in poultry production. This has also resulted in an urgent necessity to find feasible alternatives to maintain poultry health, in order to sustain poultry as an economically viable source of animal protein for human consumption (Alvarez-Olmos and Oberhelman, 2001; Boyle et al., 2007). In this regard, the use of bacterial spores from selected *Bacillus* strains as direct-fed microbials (DFM) have gained recognition as feed and food supplementation. Their capacity to resist rough environmental conditions, with survival during feed pelletization procedure with extreme temperatures, as well as tolerance to extremes of pH, dehydration, high pressures, caustic chemicals and long storage life, have made them suitable for commercialization and distribution (Vreeland et al., 2000; Cartman et al., 2007). During the last 15 years, our laboratories have worked toward the identification and application of probiotic candidates for poultry, which, in addition to nutritional benefits, can actually displace *Salmonella* and other enteric pathogens which have colonized the gastrointestinal tract (GIT) of chickens and turkeys. Different studies have been focused on specific pathogen reduction (Farnell et al., 2006; Vicente et al., 2008; Higgins et al., 2007, 2008, 2010; Menconi et al., 2011), evaluation of performance parameters under commercial conditions (Torres-Rodriguez et al., 2007a, 2007b), and effects on both idiopathic (Higgins et al., 2005) and defined enteritis (Wolfenden et al., 2007). These studies have indicated that, selection of therapeutically efficacious probiotic cultures with marked performance benefits in poultry is possible and that, defined cultures can provide an efficient alternative for conventional

antimicrobial therapy (Tellez et al., 2001, 2006, 2012; Higgins et al., 2011). On the other hand, studies have indicated that *Bacillus* spores are involved in rapid activation of host innate immune functions (Rhee et al., 2004). Furthermore, some B*acillus* species have the capacity to produce different exogenous enzymes including protease, lipase, cellulase, xylanase, phytase and keratinase (Hendricks et al., 1995; Monisha et al., 2009; Mazotto et al., 2011; Mittal et al., 2011; Shah and Bhatt, 2011; Jani et al., 2012). These enzymes may help to decompose complex feed molecules, improve absorption of nutrients, diminish intestinal viscosity in non-starch polysaccharide diets (NSP), and decrease the amount of substrates available for growth of pathogenic bacteria. Additionally, it has been shown that the presence of *Bacillus* isolates like *Bacillus subtilis*, enhance growth of other beneficial microorganisms such as *Lactobacillus* by production of subtilisin, catalase and decreasing intestinal pH (Hosoi et al., 2000). These studies, in total, have opened an exciting possibility for identification of vastly superior and more potent probiotics. A probiotic is effective against enteropathogens in various ways, including enhancing immune exclusion, competing for essential nutrients, competing for attachment regions, or secreting antimicrobial compounds against various enteropathogens (Cartman et al., 2007, 2008). However, for most of these actions to be effective, there is an inherent requirement for a metabolically active cell, implicating that germination of spores within the gastrointestinal tract could be a major factor to be considered to employ *Bacillus* spore-based DFM. In this context, studies in our laboratory have confirmed that selected heat-resistant spore-forming *Bacillus* species can markedly sporulate in high numbers (Wolfenden et al., 2010, 2011; Shivaramaiah et al., 2011). There is a growing body of evidence supporting the idea that some *Bacillus* species produced spores can germinate in the GIT of chickens, mice, pigs, dogs and humans, thus potentially being metabolically active and possibly eliciting a mechanism of action similar to

other probiotic bacteria (Hoa et al., 2000; Duc et al., 2004; Tam et al., 2006). However, it is of prime importance to understand the factors that affect spore germination along with their distribution pattern throughout the GIT. The complete mode of action of bacterial endospores is not understood comprehensively, but is presumed to be affected by physiological conditions (temperature, pH, and humidity), anatomical distribution and pattern of germination in these anatomical segments of the GIT. Earlier studies have shown that *Bacillus* spores germinate by the effects of nutritional and non-nutritional factors known as germinants, for instance: availability of L-alanine, asparagine, glucose, fructose, potassium chloride, and the effect of a non-lethal heat treatment under different pressures (Setlow, 2003). However, due to anatomical differences in the GIT of avian systems, it may not be acceptable to completely apply the results obtained in other animal and mammalian models. Therefore, the objectives of the present study were to evaluate the *in vitro* germination and growth rate of the *Bacillus subtilis* spores, as well as the evaluation of *in vivo* distribution and germination of *B. subtilis* spores in different anatomical regions of GIT in a chicken model, as an extension to enrich comprehension of the mechanism of action involving *Bacillus* based DFM in poultry.

MATERIALS AND METHODS

Animal Source and Diet

Day-of-hatch, off-sex broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR) and were placed in isolators, in a controlled age-appropriate environment. Chickens were provided ad libitum access to water and a balanced unmedicated corn-soybean diet meeting the nutritional requirements of poultry recommended by NRC (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc., 2013). The common starter diet was an antibiotic-free

corn-soybean meal diet (Table 1). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas. For experiments 2 and 3, 6 chickens were humanely killed with carbon dioxide asphyxiation upon arrival, and confirmed negative for *Bacillus* spp. vegetative cells and spores. Briefly, tissue samples from crop, ileum, and cecae were aseptically removed from 12 chicks, collected in sterile bags, homogenized, weighed, and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten-fold dilutions of these samples were plated on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD), incubated at 37°C for 24 h to confirm the absence of any aerobic vegetative cells. Additionally, all samples from each region received heat treatment in a water bath at 75°C for 10 min to eliminate counting of vegetative cells. Ten-fold dilution of these samples were plated on TSA, incubated at 37°C for 24 h to confirm the absence of *Bacillus* spp. spores per gram of sample.

Direct-fed Microbial

Several spore-based *Bacillus* spp. were isolated and studied from various environmental and poultry sources (Wolfenden et al., 2010) in our laboratory. For the present study, *B. subtilis* PHL-NP122 was chosen based on its consistent in vitro activity against *Salmonella* spp., *Clostridium* spp., and *Campylobacter* spp. In addition, *B. subtilis* PHL-NP122 has demonstrated the ability to grow and sporulate in high numbers (~10^9 to 10^{11} spores/g) in solid-state fermentation media (Shivaramaiah et al., 2010; Wolfenden et al., 2011) consisting of a mixture of 70% rice straw and 30% wheat bran. The original spore inoculum used in both *in vitro* and *in vivo* experiments were tested to be at 4.3×10^{10} spores per gram of solid media.

In vitro Determination of Germination and Growth Rate of Spores. Experiment 1.

Experiment 1 employed an in vitro crop assay to evaluate the germination and growth rate of the *B. subtilis* PHL-NP122 spores. Briefly, 1.25 g of unmedicated chick starter feed was measured into sixty 13 × 100 mm borosilicate tubes and autoclaved. Post-sterilization, the feed was suspended in 4.5 mL of sterile saline and inoculated with 0.5 g of *Bacillus* spores with a final concentration of 10^7 spores per gram of feed. After inoculation of feed with the spores, the tubes were vortexed and incubated at 40°C for 0, 10, 15, 30, 40, and 60 min. At each time point, 5 tubes were removed from the incubator and 0.2 mL per tube was immediately loaded on to a sterile 96-well flat bottom plate, which served as samples for counting spores and viable vegetative cells as previously described (Wolfenden et al., 2010; Shivaramaiah et al., 2011). The tubes were then heat treated at 75°C for 10 min to eliminate the presence of vegetative cells, and samples were again loaded on to another 96-well plate, which served for the actual spore count (Barbosa et al., 2005). Overall, each time point mentioned had 5 replicates per treatment, with or without heat treatment. Ten-fold dilutions of all samples (pre or post-heat treatment) were plated on TSA plates and incubated 12 h at 37°C for enumeration and spore count. The calculated difference in the number of cfu between the heat treated and non-heat treated groups, at each time point, was considered as the amount of spores that germinated over time.

In vivo Evaluation of Distribution, Persistence and Germination of Spores. Experiment 2.

Experiment 2 involved a total of 60 chickens that were randomly divided in 2 groups of 30 chicks per treatment (constant feed vs. single gavage dose). Each group of 30 chickens were allocated in isolation chambers with a wire floor (90 cm × 80 cm) with space underneath for excreta to minimize coprophagia and offered feed and water ad libitum. For the first group of

birds receiving spores in feed, *B. subtilis* PHL-NP122 spores were thoroughly mixed with previously autoclaved feed in a rotary mixer for 15 min to ensure thorough distribution. The final concentrations of spores were also determined retrospectively by serial dilution and further plating on TSA for enumeration of actual cfu/gram and ensured to be 10^6 spores per gram of feed. The second group of chicks was gavaged with a single dose of 10^6 spores suspended in 0.25 mL of PBS per chick using a sterile ball-ended gavage needle, just before placement. Chickens were randomly selected, humanely killed with carbon dioxide asphyxiation, and tissue samples from the crop, ileum (from Meckel's diverticulum to the ileocecal junction), and ceca were aseptically removed from 5 chicks at 24, 48, 72, 96, and 120 h after spore consumption by constant feed or single gavage, collected in sterile bags, homogenized, and weighed, and 1:4 wt/ vol dilutions were made with sterile 0.9% saline. All samples from each treatment received heat treatment in a water bath at 75°C during 10 min to eliminate counting of vegetative cells. Ten-fold dilution of these samples were plated on TSA and incubated at 37°C for 24 h to enumerate total cfu of *B. subtilis* spores per gram of tissue. Feces samples were also collected during the same time points, and viable spore counts were determined employing a similar dilution plate method as described above.

In vivo Evaluation of Persistence and Distribution of Vegetative Cells and Spores Throughout the GIT. Experiment 3.

Similarly to experiment 2, this experiment involved a total of 60 chickens that were randomly divided in 2 groups of 30 chicks per treatment (constant feed vs. single gavage dose). Each group of 30 chickens were allocated in isolation chambers with a wire floor (90 cm × 80 cm) with space underneath for excreta to minimize coprophagia and offered feed and water ad

libitum. In this experiment, along with determining just the final viable spore count, vegetative cells were also measured to evaluate the extent of germination of spores in each of section of GIT sampled, at each time point mentioned before in experiment 2. Briefly, ingesta samples collected at each time point and loaded on to 96 well sterile plates, both pre- and postheat treatment (only heat-treated samples in experiment 2). Ten-fold dilutions of all samples were then plated on TSA and incubated for 24 h at 37°C to determine the spore count pre- and postheat treatment. The difference in the cfu between heat-treated and non-heat-treated samples was counted to be the amount of spores that germinated at each time point.

Germination/Sporulation Rate of a Bacillus-DFM in Different Sections of the GIT in Broiler Chickens Consuming Bacillus Spores Constantly in the Feed. Experiment 2 and 3.

The germination rate of *B. subtilis* spores between crop and ileum was calculated as follows:

Germination rate = Crop spores – Ileum spores

Additionally, the sporulation rate between ileum and ceca was calculated as follows:

Sporulation rate = Cecal spores - Ileum spores

Statistical Analysis

Colony-forming units in all experiments were converted to log10 values. Comparison between total aerobic vegetative cells versus *B. subtilis* spores (log10 cfu/g) after heat shock in the crop, ileum, and ceca of broiler chickens after a single gavage dose or constant administration in the feed were subjected to one-way analysis of variance (ANOVA). Germination/sporulation rate of a *Bacillus*-DFM in different sections of the GIT in broiler chickens consuming *Bacillus* spores constantly in the feed were subjected to ANOVA. All data were compared using the GLM

procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC). Significant differences among the means were determined by using Duncan's multiple-range test at $P \leq 0.05$. Five replicates were evaluated per time point, using a complete randomized design.

RESULTS AND DISCUSSION

In spite of the success showed by the development of the *Lactobacillus* probiotic for use in commercial poultry, there is still an urgent need for commercial probiotics that are shelf-stable, cost-effective, and feedstable (tolerant to heat pelletization procedures) to increase compliance and widespread utilization. Among the large number of probiotic products in use today, some are bacterial spore formers, mostly of the genus *Bacillus*. Used primarily in their spore form, some have been shown to prevent selected gastrointestinal disorders along with having numerous nutritional benefits (Mazza, 1994; Hoa et al., 2000; La Ragione and Woodward, 2003; Duc et al., 2004; Williams, 2007; Hong et al., 2008). Several studies have shown that either live vegetative cells or endospores of some isolates can prevent colon carcinogenesis (Park et al., 2007) or discharge antimicrobial substances against gram-positive bacteria, such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile* (Hoa et al., 2000; Hong et al., 2008). However, distribution and germination-dependent mechanisms of action of *Bacillus* spores across the GIT are not completely understood in humans or any other animal models, suggesting that a metabolically active cell could be a major factor to be considered to employ *Bacillus* spore-based DFM. The present study provides several supporting evidences in this direction by employing both an *in vitro* crop assay and an *in vivo* chicken model.

Experiment 1 involving *in vitro* crop assay provided preliminary idea about the germination of spores under study, the data of which are summarized in Table 2. Approximately 90% of the *B.*

subtilis spores germinated within 60 min under *in vitro* crop and GIT conditions, although significant differences were recorded just after 30 min of incubation. The data suggest that this short rate at which spores germinate is an important factor, considering the rapid passage rate of the digesta, and hence the spores, through the GIT with varying physiological conditions. In addition, the data are suggestive that spores germinate into metabolically and functionally active vegetative cells, within similar time frame, to produce beneficial metabolic effects. Similar results were obtained by Leser et al. (2008) using a nutrient-rich medium, where germination of *B. licheniformis* CH200 and *B. subtilis* CH201 took between 60 to 90 min to germinate under *in vitro* conditions. On the other hand, distribution and persistence of spores across the GIT play an important factor for them to elicit their important functions, and it is believed that the germination rate described above place a major role here. Experiment 2, involving *in vivo* trials with broiler chickens, provided more understanding in this regard, the results of which are shown in Figure 1.

To begin with, *B. subtilis* spores were recovered from sample tissues of crop, ileum, ceca, and collected feces from broiler chickens, either given as a single gavage dose or constant administration in the feed. Recovered spores count was between 10^4 and 10^5 per gram of ingesta at all times in crop, ileum, and ceca when spores were constantly administered in the feed. Further, recorded reduction of about 1-$\log_{10}$, from the original spore count in the feed to the recovered count from GIT, suggested that germination of 90% of spores occurred, which is consistent with the results obtained in the *in vitro* crop assay (Table 2; Figure 1). Recovered spores from chicks that received a single gavage dose also followed the same pattern of 1-$\log_{10}$ reduction, but decreased over time. Further, spores administered either continuously in the feed or by single gavage followed a consistent pattern of change in the number of spores recovered

from the crop to the cecae. A numerical reduction tendency in the spore count was observed from crop to ileum followed by a numerical increment from the ileum to the cecae, at all sampling time points (Table 4). The results of experiment 2 were confirmed and extended in experiment 3 (Table 3). Overall, our results are in agreement with Jadamus et al. (2001), arriving at similar conclusions, that the amount of spores recovered tends to diminish from the crop to the ileum, and increase again from the ileum to the cecae (Tables 3 and 4). This change could be related to the varying physiological, nutritional, and microbiological conditions of the GIT. The reduction of spore cfu in the small intestine could be as a result of germination in the crop, duodenum, or jejunum due to abundance of nutrients and favorable conditions in these anatomical segments. On the other hand, the increment of spores in the cecae could be a response due to the competitive microbial population, contending for oxygen and nutrients. This, accompanied by elevated concentrations of bacterial metabolites, such as NH_3 (Preston and Douthit, 1984; Jadamus et al., 2001), could stimulate sporulation of vegetative cells entering the ceca, providing increased chances of survival, before being excreted into the environment. The above observations, also suggest that spores transiting through the GIT, could potentially undergo a full life-cycle of germination and resporulation, also suggested by previous studies (Barbosa et al., 2005; Cartman et al., 2008). In the present study, when neonatal chickens received a single gavage dose of spores, a gradual decreased in the amount of spores recovered throughout different sampled sections of the GIT over time was observed (Figure 1; Tables 3 and 4); however, the long persistence of spores observed in feces in experiment 2 and as well as spores detected in each GIT organ evaluated in experiments 2 and 3, following one single gavage of spores, was longer than the estimated half-life, based on gut-passage time, which in chickens is around of 6.5 h (Shires et al., 1987), suggesting that some full life-cycle development may

occurs within the GIT (Table 4). This is supported by the study showing the presence of a larger amount of spores excreted in the feces compared with the original inoculum administered to mice, suggested germination, growth and resporulation of the initial spore dose (Hoa et al., 2001).

To the contrary, Spinosa et al. (2000) have argued that the germination of *B. subtilis* spores and generation of beneficial effects by vegetative cells is highly improbable, due to the presence of low pH and secretory bile salts in certain regions of the GIT of mice, inhibiting spore germination or killing the new vegetative cells. Nevertheless, other authors have shown that heat and low pH are conditions that instead of attenuating, could actually stimulate *Bacillus* spore germination, therefore providing evidence of survival within the GIT (Faille et al., 2002), partially supported by the results obtained in the present study.

The germination rate, distribution, and suggested full-cycle development of spores, along with the spore recovery data, even after 5 d (120 h) of single gavage dose administration in experiments 2 and 3, demonstrate the persistence of these *Bacillus* spores in the gut. A study by Hoa et al. (2001), in a murine model, also showed that after a single oral dose of 5.97×10^8 spores of *B. subtilis* SC1712, endospores were detected in feces even after 7 d of sampling. Prolonged spore persistence was also reported in mice by Tam et al. (2006), where *Bacillus* spores were detected in feces 27 d after a single gavage dose of 2 natural *Bacillus* strains, overall indicating prolonged persistence in the GIT. This germination and persistence of *Bacillus* has been attributed to the ability of some strains to produce biofilms. The extracellular matrix within these biofilms is theorized to improve the adherence of vegetative cells or spores to the mucosal surface, as well as protect them against undesirable conditions present in the gut, therefore improving the possibilities to persist and thrive within the GIT (Barbosa et al., 2005).

In the present study, the disappearing of spores from the different segments of the GIT was not abrupt, even from chicks that received a single gavage dose (Figure 1). At 24 h after gavage, there were no significant differences of cfu between treatments; however, at 48 h the group of birds receiving a single oral dose started to show a significant diminishment in the presence of spores throughout the GIT, having at the last sample time (120 h) almost a 3 $\log_{10}$ difference with the group consuming spores constantly in the feed. Similarly, the presence of spores in the feces of broiler chickens given a single gavage dose gradually but consistently decreased over time compared with the feed-supplemented animals (Figure 1). The steady decrease and the rate of disappearance of spores from the gut after a single oral gavage in experiments 2 and 3 confirm that *B. subtilis* is transiently present in the GIT of chickens and that a continuous administration is advisable for continued intestinal benefits (Tables 3 and 4). Nevertheless, the further evaluation in the intended direction of using *Bacillus* spores as DFM and vaccine delivery vehicles is currently ongoing in our laboratory, employing various molecular techniques for differentiation and quantification of vegetative cells and spores present in different segments of the gastrointestinal tract of poultry.

Table 1. Ingredient composition and nutrient content of the starter diet for broiler chickens used in all experiments on as-is basis

Item	Amount per kg (%)
Ingredients	
Corn	56.59
Soybean meal	35.74
Vegetable oil	3.29
Dicalcium phosphate	1.81
Calcium carbonate[1]	1.12
Salt	0.38
DL-Methionine	0.31
Vitamin premix[2]	0.10
L-Lysine HCl	0.19
Choline chloride 60%	0.10
Mineral premix[3]	0.10
L-Threonine	0.06
Antioxidant[4]	0.15
Total	100
Calculated analysis	
Metabolizable energy (kcal/ kg)	3,035
Crude protein (%)	21.70
Lysine (%)	1.32
Methionine (%)	0.63
Met + Cys (%)	0.98
Threonine (%)	0.86
Tryptophan (%)	0.25
Total calcium (%)	0.90
Available phosphorus (%)	0.45
Sodium (%)	0.16

[1]Inclusion of 10^6 spores/g of feed mixed with Calcium carbonate.
[2]Vitamin premix supplied the following per 1000 kg: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO 64850).
[3]Mineral premix supplied the following per 1000 kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO 64850).
[4]Ethoxyquin.

Table 2. Evaluation of germination and growth of *Bacillus* PHL-NP122 (log_{10} cfu / g) spores in an *in vitro* crop assay using a corn and soybean feed with or without heat shock (Experiment 1)[1]

Time (min)	No heat shock (log_{10} cfu /g)	Heat shock (log_{10} cfu /g)
0	6.98±0.15[a]	6.78±0.14[a]
10	6.58±0.23[a]	6.52±0.17[a]
15	6.78±0.19[a]	6.56±0.21[a]
30	7.06±0.06[a]	6.66±0.09[b]
40	7.12±0.07[a]	6.58±0.15[b]
60	7.16±0.10[a]	6.33±0.20[b]

[a–b]Means within a row with different superscripts differ ($P < 0.05$).
[1]Data is expressed as mean ± SE of 5 replicates

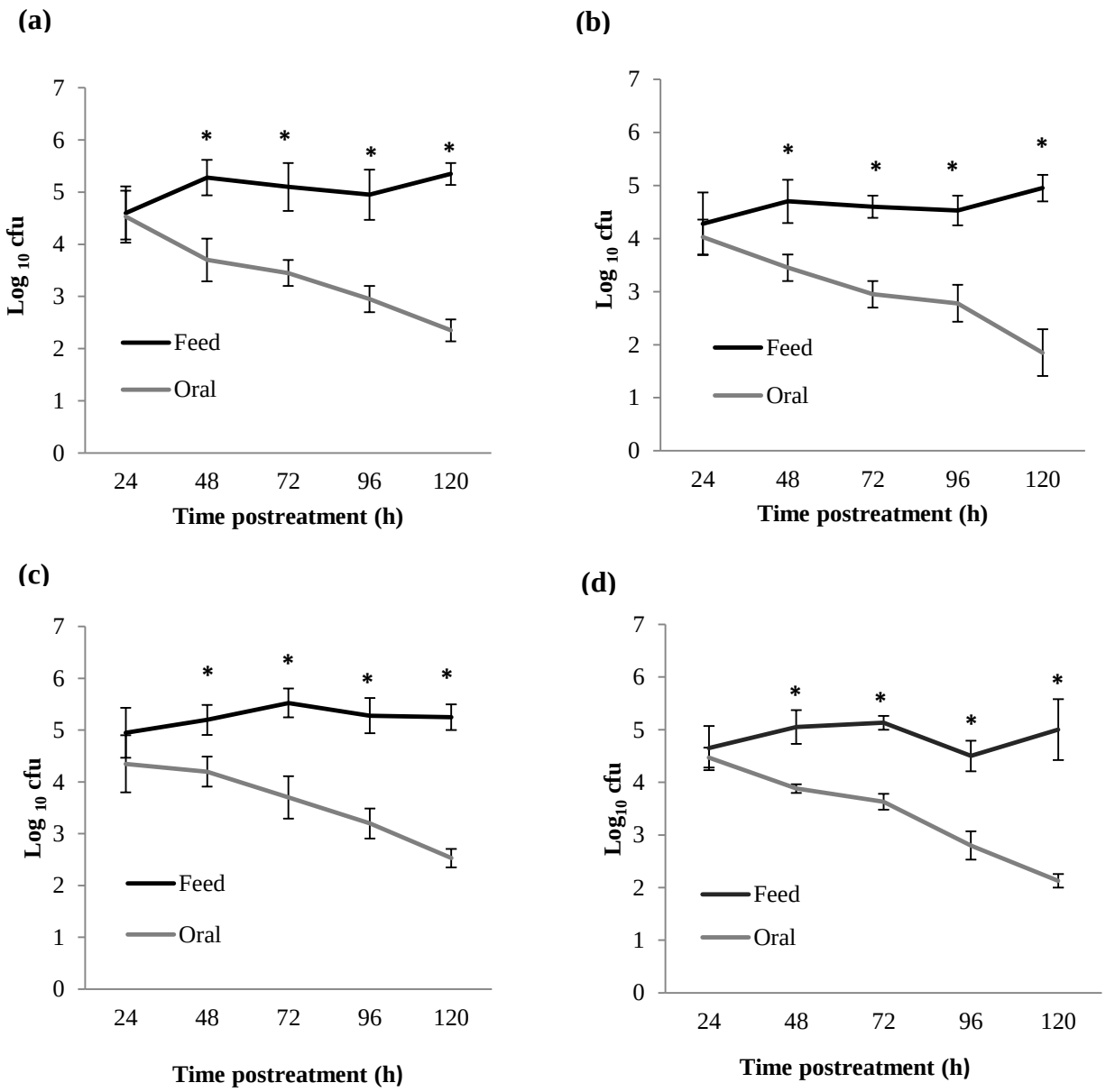

Figure 1. *Bacillus subtilis* (Log_{10} CFU g^{-1}) crop (a), ileum (b), ceca (c) and feces (d) of broiler chickens given a single oral dose or constant administration of spores in the feed (Experiment 2). *Data is expressed as mean and SE of 5 replicates ($P < 0.05$).

Table 3. Comparison between total aerobic vegetative cells *versus Bacillus subtilis* spores after heat shock[1] (HS) in crop, ileum, and ceca of broiler chickens after a single gavage dose[2] or constant administration in the feed[3] (Experiment 3)

	Feed			Gavage		
Item	**Before HS**	**After HS**	**Difference**	**Before HS**	**After HS**	**Difference**
Crop (log_{10} cfu / g)						
24 h	8.15 ± 0.32[a]	4.65 ± 0.43[b]	3.50	7.68 ± 0.39[a]	4.47 ± 0.19[b]	3.21
48 h	7.80 ± 0.12[a]	5.05 ± 0.38[b]	2.75	6.00 ± 0.28[a]	3.88 ± 0.09[b]	2.12
72 h	6.95 ± 0.38[a]	5.13 ± 0.13[b]	1.82	5.48 ± 0.07[a]	3.63 ± 0.19[b]	1.85
96 h	6.75 ± 0.10[a]	4.50 ± 0.29[b]	2.25	5.45 ± 0.18[a]	2.80 ± 0.27[b]	2.65
120 h	6.95 ± 0.13[a]	5.00 ± 0.58[b]	1.95	5.15 ± 0.35[a]	2.13 ± 0.13[b]	3.02
Ileum (log_{10} cfu / g)						
24 h	7.33 ± 0.52[a]	4.15 ± 0.17[b]	3.18	6.52 ± 0.48[a]	4.10 ± 0.09[b]	2.42
48 h	7.00 ± 0.24[a]	4.45 ± 0.21[b]	2.55	6.00 ± 0.21[a]	3.70 ± 0.10[b]	2.30
72 h	7.85 ± 0.47[a]	4.45 ± 0.17[b]	3.40	5.40 ± 0.16[a]	3.53 ± 0.17[b]	1.87
96 h	7.03 ± 0.26[a]	5.33 ± 0.24[b]	1.70	5.10 ± 0.29[a]	3.00 ± 0.00[b]	2.10
120 h	7.15 ± 0.30[a]	5.00 ± 0.41[b]	2.15	4.98 ± 0.19[a]	2.25 ± 0.14[b]	2.73
Ceca (log_{10} cfu / g)						
24 h	9.75 ± 0.05[a]	5.13 ± 0.30[b]	4.62	9.40 ± 0.14[a]	4.42 ± 0.21[b]	4.98
48 h	9.50 ± 0.09[a]	5.65 ± 0.24[b]	3.85	9.00 ± 0.24[a]	4.28 ± 0.10[b]	4.72
72 h	9.40 ± 0.18[a]	5.95 ± 0.05[b]	3.45	9.15 ± 0.09[a]	4.25 ± 0.25[b]	4.90
96 h	8.90 ± 0.27[a]	5.03 ± 0.31[b]	3.87	8.58 ± 0.17[a]	3.75 ± 0.14[b]	4.83
120 h	9.10 ± 0.09[a]	5.50 ± 0.29[b]	3.60	7.78 ± 0.43[a]	2.70 ± 0.38[b]	5.08

[a–b]Means within a row with different superscripts differ between feed or gavage treatments before and after heat shock respectively ($P < 0.05$).

[1]Heat shock was induced by placing a sample of each respective GIT sample in a water bath at 75ºC for 10 min. Data correspond to the means ± SE of results of n = 5 birds.

[2]10^6 spores per 0.25 ml

[3]10^6 spores per gram of feed.

Table 4. Germination/sporulation rates of a *Bacillus*-DFM in different sections of the gastro intestinal tract in broiler chickens consuming *Bacillus* spores constantly in the feed (Experiment 2 and 3)

Item	Crop[1] (log_{10} cfu /g)	Ileum[1] (log_{10} cfu /g)	Ceca[1] (log_{10} cfu /g)	Germination (Cp-Il)[2]	Sporulation (Cc-Il)[3]
Experiment 2					
24 h	4.60 ± 0.51[a]	4.28 ± 0.59[a]	4.95 ± 0.48[a]	0.32	0.67
48 h	5.28 ± 0.34[a]	4.70 ± 0.41[a]	5.20 ± 0.29[a]	0.58	0.50
72 h	5.11 ± 0.46[a]	4.60 ± 0.21[a]	5.53 ± 0.28[a]	0.51	0.93
96 h	4.95 ± 0.48[a]	4.53 ± 0.28[a]	5.28 ± 0.34[a]	0.42	0.75
120 h	5.35 ± 0.21[a]	4.95 ± 0.25[a]	5.25 ± 0.25[a]	0.40	0.30
Experiment 3					
24 h	4.65 ± 0.42[a]	4.15 ± 0.17[a]	5.13 ± 0.03[a]	0.50	0.98
48 h	5.05 ± 0.32[ab]	4.45 ± 0.21[b]	5.65 ± 0.24[a]	0.60	1.20
72 h	5.13 ± 0.13[b]	4.45 ± 0.17[c]	5.95 ± 0.05[a]	0.68	1.50
96 h	4.50 ± 0.29[c]	5.30 ± 0.24[b]	5.03 ± 0.31[a]	-0.80	-0.27
120 h	5.00 ± 0.58[a]	5.00 ± 0.41[a]	5.50 ± 0.29[a]	0.00	0.50

[a–c]Means within a row with different superscripts differ ($P < 0.05$).
[1]Data is expressed as mean ± SE of 5 replicates
[2]Cp-Il = Difference in spore count between crop and ileum
[3]Cc-Il = Difference in spore count between ceca and ileum

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IV. CHAPTER II

Evaluation and selection of *Bacillus* species based on enzyme production, antimicrobial activity and biofilm synthesis as direct-fed microbials candidates for poultry

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ABSTRACT

Social concern about misuse of antibiotics as growth promoters (AGP) and generation of multidrug-resistant bacteria have restricted the dietary inclusion of antibiotics in livestock feed in several countries. Direct-fed microbials (DFM) are one of the multiple alternatives commonly evaluated as substitutes of AGP. Sporeformer bacteria from the genus *Bacillus* have been extensively investigated because of their extraordinary properties to form highly-resistant endospores, production of antimicrobial compounds and synthesis of different exogenous enzymes. The purpose of the present study was to evaluate and select *Bacillus spp.* from environmental and poultry sources as DFM candidates, considering their enzyme production profile, biofilm synthesis capacity and pathogen-inhibition activity. Thirty one *Bacillus* isolates were screened for *in vitro* relative enzyme activity of amylase, protease, lipase and phytase using a selective media for each enzyme, with 3/31 strains selected as superior enzyme producers. These three isolates were identified as *B. subtilis* (1/3), and *B. amyloliquefaciens* (2/3) based on biochemical tests and 16S rRNA sequence analysis. In addition, the three selected strains have previously being tested for resistance of different simulated gastrointestinal conditions. For evaluation of biofilm synthesis, the generation of an adherent crystal violet-stained ring was determined in polypropylene tubes, resulting in 11/31 strains showing a strong biofilm formation. Moreover, all *Bacillus* strains were evaluated for growth inhibition activity against *S.* Enteritidis (26/31), *E. coli* (28/31) and *C. difficile* (29/31). The results of this study suggest that the selection and consumption of *Bacillus*-DFM, producing a variable set of enzymes and antimicrobial compounds may contribute to enhance performance through improving nutrient digestibility, reducing intestinal viscosity, maintaining a beneficial gut microflora and promoting a healthy intestinal integrity in poultry.

Keywords: *Bacillus*, direct-fed microbial, enzyme, antimicrobial, biofilm.

INTRODUCTION

The continuous tendency to reduce the use of antibiotic growth promoters (AGP) in poultry production due to social concern about generation of antibiotic resistant bacteria, have resulted in the crucial necessity to find economically viable alternatives that can maintain optimal health and performance parameters under commercial conditions (Alvarez-Olmos and Oberhelman, 2001; Boyle et al., 2007). One possible substitute for AGP that has been extensively studied is the utilization of probiotics to prevent and treat gastrointestinal infections (Higgins et al., 2011). The most common microorganisms used as probiotics are lactic acid bacteria (LAB) from the genus *Lactobacillus* and *Pediococcus*, however, these microorganisms required refrigeration or lyophilization to survive for long storage periods, and microencapsulation to withstand feed application, therefore adding cost to their industrial production (Tellez et al., 2012). Among the microorganisms used as direct-fed microbials (DFM), *Bacillus* spores have been increasingly included as feed additives in poultry diets, due to their remarkable resistance to harsh environmental conditions, and also have a long shelf life. (Cartman et al., 2007; Vreeland et al., 2000). Bacteria from the genus *Bacillus* are Gram-positive, rod shaped and usual inhabitants of the soil. However, different studies have shown that *Bacillus* spores can also be present, germinate and survive in the gastrointestinal tract (GIT) of different animal species, suggesting that these bacteria could be considered facultative anaerobes and part of the metabolically active host microflora (Hoa et al., 2001; Hong et al., 2009; Latorre et al., 2014). Rate of survival and persistence of some *Bacillus* strains in the GIT may be related to their capacity to synthesize biofilms, thereby, protecting themselves against the harsh environmental conditions present in

the gut (Barbosa et al., 2005). Moreover, one of the principal sources of enzymes and antibiotics from bacterial origin used by biotechnology companies are produced by different *Bacillus* strains, making this multifunctional microorganism useful inside or outside a host (Priest, 1977; Azevedo et al., 1993).

On the other hand, the increasing consumption of poultry meat globally, along with utilization of grains such as corn for biofuel production has led to the use of less digestible energy sources in poultry diets. Alternative cereals such as wheat, barley, triticale or rye have been previously included in poultry feed. (Lesson and Proulx, 1994; Bedford, 1998; Mendes et al., 2013). However, the incorporation of these raw materials in monogastric diets have a negative impact on growth performance due to an elevated concentration of antinutritional factors such as non-starch polysaccharides (NSP) in comparison to corn-based feed (Choct et al., 1996). Diets rich in NSP generate an increase in intestinal viscosity, affecting digestibility and absorption of nutrients by the intestinal surface (Annison, 1993). An alternative to reduce the negative effects generated by NSP is the inclusion of microbial enzymes, such as xylanase, which have been shown to reduce intestinal viscosity and *Clostridium*-associated enteritis (Guo et al., 2014). Additionally, utilization of other microbial enzymes such as α-amylase, protease, lipase and phytase have demonstrated to increase degradation of low quality proteins, improve bone quality and enhance absorption of carbohydrates and fatty acids (Meng et al., 2004; Woyengo and Nyachoti, 2011; Murugesan et al., 2014). In this regard, the exogenous enzymes produced by *Bacillus spp.* that may help to degrade complex antinutritional factors in poultry diets and improve nutrient absorption include cellulase (Hendricks et al., 1995), α-amylase (Ibrahim et al., 2012), β-glucanase (Aono et al., 1992), α-galactosidase, β-mannanase (Talbot and Sygusch, 1990), xylanase (Monisha et al., 2009), protease (Olejuyigbre and Ajele, 2005), lipase (Shah and

Bhatt, 2011), keratinase (Mazzoto et al., 2011) and phytase (Choi et al., 2001). Nonetheless, it is important to mention that not all *Bacillus* bacteria synthesize the same type of enzymes, therefore, requiring selection and characterization of adequate isolates according to the specific target substrates in the diet.

Besides the capacity of certain *Bacillus spp*. to produce enzymes and increase utilization of nutrients from different feedstuffs, spores from various *Bacillus* strains have also been included in poultry diets to control the incidence of different gastrointestinal diseases through the production of antimicrobial compounds or acting as competitive exclusion agents against *Salmonella* Typhimurium (Shivaramaia et al., 2011), *Clostridium perfringens* (Tactacan et al., 2013), *Escherichia coli* (La Ragione et al., 2001) and *Campylobacter jejuni* (Svetoch et al., 2005). Additionally, *Bacillus*-DFM have shown to enhance cellular and humoral immune responses by increasing the number of solitary lymphoid follicles in the intestinal mucosa, influencing the development of the gut-associated lymphoid tissue (GALT), enhancing antibody responses after vaccination, and augmenting macrophage function (Rhee et al., 2004; Kyung-Woo et al., 2011; Molnar et al., 2011). Dietary supplementation with *Bacillus* spores may also has a positive effect on other beneficial bacteria populations such as LAB through production of subtilisin and catalase, as well as, reducing pH and oxygen concentration in the gut to generate a more favorable environment (Hosoi et al., 2000; Jeong and Kim, 2014). In the case of intestinal epithelial integrity, it has been shown *in vitro* (Caco2 cells) and *ex vivo* that a *B. subtilis* quorum-sensing signal molecule known as the competence and sporulation-stimulating factor (CSF), induces expression of the heat shock protein Hsp27. Therefore, enhancing protection of enterocytes against oxidative damage, and preventing detrimental effects on the intestinal barrier (Okamoto et al., 2012). At the end, all the characteristics mentioned before support the

utilization of selected *Bacillus spp.* spores as a feasible alternative to AGP, improving performance parameters through production of enzymes and maintaining an optimal health status by synthesis of antimicrobial compounds. Therefore, the purpose of the present study was to evaluate and select *Bacillus* isolates from environmental and poultry sources as candidate direct-fed microbials (DFM) based upon enzyme production profiles, pathogen inhibition capacity and biofilm synthesis, hence, extending our understanding of the mechanism of action of *Bacillus*-DFM and its applicability in the poultry industry.

MATERIALS AND METHODS

Bacillus spp. isolation

Previous research conducted in our laboratory focused on isolation of several *Bacillus spp*. from environmental and poultry sources as described by Wolfenden et al. (2010). Briefly, samples from intestinal content, fecal material and environmental sources were collected using sterile cotton swabs and placed into sterile borosilicate tubes for transport. All samples were pasteurized by heat treatment at 70° C for 15 min to eliminate the presence of vegetative cells and allow the isolation of sporeformers only. Swabs were then plate struck on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD) to be able to collect individual colonies after 24 h of incubation at 37°C. Additionally, all the strains used in the present study were previously selected as negative for alpha and beta hemolysis after being inoculated on TSA plates containing 50 ml/L of defibrinated sheep blood (catalog no. R54012, Remel, Lenexa, KS).

In vitro determination of enzyme activity

Thirty one *Bacillus spp.* isolates obtained from the Poultry Health Laboratory at the University of Arkansas were screened for production of α-amylase, protease, lipase and phytase. All *Bacillus* strains were growth in tryptic soy broth (TSB, Becton Dickinson, Sparks, MD) at 37ºC for 24 h. Then the isolates were washed with a saline solution (0.9%) and centrifuged three times at 3500 RPM for 15 min to prepare a clean inoculum. During the screening process, 10 µl of each *Bacillus* strain were placed on the center of a selective media according to the enzyme under evaluation. After incubation, all plates were evaluated and the diameters of the zones of clearance were measured. The relative enzyme activity (REA) was determined by using the formula: REA = diameter of zone of clearance divided by the diameter of the bacterial colony in millimeters. Based on REA test organisms were categorized into excellent (REA > 5.0), good (REA > 2.0 to 5.0) or poor (REA < 2.0) enzyme producers (Jani et al., 2012). Each *Bacillus* strain was evaluated by triplicate, and values are presented in table 1. More details about the composition of each selective media and incubation periods used to evaluate the capacity to produce a particular enzyme are described below.

Production of Amylase

To determine amylase enzyme activity, a starch agar media was used and consisted of 10 g of tryptone, 3 g of soluble starch, 5 g of KH_2PO_4, 10 g of yeast extract, 15 g of noble agar and 1000 ml of distilled water. The starch media was autoclaved at 121ºC for 15 min and poured in petri dishes when the temperature reach 50ºC. Then each tested *Bacillus* strain was inoculate and incubated at 37ºC for 48 h. For visualization of the zone of clearance all petri dishes were flooded with 5 ml of Gram's iodine solution (Ibrahim et al., 2012).

Production of Protease

For evaluation of protease activity, a skim milk agar media was prepared containing 25 g of skim milk, 25 g of noble agar and 1000 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 121°C for 15 min. For plating, the skim milk agar solution was held in a water bath at 50°C and then it was poured quickly into plates. Each *Bacillus* strain was inoculate on petri dishes and incubated at 37ºC for 24 h to observe if a zone of clearance was developed (Pailin et al., 2001).

Production of Lipase

Lipase activity was assessed using the Spirit blue agar media (Difco Laboratories, Detroit, MI, USA) composed by 10 g of pancreatic digest of casein, 5 g of yeast extract, 20 g of noble agar, and 0.15 g of the die spirit blue. A total of 35 g spirit blue agar were used per 1000 ml of distilled water. The media was sterilized at 121ºC for 15 min and let to reach 50ºC in a water bath, before being mixed with 30 ml of a lipoidal solution prepared with 100 ml of olive oil, 1 ml of polysorbate 80 and 400 ml warm water (60ºC). Plates were inoculated and incubated at 37ºC for 24 h, before the determination of a zone of clearance around each bacterial colony.

Production of Phytase

For determination of phytase activity, *Bacillus* isolates were screened in a medium that contained: 10 g dextrose, 0.3 g $(NH_4)_2SO_4$, 0.5g $MgSO_4$, 0.1 g $CaCl_2$, 0.01 g $MnSO_4$, 0.01 g $FeSO_4$, 5 g Na-phytate, and 20 g of noble agar per 1000 ml of distilled water. The phytate media was autoclaved at 121ºC for 15 min and poured into petri dishes when the temperature reach 50ºC. Isolates were inoculated and incubated at 37ºC for a maximum of 120 h to evaluate if a

zone of clearance was generated surrounding the tested bacterial strains (Gulati et al., 2007; Mittal et al., 2011).

In vitro Assessment of Antimicrobial Activity against Salmonella Enteritidis and Escherichia coli

Thirty one *Bacillus spp*. strains were screened by triplicate for *in vitro* antimicrobial activity against *Salmonella* Enteritidis and *Escherichia coli* as reported previously (Wolfenden et al., 2010). Briefly, 10 µl of each *Bacillus* isolate were placed on the center of TSA plates, and incubated for 24 h at 37ºC. Then, the petri dishes with visible *Bacillus* colonies were overlaid with a TSA soft agar containing either 10^6 cfu/ml of *S*. Enteritidis or *E. coli*. After aerobic incubation for 24 h at 37ºC, all plates were observed and the diameters of the zones of inhibition were measured removing the diameter of the bacterial colony.

In vitro Assessment of Antimicrobial Activity against Clostridium difficile

All tested *Bacillus spp.* isolates were cultured aerobically overnight on TSA plates and screened for *in vitro* antimicrobial activity against *Clostridium difficile*. Briefly, 10 µl of each *Bacillus* strains were placed in the centre of TSA plates. After 24 h of incubation at 37 °C, the plated samples were overlaid with TSA containing 10^6 cfu/mL of *C. difficile* and plates were incubated anaerobically. After 24 h of incubation at 37 °C, all plates were evaluated for the presence of zones of inhibition, and the diameter of the inhibition zone was measured as mentioned above for *S.* Enteritidis and *E. coli* antimicrobial activity evaluation.

Biofilm assay

To determine biofilm synthesis a previously published crystal violet staining method was used with slight modifications (O'Toole and Kolter, 1998). Briefly, *Bacillus* isolates were growth in TSB overnight at 37ºC, and 10 µl of each strain were inoculated in 0.5 ml of Casein-Manitol broth in 1.5 ml polypropylene tubes. After 12 h of incubation at 37ºC, the liquid supernatant was removed and the tubes were gently rinsed with distilled water. Then, 1 ml of a 1% w/v crystal violet solution was added to the tubes to stained the cells adhered to the walls forming a ring. After 25 min, the crystal violet solution was removed and the tubes were washed with distilled water. The qualitative measurement of biofilm synthesis was based on color intensity and size of the adherent crystal violet ring with a score ranging from negative (-) to strong (++) biofilm formation.

Identification of Bacillus-DFM candidates

Bacillus spp. strains laboratory identified as AM1002, AM0938 and JD17 were selected as superior enzyme producers based on their enzyme activity profile. These candidates were identified and characterized based on biochemical evaluation tests using a bioMerieux API 50 CHB test kit (bioMerieux, Marcy l'Etoile, FRA). Selected candidates were also subjected to 16S rRNA sequence analysis in a specialized laboratory (Midi labs, Newark, DE, USA). Generally recognized as safe (GRAS) status of these three isolates were affirmed as described by Wolfenden et al., (2011). One of the three *Bacillus* strains (AM1002) was identified as *B. subtilis*, and the other two isolates (AM0938 and JD17) were identified as *B. amyloliquefaciens*.

Statistical analysis

Data from all measurements were subjected to One-way analysis of variance as a completely randomized design using the General Linear Models procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC) (SAS, 2002). Means were separated with Duncan's multiple-range test and considered significant at $P < 0.05$. Data were reported as mean ± standard error.

RESULTS

Determination of in vitro enzyme activity

Bacillus spores were isolate by heat treatment of intestinal, fecal and environmental samples eliminating the presence of vegetative cells. Although enzyme activity was detected for the majority of the strains, there were considerable differences in their relative enzyme activity values. Three of the thirty one screened *Bacillus spp.* strains showed a significantly higher REA value for amylase production in comparison to other bacterial colonies. Isolates AM1002, AM1012 and AM0905 obtained REA values of 6.3, 6.1 and 5.8 respectively, all of them categorizing these *Bacillus* isolates as excellent amylase producers (REA > 5.0). In the case of protease activity, strain AM0938 showed a REA value of 3.4 which is considered good (REA >2.0-5.0), surpassing the enzyme activity values of the other screened strains. Lipase synthesis was superior in the isolate AM1002 (REA = 3.0), meanwhile, phytase production was classified as good for the strains JD17 (REA = 2.3) and MM65 (REA = 2.5). A complete description of the enzyme activity profile of all the evaluated isolates and the appearance of each selective media are presented in Table 1 and Figure 1 respectively.

In vitro evaluation of antimicrobial activity

An overlay method was used to assess the production of antimicrobial compounds by the thirty one *Bacillus* strains against Gram-positive and Gram-negative enteropathogens (Table 2; Figure 2). Although antimicrobial activity was observed in a greater number of isolates, individual differences were evident in the degree of inhibition and spectrum of activity. In the case of *S.* Enteritidis, isolate NP122 generated the largest diameter of the zone of inhibition with 13.7 mm, followed by the strain AM0904 with a diameter of 12.0 mm. Activity against *E. coli* was more evident in isolates AM1010 and AM1012, both with a diameter of clearance of 20 mm. Interestingly, *C. difficile* was the most susceptible microorganism in presence of almost all *Bacillus spp.* strains, with an average zone of inhibition of 19 mm for the thirty one isolates, where the strain AM1010 produced larger pathogen inhibition activity with a diameter of clearance of 28 mm.

Biofilm synthesis

Biofilm production was evaluated by generation of an adherent crystal violet-stained ring in polypropylene tubes. All the screened *Bacillus spp.* strains produced biofilms, however, isolates AM0905, AM0933, AM0940, AM0941, AM1002, AM1011, AM1012, AM1109A, AM1109B, NP122 and MM65 were identified as strong biofilm formers with a wider and more colorful intense ring of adherence present on the wall of the test tubes (Table 2; Figure 3).

Characterization and selection of Bacillus-DFM candidates

Based on the REA results, three *Bacillus*-DFM candidates were selected with excellent to good REA values for each of the evaluated enzymes. These candidates were then identified and characterized using a bioMerieux API 50 CHB test kit (bioMerieux, Marcy l'Etoile, FRA). This

set of biochemical tests classified *Bacillus spp*. strains based on their capacity to metabolize 49 different carbohydrates (Table 3). According to the fermentation profile, all isolates were categorized as *Bacillus subtilis/amyloliquefaciens* with an identification percentage of 99.0 % or higher. To further assist in identification of the strains, each isolate was also subjected to 16S rRNA sequence analysis in a specialized laboratory (Midi labs, Newark, DE, USA). One of the three *Bacillus spp*. isolates (AM1002) was identified as *B. subtilis*, and the other two isolates (AM0938 and JD17) were identified as *B. amyloliquefaciens* (Table 4). Generally recognized as safe (GRAS) status of these three isolates were affirmed as described by Wolfenden et al. (2010).

DISCUSSION

Nowadays, poultry diets include a variety of ingredients from different plant and animal sources. Due to an increasing demand of cereal grains for production of biofuels, rising corn prices have had a direct impact on diet costs (Donohue and Cunninghan, 2009). Consequently, the necessity to reduce costs of production have required the inclusion of less digestible and more available raw materials in poultry diets. Distillers' dried grains with solubles (DDGS) are usually available to be included in the ingredient matrix, as a result of the continuous development of the ethanol industry (Loar et al., 2010). Additionally, alternative grains such as wheat, barley and sorghum have also increased their participation in the composition of poultry diets, however, it is important to mention that these feedstuffs often contain a higher concentration of non-starch polysaccharides in comparison to corn (Kundsen, 1997). To improve nutrient utilization and increase flexibility of the ingredient matrix used in poultry diets, multiple research have been performed evaluating the inclusion of different exogenous enzymes either alone or in diverse combinations (Choct et al., 1995; Avila et al., 2012). It has been well established that

incorporation of carbohydrases (xylanase, β-glucanase, or amylase), and phytase can reduce the adverse impact of anti-nutritional factors in monogastric animals fed with diets containing alternative grains (Cowieson and Adeola, 2005). Additionally, a growing interest based on the reduction of environmental pollution generated by livestock production has been one of the principal targets supporting the inclusion of enzymes in animal feed (Ghazi et al., 2003). Nevertheless, research results have been variable due to the different sources of exogenous enzymes under evaluation. Some of this enzymes are denature at acidic pH (Proventriculus) or do not resist high temperatures commonly used during feed pelletization. One of the principal sources of microbial enzymes are produced by bacteria from the genus *Bacillus* (Monisha et al., 2009; Ibrahim et al, 2012). For this reason in the present study, thirty one *Bacillus spp.* were screened for production of amylase, protease, lipase and phytase (Table 1). Three strains were selected based on superior REA values on at least one of the enzymes under evaluation. These results demonstrate that not all *Bacillus spp.* synthesize the same type of enzymes over time, suggesting that this capacity is a strain-specific characteristic (Figure 1). Additionally, the three selected *Bacillus spp*. strains candidates were identified as GRAS by biochemical tests and 16s rRNA sequencing analysis (Table 4), suggesting that they could be included in poultry diets under commercial conditions.

On the other hand, despite of the success showed by the development of the LAB probiotics for use in commercial poultry, there is still an urgent necessity for commercial DFM that are shelf-stable, cost-effective and feed-applicable to increase widespread utilization of viable substitutes of AGP in the poultry industry. In this regard, *Bacillus spp*. spores have been isolated from the gastrointestinal tract of multiple animal species, including poultry and pigs suggesting that this microorganism could be an active member of the host microflora (Barbosa et al., 2005; Guo et

al., 2006). Moreover, some *Bacillus spp.* endospores have been extensively studied as DFM, showing to be a safe and reliable prophylactic tool to diminish the presentation of gastrointestinal diseases in livestock and humans (La Ragione and Woodward, 2003; Duc et al., 2004; Hong et al., 2008). In the present study, the majority of the tested *Bacillus spp*. strains showed antimicrobial activity against different food-borne pathogens, including *S.* Enteriditis (25/31) and *E. coli* (27/31). This could be the result of the capacity of some *Bacillus* to synthesize antimicrobial compounds, compete for nutrients, and/or change the environmental conditions of the media (Figure 2). Furthermore, it was remarkable to observe that the most susceptible enteropathogen to the presence of almost all *Bacillus* isolates was *C. difficile* (28/31). This anaerobic sporeformer bacteria is the principal aetiological agent of nosocomial diarrhea in patients under antibiotic therapy, and it has also been isolated from animals and retail meat (Harvey et al., 2011; Colenutt and Cutting, 2014). Therefore, these results suggest that utilization of selected *Bacillus*-DFM may be a suitable alternative to reduce the incidence of bacterial gastrointestinal diseases in humans and animals, including cases of *C. difficile* infection. However, as observed in the enzyme production profile, the ability to produce antimicrobial compounds appear to be a specific feature for each *Bacillus spp.* isolate (Table 2).

In the case of biofilm formation, it is possible that this polysaccharide structure serve as a mechanism of survival for some *Bacillus* isolates to resist the harsh environmental conditions of the gastrointestinal tract. Additionally, generation of biofilms could help *Bacillus* cells to be attached to the gut epithelia, therefore, increasing their persistence in the intestinal mucosa, as well as, preventing adherence of enteropathogens. (Barbosa, et al., 2005). Results of the biofilm assay in the present study classified (11/31) *Bacillus spp.* strains as superior biofilm formers,

suggesting that these isolates could probably remain for a longer period in the gastrointestinal tract (Table 2; Figure 3).

In summary, our results confirm that *Bacillus spp*. isolates differ in their capacity to produce enzymes, antimicrobial compounds and biofilms even if they are from the same species. Therefore, an exhaustive selection process must be performed according to the purpose the DFM is going to be used. *Bacillus* strains selected as superior enzyme producer were different from the isolates showing the highest antimicrobial activity, however, all *Bacillus* isolates showed certain pathogen-inhibition activity. As a result, it is expected that the consumption of the *Bacillus*-DFM candidate selected in this study based on enzyme activity profiles, may contribute to enhanced performance parameters by improving nutrient digestibility, maintaining a balanced microflora and promoting healthy intestinal integrity in poultry consuming conventional and high NSP diets.

Table 1. Relative enzyme activity (REA)[a] values produced by *Bacillus spp*. strains evaluated as enzyme producer candidates

***Bacillus* isolates[b]**	**Amylase**	**Protease**	**Lipase**	**Phytase**
AM0902	1.0 ± 0.00	1.0 ± 0.00	1.9 ± 0.15	1.0 ± 0.00
AM0904	5.3 ± 0.19	2.7 ± 0.08	2.3 ± 0.06	1.2 ± 0.07
AM0905	5.8 ± 0.44 *	3.0 ± 0.26	2.7 ±0.17	1.6 ± 0.24
AM0908	5.3 ± 0.06	2.1 ± 0.08	2.3 ±0.07	1.4 ± 0.10
AM0923	5.7 ± 0.19	2.8 ± 0.04	2.2 ±0.26	1.5 ± 0.02
AM0933	5.3 ± 0.21	2.3 ± 0.09	2.1 ±0.07	1.3 ± 0.07
AM0934	4.5 ± 0.18	3.1 ± 0.34	2.4 ±0.35	1.2 ± 0.08
AM0938	5.0 ± 0.50	3.4 ± 0.30 *	2.7 ±0.17	2.1 ± 0.08
AM0939	3.9 ± 0.12	2.9 ± 0.44	2.2 ±0.12	1.4 ± 0.13
AM0940	5.9 ± 0.27	1.8 ± 0.19	2.4 ±0.21	1.4 ± 0.12
AM0941	1.0 ± 0.00	1.7 ± 0.40	2.8 ±0.27	2.0 ± 0.12
AM1002	6.3 ± 0.12 *	2.8 ± 0.15	3.0 ±0.35 *	2.1 ± 0.11
AM1010	5.7 ± 0.16	2.1 ± 0.11	2.6 ±0.21	1.5 ± 0.12
AM1011	4.4 ± 0.30	3.0 ± 0.13	2.5 ±0.29	1.3 ± 0.10
AM1012	6.1 ± 0.18 *	2.5 ± 0.15	2.3 ±0.17	1.4 ± 0.02
AM1013	4.1 ± 0.08	2.3 ± 0.09	2.0 ±0.09	1.3 ± 0.05
AM1109A	2.7 ± 0.27	1.8 ± 0.10	2.2 ±0.11	1.4 ± 0.11
AM1109B	1.8 ± 0.42	1.0 ± 0.00	2.4 ±0.21	1.4 ± 0.07
B2/53	4.0 ± 0.64	2.7 ± 0.16	2.5 ±0.08	1.6 ± 0.05
BL	2.2 ± 0.13	1.0 ± 0.00	1.0 ±0.00	1.0 ± 0.00
JD17	4.0 ± 0.29	2.9 ± 0.20	2.6 ±0.11	2.3 ± 0.15 *
JD19	3.4 ± 0.33	2.1 ± 0.17	2.2 ±0.12	1.5 ± 0.01
NP001	4.3 ± 0.19	2.3 ± 0.14	1.9 ±0.11	1.1 ± 0.04
NP002	3.0 ± 0.40	2.3 ± 0.29	2.1 ±0.11	1.2 ± 0.12
NP117B	2.7 ± 0.48	3.0 ± 0.06	2.1 ±0.14	1.3 ± 0.12
NP121	3.1 ± 0.46	2.2 ± 0.13	2.0 ±0.09	1.5 ± 0.14
NP122	4.7 ± 0.36	2.8 ± 0.40	2.3 ±0.15	1.3 ± 0.12
NP124	1.6 ± 0.40	2.1 ± 0.29	2.2 ±0.12	1.1 ± 0.00
NP126	3.3 ± 0.23	2.5 ± 0.15	2.2 ±0.12	1.2 ± 0.07
MM65	3.8 ± 0.31	1.0 ± 0.00	3.0 ±0.22	2.5 ± 0.06 *
RW41	4.2 ± 0.88	1.3 ± 0.11	2.0 ±0.04	1.2 ± 0.04

* Identified bacterial strains as superior enzyme producers with a higher REA value $P < 0.05$

[a] REA was calculated dividing the diameter of area of clearance by the diameter of the *Bacillus* colony. Organism were classified as excellent (REA > 0.5), good (REA >2.0-5.0), or poor (REA < 2.0) enzyme producers. Data expressed as mean ± SE.

[b] All *Bacillus spp*. isolates were tested by triplicate.

(a) (b) (c) (d)

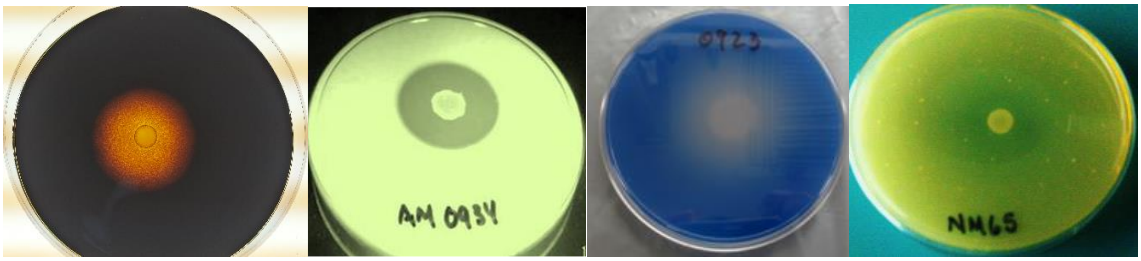

Figure 1. Representative examples of microbial enzyme activity using a different selective media for each enzyme under evaluation. An area of clearance around a bacterial colony can be observed, representing enzyme production of (a) Amylase, (b) Protease, (c) Lipase, and (d) Phytase. All *Bacillus spp*. strains were screened by triplicate.

(a) (b) (c)

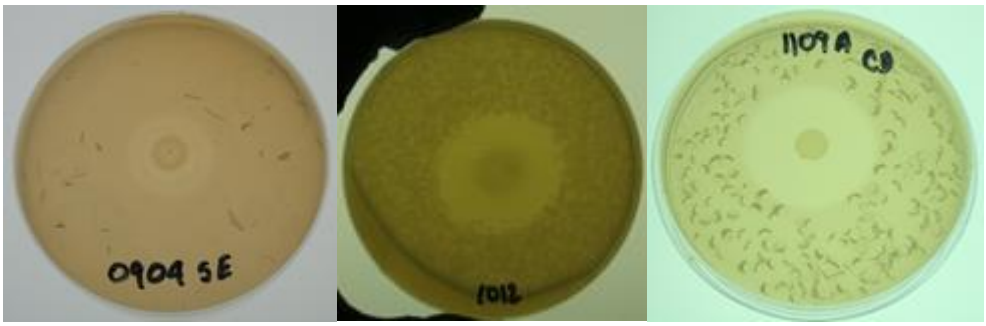

Figure 2. Evaluation of antimicrobial activity from different *Bacillus spp.*isolates using an overlay method. A zone of inhibition is shown surrounding a tested bacterial colony located in the middle of the plate against (a) *S.* Enteritidis, (b) *E. coli* and (c) *C. difficile*. All *Bacillus spp*. strains were screened by triplicate.

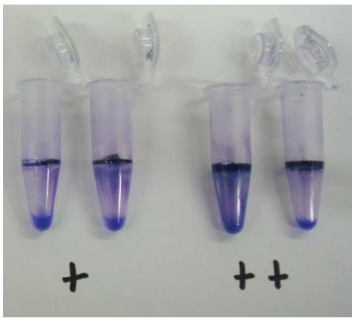

Figure 3. Determination of biofilm synthesis was performed using a crystal violet staining method. Measurement of biofilm synthesis was based on color intensity and size of the adherent crystal violet ring with a score ranging from negative (-) to strong (++) biofilm formation. All *Bacillus spp*. strains were screened by triplicate.

Table 2. Evaluation of Antimicrobial activity[a] and biofilm synthesis[b] of different *Bacillus spp*. isolates.

Bacillus isolates	*S*. Enteritidis (mm)	*E. coli* (mm)	*C. difficile* (mm)	Biofilm formation
AM0902	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	+
AM0904	12.0 ± 0.38 *	16.0 ± 2.31	26.0 ± 1.86	+
AM0905	6.7 ± 0.67	14.0 ± 1.15	20.3 ± 1.67	++
AM0908	6.0 ± 0.56	4.3 ± 0.33	22.0 ± 2.31	+
AM0923	7.7 ± 0.30	10.0 ± 3.06	24.0 ± 3.06	+
AM0933	1.3 ± 0.33	4.0 ± 0.58	10.0 ± 1.15	++
AM0934	6.3 ± 0.40	8.7 ± 1.76	22.7 ± 2.40	+
AM0938	8.0 ± 1.15	10.0 ± 2.00	22.0 ± 2.00	+
AM0939	6.3 ± 0.88	8.3 ± 1.33	26.0 ± 2.60	+
AM0940	8.0 ± 1.12	10.3 ± 1.67	21.0 ± 1.76	++
AM0941	0.7 ± 0.27	0.0 ± 0.00	0.0 ± 0 .00	++
AM1002	5.7 ± 0.58	8.7 ± 1.76	16.0 ± 2.08	++
AM1010	8.0 ± 1.10	20.0 ± 1.45 *	28.0 ± 2.67 *	+
AM1011	8.5 ± 0.90	10.7 ± 1.76	20.3 ± 2.33	++
AM1012	8.7 ± 0.88	20.0 ± 2.19 *	10.0 ± 1.75	++
AM1013	4.0 ± 1.15	10.0 ± 1.15	22.0 ± 1.15	+
AM1109A	10.3 ± 1.20	12.0 ± 1.50	24.0 ± 1.11	++
AM1109B	0.3 ± 0.33	0.0 ± 0.00	14.7 ± 1.62	++
B2/53	10.3 ± 1.20	12.0 ± 0.58	26.0 ± 3.08	+
BL	0.0 ± 0.00	4.0 ± 0.52	10.0 ± 2.00	+
JD17	6.3 ± 0.33	10.0 ± 1.15	20.6 ± 3.53	+
JD19	2.0 ± 0.58	2.7 ± 0.67	19.0 ± 1.72	+
NP001	8.0 ± 0.88	6.0 ± 0.58	12.0 ± 1.13	+
NP002	4.3 ± 1.33	6.0 ± 1.10	20.7 ± 2.40	+
NP117B	2.7 ± 0.67	6.0 ± 1.15	18.0 ± 3.46	+
NP121	2.3 ± 0.33	14.0 ± 3.06	16.0 ± 2.31	+
NP122	13.7 ± 1.86 *	12.0 ± 2.00	26.0 ± 4.16	++
NP124	6.0 ± 1.73	12.0 ± 1.86	22.0 ± 2.03	+
NP126	0.3 ± 0.30	2.0 ± 1.89	21.7 ± 1.76	+
MM65	8.0 ± 0.55	10.0 ± 1.15	20.3 ± 1.45	++
RW41	5.7 ± 0.88	10.0 ± 2.00	22.0 ± 2.28	+

* Identified bacterial strains with the enhanced antimicrobial activity $P < 0.05$

[a] Represents the diameter of the zone of inhibition observed at 24 h of incubation without the diameter of the bacterial colony. Data expressed as mean ± SE.

[b]The qualitative measurement of biofilm synthesis was based on color intensity and size of the adherent crystal violet ring with a score ranging from negative (-) to strong (++) biofilm formation. All *Bacillus spp*. isolates were tested by triplicate.

Table 3. Characterization of selected *Bacillus*-DFM candidate strains based on biochemical carbohydrate metabolism tests[ab]

Item	AM1002	AM0938	JD17
Amidon (starch)	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
D-Adonitol	-	-	-
D-Arabinose	-	-	-
D-Arabitol	-	-	-
D-Cellobiose	+	+	+
D-Fructose	+	+	+
D-Fucose	-	-	-
D-Galactose	-	-	-
D-Glucose	+	+	+
D-Lactose (bovine origin)	+	+	+
D-Lyxose	-	-	-
D-Maltose	+	+	+
D-Mannitol	+	+	+
D-Mannose	+	+	+
D-Melezitose	-	-	-
D-Melibiose	+	-	+
D-Raffinose	+	+	+
D-Ribose	+	+	+
D-Saccharose (sucrose)	+	+	+
D-Sorbitol	+	+	-
D-Tagatose	-	-	-
D-Trehalose	+	+	+
D-Turanose	-	-	-
Dulcitol	-	-	-
D-Xylose	+	+	+
Erythritol	-	-	-
Esculin (ferric citrate)	+	+	+
Gentibiose	+	+	-
Glycerol	+	+	+
Glycogen	+	+	+
Inositol	+	+	+
Inulin	+	-	-
L-Arabinose	+	+	+
L-Arabitol	-	-	-
L-Fucose	-	-	-
L-Rhamnose	-	-	-
L-Sorbose	-	-	-
L-Xylose	-	-	-

Table 3. Characterization and identification of selected *Bacillus*-DFM candidate strains based on biochemical carbohydrate metabolism tests[ab] (Continue)

Item	AM1002	AM0938	JD17
Methyl-αD-glucopyranoside	+	+	+
Methyl-αD-mannopyranoside	-	-	-
Methyl-βD-xylopyranoside	-	-	-
N-Acetylglucosamine	-	-	-
Potassium 2-Ketogluconate	-	-	-
Potassium 5-Ketogluconate	-	-	-
Potassium gluconate	-	-	-
Salicin	+	+	+
Xylitol	-	-	-

[a]BioMerieux API50 CHB test kit (bioMerieux, Marcy l'Etoile, FRA)
[b]Different scores (+ or -) reflect the capacity of the tested *Bacillus spp*. isolate to ferment an specific carbohydrate or carbohydrate derivative.

Table 4. Identification of *Bacillus*-DFM candidates showing the highest enzyme by bioMerieux API 50 CHB[a] and 16S rRNA sequence analyses[b]

Isolate	API50 CHB		16S rRNA sequence analysis	
	Taxon	**% ID**	**Closest match**	**% ID**
AM1002	*Bacillus subtilis/amyloliquefaciens*	99.2	*Bacillus subtilis*	100.0
AM0938	*Bacillus subtilis/amyloliquefaciens*	99.0	*Bacillus amyloliquefaciens*	99.7
JD17	*Bacillus subtilis/amyloliquefaciens*	99.4	*Bacillus amyloliquefaciens*	99.6

[a] BioMerieux API 50 CHB test kit.
[b] 16S rRNA sequence analysis.

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V. CHAPTER III

Selection of *Bacillus spp.* for cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and *Clostridium perfringens* proliferation using an *in vitro* digestive model in different poultry diets

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ABSTRACT

Previously, our laboratory has screened and identified *Bacillus spp.* isolates as direct-fed microbials (DFM). The purpose of the present study was to evaluate the cellulase and xylanase production of these isolates and select the most appropriate *Bacillus spp.* candidates for DFM. Furthermore, an *in vitro* digestive model, simulating different compartments of the gastrointestinal tract, was used to determine the effect of these selected candidates on digesta viscosity and *Clostridium perfringens* proliferation in different poultry diets. Production of cellulase and xylanase were based on their relative enzyme activity. Analysis of 16S rRNA sequence classified two strains as *B. amyloliquefaciens* and one of the strains as *B. subtilis*. The DFM was included at a concentration of 10^8 spores/g of feed in 5 different sterile soybean-based diets containing corn, wheat, rye, barley, or oat. After digestion time, supernatants from different diets were collected to measure viscosity, and *C. perfringens* proliferation. Additionally, from each *in vitro* simulated compartment, samples were taken to enumerate viable *Bacillus*-spores using a plate count method after heat-treatment. Significant ($P<0.05$) DFM-associated reductions in supernatant viscosity and *C. perfringens* proliferation were observed for all non-corn diets. These results suggest that antinutritional factors such as non-starch polysaccharides from different cereals can enhance viscosity and *C. perfringens* growth. Remarkably, dietary inclusion of the DFM that produce cellulase and xylanase reduced both viscosity and *C. perfringens* proliferation compared with control diets. Regardless of diet composition, 90% of the DFM spores germinated during the first 30 min in the crop compartment of the digestion model, followed by a noteworthy increased in the intestine compartment by ~2 $\log_{10}$, suggesting a full-life cycle development. Further studies to evaluate *in vivo* necrotic enteritis effects are in progress.

Keywords: *Clostridium perfringens*, *Bacillus*-DFM, spore, enzymes, viscosity

INTRODUCTION

Necrotic enteritis (NE) in broilers is a multi-factorial disease with severe economic implications (Hofacre, 2001). It is caused by type A strains of *Clostridium perfringens* that are specific to poultry with toxin types alpha and NetB (Keyburn et al., 2006; Keyburn et al., 2008). Coccidia infections are the most common pre-requisite for NE to occur (Schoepe et al., 2001), however, dysbacteriosis associated with diet ingredients, changes in feed ration, immunosuppression, *Salmonella* infections, and/or removal of the use of quimioterapeutics are known to predispose birds to NE (Shivaramaiah et al., 2011b). Antibiotic growth promoters (AGPs) are commonly used to mitigate the incidence of enteric diseases such as NE. Nevertheless, concerns regarding the development of antibiotic-resistant microorganisms and social pressures have led to a tendency to ban AGPs in poultry production (Castanon, 2007). In this scenario, there is an imperative necessity to find feasible alternatives for AGPs to maintain poultry health (Alvarez-Olmos and Oberhelman, 2001). In fact, the use of selected strains of various beneficial microorganisms from the genus *Bacillus* and *Lactobacillus* have shown to be a suitable option for the poultry industry (Tellez et al., 2012). *Bacillus spp.* are gram-positive, aerobe, motile, and usually found in soil and water sources, as well as in the gastrointestinal tract of animals and humans (Hong et al., 2009). Different *Bacillus spp.* have already been studied and extensively used as a source of industrial enzymes as well as antibiotics by biotechnology companies (Kunst, 1997). However, the production of most of these enzymes depends on the intense metabolic changes associated with environmental conditions (Gonzalez-Pastor et al., 2003; Hong et al., 2005; López et al., 2009). During extreme environmental conditions, vegetative cells of *Bacillus*

spp. form endospores, which are considered, the toughest way of life on Earth (Vreeland et al., 2000).

The use of spores from selected *Bacillus* strains, as direct-fed microbials (DFM), are shown to have the capacity to germinate and sporulate in the gastrointestinal tract of different animal species including poultry. Thus, they become metabolically active *in vivo*, imparting numerous nutritional benefits including the production of extracellular enzymes such as protease, lipase, cellulase, xylanase, phytase and keratinase (Hendricks et al., 1995; Sen et al., 2012) and other chemical compounds beneficial for the host (Jadamus et al., 2001).

In most of the U.S.A. and other countries, including Brazil, broiler feed is based primarily on corn and soybean meal. However, sometimes it is difficult to formulate least-cost diets using corn. Consequently, other cereals or ethanol by-products with variable concentrations of antinutritional factors are used as alternatives. When chickens are fed alternative grains with high levels of non-starch polysaccharides (NSP), an increase in digesta viscosity, poor nutrient digestibility, reduced bone mineralization and occurence of enteric diseases such a NE have been reported (MacAuliffe et al., 1976; Choct et al., 1996). Hence, utilization of these feedstuffs in poultry diets usually result in decreased growth performance, intestinal dysbacteriosis, and detrimental litter conditions caused by sticky droppings (Fengler and Marquardt, 1988; Bedford and Classen, 1993). For that reason, the inclusion of enzymes such as carbohydrases is a routine practice in poultry diets that contain grains with elevated NSP concentration values in comparison to corn (Adeola and Cowieson, 2011; Slominski, 2011). However, there are inconveniences related to dietary inclusion of some enzymes, due to denaturation and lost of activity under high pelletization temperatures commonly used in poultry rations. Therefore, the objective of the present study was to perform a selection of *Bacillus spp.* for cellulase and

xylanase production as direct-fed microbials, and evaluate them on digesta viscosity and *Clostridium perfringens* proliferation in different poultry diets using an *in vitro* digestive model. The practical implication of the results will be to utilize cost effective alternative grains in poultry feed formulation, and at the same time improve digestibility as well as production performance in birds using a more thermostable DFM product.

MATERIALS AND METHODS

Diets

Five mash soybean-based broiler grower diets containing different cereals such as corn, wheat, rye, barley or oat were used as substrate for bacterial growth during the *in vitro* digestive model. Experimental diets were formulated to approximate the nutritional requirements of broiler chickens as recommended by the NRC (NRC, 1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc,. 2013). No antibiotics, coccidiostats or enzymes were added to the feed (Table 1). All diets were autoclaved and confirmed negative for *Bacillus* spp. spores. Later, these diets were inoculated with the respective spores (10^8 spores/g of feed) of the *Bacillus*-DFM candidate according to various treatments.

In vitro assessment of cellulase and xylanase production

Previous research conducted in our laboratory focused on isolation of several *Bacillus spp.* from environmental and poultry sources (Shivaramaiah et al., 2011a; Menconi et al., 2013). Isolates were then screened for production of cellulase and xylanase. For evaluation of cellulase activity, the cellulose-Congo red agar was used and consisted of 0.50 g of K_2HPO_4 (Fisher Scientific, San Francisco, CA, USA), 0.25 g of $MgSO_4$ (Sigma Chemical Co, St. Louis, MO, USA), 1.88 g of

ashed, acid-washed cellulose powder (J. T. Baker Chemical Inc, Phillipsburg, NJ, USA), 0.20 g of Congo red (J. T. Baker Chemical Inc, Phillipsburg, NJ, USA), 20 g of noble agar (Difco Laboratories, Detroit, MI, USA), and 1000 mL of distilled water (Hendricks et al., 1995). For evaluation of xylanase activity, the medium used to screen *Bacillus* isolates contained 3 g of $NaNO_3$, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.02 g of $MnSO_4.H_2O$, 0.02 g of $FeSO_4.H_2O$, 0.02 g of $CaCl_2.2H_2O$ with 20 g of noble agar (Difco Laboratories, Detroit, MI, USA), and 1000 mL of distilled water. Besides, 1 g yeast extract and 5 g beechwood xylan (Sigma Chemical Co, St. Louis, MO, USA) were used as carbon sources (Monisha et al., 2009). During the screening process, 10 µL of each *Bacillus* isolate were placed on the centre of each plate containing cellulose or xylan media. After 24 h of incubation at 37 °C, all plates were evaluated and the diameters of the zones of clearance were measured removing the diameter of the bacterial colony. The relative enzyme activity (REA) was calculated by using the formula: REA = Diameter of zone of clearance divided by the diameter of the bacterial colony in millimetres (mm). Based on REA test in each group, organisms were categorized in to excellent (REA>5.0), good (REA>2.0 to 5.0) or poor (REA<2.0) relative enzyme activity (Jani et al., 2012). Each *Bacillus* strain was evaluated by triplicate, and the average measurements are presented in Table 2.

DFM culture identification

Based on the REA results, three *Bacillus*-DFM candidates with excellent to good REA were selected. These candidates were then identified and characterized using a bioMerieux API 50 CHB test kit (bioMerieux, Marcy l'Etoile, FRA). Individual strain were also subjected to 16S rRNA sequence analysis to a specialized laboratory (Midi labs, Newark, DE, USA). Generally

recognized as safe (GRAS) status of these three isolates were affirmed as described by Wolfenden et al. (2011). One of the three *Bacillus* strains (AM1002) was identified as *B. subtilis*, and the other two isolates (AM0938 and JD17) were identified as *B. amyloliquefaciens* (Table 3). Following the identification, all three *Bacillus* candidate strains were sporulated and mixed in equal amounts during the *Bacillus*-DFM preparation process as described below and incorporated to the experimental diets.

Preparation of Spore-based DFM

In an effort to grow high numbers of viable spores, modified version of a solid state fermentation media (SS) developed by Zhao et al. (2008) was used. Briefly, to prepare the SS fermentation media, ammonia broth was added to a mixture of 70% rice straw and 30% wheat bran at the rate of 40% by weight. Then, the SS fermentation media was added to 250 mL Erlenmeyer flasks and sterilized by autoclaving for 30 min at 121°C. Each of the three *Bacillus* strains candidates was grown, individually, overnight at 37 °C in test tubes containing 10 mL of tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA). After incubation, 2 mL of each candidate culture were added separately to the previously prepared SS fermentation media flasks. The inoculated flasks were incubated for 24 h at 37 °C to promote growth of the *Bacillus spp.* candidates, and incubated for another 72 h at 30 °C to trigger the initiation of the sporulation process. Following this, the inoculated SS fermentation media was removed from the Erlenmeyer flasks, placed onto Petri dishes, and dried at 60 °C for 18 h. Then, the SS fermentation media was aseptically ground into a fine powder that contained stable *Bacillus* spores (~ 10^{11} spores/g). 1 g of spores from each isolate (1:1:1) was combined to produce the *Bacillus*-DFM candidate final product containing ~ 3 x 10^{11} spores/g. *Bacillus*-DFM candidate was included into each experimental diet to reach a

final concentration of 10^8 spores/g using a rotary mixer for 15 min. Samples of feed containing the DFM candidate were subjected to 100°C for 10 min to eliminate vegetative cells and validate the amount of spores per g of feed after inclusion and mixing steps. Following heat-treatment, 10-fold dilutions of the same feed samples from the glass tubes were plated on tryptic soy agar plates (TSA, Becton Dickinson, Sparks, MD, USA); letting spores in the feed sample germinate to vegetative cells after incubation at 37 °C for 24 h, hence representing the number of spores present per g of feed.

Clostridium perfringens strain

A strain of *C. perfringens* previously described in a NE challenge model was kindly donated by Dr. Jack. L. McReynolds, USDA-ARS, College Station, TX (McReynolds et al., 2004). A frozen aliquot was shipped on ice to our laboratory and was amplified in TSB with sodium thioglycolate (Sigma-Aldrich, St Louis, MO, USA). The broth culture was plated on phenylethyl alcohol agar plates (PEA, Becton Dickinson, Sparks, MD, USA) with 5 % sheep blood (Remel, Lenexa, KS, USA) to confirm purity, aliquots were made with 25 % sterile glycerol and stored at -80 °C until further use. A single aliquot was individually amplified in TSB with sodium thioglycolate overnight for the *in vitro* proliferation studies and the final dose was confirmed by plating 10-fold dilutions on TSA plates with sodium thioglycolate.

In vitro assessment of antimicrobial activity against Clostridium perfringens

The three *Bacillus* isolates present in the *Bacillus*-DFM candidate treatment were individually cultured aerobically overnight on TSA and screened for *in vitro* antimicrobial activity against *C. perfringens* as reported previously (Layton et al., 2013). Briefly, 10 µL of each *Bacillus* isolate

were placed on the centre of TSA plates, and incubated for 24 h at 37 °C. Then the plates with visible *Bacillus* colonies were overlaid with TSA with sodium thioglycolate containing 10^6 colony forming units (cfu) per mL of *C. perfringens*, and all plates were incubated anaerobically at 37 °C. After 24 h of incubation, all plates were evaluated and the diameters of the zones of inhibition were measured removing the diameter of the bacterial colony. Each *Bacillus* strain was evaluated by triplicate, and the average measurements of antimicrobial activity against *C. perfringens* are presented in Table 2.

In vitro digestion assay

The *in vitro* digestion model used in the present study was based on previous publications, with minor modifications (Zyla et al., 1995; Annett et al., 2002), and the assay was performed with five different experimental diets, with or without *Bacillus*-DFM candidate, in quintuplicates. Briefly, for all the gastrointestinal compartments simulated during the *in vitro* digestion model, a biochemical oxygen demand incubator (VWR, Houston, TX, USA) set at 40^{o}C (to simulate poultry body temperature), customized with an standard orbital shaker (19rpm; VWR, Houston, TX, USA) was used for mixing the feed content. Additionally, all tube samples were held at an angle of 30^o inclination to facilitate proper blending of feed particles and the enzyme solutions in the tube. The first gastrointestinal compartment simulated was the crop, where 5 g of feed and 10 ml of 0.03 M hydrochloric acid (HCL, EMD Millipore Corporation, Billerica, MA, USA) were placed in 50 mL polypropylene centrifuge tubes and mixed vigorously reaching a pH value around 5.2. Tubes were then incubated for 30 min. Following this time, all tubes were removed from the incubator. To simulate the proventriculus as the next gastrointestinal compartment, 3000 U of pepsin per g of feed (Sigma-Aldrich, St Louis, MO, USA) and 2.5 mL of 1.5 M HCl

were added to each tube to reach a pH of 1.4 to 2.0. All tubes were incubated for additional 45 min. The third and final step was intended to simulate the intestinal section of the gastrointestinal tract. For that, 6.84 mg of 8 x pancreatin (Sigma-Aldrich, St Louis, MO, USA) in 6.5 mL of 1.0 M sodium bicarbonate (Sigma-Aldrich, St Louis, MO, USA) were added, and the pH was adjusted to range between 6.4 and 6.8 with 1.0 M sodium bicarbonate. All tube samples were further incubated for 2 h. Hence, the complete *in vitro* digestion process took 3 h and 15 min. After the digestion, supernatants from all the diets were obtained by centrifugation for 30 min at 2000 g. All supernatants were then tested for viscosity and *C. perfringens* proliferation as described below.

Viscosity

Viscosity was measured using a Brookfield digital cone-plate viscometer fitted with a CP-40 spindle (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA). From each supernatant, 0.5 mL were taken to measure viscosity at a shear rate of 42.5 sec^{-1} at 40 °C to mimic body temperature of poultry. Viscosity was evaluated by quintuplicate per diet with or without inclusion of the *Bacillus*-DFM candidate and reported in centipoise (cP = 1/100 dyne s/cm^2).

Clostridium perfringens proliferation

Proliferation of *C. perfringens* was performed according to previously published methods with minor modifications (Annett et al., 2002). A suspension of 10^5 cfu per mL of *C. perfringens* was added to five replicates of each of the following groups: 1) 6 mL TSB with sodium thioglycolate as a positive control group; 2) 3 mL TSB with sodium thioglycolate plus 3 mL supernatant from

each digested control non-DFM diet; 3) 3 mL TSB with sodium thioglycolate plus 3 mL supernatant from digested diets supplemented with *Bacillus*-DFM. Samples were incubated anaerobically at 40 °C, with tubes set at 30^{o} angle with constant shaking (200 rpm) for 4h. After incubation, 10-fold serial dilutions were made from all treatment groups in 0.85 % sterile saline. Then, 10 µL was plated on TSA with sodium thioglycolate and incubated for 24 h at 40 °C, anaerobically. Results were expressed as log10 cfu of *C. perfringens*/mL.

In vitro determination of spore persistence

Persistence of the *Bacillus*-DFM spores in the *in vitro* digestive model was also evaluated (five replicates per diet treatment). At each time point during the digestive simulation process (crop, proventriculus and intestine) 0.2 mL were immediately loaded into 0.5 mL sterile centrifuge tubes and heat-treated (pasteurized) at 75 °C for 10 min to eliminate the presence of vegetative cells (Barbosa et al., 2005). After pasteurization, samples were loaded into sterile 96-well flat bottom plate and 10-fold dilutions were made and plated on TSA. Plates were incubated for 24 h at 37^{o}C on aerobic conditions to enumerate spores/g of sample.

Statistical analysis

Data from all measurements were subjected to One-way analysis of variance as a completely randomized design using the General Linear Models procedure of SAS (SAS version 9.1) (SAS, 2002). Means were separated with Duncan's multiple-range test at $P < 0.05$ considered as significant. Data were reported as mean ± standard error.

RESULTS

Isolates AM1002, AM0938, and JD17 were selected from a pooled of *Bacillus* isolates in our laboratory, based on the REA values for cellulose and xylanase, and the zone of inhibition for *C. perfringens* (Table 2). Isolate AM1002 showed a REA value of 6.2 and AM0938 showed a REA value of 5.1, both considered excellent REA values (>5.0) for cellulase activity (Jani et al., 2012); additionally, isolate JD17 showed a REA value of 4.7, which is considered good (>2.0 to 5.0) for cellulase production. A similar trend was observed for xylanase activity where isolate AM1002 showed a REA value of 6.3 (excellent); AM0938 showed a REA value of 4.8 (good), and isolate JD17 showed a REA value of 4.0 (good) for xylanase production. In the case of antimicrobial activity against *C. perfringens*, isolate AM0938 generated the largest diameter of the zone of inhibition with 14 mm, followed by isolates AM1002 and JD17 with 12 and 8 mm respectively. Although enzyme production and antimicrobial activity were observed for all the isolates, individual differences were evident even in bacteria of the same species (Table 2). The API 50 CHB system characterized all three isolates as *B. subtilis/amyloliquefaciens* (Table 3). Analysis of 16S rRNA sequence classified two strains (AM0938, JD17) as *B. amyloliquefaciens* and one of the strains (AM1002) as *B. subtilis*, which was consistent with the results observed by the carbohydrate fermentation profile of the biochemical test.

The results of the evaluation of digesta viscosity of different diets with or without inclusion of a *Bacillus*-DFM candidate after *in vitro* digestion are summarized in Table 4. An evident increase in viscosity was observed in soybean-based diets containing wheat, barley, rye and oats when compared to corn, being rye and oat diets with the highest viscosity values. However, it was noteworthy to observe that dietary inclusion of the *Bacillus*-DFM candidate significantly ($P < 0.05$) reduced viscosity in all diets containing cereals different to corn in comparison to control diets without DFM inclusion (Table 4).

Table 5 summarizes the results of the proliferation of *C. perfringens* in the supernatant from different digested diets with or without inclusion of a *Bacillus*-DFM candidate. A significant increase in *C. perfringens* proliferation was observed in supernatants collected from control diets that contained wheat, barley, rye and oat compared to the TSB positive control group. Startlingly, dietary inclusion of a *Bacillus*-DFM candidate in non-corn diets significantly reduced *C. perfringens* proliferation when compared to the control non-DFM supplemented diets. The corn-based diet showed similar cfu values of *C. perfringens* with or without inclusion of the *Bacillus*-DFM candidate.

Persistence of the *Bacillus*-DFM candidate spores in the different gastrointestinal compartments simulated in the *in vitro* digestive model is presented in Table 6. Regardless of diet composition, on average, a reduction of more than half of a $\log_{10}$ was observed in the crop compartment during the first 30 min of incubation, and it was followed by a further significant ~2 $\log_{10}$ reduction of spore counts in the proventriculus. Remarkably, in all diets, a significant increment in spore numbers, ~2 $\log_{10}$ was observed during the final digestion step simulating intestinal conditions (Table 6).

DISCUSSION

High-energy diets have been utilized to maximize growth during starter, grower and finisher phases of production. Consequently, the primary dietary energy sources in commercial broiler diets have been traditional cereal grains such as corn and sorghum. However, with the recent price volatility of common feed ingredients, the animal industry seeks alternative grains or industry by-products to include in diet formulations (Friesen et al., 1992; Kiarie et al., 2013). Wheat, barley, rye, and oat, contain lower bioavailable energy, and elevated NSP levels in

comparision to corn are alternative options (Bach Kundsen, 1997). However, these cereals have limited use in monogastric diets, because often high inclusion results in relatively poor performance, detrimental litter conditions, and increase predisposition for NE (Bedford et al., 1991; Choct, et al., 1995; Mahmood et al., 2014). Hence, supplemental carbohydrases such as NSP-degrading enzymes have allowed to increase the utilization of these alternative ingredients by reducing their antinutritional effects (Shirzadi et al., 2010; Zou et al., 2013). The carbohydrase market is accounted by two dominant enzymes: xylanases and cellulases. Other commercially available carbohydrases include α-amylase, α-galactosidase, β-glucanase, β-mannanase, and pectinase (Bedford and Schulze, 1998).

In the present study, the *Bacillus spp.* strains that conform the DFM candidate treatment were identified as either *B. subtilis* or *B. amyloliquefaciens* (Table 3), therefore being feasible for *in vivo* evaluation studies as they are generally recognized as safe (GRAS) candidates (Shivaramaiah et al., 2011a). Furthermore, the three selected *Bacillus spp.* isolates showed a variable ability to produce cellulase and xylanase (Table 2), hence, in addition to the benefits that spores or vegetative cells can provide as probiotics (Hong et al., 2009), they may improve the digestibility of cereals with high soluble NSP (Wang et al., 2005).

The *Bacillus*-DFM candidate treatment also demonstrated effective antimicrobial properties against *C. perfringens* which could be due to production of antimicrobial like-compounds and/or competition for nutrients (Tables 2 and 5). Little is known about the mechanisms underlying the higher incidence of NE in broilers fed diets containing cereals with elevated levels of NSP, but it could be related to a prolonged feed rate of passage and a reduction in the digestion of nutrients that later in the hind gut will be available for bacteria to growth (Palliyeguru and Rose, 2014).

For *in vitro* evaluation of *C. perfringens* proliferation, TSB with sodium thioglycolate (positive

control) groups were included. In the TSB group (positive control) the *C. perfringens* inoculum was increased ~ 0.5 $\log_{10}$, after 4 h of incubation. However, it was interesting to observe a significant increase in *C. perfringens* proliferation in the supernatants collected from control non-DFM diets that contained wheat, barley, rye and oat, compared with the enrichment TSB medium with sodium thioglycolate group (Table 5).

These results suggest that partial digestion of NSP grains and increased digesta viscosity provides a favorable nutritional environment that supports the growth of *C. perfringens*. Interestingly, dietary inclusion of a *Bacillus*-DFM candidate in non-corn diets significantly reduced both viscosity (Table 4) and *C. perfringens* proliferation (Table 5), when compared to control diets without DFM inclusion. This result shows the capacity of certain *Bacillus* isolates to inhibit the growth of pathogenic bacteria like *C. perfringens,* probably due to competition for nutrients, production of antimicrobial-like compounds or changes in environmental conditions. Proliferation of *C. perfringens* in the corn-based diet remained constant with or without the inclusion of the *Bacillus*-DFM candidate and in the TSB positive control group (Table 5). This outcome could be related to the lower concentration of NSP usually found in corn grains in comparison to other cereals, which was also supported by low digesta viscosity values (Table 4). These results are in accordance with previous reports (Annett et al., 2002), however, it is important to mention that diet ingredients are just one of the multiple predisposing factors that could affect the incidence of NE in commercial conditions (Murphy et al., 2009; Lee et al., 2011).

Beneficial bacterial spores are popular as DFM, though little is known about their mode of action. Previous studies conducted in our laboratory, have demonstrated that ~ 90% of *Bacillus* spores of a selected strain germinate within 30 min under *in vitro* and *in vivo* model conditions,

with relatively constant numbers of spores in each gastrointestinal compartment evaluated, hence, suggesting that full life-cycle may occurs (Latorre et al., 2014). In the present study, regardless of the diet, similar *in vitro* persistence of the *Bacillus*-DFM candidate spores was observed in the different simulated compartments (Table 6). On average, a half $\log_{10}$ reduction in spore numbers were detected in the crop compartment suggesting spore germination. In the proventriculus compartment, a further ~2 $\log_{10}$ reduction was shown, supporting our previous findings (Menconi et al., 2013; Latorre et al., 2014), which suggest that further germination of spores occurs even at low pH enviroments. However, it was particularly interesting to observed a ~2 $\log_{10}$ increment in spore counts in the intestinal simulated compartment (Table 6). The increment in the numbers of spores could be a response to bacterial metabolites, competition for oxygen and nutrients available resulting in resporulation (Jadamus et al., 2001). The above observations also support previous reports suggesting that spore transiting through the gastrointestinal tract could potentially undergo a full life-cycle of germination and resporulation (Barbosa et al., 2005; Cartman et al., 2008). Moreover, it has been demonstrated that germination of spores into metabolically and functionally active vegetative cells, within a similar time frame, produced beneficial metabolic and immunological effects in different animal species (Hoa et al., 2001; La Ragione et al., 2003; Leser et al., 2008; Huang et al., 2009; Xu et al., 2012).

In summary, our results confirm that poultry diets containing cereal grains with a higher content of NSP in comparison to corn can enhance viscosity and *C. perfringens* growth (Bedford et al., 1993; Choct et al., 1996). Remarkably, the dietary inclusion of a selected *Bacillus*-DFM candidate in non-corn based diets significantly reduced both viscosity and *C. perfringens* proliferation when compared with the control non supplemented-diets. Additionally, *Bacillus*-DFM candidate spores persisted and change their amount according to the variable biochemical

conditions of the *in vitro* digestive model; therefore, supporting the hypothesis of the possible full-life cycle development in the gastrointestinal tract. The results from the present *in vitro* study encourage us to further evaluate the utilization of this *Bacillus*-DFM candidate in an *in vivo* NE model that we have developed in our laboratory (Shivaramaiah et al., 2011b), as well as to purify, characterize, and measure the international units of cellulase and xylanase that these *Bacillus* isolates produce. This knowledge will provide a valuable tool to use a stable DFM that produce exogenous enzymes in poultry diets.

Table 1. Ingredient composition and nutrient content of different broiler chicken diets used for *in vitro* digestion with or without inclusion of *Bacillus*-DFM candidate spore on as-is basis[a].

Item	Corn-based diet	Wheat-based diet	Barley-based diet	Rye-based diet	Oat-based diet
Ingredients (g/kg)					
Corn (80 g/kg CP)	619.6	-	-	-	-
Wheat (135 g/kg CP)	-	711.0	-	-	-
Barley (113 g/kg CP)	-	-	654.3	-	-
Rye (126 g/kg CP)	-	-	-	622.6	-
Oats (98 g/kg CP)	-	-	-	-	638.0
Soybean meal (475 g/kg CP)	298.2	203.9	241.9	264.6	260.0
Poultry oil	39.1	42.8	65.0	70.0	70.0
Dicalcium phosphate	16.9	17.1	17.0	16.6	16.4
Calcium carbonate	10.6	8.5	8.2	10.4	10.0
Salt	3.8	3.0	3.0	5.7	2.0
DL-Methionine	3.3	2.5	3.0	3.0	3.2
L-Lysine HCL	2.8	4.6	2.0	2.0	1.6
L-Threonine	1.2	2.1	1.1	0.6	0.6
Choline chloride 60 %	2.0	2.0	2.0	2.0	2.0
Vitamin premix[b]	1.0	1.0	1.0	1.0	1.0
Mineral premix[c]	1.0	1.0	1.0	1.0	1.0
Antioxidant[d]	0.5	0.5	0.5	0.5	0.5
Calculated analysis					
Metabolizable energy (MJ/kg)	13.0	13.0	12.3	12.2	11.9
Crude protein (g/kg)	195.0	200.0	190.0	205.0	186.4

[a]Inclusion of 10^8 spore/g of feed mixed with calcium carbonate.
[b]Vitamin premix supplied per kg of diet: Retinol, 6 mg; cholecalciferol, 150 µg; dl-α-tocopherol, 67.5 mg; menadione, 9 mg; thiamine, 3 mg; riboflavin, 12 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.3 mg; cyanocobalamin, 0.4 mg.
[c]Mineral premix supplied per kg of diet: Mn, 120 mg; Zn, 100 mg; Fe, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.2 mg; and cobalt, 0.2 mg.
[d]Ethoxyquin.

Table 2. Relative enzyme activity values (REA) and *Clostridium perfringens* zone of inhibition produced by different *Bacillus* spp. strains present in the *Bacillus*-DFM candidate treatment.

Measurements	AM1002	AM0938	JD17
Cellulase activity at 24 h			
Colony size (mm)	5.7 ± 0.33 [a]	6.0 ± 0.58 [a]	6.3 ± 0.33 [a]
Zone of clearance (mm)	35.2 ± 1.76 [a]	30.7 ± 0.67 [ab]	29.3 ± 2.19 [b]
REA[c]	6.2 ± 0.12 [a]	5.1 ± 0.49 [ab]	4.7 ± 0.29 [b]
Xylanase activity at 24 h			
Colony size (mm)	5.0 ± 0.58 [b]	6.7 ± 0.33 [ab]	7.3 ± 0.67 [a]
Zone of clearance (mm)	31.7 ± 0.88 [a]	32.0 ± 1.15 [a]	29.0 ± 1.53 [a]
REA[c]	6.3 ± 0.87 [a]	4.9 ± 0.43 [ab]	4.0 ± 0.15 [b]
***C. perfringens* at 24 h**			
Zone of inhibition (mm)[d]	12.3 ± 1.45 [a]	14.0 ± 1.00 [a]	8.0 ± 1.15 [b]

[a-b] Superscripts within a row with no common superscript differ significantly $P < 0.05$.
[c] Relative enzyme activity values (REA) reflect the capacity to produce cellulase and xylanase enzymes by *Bacillus spp*. REA was calculated dividing the diameter of area of clearance by the diameter of the *Bacillus* colony. Based on REA test, organism can be categorized into three groups showing excellent (REA>5.0), good (REA>2.0 to 5.0), or poor (REA<2.0) relative enzyme activity. All *Bacillus spp*. isolates were tested by triplicate. Data expressed as mean ± SE.
[d] Represents the diameter of the zone of inhibition observed at 24 h of incubation without the diameter of the bacterial colony. All *Bacillus spp*. isolates were tested by triplicate. Data expressed as mean ± SE

Table 3. Identification of *Bacillus spp.* isolates by bioMerieux API 50 CHB[a] and 16S rRNA sequence analyses[b] present in the *Bacillus*-DFM candidate treatment.

	API50 CHB		16S rRNA sequence analysis	
Isolate	**Taxon**	**% ID**	**Closest match**	**% ID**
AM1002	*Bacillus subtilis/amyloliquefaciens*	99.2	*Bacillus subtilis*	100.0
AM0938	*Bacillus subtilis/amyloliquefaciens*	99.0	*Bacillus amyloliquefaciens*	99.7
JD17	*Bacillus subtilis/amyloliquefaciens*	99.4	*Bacillus amyloliquefaciens*	99.6

[a] BioMerieux API 50 CHB test kit.
[b] 16S rRNA sequence analysis.

Table 4. Evaluation of *in vitro* viscosity of different diets with or without inclusion of a *Bacillus*-DFM candidate.

Diet	Viscosity (cP)[c]	
	Control	***Bacillus*-DFM**
Corn-based	0.96 ± 0.01 [a]	0.97 ± 0.01 [a]
Wheat-based	1.55 ± 0.02 [a]	1.28 ± 0.01 [b]
Barley-based	1.75 ± 0.02 [a]	1.34 ± 0.03 [b]
Rye-based	8.40 ± 0.37 [a]	2.39 ± 0.04 [b]
Oat-based	36.9 ± 2.15 [a]	1.34 ± 0.01 [b]

[a-b]Superscripts within a row with no common superscript differ significantly $P < 0.05$.
[c] Viscosity was measured after 3 h and 15 min of *in vitro* digestion at 40 ^{o}C. Data expressed as mean ± standard error.

Table 5. Proliferation of *Clostridium perfringens*[d] in different digested diets with or without inclusion of *Bacillus*-DFM candidate spore[e].

Diet[c]	TSB+Thio	Control Diet	*Bacillus*-DFM
Corn-based	6.38 ± 0.13 [a]	6.44 ± 0.19 [a]	6.68 ± 0.08 [a]
Wheat-based	6.12 ± 0.24 [b]	7.12 ± 0.07 [a]	5.20 ± 0.18 [b]
Barley-based	6.36 ± 0.06 [c]	7.50 ± 0.13 [a]	6.86 ± 0.11 [b]
Rye-based	6.05 ± 0.21 [c]	7.15 ± 0.09 [a]	6.68 ± 0.12 [b]
Oat-based	6.12 ± 0.07 [b]	6.96 ± 0.13 [a]	5.76 ± 0.07 [c]

[a-b] Superscripts within a row with no common superscript differ significantly $P < 0.05$.
[c] Supernatant from each diet was used as part of the broth for *C. perfringens* growth. Data expressed as mean ± standard error.
[d] Inoculum used 10^5 cfu of *C. perfringens* and 10^8 spores/g of *Bacillus*-DFM candidate.
[e] Data expressed in $\log_{10}$ cfu/mL.

Table 6. Persistence of *Bacillus*-DFM candidate[c] spore during *in vitro* digestion[f] in different diets under variable biochemical conditions simulating different sections of the gastrointestinal tract of poultry[e].

Diet[d]	Crop (30 min)	Proventriculus (45 min)	Intestine (120 min)
Corn-based	7.32 ± 0.10 [a]	5.43 ± 0.17 [b]	7.20 ± 0.09 [a]
Wheat-based	7.54 ± 0.06 [a]	5.58 ± 0.10 [b]	7.33 ± 0.19 [a]
Barley-based	7.45 ± 0.16 [a]	4.95 ± 0.21 [b]	7.27 ± 0.08 [a]
Rye-based	7.28 ± 0.10 [a]	5.60 ± 0.22 [b]	7.09 ± 0.17 [a]
Oat-based	7.66 ± 0.07 [a]	5.06 ± 0.15 [b]	7.30 ± 0.15 [a]

[a-b] Superscripts within a row with no common superscript differ significantly $P < 0.05$.
[c] Inclusion of 10^8 spore/g of feed
[d] Heat shock was induced by placing a sample of each simulated compartment in a water bath at 75 ºC for 10 min. Data expressed as mean ± standard error.
[e] Data expressed in Log_{10} cfu/mL.
[f] pH and time of incubation varied according to the simulated organ.

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VI. CHAPTER IV

Role of a *Bacillus subtilis* direct-fed microbial on digesta viscosity, bacterial translocation, and bone mineralization in turkey poults fed with a rye-based diet

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ABSTRACT

Rye contains high concentrations of non-starch polysaccharides (NSPs), leading to reduced digestibility. Since poultry have little or no endogenous enzymes capable of hydrolyzing these NSP, exogenous carbohydrases as feed additives are used in an attempt to reduce the anti-nutritional effects of these polysaccharides. Previously, an *in vitro* study conducted in our laboratory showed that inclusion of certain *Bacillus* direct-fed microbial (DFM) candidates that produce exogenous phytase, lipase, protease, cellulase, and xylanase in high-NSP diets significantly reduced both digesta viscosity and *Clostridium perfringens* proliferation. In the present study, rye-based turkey starter diets with or without *Bacillus*-DFM were administered *ad libitum* to day-of-hatch turkey poults in two independent experiments. In both experiments, day-of-hatch turkey poults were randomly assigned to either a control diet (CON) or a DFM treated diet (n = 25 birds/group). At 10 days-of-age, all turkey poults from experiments 1 and 2 were weighted and 12 turkey poults/group were randomly selected and humanely killed. Liver samples were aseptically collected to evaluate bacterial translocation, and intestinal digesta samples were individually collected to evaluate viscosity. Additionally, in experiment 2 both tibias were removed for assessment of bone parameters. In both experiments, the treated group showed a reduction in the total number of coliforms in the liver and a reduced digesta viscosity when compared to the CON group (P <0.05).Turkey poults fed the *Bacillus*-DFM candidate had increased tibia diameter, breaking strength, ash content, calcium content, and phosphorus content when compared with CON turkey poults. In summary, turkey poults fed with a rye-based diet without DFM showed an increase in bacterial translocation and digesta viscosity, accompanied by a reduction in bone mineralization; however, these adverse effects can be prevented by the inclusion of selected a *Bacillus*-DFM candidate in high-NSP diets.

Keywords: *Bacillus subtilis*, digesta viscosity, bone mineralization, turkey poults, rye

INTRODUCTION

Maize is usually the main energy source in poultry diets, but in some countries, at times, it is difficult to formulate least cost diets using this cereal. In addition, the unpredictable price of maize suggests that the global poultry industry will continue to seek alternative cost-effective feed ingredients such as cereal by-products from biofuel and milling industries. Nevertheless, the successful inclusion of alternative ingredients in poultry diets will depend on the characterization of the nutritive value of these feed ingredients. Additionally, there is an increasing interest for technologies that can mitigate problems associated with anti-nutritional factors such as non-starch polysaccharides (NSP) and phytates in poultry feed ingredients considering that chickens lack the endogenous enzymes necessary for digesting these components (Moran et al., 1969; Bedford et al., 1991; Friesen et al., 1992; Bedford and Classen, 1993; Kiarie et al., 2013). Rye has been recognized as one of the cereals with the highest negative impact on performance parameters when included in poultry diets. It has been well documented that the high concentration of soluble NSP in rye increases digesta viscosity and stickiness of droppings, which results in poor performance (Choct et al., 1995; Bedford and Schulze, 1998; Lázaro et al., 2004). Furthermore, malabsorption of lipids and fat-soluble vitamins, deterioration of bone mineralization, and reduced leg soundness are all associated with rye utilization in poultry feeds (MacAuliffe and McGinnis, 1971). Soluble NSP in rye mainly comprised highly branched arabinoxylans, increasing digesta viscosity being responsible for poor digestibility through interference with the movement of particles and solutes across the intestinal lumen, preventing the access of digestive enzymes to the endosperm contents, and reducing intestinal absorption of

sodium and calcium (Fengler and Marquardt, 1988). Increased digesta viscosity reduces conjugated bile acids, affecting fat emulsification and fat digestibility (Langhout et al., 1997). Recent studies published by our laboratory have shown that the significant reduction in bone strength and mineralization in chickens fed with rye (Tellez et al., 2014), or gluten intolerance in human beings (Bianchi and Bardella, 2008; Capriles et al., 2009), are also associated with malabsorption of minerals and fat-soluble vitamins. Since poultry has no enzymes capable of hydrolyzing NSP, exogenous xylanases are used as feed additives in an attempt to reduce the effect of this anti-nutritive factor (Bedford and Schulze, 1998). Previously, we have evaluated the inclusion of a selected *Bacillus* direct-fed microbial (DFM) candidate that produces exogenous enzymes (protease, phytase, lipase, xylanase, and cellulase) in high NSP diets (rye, wheat, barley, and oat). In those studies, a significant reduction in both viscosity and *Clostridium perfringens* proliferation was observed between high-NSP control non-treated diets and the same diets supplemented with *Bacillus*-DFM *in vitro* (Latorre et al., 2014; Tellez et al., 2014a). In addition, studies conducted in broiler chickens using rye as an energy source in our laboratories also have shown that rye increased digesta viscosity, bacterial translocation, and leakage of fluorescein isothiocyanate dextran (FITC-d), altering the microbiota composition as well as bone mineralization in chickens (Tellez et al., 2014b). The objective of the present study was to evaluate the role of a *Bacillus subtilis* based DFM candidate, which was selected for enzyme production on digesta viscosity, bacterial translocation, and bone mineralization in turkey poults fed with a rye-based diet.

MATERIALS AND METHODS

Animal source and diets

In order to show that similar results can be achieved independently, two experiments were conducted in the present study. In each experiment, 50 day-of-hatch turkey poults were obtained from a commercial hatchery (Cargill, Gentry, AR, USA), and placed in isolators chambers with a controlled age-appropriate environment and *ad libitum* access to feed and water for 10 days. The number of animals used was based on published studies in which similar outcomes were measured (Campbell et al., 1983; Zhang and Coon, 1997; Higgins et al., 2010a; Higgins et al., 2010b). Turkey poults were randomly assigned to either a control group (CON) with a rye-based diet meeting the nutritional requirements for turkey poults as recommended by the National Research Council (NRC, 1994), or a treated group (TRT) fed with a rye-based diet supplemented with 10^6 spores/g of feed of a specifically selected *Bacillus*-DFM candidate. No antibiotics were added to any of the diets (Table 1). All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville. Specifically, the IACUC approved this study under the protocol #11047 – "evaluation of direct-fed microbials and prebiotics in poultry."

DFM Preparation

In an effort to grow high numbers of viable spores, a solid state fermentation media (SS) developed by Zhao et al. (2008) was selected and modified for use in these experiments. Briefly, a liquid media component was added to a mixture of 70 % rice straw and 30 % wheat bran at a rate of 40 % by weight. The SS media was added to a 250ml Erlenmeyer flask and sterilized by autoclaving for 30 min at 121°C. Candidate isolates were grown individually overnight at 37°C in TSB, then 2ml of a candidate culture were added to the prepared SS media. The inoculated flasks were incubated for 24 h at 37°C and then incubated for another 72h at 30°C. The cultures

were removed from their flasks, placed onto petri dishes, and then dried at 60°C. Following this, the cultures were aseptically ground into a fine powder to generate stable spores (~10^{11} spores/g). Spores were mixed into the feed using a rotary mixer for15min. Samples of feed containing the *Bacillus*-DFM culture were taken and a 1:10 dilution was made with saline. All samples were subject to 100°C for 10 min, enumerated using 10- fold dilutions and plate-counted following overnight incubation at 37°C on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD, USA) (Data not shown).

Experimental design

In experiments 1 and 2, 50 day-of-hatch, turkey poults were randomly assigned to 1 of 2 groups (n = 25). At 10 days-of-age, all turkey poults in both experiments were weighed and 12 turkey poults were randomly selected and humanely killed using carbon monoxide asphyxiation method. The right half of the liver was collected aseptically to evaluate bacterial translocation. Additionally, digesta samples were individually collected to evaluate viscosity and, and in experiment 2, both tibias were used to analyze bone parameters as described below.

Viscosity

Total intestinal contents were collected from Meckel's diverticulum to the ileocecocolonic junction. For viscosity analysis, approximately 1.5 g (wet weight) of the fresh digesta were immediately centrifuged (12,000 x *g*) for 5 min. The supernatant was collected and stored on ice until viscosity was determined using a LVDV-I Brookfield digital cone-plate viscometer fitted with a CP-40 spindle (Brookfield Engineering, Middleboro, MA). The analyzed samples and the viscometer cup were maintained at a temperature of 40°C during viscosity measurement.

Viscosity was measured in centipoise (cP = 1/100 dyne s/cm^2) and the results were reported as $\log_{10}$ cP.

Bacterial translocation

Briefly, the right half of the liver was removed from each poult, collected into sterile bags, homogenized, weighed and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample were made in a sterile 96 well Bacti flat bottom plate, and the diluted samples were plated on MacConkey Agar (VWR Cat. No. 89429-342 Suwanee, GA 30024). Biochemical evaluation tests as well as identification of isolated colonies were carried out using a bioMerieux API 20E test kit (catalog no. 50430, bioMerieux, Marcy l'Etoile, France). Bacterial translocation was expressed in colony forming units ($\log_{10}$ cfu/g of tissue).

Bone parameters

In experiment 2, bone parameters were measured according to the methods described by Zhang and Coon (1997). Tibias from each poult were cleaned of attached tissues. Bones from the left leg were subjected to conventional bone assays as described below, and the tibias from the right legs were used to determine breaking strength. The bones from the left tibia were dried at 100°C for 24 h and again weighed. Then the samples were ashed in a muffle furnace (Isotemp muffle furnace, Fisher Scientific, Pittsburgh, PA) at 600°C for 24 h in crucibles, cooled in a desiccator, and weighed. Finally, the content of calcium and phosphorus in the tibia were determined using standard methods (AOAC International, 2000). The right tibial diaphyses from individual birds were cleaned of adherent tissues, the periosteum was removed, and the biomechanical strength of each bone was measured using an Instron 4502 (Norwood, MA, USA) material testing machine

with a 509 kg load cell. The bones were held in identical positions and the mid-diaphyseal diameter of the bone at the site of impact was measured using a dial caliper. The maximum load at failure was determined using a three-point flexural bend fixture with a total distance of 30 mm between the two lower supporting ends. The load, defined as force in kg per mm^2 of cross-sectional area (kg/mm^2), represents bone strength. The rate of loading was kept constant at 20 mm/min collecting 10 data points per second using Instron's Series IX Software (Norwood, MA, USA).

Statistical analysis

All data were subjected to one-way analysis of variance as a completely randomized design using the General Linear Models procedure of SAS (SAS Institute, 2002). Data are expressed as mean ± standard error. A *P*-value of $P < 0.05$ was set as the standard for significance.

RESULTS AND DISCUSSION

Thousands of years of evolution shaped the digestive system of the jungle fowl to deal with the dietary ingredients they encounter in an efficient manner. More recently, through intensive genetic manipulation, nutrition, and health programs, we have altered the biology and growth potential of poultry among other productive animals (Muramatsu et al., 1990; Fuller et al., 1995). In the wild, the diets of these animals would be made up of many different ingredients, few of which would ever reach more than 30% of total intake on a life time basis. The range in types and relative quantities of ingredients that can be presented to the modern commercial monogastric animals, while complex, tend to result in a diet in which two or three ingredients may constitute around 75% of intake (NRC, 1994). Such change is driven by least cost

formulation processes, and endeavors to provide maximum nutrient density for minimum cost (Bedford and Schulze, 1998). Maize is usually the main source of energy in poultry diets, but at times it is difficult to formulate least cost diets using maize and unconventional grains have to be used. When poultry are fed alternative grains such wheat or rye that are high in NSPs, poor performance, and unmanageable litter conditions caused by sticky droppings are reported (Campbell et al., 1983; Fengler and Marquardt, 1988; Choct et al., 1995).Wheat or rye contains high concentrations of NSP, leading to reduced digestibility. In addition, high-NSP diets have also been associated with necrotic enteritis, a multifactorial disease caused by *C. perfringens* that is probably the most important bacterial disease in terms of economic implications in broiler chickens (Hofacre, 2001; Annett et al., 2002; Timbermont et al., 2011). Therefore, feeding enzymes for swine and poultry have made the largest impact in the past decade to solve problems associated with grains rich in NSP (Kiarie et al., 2013). NSP-degrading enzymes (NSPases, xylanase, β-glucanase, β-mannanase, α-galactosidase, and pectinase) improve nutrient digestibility and reduce digesta viscosity (Esteve-Garcia et al., 1997). In the present study, a significant improvement in body weight by day10-of-age was observed in turkey poults fed rye with added *Bacillus*-DFM candidate when compared to turkey poults that were fed an unsupplemented rye-based diet (P <0.05). Turkey poults from the TRT group in both experiments showed a significant reduction in digesta viscosity, which was associated with a decrease in bacterial translocation to the liver (Table2). The lactose positive colonies obtained from liver samples were identified as *E. coli*. Feeding diets that are high in NSP and high viscosity may be the pathological mechanism underlying bacterial translocation of gut microflora from the intestinal lumen, which predisposes poultry to systemic bacterial infections (Yegani and Korver, 2008; Salzman, 2011; Ilan, 2012; Seo and Shah, 2012). Inflammatory responses to gut-

derived and blood-borne pathogens typically occur in the liver and spleen, which are the major organs that remove bacteria and toxins, including lipopolysaccharides (LPS), from the blood stream (Yoshikawa et al., 2008). Particularly, the levels of LPS, a component of Gram-negative bacteria, are increased in the portal and/or systemic circulation with impaired gut epithelial integrity and dysbacteriosis (Tlasklova et al., 2011). Strong evidence suggests that pathogen-derived compounds from the gut have a major role and modulating effect on liver diseases and chronic inflammation (Silva et al., 2012; Sjöberg et al., 2013). Diet ingredients, integrity of the gut epithelium, immune defense in the gut and in the liver, as well as the composition of the microbiome in the intestinal tract all appear to play an integrated role in the maintenance of health and balance in the gut–liver axis (Keita and Söderholm, 2012). The results of the present study suggest that rye-based diets can both enhance bacterial translocation and digesta viscosity, but these adverse effects can be prevented by the inclusion of this specific *Bacillus*-DFM candidate (Table2).

Bone parameters were measured in experiment 2 to determine whether the addition of the specifically selected *Bacillus*-DFM candidates would counteract the loss of mineral and vitamin utilization that occurs when feeding a rye-based diet. There was a significant increase (P <0.05) in tibial diameter, tibial breaking strength, as well as the ash, calcium, and phosphorus content of the tibias observed when the selected candidate DFM was added to the rye-based diet in comparison to rye-based diet fed turkey poults without the *Bacillus*-DFM (Table3). The significant reduction in bone mineralization observed in the control group confirmed previous studies that have shown that high-NSP diets in poultry or gluten intolerance in human beings is also associated with deterioration of bone mineralization and leg soundness (MacAuliffe et al., 1976; Kotake et al., 2009; Schuppan et al., 2009; Wideman and Prisby, 2011). In the present

study, dietary supplementation of a selective DFM candidate, which has the ability to secrete exogenous enzymes (xylanase, cellulose, protease, phytase, and lipase) was able to significantly improve performance, decrease digesta viscosity, and improve bone mineralization (experiment 2), suggesting that the possibility of using this feed additive as an alternative to feed enzymes. These results also confirm in some extent our earlier findings that have shown significant reduction in both viscosity and *C. perfringens* proliferation between high-NSP control non-treated diets or the same diets supplemented with the selective DFM candidate *in vitro* (Latorre et al., 2014, Tellez et al., 2014b). Together, they represent a step toward the application of nutrigenomics in the context of a poultry model. The incorporation of one or more "omics" techniques (in particular, assessment of the microbiome) will provide a better understanding of how dietary food components can affect physiological functions and the fundamental cellular and molecular mechanisms implicated in the digestive process of high-NSPs diets in poultry. In summary, the results of the present study confirm our previous investigation conducted in chickens fed with rye, which showed a significant increase in digesta viscosity that has been associated with low performance, increased enteric bacterial translocation to liver, and decreased bone strength (Tellez et al., 2014a). Large scale commercial studies to evaluate dietary inclusion of selected *Bacillus*-DFM candidates that produce exogenous enzymes in high-NSP diets in poultry, on performance parameters, micro- biome composition, and incidence of necrotic enteritis caused by *C. perfringens* are currently being evaluated.

Table 1. Composition of the experimental diets (g/kg)

Diet	**Rye-based diet**
Rye (126 g/kg CP)	372.4
Soybean meal (474.2 g/kg CP)	482.2
Poultry oil	79.5
Dicalcium phosphate	36.6
Ground limestone	11.3
Sodium chloride	4.1
DL-Methionine	4.3
Vitamin premix[a]	1.0
L-Lysine HCl	5.0
Choline chloride 60 %	1.0
Mineral premix[b]	1.0
Threonine	1.2
Selenium	0.2
Antioxidant[c]	0.2
Calculated analysis	
ME, MJ/kg	11.9
Crude protein, g/kg	285
Crude fat, g/kg	96.2
Calcium, g/kg	14.9
Total phosphorus, g/kg	10.9
Lysine, g/kg	18.2
Methionine, g/kg	7.9
Methionine + cystine, g/kg	11.8

[a]Vitamin premix supplied per kilogram of diet: Retinol, 9.2 mg; cholecalciferol, 100 µg; dl-α-tocopherol, 90 mg; menadione, 6 mg; thiamine, 6.2 mg; riboflavin, 26.5 mg; pantothenic acid, 39.7 mg; niacin, 100 mg; pyridoxine, 11 mg; folic acid, 4 mg; biotin, 0.3 mg; cyanocobalamin, 0.1 mg.
[b]Mineral premix supplied per kilogram of diet: Mn, 70 mg; Zn, 40 mg; Fe, 37 mg; Cu, 6 mg; I, 0.7mg; Co, 0.2 mg.
[c]Ethoxyquin.

Table 2. Evaluation of body weight, digesta viscosity, and bacterial translocation to the liver in neonatal turkey poults fed with a rye-soybean based diet with or without dietary inclusion of a *Bacillus* direct-fed microbial (DFM) in experiment 1 and 2.

	Experiment 1			Experiment 2		
	Body weight (g)	**Digesta viscosity (cP Log_{10})**	**Bacterial translocation (cfu Log_{10})**	**Body weight (g)**	**Digesta viscosity (cP Log_{10})**	**Bacterial translocation (cfu Log_{10})**
CON[c]	65.91 ± 3.61[b]	2.03 ± 0.31[a]	3.03 ± 0.51[a]	74.47 ± 1.59[b]	1.80 ± 0.45[a]	2.13 ± 0.67[a]
TRT[d]	82.85 ± 4.23[a]	1.54 ± 0.22[b]	1.24 ± 0.51[b]	95.60 ± 2.17[a]	1.62 ± 0.53[b]	0.35 ± 0.40[b]

[a-b]Superscripts within columns indicate significant difference at $P < 0.05$.
[c]Control rye based diet
[d]Control rye based diet with candidate DFM.
Body weight n=25; Intestinal viscosity and bacterial translocation n= 12. Data is express as mean ± SE.
Intestinal viscosity is expressed in Log_{10} (in centipoise, cP = 1/100 dyne s/cm^2).
Liver bacterial translocation (expressed in cfu Log_{10} /g of tissue).

Table 3. Evaluation of bone strength and bone composition in neonatal turkey poults fed with a rye-soy based diet with or without dietary inclusion of a *Bacillus* direct-fed microbial (DFM) in experiment 2.

	Tibia strength load at yield (kg/mm^2)	**Tibia diameter (mm)**	**Total ash from tibia (%)**	**Calcium (% of ash)**	**Phosphorus (% of ash)**
CON[c]	0.26 ± 0.02[b]	4.45 ± 0.32[b]	35.61 ± 0.81[b]	27.35 ± 0.07[b]	16.35 ± 0.52[b]
TRT[d]	0.44 ± 0.03[a]	5.82 ± 0.78[a]	50.87 ± 0.75[a]	40.31 ± 0.46[a]	22.67 ± 0.29[a]

[a-b] Superscripts within columns indicate significant difference at $P < 0.05$.
[c]Control rye based diet
[d]Control rye based diet with candidate DFM.
Tibias from twelve poults were collected to evaluate bone qualities. Data is expressed as mean ± SE.

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VII. CHAPTER V

Evaluation of a *Bacillus* direct-fed microbial candidate on digesta viscosity, bacterial translocation, microbiota composition and bone mineralization in broiler chickens fed on a rye-based diet

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ABSTRACT

1. The effects of the dietary inclusion of a *Bacillus* based direct-fed microbial (DFM) candidate on digesta viscosity, bacterial translocation, microbiota composition, and bone mineralization were evaluated in broilers consuming rye-based diets.

2. In the present study, control mash rye-based diets (CON) or *Bacillus*-DFM supplemented diets (TRT) were administered *ad libitum* to male broilers in three independent experiments.

3. In experiments 1 and 2 (n = 25/group) liver samples were taken to evaluate bacterial translocation, digesta samples were used for viscosity measurements, and the intestinal microbial flora was evaluated from different intestinal sections to enumerate total recovered gram-negative bacteria, lactic acid bacteria and anaerobic bacteria, additionally both tibias were removed for assessment of bone quality.

4. In Experiment 3, each experimental group had 8 replicates of 20 chickens (n = 160/group). Weekly, body weight (BW), feed intake and feed conversion ratio (FCR) were evaluated. At d 28-of-age, samples were taken to determine bacterial translocation, digesta viscosity, and bone quality characteristics.

5. In all experiments, consumption of *Bacillus*-DFM reduced bacterial translocation to the liver and digesta viscosity ($P < 0.05$). Additionally, DFM supplementation improved BW, bone quality measurements and FCR ($P < 0.05$). Moreover, chickens fed the *Bacillus*-DFM diet in experiments 1 and 2 showed a significant reduction in the number of gram-negative and anaerobic bacteria in the duodenal content compared to control.

6. In summary, chickens fed a rye-based diet without DFM inclusion showed an increase in bacterial translocation and digesta viscosity, accompanied by reduced performance and bone quality variables relative to the *Bacillus*-DFM candidate group. Hence, incorporation into the

feed of a selected DFM ameliorated the adverse antinutritional effects related to utilization of rye-based diets in broilers chickens.

Keywords: *Bacillus spp.*, viscosity, microbiota, bone quality, broilers, rye

INTRODUCTION

Concerns regarding development of antibiotic-resistant microorganisms and social pressures have continued the trend to ban the use of antibiotics as growth promoters in poultry production (Castanon, 2007). This has also resulted in an urgent necessity to find feasible alternatives to maintain poultry health, in order to sustain poultry as an economically viable source of animal protein for human consumption (Alvarez-Olmos and Oberhelman, 2001). In this regard, the use of selected strains from different beneficial microorganism from the genus *Bacillus* and *Lactobacillus* have shown to be a suitable option for the poultry industry (Tellez et al., 2012). *Bacillus spp.* are a gram-positive, facultative aerobe, endospore-forming, rod shaped bacterium normally found in soil and water sources, as well as in the gastrointestinal tract of animals and humans (Hong et al., 2009). Its multiple flagella, allows it to move quickly in liquids. *Bacillus spp.* are the most investigated gram-positive bacteria and a model organism to study bacterial chromosome replication and cell differentiation and together with other beneficial microbes have been extensively used as a source of industrial enzymes and antibiotics by biotechnology companies (Hendricks et al., 1995; Kunst et al., 1997; Monisha et al., 2009). When environmental conditions are not favorable for growth and replication of bacteria from the genus *Bacillus*, dramatic metabolic changes occur, such as; the induction of chemotaxis, cannibalism, production of macromolecular hydrolases (proteases and carbohydrases), as well as the

formation of endospores (González-Pastor et al., 2003; Hong et al., 2005; López et al., 2009; Higgins and Dworkin, 2012). Due to the capacity of bacterial spores to resist harsh environmental conditions and long storage periods, endospores from selected *Bacillus* strains have been used as reliable direct-fed microbials (DFM) in animal production (Tellez et al., 2013). Additionally, *Bacillus*-DFM have previously been shown to prevent gastrointestinal disorders and impart numerous nutritional benefits for animals and humans (Duc et al., 2004; Cartman et al., 2007; Sen et al., 2012). Recent studies published by our laboratory have shown that approximately 90% of *B. subtilis* spores germinate within 60 min in presence of feed *in vitro* and *in vivo* in different segments of the gastrointestinal (Latorre et al., 2014a). After spore germination into vegetative cells, *Bacillus spp.* bacteria become metabolically active to produce chemical compounds that are beneficial to the host and the intestinal microflora (Jadamus et al., 2001; Leser et al., 2008).

In most of the U.S. and in other countries, including Brazil, broiler feed is based primarily on maize and soybean meal, which supplies the majority of energy and protein in the diet. Utilization of the nutrients contained in maize by broilers is generally considered to be high. Nevertheless, at times it is difficult to formulate least cost diets using maize and unconventional grains with variable concentrations of antinutritional factors have to be used. Rye (*Secale cereale*) is a cereal member of the wheat tribe (*Triticeae*) and has been reported to contain 152 grams of total non-starch polysaccharides (NSP) per kilogram of dry matter (Antoniou et al., 1981; Bach Knudsen, 1997). When chickens are fed alternative cereal grains such as rye that are high in soluble NSP; high digesta viscosity, poor nutrient digestibility and reduced bone mineralization have been reported, resulting in decreased growth performance and reduced litter quality conditions caused by sticky droppings (Campbell et al, 1983; Fengler and Marquardt,

1988). However, different studies have shown that the inclusion of carbohydrases such as xylanase in rye-based diets significantly improved all these negative factors reducing the impact of the antinutritional components present in the rye grain. (Bedford and Classen, 1993; Dänicke et al., 1997; Silva and Smithard, 2002). Previously, we have evaluated the inclusion of selected *Bacillus*-DFM candidates that produce a different set of extracellular enzymes using different poultry diets *in vitro* (rye, wheat, barley, and oat based-diets), resulting in a significant reduction in both digesta viscosity and *Clostridium perfringens* proliferation between control diets and *Bacillus*-DFM supplemented diets (Latorre et al., 2015). The objective of the present study was to evaluate the role of a multiple enzyme producing *Bacillus*-based DFM on growth performance, digesta viscosity, bacterial translocation, microbiota composition, and bone mineralization in broiler chickens fed with a rye-based diet.

MATERIALS AND METHODS

Isolation and characterization of Bacillus spp.

Previous research conducted in our laboratory focused on isolation of several *Bacillus spp.* from environmental and poultry sources (Shivaramaiah et al., 2011; Wolfenden et al., 2011; Menconi et al., 2013). Identification and characterization of the different isolates was carried out using a bioMerieux API 50 CHB test kit (catalog no. 50430, bioMerieux, Marcy l'Etoile, France), and individual plates of each strain were also sent for 16S rRNA sequence analysis to a specialized laboratory (Midi labs, Newark, DE, USA). One of the three *Bacillus* strains (AM1002) was identified as *B. subtilis*, and the other two isolates (AM0938 and JD17) were identified as *B. amyloliquefaciens* (Table 1). These *Bacillus* strains were selected as superior producers of cellulase and xylanase based on a qualitative enzyme activity evaluation performed using a

different selective media for each evaluated enzyme (Latorre et al., 2014b; Latorre et al., 2015). Then, the three *Bacillus spp.* selected strains were sporulated and mixed during the DFM-candidate preparation process before dietary inclusion.

DFM preparation

In an effort to grow high numbers of viable spores, a solid state fermentation media (SS) developed by Zhao et al. (2008) was selected and modified for use in these experiments. Briefly to prepare the SS fermentation media, ammonia broth was added to a mixture of 70% rice straw and 30% wheat bran at a rate of 40% by weight. Then, the SS fermentation media was added to 250 mL Erlenmeyer flasks and sterilized by autoclaving for 30 min at 121°C. Each of the three *Bacillus spp.* isolates was grown individually overnight at 37°C in test tubes containing 10 mL of tryptic soy broth (TSB, catalog no. 211822, Becton Dickinson, Sparks, MD). Following incubation, 2 mL of each isolate culture were added separately to the previously prepared SS fermentation media flask. The inoculated flasks were incubated for 24 h at 37°C to promote growth of the *Bacillus spp.* vegetative cells, and then incubated for another 72 h at 30°C to trigger the initiation of the sporulation process. Following this, the inoculated SS fermentation media was removed from the Erlenmeyer flasks, placed onto petri dishes, and dried at 60°C. Then, the SS fermentation media was aseptically ground into a fine powder that contained stable *Bacillus* spores (~ 10^{11} spores/g). *Bacillus spp.* spores from each of the three selected strains were combined in equal amounts to conform the *Bacillus*-DFM candidate treatment. Next, the DFM was included into the feed to reach a concentration of 10^6 spores per gram of feed using a rotary mixer for 15 minutes. Samples of feed containing the *Bacillus*-DFM candidate were taken to validate the amount of spores per gram of feed after the inclusion and mixing steps, a 1:10

dilution was made with saline in glass sterile tubes and all feed samples were incubated at 100°C for 10 min to eliminate the presence of vegetative cells present in the feed allowing the enumeration of spores only. Following heat-treatment, 1:10 dilution of the feed samples from the glass tubes were plated on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD); letting spores in the feed sample germinate into vegetative cells after incubation at 37°C for 24 h, hence representing the number of spores present per gram of feed.

Animal Source and Diets

In the present study, three independent experiments were conducted. For all experiments d-of-hatch male broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR, USA). In experiments 1 and 2, chicks were placed in isolation chambers (90 cm x 80 cm) with a controlled age-appropriate environment. Meanwhile in experiment 3, birds were neck-tagged and randomly located to one of sixteen floor pens (300 cm x 150 cm) with new pine shavings as litter in an environmentally controlled room. Temperature was maintained at 34°C for the first 5 d and was then gradually reduced according to normal management practices, until a temperature of 23°C was achieved at day 21 of age. In all experiments, broilers chicks were randomly assigned to either a control group (CON) consuming a mash rye-based diet or a treated group (TRT) fed with a mash rye-based diet supplemented with the *Bacillus*-DFM candidate (10^6 spores/gram of feed). In experiments 1 and 2, a starter diet was fed throughout the experimental period (0 to 10 d). However, in experiment 3 due to a prolonged duration, starter (0 to 7 d) and grower (8 to 28 d) diets were offered. Prior to formulating the experimental diets, it was determined that the rye grain contained: moisture 109 g/kg, crude protein 124 g/kg, crude fat 18 g/kg, crude fiber 29 g/kg, calcium 0.9 g/kg, and phosphorus 3.1 g/kg. The experimental diets were formulated to

approximate the nutritional requirements of broiler chickens as recommended by the National research council (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc., 2013). No antibiotics or coccidiostats were added to the feed (Table 2). The chemical composition of the experimental diets was determined by AOAC international (2000) methods for moisture (930.15), crude protein (984.13), crude fat (920.39), crude fiber (978.10), calcium (968.08) and phosphorus (965.17). All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas, Fayetteville, USA.

Experimental Design of Experiments 1 and 2

In order to show that similar results can be achieved independently, two experiments were conducted in the present study in broiler chickens that were raised for 10 days. In each experiment, 50 d-of-hatch, chickens were randomly assigned to one of two groups: rye-based diet or a rye-based diet supplemented with *Bacillus*-DFM (n = 25/group); the number of animals used was based on published studies in which similar variables were measured (Bedford et al., 1991; Latorre et al., 2014b; Tellez et al., 2014). At 10 d-of-age, in both experiments, all chickens were weighed and humanely killed by CO_2 asphyxiation. Samples were obtained from randomly selected individual broilers and analyzed separately. The right half of the liver was aseptically removed to evaluate bacterial translocation (n = 12/group). Additionally, digesta samples were taken to evaluate viscosity (*n* = 8/group) and both tibias were removed to analyze bone quality characteristics (n = 12/group). Moreover, in both experiments duodenal, ileal, and cecal gut sections were obtained to enumerate different bacterial populations (n = 12/group). Details about measurement techniques are described below.

Experimental Design of Experiment 3

As an extension of experiments 1 and 2, and for evaluation of the rye-based diet during the starter and grower production periods, 320 d-of-hatch chickens were neck tagged and randomly allotted based on initial body weight (BW) to one of two groups; control rye-based diet or *Bacillus*-DFM supplemented rye-based diet. Each treatment was comprised of eight replicates of 20 chicks each (n = 160/group) and for evaluation of growth performance each replicate was used as experimental unit. Weekly, all broilers were individually weighed and BW, body weight gain (BWG) and pen feed intake (FI) were noted at the end of each phase to calculate the feed conversion ratio (FCR) for starter (0 to 7 d), grower (8 to 28 d), and overall (0 to 28 d) experimental phases. At 28 d-of-age, all chickens were weighed and humanely killed by CO_2 asphyxiation. Samples were obtained from two randomly selected broilers for bacterial translocation and bone quality determination (n = 16/group). In the case of evaluation of digesta viscosity, one bird per replicate was selected to collect the intestinal content (n = 8/group). Details about measurement techniques are described below.

Digesta Viscosity

Total intestinal contents were obtained from duodenum to cloaca in experiment 1 and 2 to evaluate digesta viscosity. In experiment 3, digesta was taken from duodenum to Meckel's diverticulum. Approximately 1.5 grams (wet weight) of the fresh digesta were immediately centrifuged (12,000 x *g*) for 5 min. The supernatant was obtained and stored on ice until viscosity was determined using a LVDV-I Brookfield digital cone-plate viscometer fitted with a CP-40 spindle (Brookfield Engineering, Middleboro, MA). The analyzed samples and the viscometer cup were maintained at a temperature of 40°C during viscosity measurements to

simulate broiler's body temperature conditions. Viscosity was measured in centipoise (cP = 1/100 dyne s/cm^2).

Bacterial Translocation

Briefly, the right half of the liver was removed from each chicken, collected into sterile bags, homogenized, weighed and 1:4 w/v dilutions were made with sterile 0.9% saline. Then, ten-fold dilutions of each sample were made in a sterile 96 well Bacti flat bottom plate, and the diluted samples were plated on MacConkey Agar (VWR Cat. No. 89429-342 Suwanee, GA 30024). Biochemical evaluation tests as well as identification of isolated colonies that grew on the MacConkey agar plates were carried out using a bioMerieux API-20E test kit (catalog no. 20100, bioMerieux, Marcy l'Etoile, France). Bacterial translocation was expressed in colony forming units (Log_{10} cfu/gram of tissue).

Intestinal microflora

Whole duodenum, ileum, and cecae were aseptically removed, separated into sterile bags, and homogenized. Samples were weighed and 1:4 w/v dilutions were made with sterile 0.9% saline. Then, ten-fold dilutions of each sample, from each group were made in a sterile 96 well Bacti flat bottom plate and the diluted samples were plated on three different culture media; for enumeration of total recovered lactic acid bacteria (LAB) on de Man Rogosa Sharpe agar (Difco™ Lactobacilli MRS Agar VWR Cat. No. 90004-084 Suwanee, GA 30024); total recovered gram negative bacteria (TGB) on MacConkey agar; and total recovered anaerobes (TAB) on tryptic soy agar plates containing sodium thioglycolate (catalog no. 212081, Becton

Dickinson, Sparks, MD). Bacteria enumeration was expressed in colony forming units (Log_{10} cfu/gram of tissue), and all plates were incubated during 18 h at 37°C before bacterial count.

Bone Quality

Bone quality measurements were made according to the methods described by Zhang and Coon (1997). Tibias from each chicken were cleaned of adherent tissues. Bones from the left leg were subjected to conventional bone assays as described below, and tibias from the right legs were used to determine breaking strength. Bones from the left tibia were dried at 100°C for 24 h and weighed. Then the samples were ashed in a muffle furnace (Isotemp muffle furnace, Fisher Scientific, Pittsburgh, PA) at 600°C for 24 h in crucibles, cooled in a desiccator, and weighed. From the left tibia, total calcium content was obtained by inductively coupled plasma determination (968.08; AOAC international, 2000), and total phosphorus contend was determined by colorimetry using the molybdo-vanadate method (965.17; AOAC international, 2000). In the case of the right tibia samples, the tibial diaphysis from individual birds were cleaned of adherent tissues, the periosteum was removed, and the biomechanical strength of each bone was measured using an Instron 4502 material testing machine (Norwood, MA) with a 509 kg load cell. The bones were held in identical positions and the mid-diaphyseal diameter of the tibial midshaft, which was also the site of impact, was measured using a dial caliper. The maximum load at failure was determined in the tibial midsection between epiphyses, using a three-point flexural bend fixture with a total distance of 30 mm between the two lower supporting ends. The load, defined as force in kilograms per square millimeter of cross-sectional area (kg/mm^2), represents bone strength. The rate of loading was kept constant at 20 mm/min

collecting 10 data points per second. The data were automatically calculated using Instron's Series IX Software (Norwood, MA).

Statistical Analysis

In all experiments, data were subjected to one-way ANOVA as a completely randomized design using the GLM procedure of SAS (SAS Institute, 2002). In experiments 1 and 2, each measurement obtained from individual broilers from each experimental group was considered as the experimental unit for BW (n=25/group), digesta viscosity (n=8/group), liver bacterial translocation (n=12/group), microbiota composition (n=12/group) and bone quality parameters (n=12/group). Additionally, as an extension of experiments 1 and 2, in experiment 3 for the evaluation of growth performance (BW, BWG, FI and FCR) each of the 8 replicates of 20 chickens was considered as the experimental unit, whereas data on digesta viscosity (n=8/group) bacterial translocation (n=16/group), and bone quality (n=16/group) were based on randomly selected broilers from all replicates of each group. Data are expressed as mean ± SE and a *P*-value of $P < 0.05$ was set as the standard for significance.

RESULTS

The results of the evaluation of BW, digesta viscosity, and liver bacterial translocation in broiler chickens consuming a rye-based diet with or without dietary inclusion of a selected *Bacillus*-DFM candidate in experiments 1 and 2 are summarized in table 3. Chickens that received the rye-based diet supplemented with the DFM had a significant increase in BW at d 10 of age compared with the control group ($P < 0.05$), and also showed a significant reduction in digesta viscosity and bacterial translocation in both experiments (Table 3). Identification of the gram-

negative, lactose positive bacteria translocated to the liver in all experiments was confirmed to be *Escherichia coli* using the bioMerieux API-20E test kit.

Table 4 summarizes the results of the evaluation of total bacterial counts recovered from duodenum, ileum, and cecae in 10 d-old broiler chickens from experiments 1 and 2. In both trials, DFM supplemented chickens had a significant decrease in the number of total gram-negative bacteria recovered from the duodenum when compared to the control group. Similarly, in both experiments, a reduction in the number of total anaerobic bacteria from the duodenum of chickens consuming the *Bacillus*-DFM was observed in comparison to the unsupplemented group. Additionally, a reduction in the number of total anaerobic bacteria was also showed in the ileum from the DFM group in experiment 2 ($P < 0.05$). Nevertheless, similar amounts of lactic acid bacteria were recovered from each intestinal section evaluated in both experiments from both experimental groups. Moreover, comparable bacterial counts were observed between experimental groups in the cecae in both trials for total gram-negative, lactic acid and anaerobic recovered bacteria.

In experiment 3, during the starter phase (0 to 7d), broilers consuming the diet supplemented with *Bacillus*-DFM showed similar values in all the growth performance variables (BW, BWG, FI and FCR) that were evaluated in comparison to the control group. On the other hand, during the grower phase (8 to 28d) and the overall study period (0 to 28d), broilers consuming the *Bacillus*-DFM candidates had a significantly higher BW and BWG coupled with a more efficient FCR compared to the control group ($P < 0.05$). Feed intake was similar between both experimental groups throughout the study. In the case of digesta viscosity and bacterial translocation, inclusion of the *Bacillus*-DFM in the rye-based diet resulted in a significant reduction of viscosity in the intestinal content, together with a decrease in the number of gram-

negative, lactose positive bacteria present in the liver, which was confirmed to be *Escherichia coli* (Table 5).

The effects of the dietary inclusion of the *Bacillus*-DFM candidate on bone quality in chickens consuming a rye-based diet are shown in Table 6. In experiments 1 and 2, bone strength and composition were measured in 10 d-old broilers, showing a significant improvement in all bone quality measurements in chickens consuming the *Bacillus*-DFM in comparison to the control group ($P < 0.05$). Similar results were also obtained in experiment 3, where bone quality variables were measured in 28 d-old broilers, showing an increase in bone strength, and percentage of ash, calcium and phosphorus when the *Bacillus*-DFM was included in the diet ($P < 0.05$). However, in all experiments similar tibia diameters were observed between experimental groups.

DISCUSSION

When chickens are fed with diets containing grains such as rye instead of maize, poor performance and detrimental litter conditions caused by sticky droppings occurred (Campbell et al., 1983; Fengler and Marquardt, 1988). Rye has an elevated concentration of highly branched arabinoxylans in comparison to other cereals like wheat or maize (Bach Knudsen, 1997). The presence of soluble NSP from rye in the intestinal lumen increase digesta viscosity affecting nutrient availability and absorption (Bedford and Classen, 1993; Choct et al., 1995;). The high concentration of soluble NSP in rye-based diets also have an impact on the intestinal bacterial population, probably as a consequence of the increase digesta viscosity and prolonged feed passage time (Choct et al., 1996; Bedford and Schulze, 1998; Kiarie et al., 2013). Furthermore, utilization of rye in poultry diets has also been related to malabsorption of lipids, deterioration of

bone mineralization, and reduced leg soundness (MacAuliffe and McGinnis, 1971). This negative effect on bone quality could be also be related to an elevated digesta viscosity, therefore, enhancing the deconjugation of bile acids by the overgrowth intestinal microflora, resulting in a reduction of micelle formation, affecting fat solubilization and absorption of fat soluble vitamins and minerals (Grammer et al., 1982). Since monogastric animals do not have endogenous enzymes capable of hydrolyzing the β-linkages present in soluble NSP, exogenous carbohydrases (xylanase, β-glucanase, β-mannanase, α-galactosidase and pectinase) have been used in poultry diets as feed additives in attempt to reduce the adverse impact of these anti-nutritional factors (Bedford et al., 1991; Bedford and Classen, 1993; Esteve-Garcia et al., 1997). It has been well documented that inclusion of xylanase in rye-based diets significantly improve viscosity of digesta supernatant, accelerate feed passage time through the gastrointestinal tract, and enhance digestibility of dietary protein and fat sources resulting in an improvement in growth performance (Dänicke et al., 1997; Langhout et al., 1997; Lázaro et al., 2004; Lee, 2014). The results of the present study support previous findings published by our laboratory in turkey poults fed with rye-based diets (Latorre et al., 2014b). Similarly, digesta viscosity was considerably higher in chickens fed with rye-based diets without the DFM when compared to broilers consuming the DFM-supplemented diet, the supernatant being more semi-solid than liquid in the control group, suggesting that viscosity alone could be directly responsible for poor performance. The increase in digesta viscosity observed in the control group was also associated with elevated bacterial translocation to the liver and overgrowth of gram-negative and anaerobic bacteria in the duodenal section when compared with chickens that consumed the *Bacillus*-DFM diet. These differences could be due to less substrates available for bacterial growth, generating lower intestinal inflammation, and translocation of bacteria when the intestinal viscosity was

reduced by the inclusion of the DFM candidate, suggesting more absorption of nutrients by the intestinal brush border of supplemented broilers. It has been previously reported that alterations in gut permeability are connected with bacterial translocation in the portal and/or systemic circulation during several types of "leaky gut" syndromes leading to bacterial septicemia (Ilan, 2012; Seki and Schnabl, 2012). On the other hand, significant improvements in BW, BWG and FCR were observed in chickens consuming the *Bacillus*-DFM supplemented diet when compared to chickens from the control group, suggesting that the production of enzymes from the combined *Bacillus spp.* strains used as DFM could increase the absorption of nutrients promoting growth performance and a more efficient feed conversion ratio in addition to enhancing the physical and bacteriological conditions of the intestinal content. Furthermore, the significant reduction in bone strength and mineralization generated by consumption of rye-based diets confirmed previous research from different authors that have shown that the inclusion of rye in poultry diets is associated with malabsorption of minerals and fat-soluble vitamins (MacAuliffe et al., 1976; Campbell et al., 1983; Wideman et al., 2013). However, the results from the present study suggest that the reduction of digesta viscosity together with the production of phytase by the *Bacillus*-DFM candidate could enhance the absorption of nutrients including minerals, hence improving bone strength and bone mineralization.

In conclusion, the present study showed that chickens fed on rye-based diets have an increased in digesta viscosity and bacterial translocation associated with overgrowth of gut microflora, low performance, and decreased bone mineralization. However, this is one of the first studies reporting that these adverse effects caused by the utilization of rye in poultry diets can be minimized by the inclusion of a selected *Bacillus*-DFM candidate, thereby enhancing intestinal integrity and absorption of nutrients resulting in an improvement of production performance.

Large-scale commercial studies to evaluate the dietary supplementation of different poultry diets with the combination of these *Bacillus spp.* candidate strains are currently being evaluated.

Table 1. Identification of *Bacillus spp.* isolates by bioMerieux API 50 CHB[1] and 16S rRNA sequence analyses[2] present in the *Bacillus*-DFM candidate treatment.

Isolate	API50 CHB		16S rRNA sequence analysis	
	Taxon	**% ID**	**Closest match**	**% ID**
AM1002	*Bacillus subtilis/amyloliquefaciens*	99.2	*Bacillus subtilis*	100.0
AM0938	*Bacillus subtilis/amyloliquefaciens*	99.0	*Bacillus amyloliquefaciens*	99.7
JD17	*Bacillus subtilis/amyloliquefaciens*	99.4	*Bacillus amyloliquefaciens*	99.6

[1] BioMerieux API 50 CHB test kit.
[2] 16S rRNA sequence analysis.

Table 2. Ingredient composition and nutrient content of broiler chicken rye-based diets used in all experiments on as-is basis

Item	Starter diet	Grower diet
Ingredients (g/kg)		
Rye	583.4	619.1
Soybean meal	311.6	269.3
Poultry fat	63.0	70.0
Dicalcium phosphate	18.0	16.6
Calcium carbonate[1]	11.0	10.4
Salt	3.8	5.7
DL-Methionine	3.5	3.0
Vitamin premix[2]	1.0	1.0
L-Lysine HCl	2.2	2.0
Choline chloride 60%	1.0	1.0
Mineral premix[3]	1.0	1.0
Threonine	0.8	0.6
Antioxidant[4]	0.2	0.2
Calculated analysis		
Metabolizable energy (MJ/ kg)	11.93	12.18
Crude protein	223.8	207.9
Lysine	13.2	11.9
Methionine	6.4	5.8
Methionine + Cystine	9.8	8.9
Threonine	8.6	7.8
Tryptophan	3.0	2.8
Crude fat	77.0	88.4
Crude fibre	27.2	26.7
Total calcium	9.0	8.6
Available phosphorus	4.5	4.2
Determined analysis		
Crude protein	220.0	203.0
Crude fat	76.0	88.2
Crude fibre	27.0	26.4
Calcium	9.1	8.4
Total phosphorus	7.2	7.0

[1]Inclusion of 10^6 spores/g of feed mixed with calcium carbonate.
[2]Vitamin premix supplied per kilogram of diet: Retinol, 6 mg; cholecalciferol, 150 µg; DL-α-tocopherol, 67.5mg; menadione, 9 mg; thiamine, 3 mg; riboflavin, 12 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.3 mg; cyanocobalamin, 0.4 mg.
[3]Mineral premix supplied per kilogram of diet: Mn, 120 mg; Zn, 100 mg; Fe, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.2 mg; and cobalt, 0.2 mg.
[4]Ethoxyquin.

Table 3. Evaluation of body weight (BW), digesta viscosity, and bacterial translocation to the liver in broiler chickens consuming a rye-based diet with or without dietary inclusion of a selected *Bacillus* direct-fed microbial candidate (experiments 1 and 2)[1]

Item	BW[2] (g)	Digesta viscosity[3] (cP)	Bacterial translocation[4] (Log_{10} cfu/g)
Experiment 1			
Rye diet	111 ± 5.2^{b}	501 ± 71.17^{a}	2.4 ± 0.45^{a}
Rye diet + DFM	138 ± 4.9^{a}	271 ± 12.69^{b}	1.0 ± 0.27^{b}
Experiment 2			
Rye diet	141 ± 5.2^{b}	591 ± 51.72^{a}	2.4 ± 0.73^{a}
Rye diet + DFM	168 ± 6.9^{a}	306 ± 14.70^{b}	1.1 ± 0.57^{b}

[a,b]Superscripts within colums and experiment indicate significant difference at $P < 0.05$.
[1]Data are expressed as mean ± SE.
[2]Body weight, n = 25/group.
[2]Digesta viscosity is expressed in centipoise (cP), n = 8/group.
[3]Liver bacterial translocation is expressed in colony forming units, n = 12/group.

Table 4. Evaluation of total bacterial counts in duodenum, ileum, or cecae in neonatal broiler chickens consuming a rye-based diet with or without dietary inclusion of a selected *Bacillus* direct-fed microbial candidate (Experiments 1 and 2)[1]

	Duodenum[2]			Ileum[2]			Cecae[2]		
Item	**TGB**	**LAB**	**TAB**	**TGB**	**LAB**	**TAB**	**TGB**	**LAB**	**TAB**
Experiment 1									
Rye diet	3.9 ± 0.08[a]	6.1 ± 0.21	5.6 ± 0.31[a]	5.4 ± 0.74	6.8 ± 0.12	5.9 ± 0.65	7.5 ± 0.19	8.2 ± 0.15	7.9 ± 0.37
Rye diet + DFM	2.2 ± 0.69[b]	5.8 ± 0.08	4.7 ± 0.12[b]	4.9 ± 0.91	6.4 ± 0.38	5.4 ± 0.63	7.2 ± 0.28	7.9 ± 0.09	7.9 ± 0.19
Experiment 2									
Rye diet	4.0 ± 0.25[a]	7.3 ± 0.21	7.9 ± 0.22[a]	3.7 ± 0.34	7.9 ± 0.23	8.6 ± 0.08[a]	7.7 ± 0.22	8.9 ± 0.12	9.0 ± 0.12
Rye diet + DFM	3.0 ± 0.23[b]	7.0 ± 0.25	6.5 ± 0.25[b]	4.0 ± 0.25	8.1 ± 0.14	7.9 ± 0.07[b]	7.4 ± 0.23	8.8 ± 0.15	8.8 ± 0.11

[a,b]Superscripts within columns and experiment indicate significant difference at $P < 0.05$.

[1]Data are expressed as Log_{10} cfu/g mean ± SE.

[2]TGB: Total Gram negative bacteria recovered, LAB: Total lactic acid bacteria recovered, TAB: Total anaerobic bacteria recovered from different intestinal sections in each experimental group, n = 12/group.

Table 5. Evaluation of body weight (BW), body weight gain (BWG), feed intake, feed conversion ratio (FCR), digesta viscosity, and bacterial translocation to the liver in broiler chickens consuming a rye-based diet with or without dietary inclusion of a selected *Bacillus*-direct-fed microbial candidate (Experiment 3)[1]

Item	Rye diet	Rye diet + DFM
BW, g/broiler		
d 0	39 ± 0.2	38 ± 0.3
d 7	96 ± 3.2	102 ± 3.1
d 28	804 ± 7.3[b]	830 ± 6.4[a]
BWG, g/broiler		
d 0 to 7	57 ± 3.1	64 ± 2.9
d 7 to 28	708 ± 6.2[b]	728 ± 6.2[a]
d 0 to 28	766 ± 7.4[b]	790 ± 6.5[a]
Feed intake, g/broiler		
d 0 to 7	118 ± 2.8	116 ± 2.3
d 7 to 28	1733 ± 17.8	1694 ± 16.8
d 0 to 28	1851 ± 19.6	1810 ± 17.0
Feed conversion ratio		
d 0 to 7	2.10 ± 0.072	1.85 ± 0.122
d 7 to 28	2.45 ± 0.025[a]	2.34 ± 0.023[b]
d 0 to 28	2.42 ± 0.019[a]	2.30 ± 0.021[b]
Evaluation at 28 d		
Digesta viscosity (cP)[2]	96.2 ± 2.95[a]	61.5 ± 2.34[b]
BT (Log_{10} cfu/g)[3]	1.5 ± 0.18[a]	0.9 ± 0.15[b]

[a,b]Superscripts within rows indicate significant difference at $P < 0.05$
[1]Data are expressed as mean ± SE.
[2]Digesta viscosity evaluated in 28-d old broilers expressed in centipoise (cP), n = 8/group
[3]BT = Bacterial translocation was evaluated in 28-d old broilers, n = 16/group

Table 6. Evaluation of bone breaking strength and bone composition in broiler chickens consuming a rye-based diet with or without dietary inclusion of a selected *Bacillus* direct-fed microbial candidate (Experiments 1, 2 and 3)[1]

Item	Load at break (kg)	Tibia diameter (mm)	Breaking strength (kg/mm^2)	Total ash (%)	Calcium (%)	Phosphorus (%)
Experiment 1[2]						
Rye diet	1.7 ± 0.01^{b}	2.6 ± 0.28	0.6 ± 0.02^{b}	34.9 ± 0.35^{b}	18.5 ± 0.27^{b}	13.1 ± 0.12^{b}
Rye diet + DFM	2.7 ± 0.01^{a}	2.9 ± 0.28	0.9 ± 0.01^{a}	54.7 ± 0.39^{a}	36.5 ± 0.87^{a}	26.1 ± 0.82^{a}
Experiment 2[2]						
Rye diet	1.7 ± 0.03^{b}	2.9 ± 0.78	0.6 ± 0.03^{b}	30.9 ± 0.75^{b}	21.3 ± 0.46^{b}	15.7 ± 0.29^{b}
Rye diet + DFM	2.8 ± 0.09^{a}	2.9 ± 0.28	1.0 ± 0.09^{a}	56.6 ± 0.44^{a}	40.3 ± 0.21^{a}	29.8 ± 0.10^{a}
Experiment 3[3]						
Rye diet	22.2 ± 0.93^{b}	5.5 ± 0.08	4.1 ± 0.23^{b}	44.9 ± 0.95^{b}	17.5 ± 0.26^{b}	9.2 ± 0.11^{b}
Rye diet + DFM	26.5 ± 1.68^{a}	5.6 ± 0.20	4.8 ± 0.18^{a}	55.0 ± 0.61^{a}	29.5 ± 0.27^{a}	15.2 ± 0.13^{a}

[a,b]Superscripts within columns indicate significant difference at $P < 0.05$ within each experiment.
[1]Data are expressed as mean ± SE.
[2]Bone measurements evaluated from 10 d-old broilers, n = 12/group
[3]Bone measurements evaluated from 28 d-old broilers, n = 16/group

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VIII. CHAPTER VI

Effects of the inclusion of a *Bacillus* direct-fed microbial on performance parameters, bone quality, gut microflora, and intestinal morphology in broilers consuming a grower diet containing corn distillers' dried grain with solubles.

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ABSTRACT

Distiller's dried grains with solubles (DDGS) have increasingly been used in poultry diets as consequence of rising grain costs. DDGS have a variable compositional value and a high inclusion of this by-product has been considered a risk factor for GI diseases such as necrotic enteritis. Presently, two experiments were conducted using a starter corn-soybean diet (0-7d) and a corn-DDGS-soybean grower diet (7-28d) with or without inclusion of a *Bacillus*-DFM. In both experiments, day-of-hatch chicks were randomly assigned to two different groups: Control group without DFM or *Bacillus*-DFM group, containing 10^6 spores/g of feed. In each experiment, eight pens of 20 chicks (n=160/group) were used. Performance parameters of body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion (FCR) were evaluated in each growth phase. Additionally, in experiment 2, one broiler per replicate was humanely killed and intestinal samples were collected to determine intestinal morphology, as well as, the microbiota population of total lactic acid bacteria (LAB), total gram negative bacteria (GNB) and total anaerobic bacteria (TAB) at 28d of age. Furthermore, both tibias were evaluated for bone strength and bone composition. In both experiments BW, BWG and FCR were improved by the DFM when compared to the control group ($P<0.05$). In experiment 2, chickens supplemented with the DFM had less TGN in the foregut intestinal segment and higher LAB counts in both foregut and hindgut sections ($P<0.05$). Small but significant increases in tibia breaking strength, and bone mineralization were observed in the DFM group when compared with control. In the case of intestinal morphology, DFM dietary inclusion increased villus height, villus width, villus area, muscular thickens and the VH:CD ratio in both duodenum and ileum sections. Therefore, results of this study suggest that consumption of a selected *Bacillus*-DFM producing a variable

set of enzymes, could contribute to enhanced performance, intestinal microbial balance and bone quality in broiler chickens consuming a grower diet that contains corn-DDGS.

Keywords *Bacillus*-DFM, DDGS, enzymes, microbiota, bone quality

INTRODUCTION

Distillers dried grains with solubles (DDGS) is a by-product of the biofuel industry produced by dry mill ethanol plants. During the fermentation process starch from cereal grains is converted to ethanol and CO_2, concentrating the remaining nutrients in DDGS (Singh et al., 2007). Corn, as an efficient source of readily fermentable starch, is the main grain used in ethanol production; but also wheat, barley, and sorghum or combinations of these grains are used. The growth of ethanol production have resulted in increased quantities of DDGS available to feed producers, therefore, making it an attractive alternative feed ingredient during elevated corn cost periods (Singh et al., 2007; Stein, 2007). DDGS may provide a rich source of protein, amino acids, phosphorus, xanthophylls and other nutrients in poultry diets (Wang et al., 2007a). High quality DDGS can be safely fed at 5-8% in starter diets for broiler chickens and turkeys, also 10-15% dietary level can be used in broiler and turkey grower-finisher diets or for feed formulation of laying hens, partially replacing in a cost effective way, soybean meal, corn and other cereals (Singh et al., 2005; Świątkiewicz and Koreleski., 2008). However, the principal limitations on use of DDGS as a feed component are the high nutritional composition variability and bioavailability of nutrients, observed especially for lysine, methionine, minerals and energy (Barekatain et al., 2013). On the other hand, Behnke (2007) reported that inclusion of 5 to 7% of DDGS could have a negative impact on pellet quality, increasing the percentage of fines per feed

batch. The majority of the reported compositional profiles of DDGS have focused mainly on common constituents such as crude protein (28.7 - 32.9%), crude fiber (5.4 – 10.4%), crude fat (8.8 – 12.4%), ash (3.0- 9.8%), phosphorus (0.42 – 0.99%), lysine (0.61 – 1.06%), methionine (0.54 – 0.76%) and tryptophan (0.18 – 0.28%)(US Grains Council, 2012). Nevertheless, non-starch polysaccharides (NSP) make up 25–30% of the DDGS, with the two major components of the NSP being arabinoxylan and cellulose (Singh et al., 2002; Singh et al., 2005; Kim et al., 2008). Therefore, targeting the indigestible components specific of DDGS with the correct blend of supplemental exogenous enzymes can allow a more efficient utilization of this co-product by poultry, as well as increased its percentage of inclusion in livestock diets, resulting in greater economic returns (Min et al., 2011). The use of spores from selected *Bacillus* strains as direct-fed microbials (DFM), have been shown to prevent gastrointestinal disorders and impart numerous nutritional benefits including the production of extracellular enzymes such as amylase, protease, lipase, cellulase, xylanase, and phytase (Hendricks et al., 1995). Previous studies published by our laboratory suggest that the dietary inclusion of a previously selected *Bacillus*-DFM based on *in-vitro* enzyme production profiles, could contribute to enhance bone quality, reduce digesta viscosity and improve both intestinal microbial balance and performance parameters in poultry consuming diets that contained a considerable percentage of soluble NSP (Latorre et al., 2014b; Latorre et al., 2015a). Therefore, the objectives of the present study were to evaluate the inclusion of a *Bacillus*-DFM in a grower broiler diet containing DDGS on performance, bone quality, intestinal microflora and intestinal epithelial morphology.

MATERIALS AND METHODS

Animal source and experimental diets

In the present study, two independent experiments were conducted. For all experiments d-of-hatch male broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR, USA). In both experiments, chicks were neck-tagged and randomly located to one of sixteen floor pens (300 cm x 150 cm) with new pine shavings as litter in an environmentally controlled room. Temperature was maintained at 34°C for the first 5 d and was then gradually reduced according to normal management practices, until a temperature of 23°C was achieved at day 21 of age. In both trials, a mash starter corn-soybean based diet (0 to 7 d) and a mash grower diet containing 8% DDGS (8 to 28 d) were offered according to the phase of production. Prior to formulating the experimental diets, it was determined that DDGS contained: moisture 13.1% , crude protein 29.2%, crude fat 11.0%, crude fiber 8.5%, calcium 0.14% and phosphorus 0.72%. The experimental diets were formulated to approximate the nutritional requirements of broiler chickens as recommended by the National research council (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc., 2013). No antibiotics or coccidiostats were added to the feed (Table 1). The chemical composition of the experimental diets was determined by AOAC international (2000) methods for moisture (930.15), crude protein (984.13), crude fat (920.39), calcium (968.08) and phosphorus (965.17). All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas, Fayetteville, USA.

Experimental design

In order to show that similar results can be achieved independently, two experiments were conducted in the present study in broiler chickens that were raised during the starter and grower production phases. In both trials, broilers chicks were randomly assigned to either a control

group or a DFM candidate group fed with a diet supplemented with 10^6 spores/gram of feed of a *Bacillus*-DFM previously selected based on *in vitro* enzyme activity (Latorre et al., 2015b). Each treatment was comprised of eight pens of 20 chicks (n=160/group) and for evaluation of growth performance, each replicate was used as experimental unit. Every week, all broilers were individually weighed and BW, body weight gain (BWG) and feed intake (FI) data per pen were obtained to calculate the feed conversion ratio (FCR) for starter , grower and the overall experimental periods. Additionally in experiment 2 at day 28 of age, one broiler per replicate (n = 8/group) was humanely killed by CO_2 asphyxiation to collect intestinal samples for determination of the recovered microbiota population of total lactic acid bacteria (LAB), as well as, total Gram negative bacteria (GNB) and total anaerobic bacteria (TAB). Additionally, intestinal sections of duodenum and ileum were obtained to evaluate morphological parameters (n = 8/group). In the case of evaluation of bone quality parameters, both tibias from one bird per replicate were collected (n = 8/group). Details about measurement procedures are described below.

DFM preparation

In an effort to grow high numbers of viable spores, a solid state fermentation media (SS) developed by Zhao et al. (2008) was selected and modified for use in these experiments. Briefly a liquid media component was added to a mixture of 70% rice straw and 30% wheat bran at a rate of 40% by weight. The SS media was added to a 250 mL Erlenmeyer flask and sterilized by autoclaving for 30 min at 121°C. Candidate isolates were grown individually overnight at 37°C in TSB, then 2 mL of a candidate culture were added to the prepared SS media. The inoculated flasks were incubated for 24 h at 37°C then incubated for another 72 h at 30°C. The cultures

were removed from their flasks, placed onto petri dishes, and then dried at 60°C. Following this, the cultures were aseptically ground into a fine powder to generate stable spores (~ 10^{11} spores/g). Spores were mixed into the feed using a rotary mixer for 15 minutes. Samples of feed containing the DFM culture were taken and a 1:10 dilution was made with saline. All samples were subject to 100°C for 10 min. Ten-fold dilutions of these samples were plate on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD), incubated at 37°C for 24 h to count the number of spores per g of feed.

Enumeration of bacteria

For determination of total recovered bacteria, intestinal sections from duodenum to Meckel's diverticulum (foregut) and from Meckel's diverticulum to ceca (hindgut) were aseptically collected, separated into sterile bags and homogenized. Samples were weighed and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Later, ten-fold dilutions of each sample, from each group were made in a sterile 96 well Bacti flat bottom plate and then plated on different culture media; for enumeration of total recovered lactic acid bacteria (LAB) on Man Rogosa Sharpe agar (Difco™ Lactobacilli MRS Agar VWR Cat. No. 90004-084 Suwanee, GA 30024); total recovered Gram negative bacteria (TGN) on MacConkey agar (VWR Cat. No. 89429-342 Suwanee, GA 30024) and total recovered anaerobes (TAB) on tryptic soy agar containing sodium thioglycolate (Becton Dickinson Cat No. 212081 Sparks, MD 21152). All plates were incubated at 37ºC for 18 h and bacterial counts were expressed in colony forming units (Log_{10} cfu/g of tissue).

Bone parameters

Bone parameters were measured according to the methods described by Zhang and Coon, (1997). Tibias from each chicken were cleaned of attached tissues. Bones from the left leg were subjected to conventional bone assays as described below and tibias from the right leg were used to determine breaking strength. The bones from left tibia were dried at 100°C for 24 h and weighed. Then the samples were ashed in a muffle furnace (Isotemp muffle furnace, Fisher Scientific, Pittsburgh, PA) at 600°C for 24 h in crucibles, cooled in a desiccator and weighed. From the left tibia, total calcium content was obtained by inductively coupled plasma (968.08; AOAC International, 2000) and total phosphorus content was determined by colorimetry using the molybdo-vanadate method (967.17; AOAC International, 2000). In the case of the right tibia samples, the tibial diaphysis from individual birds were cleaned of adherent tissues, the periosteum was removed and the biomechanical strength of each bone was measure using an Instron 4502 (Norwood, MA) material testing machine with a 590 kg load cell. The bones were held in identical positions and the mid-diaphyseal diameter of the tibial mid-shaft which was also the site of impact, was measured using a dial caliper. The maximum load at failure was determined in the tibial mid-section between epiphyses, using a three-point flexural bend fixture with a total distance of 30 mm between the two lower supporting ends. The load, defined as force in kilograms per square millimeter of cross-sectional area (kg/mm^2), represents bone strength. The rate of loading was kept constant at 20 mm/min collecting 10 data points per second. The data were automatically calculated using Instron's Series IX Software (Norwood, MA).

Intestinal Morphometric Analysis

Intestinal sections were standardized: for duodenum, a 0.5 cm section was collected from the middle of the descending duodenum; and for ileum, a 0.5 cm section was collected from the mid-ileum at the Meckel's diverticulum. Duodenal, and ileal sections were fixed in 10% neutral buffered formalin and embedded in paraffin, sectioned (5-mm thick), set on a glass slide, and stained with hematoxylin and eosin (H&E), then examined by light microscopy. Photomicrographs of random selected fields of each intestinal sample were acquired using a microscope equipped with a Leica DFC450C camera and Leica V 3.8.0. software (Leica Application Suit) and used for morphometric analysis. ImageJ 1.47v software (Rasband, 1997-2012) was used to make the measurements in the morphometric analysis of the different intestinal sections. For villus height of duodenum and ileum, an average of 10 villi per bird were measured, with a total of 8 broilers per group. Villus length was measured from the tip of the villus to the top of the lamina propria. Crypt depth was measured from the base of the invagination between villus upwards the region of transition between crypt and villus (Aptekmann et al., 2001). Data from villus height and crypt depth were used to obtain the VH:CD ratio. Moreover, villus width was measured at the base area of each villi, and the villus surface area was calculated using the formula $(2\pi)(VW/2)(VL)$, where VW = villus width, and VL = villus length (Sakamoto et al., 2000).

Statistical Analysis

In all experiments, data were subjected to one-way ANOVA as a completely randomized design using the GLM procedure of SAS (SAS Institute, 2002). In both experiments, for the evaluation of growth performance (BW, BWG, FI and FCR) each of the 8 replicates of 20 chickens was considered as the experimental unit, whereas data on bone quality (n=8/group), intestinal

microbiota (n=8/group), and intestinal morphology (n=8/group) were based on randomly selected broilers from all replicates of each group. Data are expressed as mean ± SE and a *P*-value less than 0.05 was set as the standard for significance.

RESULTS

The results of the evaluation of performance parameters (BW, BWG, FI, FCR) in broiler chickens consuming a corn-DDGS-soybean grower diet with or without dietary inclusion of a *Bacillus*-DFM candidate of experiment 1 are summarized in Table 2. In this experiment, during the starter phase (0-7d), broilers consuming the diet supplemented with the DFM showed similar values in all the growth performance variables that were evaluated in comparison to the control group. On the other hand, during the grower phase (8-28d) when 8% of DDGS was included into the diet, supplementation with the *Bacillus*-DFM improve BWG in 48 g and FCR in 9 points when compared to the control group ($P<0.05$) (Table 2). Similarly, in experiment 2, inclusion of the *Bacillus*-DFM significantly increase BWG in 34 g and improve FCR in 7 points compared to the group consuming an unsupplemented diet. Additionally, FI was reduced in 41 g in the DFM group compared to control ($P<0.05$)(Table 3). In both trials, addition of the DFM improved performance parameters in the overall experimental period (0-28d), showing consistency of the results between trials.

Table 4 shows the results of the determination of total bacterial counts recovered from the foregut and hindgut intestinal segments in 28-d old broiler chickens from experiment 2. Chickens that received the *Bacillus*-DFM had reduced counts of TGN and increased numbers of LAB in both foregut and hindgut intestinal sections ($P<0.05$). Results of the assessment of bone strength and bone composition in broiler chickens fed with a corn-DDGS-soybean grower diet with or

without dietary inclusion of a *Bacillus*-DFM candidate in experiment 2 are summarized in Table 5. Bone strength and composition were measured in 28 d old broilers, showing that supplementation with the *Bacillus*-DFM significantly improve tibial breaking strength, as well as, calcium and phosphorus content compared with chickens receiving a control diet. (Table 5). Table 6 shows the results of the intestinal morphometric analysis of duodenum and ileal sections in chickens at 28-d of age from experiment 2. A significant increase in villus height, villus width, villus area, muscular thickness and VH:CD ratio were observed in chickens that received the DFM in both duodenum and ileum sections when compared with control group (Table 6).

DISCUSSION

Distillers dried grain with solubles is a by-product that can be obtained from different cereals during biofuel production (Świątkiewicz and Koreleski, 2008). As ethanol production has expanded in recent years, the availability of DDGS as feedstuff for poultry diets has increased (Wang et al., 2007b). To choose the correct percentage of inclusion for DDGS, it is important to know the nutritional composition profile of this raw material. Although DDGS have a good nutritional value and can be included at high levels in other livestock rations such as swine, 10% has traditionally been the recommended feeding limit for broiler chickens during the grower period (Stein, 2007). This upper feeding limit is linked to the high level of indigestible fiber components present in either wheat or corn DDGS (Barekatain et al., 2013). Compared with protein sources such as soybean meal, the nutritional value of DDGS is lower, due to its inferior protein quality, partly caused by the excessive pretreatment and drying conditions during the ethanol production process, and high level of non-starch polysaccharides (~30%) (Martinez-Amezcua et al., 2007). Diets high in NSP reduce effective energy and nutrient utilization in

poultry and other monogastric animals due to the lack of endogenous enzymes needed to break down the complex cell wall polysaccharides that encapsulate other nutrients (Bedford et al., 1991; Bedford and Classen, 1993; Bedford and Schulze, 1998) Hence, exogenous enzymes have been used as feed additives in poultry diets to diminish this antinutritional cage effect (Choct, 2006; Slominski, 2011). For instance, non-starch polysaccharide-hydrolyzing enzymes may increase the accessibility of phytase to phytin, increasing phosphorus availability and absorption (Singh et al., 2007; Zijlstra et al., 2010). This hypothesis is supported by research published by different authors (Choct et al., 1995; De Vries et al., 2014). On the other hand, one of the principal sources of the exogenous enzymes used by biotechnology companies are bacteria from the genus *Bacillus* (Monisha et al., 2009; Shah and Bhatt, 2011; Ibrahim et al., 2012). *Bacillus* are Gram-positive, rod shape and facultative anaerobe bacteria with a remarkable life-cycle including generation of endospores in nutritionally limit environments (Cutting, 2011). *Bacillus spp*. spores ability to resist rough environmental conditions, surviving high temperature during the feed pelletization procedure, as well as tolerating extreme pH, dehydration, high pressures, caustic chemicals and long storage periods have made them suitable for commercialization and distribution as direct-fed microbials (Cartman et al., 2007). Previously, our laboratory has screened and identified different *Bacillus spp.* isolates as DFM candidates for the production of exogenous enzymes (cellulase, xylanase, amylase, phytase, protease, and lipase). Moreover, we have demonstrated that the inclusion of cereal grains with a higher content of soluble NSP in comparison to corn increased digesta viscosity and *C. perfringens* growth. Nevertheless, the dietary inclusion of the selected *Bacillus*-DFM candidate in non-corn based diets significantly reduced both viscosity and *C. perfringens* proliferation when compared to control non-supplemented diets (Latorre et al., 2015b). Additionally, chickens or turkeys fed a rye-based diet

without DFM showed an increase in bacterial translocation and digesta viscosity, accompanied by reduced bone mineralization; however these adverse effects were ameliorated by the inclusion of the DFM-candidate (Latorre et al., 2014b; Latorre et al., 2015a). Furthermore, it has been shown that *Bacillus* spores can persist and change their distribution according to the variable biochemical conditions of the GIT of broiler chickens; therefore, supporting the hypothesis of a possible full-lifecycle development in the gastrointestinal tract (Latorre et al., 2014a). In the present study, supplementation with the *Bacillus*-DFM in a grower diet containing 8 % of DDGS improved performance parameters, bone quality, intestinal microflora balance and intestinal morphology, therefore supporting our previous results with poultry diets including alternative feed ingredients. Chickens that received the DFM in the grower-DDGS diet, increased the surface area of absorption in both duodenal and ileal intestinal sections, and this could be related to a more efficient utilization of the diet due to the production of exogenous enzymes by the DFM. It was also interesting to observed, that broilers receiving the *Bacillus*-DFM had a higher count of LAB in the foregut and hindgut intestinal sections. Perhaps, the improvement in villi high and villi width was due to an elevated production of short chain fatty acids by LAB in the intestinal lumen. On the other hand, the reduction of TGN bacteria, may also diminished the level of intestinal inflammation, therefore, enhancing epithelial integrity and nutrient absorption. In summary, the results of this study suggest that the dietary inclusion of a previously selected *Bacillus*-DFM based on *in-vitro* enzyme production profiles, could contribute to enhance performance, bone quality, and improve both intestinal microbial balance as well as epithelial morphology in broiler chickens consuming diets that contained a considerable percentage of DDGS. Further studies to evaluate metabolomics and microbiome analysis as well as other gut

inflammation biomarkers in chickens fed with this selected *Bacillus*-DFM in different poultry diets are currently being evaluated.

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Table 1. Ingredient composition and nutrient content of a corn-soybean starter diet and a corn-DDGS-soybean grower diet used in all experiments on as-is basis

Item	Starter diet	Grower diet
Ingredients (%)		
Corn	57.34	56.68
Soybean meal	34.66	27.05
DDGS	-	8.00
Poultry fat	3.45	4.09
Dicalcium phosphate	1.86	1.59
Calcium carbonate[a]	0.99	1.03
Salt	0.38	0.34
DL-Methionine	0.33	0.26
L-Lysine HCl	0.31	0.32
Threonine	0.16	0.12
Vitamin premix[b]	0.20	0.20
Mineral premix[c]	0.10	0.10
Choline chloride 60%	0.20	0.20
Antioxidant[d]	0.02	0.02
Calculated analysis		
Metabolizable energy (kcal/ kg)	3,035	3,108
Crude protein (%)	22.16	20.73
Ether extract (%)	5.68	7.11
Lysine (%)	1.35	1.20
Methionine (%)	0.64	0.57
Methionine + Cystine (%)	0.99	0.91
Threonine (%)	0.92	0.82
Tryptophan (%)	0.28	0.24
Total calcium	0.90	0.84
Available phosphorus	0.45	0.42
Determined analysis		
Crude protein (%)	21.15	20.30
Ether extract (%)	6.05	6.78
Calcium (%)	0.94	0.90
Phosphorus (%)	0.73	0.69

[a]Inclusion of 10^6 spores/g of feed mixed with calcium carbonate.
[b]Vitamin premix supplied the following per kg: vitamin A, 20,000 IU; vitamin D3, 6,000 IU; vitamin E, 75 IU; vitamin K3, 6.0 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 µg; and ascorbic acid, 200 mg (Nutra Blend LLC, Neosho, MO 64850).
[c]Mineral premix supplied the following per kg: manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg (Nutra Blend LLC, Neosho, MO 64850).
[d]Ethoxyquin.

Table 2. Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), in broiler chickens consuming a Corn-DDGS-Soybean grower diet with or without dietary inclusion of *Bacillus*-direct-fed microbials (Experiment 1)[c]

Item	Control	*Bacillus*-DFM
BW, g/broiler		
d 0	47.2 ± 0.5[a]	47.5 ± 0.2[a]
d 7	150.6 ± 3.2[a]	148.8 ± 1.2[a]
d 28	1437.0 ± 14.4[b]	1484.0 ± 14.5[a]
BWG, g/broiler		
d 0 to 7	103.4 ± 2.9[a]	101.3 ± 1.1[a]
d 8 to 28	1286.4 ± 13.3[b]	1335.3 ± 14.1[a]
d 0 to 28	1389.8 ± 14.3[b]	1436.6 ± 14.6[a]
FI, g/broiler		
d 0 to 7	177.0 ± 6.4[a]	175.1 ± 7.3[a]
d 8 to 28	2081.8 ± 19.8[a]	2052.3 ± 20.8[a]
d 0 to 28	2212.6 ± 19.9[a]	2182.6 ± 19.6[a]
FCR		
d 0 to 7	1.17 ± 0.02[a]	1.18 ± 0.04[a]
d 8 to 28	1.62 ± 0.01[a]	1.53 ± 0.02[b]
d 0 to 28	1.54 ± 0.01[a]	1.47 ± 0.01[b]

[a,b] Means with no common superscript letter within a row differ significantly at $P < 0.05$
[c] Data are expressed as mean ± SE.

Table 3. Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), in broiler chickens consuming a Corn-DDGS-Soybean grower diet with or without dietary inclusion of *Bacillus*-direct-fed microbials (Experiment 2)[c]

Item	Control	*Bacillus*-DFM
BW, g/broiler		
d 0	39.7 ± 0.2[a]	39.9 ± 0.3[a]
d 7	115.3 ± 1.7[a]	116.2 ± 1.5[a]
d 28	1409.0 ± 7.9[b]	1444.0 ± 12.6[a]
BWG, g/broiler		
d 0 to 7	75.6 ± 1.8[a]	76.3 ± 1.6[a]
d 8 to 28	1294.1 ± 8.7[b]	1328.6 ± 12.1[a]
d 0 to 28	1369.7 ± 7.9[b]	1404.8 ± 12.6[a]
FI, g/broiler		
d 0 to 7	130.8 ± 3.2[a]	130.5 ± 2.3[a]
d 8 to 28	1879.0 ± 10.2[a]	1838.0 ± 13.3[b]
d 0 to 28	2010.0 ± 9.7[a]	1966.6 ± 13.6[b]
FCR		
d 0 to 7	1.13 ± 0.01[a]	1.12 ± 0.02[a]
d 8 to 28	1.45 ± 0.03[a]	1.38 ± 0.01[b]
d 0 to 28	1.43 ± 0.02[a]	1.36 ± 0.01[b]

[a,b] Means with no common superscript letter within a row differ significantly at $P < 0.05$
[c] Data are expressed as mean ± SE.

Table 4. Determination of total bacterial counts in the foregut and hindgut intestinal segments in broiler chickens consuming a corn-DDGS-soybean grower diet with or without dietary inclusion of *Bacillus* direct-fed microbials (Experiments 2)[c]

	Foregut[d]			Hindgut[d]		
Item	**TGB[e]**	**LAB[e]**	**TAB[e]**	**TGB[e]**	**LAB[e]**	**TAB[e]**
Control	4.70 ± 0.18[a]	5.19 ± 0.29[b]	5.24 ± 0.28[a]	6.76 ± 0.41[a]	6.10 ± 0.42[b]	7.14 ± 0.60[a]
***Bacillus*-DFM**	3.75 ± 0.17[b]	6.11 ± 0.19[a]	5.67 ± 0.49[a]	5.89 ± 0.49[a]	7.37 ± 0.04[a]	6.39 ± 0.61[b]

[a,b] Different superscripts within columns indicate significant difference at $P < 0.05$.
[c] Bacteria enumeration evaluated from 28 d-old broilers, n = 8/group
Data are expressed as Log_{10} cfu/g mean ± SE.
[d] Forgut: From duodenum to Meckel's diverticulum, Hindgut: From Meckel's diverticulum to ceca.
[e] TGB: Total Gram negative bacteria recovered, LAB: Total lactic acid bacteria recovered, TAB: Total anaerobic bacteria recovered from different intestinal sections in each experimental group.

Table 5. Assessment of bone strength and bone composition in broiler chickens fed with a Corn-DDGS-Soybean grower diet with or without dietary inclusion of *Bacillus* direct-fed microbials (Experiment 2)[c]

Item	Load at break (kg)	Tibia diameter (mm)	Breaking strength (kg/mm^2)	Calcium (%)	Phosphorus (%)
Control	35.85 ± 1.47^{b}	6.84 ± 0.21^{a}	5.26 ± 0.02^{b}	35.24 ± 0.10^{b}	16.60 ± 0.30^{b}
***Bacillus*-DFM**	42.88 ± 2.75^{a}	7.14 ± 0.31^{a}	5.99 ± 0.01^{a}	39.26 ± 0.24^{a}	20.83 ± 0.66^{a}

[a,b] Means with no common superscript letter within a column differ significantly at $P < 0.05$

[c] Data are expressed as mean ± SE. Bone measurements evaluated from 28 d-old broilers, n = 8/group

Table 6. Morphometric analysis of duodenum and ileal tissue in chickens at d 28 of age (Experiment 2)[c]

Tissue	Control	*Bacillus*-DFM
Duodenum		
Villus Height (µm)	337.20 ± 3.07[b]	457.24 ± 4.66[a]
Villus Width (µm)	40.07 ± 0.44[b]	44.43 ± 0.22[a]
Crypt depth (µm)	64.07 ± 1.14[a]	55.23 ± 0.44[b]
Area (mm^2)[d]	42.38 ± 0.52[b]	63.95 ± 0.85[a]
Muscular thickness (µm)	46.79 ± 0.82[b]	60.42 ± 0.40[a]
VH:CD[e]	5.34 ± 0.06[b]	8.32 ± 0.11[a]
Ileum		
Villus Height (µm)	140.88 ± 3.06[b]	166.90 ± 3.81[a]
Villus Width µm	33.91 ± 0.82[b]	39.62 ± 0.62[a]
Crypt depth (µm)	46.88 ± 1.64[a]	38.59 ± 1.00[b]
Area[c] (mm^2)[d]	15.32 ± 0.59[b]	21.15 ± 0.73[a]
Muscular thickness (µm)	34.86 ± 0.44[b]	43.16 ± 0.64[a]
VH:CD[e]	3.02 ± 0.03[b]	4.55 ± 0.13[a]

[a,b] Means with no common superscript letter within a row differ significantly at $P < 0.05$
[c] Data are expressed as mean ± SE. Morphometric analysis evaluated from 28 d-old broilers, n = 8/group
[d] 2π × (villus width/2) × villus height (Sakamoto et al., 2000).
[e] Villus height to crypt depth ratio.

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IX. CONCLUSIONS

In the series of studies evaluating and selecting different *Bacillus spp.* strains as DFM candidates presented in this dissertation, multiple mechanisms of action of this wonderful microorganism were investigated, including strain-specific features from different isolates, and feasibility of being used in large-scale commercial production conditions with different poultry diets.

Determination of the endospores' behavior and distribution in the GIT of chickens was crucial to provide support to following research. It was observed that approximately 90% of the spores included in the diet germinate in the GIT, suggesting that spores become metabolically active vegetative cells in the intestinal lumen, providing a different set of benefits such as production of microbial compounds and enzymes. Additionally, the persistence of spores in the GIT following a single gavage dose was much longer than the estimated half-life based on gut passage time, suggesting that some full-life cycle development occurs within the GIT. The number of spores variates in each gut section, showing a higher germination rate in compartments like the crop, and an increase in the sporulation rate in the ceca. This could reflect different phases of the live cycle preparing a new generation of bacteria to exit the host and remain viable in the environment as endospores. Moreover, these results confirm that *Bacillus* spores are transiently present in the GIT of chickens, but the persistence of vegetative cells is presently unknown. Therefore, to obtain a prolonged benefit in poultry performance parameters, continuous administration of selected *Bacillus spp.* strains as DFM is advisable.

Screening and selection of *Bacillus* isolates is an exhaustive process that depend on the purpose the DFM is going to be used. Not all *Bacillus* bacteria are the same, even within the same species there are individual differences that could affect the capacity to produce variable amounts of antimicrobial compounds, enzymes, and biofilms or immunomodulatory effects in the host. In

the case of enzyme production, from 31 different isolates 3 were selected as superior enzyme producers of amylase, protease, lipase, cellulase, xylanase, and phytase. However, it is important to mention that the three selected *Bacillus* isolates were mixed in equal proportions (1:1:1) to conform the final DFM-candidate. Furthermore, the previously selected DFM during an *in vitro* study showed to reduce digesta viscosity, *C. perfringens* proliferation and also survive different simulated biochemical conditions of various compartments of the GIT (Crop, proventriculus and small intestine) using poultry diets based on multiple high NSP cereals as principal source of energy.

On the other hand, inclusion of the *Bacillus*-DFM candidate in a rye-based diet fed to turkey poults showed an impressive reduction of the detrimental antinutritional effects reported in diets with an elevated content of arabinoxylans. The group of poults consuming the DFM had a significant decrease in digesta viscosity, as well as in the number of bacteria translocated from the intestinal lumen to the liver in comparison to control, suggesting an improvement of gut epithelial integrity related to the capacity of these selected strains to produce xylanase and antimicrobial agents to control bacterial overgrow and presentation of intestinal inflammation. Therefore, supplementation of high NSP diets with the DFM-candidate resulted in enhanced intestinal physiological conditions for absorption of nutrients, improving bone quality parameters and growth performance. A similar outcome was observed in a series of trials conducted with broiler chickens consuming rye-based diets, where the inclusion of the *Bacillus*-DFM reduced digesta viscosity, bacterial translocation, and total recovered Gram-negative bacteria as well as total recovered anaerobic bacteria in the duodenum. Decrease digesta viscosity in diets containing a higher concentration of soluble NSP is a key factor to increase performance parameters, because in a viscous intestinal environment the interaction of endogenous enzymes

and the intestinal content is limited and inefficient. Additionally, the lower passage rate and oxygen tension can benefit growth of enteropathogens such as *C. perfringens* resulting in the presentation of intestinal diseases such as necrotic enteritis. Hence, incorporation to the ingredient matrix of a feed additive taking advantage of already know benefits of probiotics on gut health and also their ability to improve nutrient utilization through synthesis of exogenous enzymes could be a new approach to maintain performance standards in diets without AGP.

Due to a continuous interest in renewable energy sources, the biofuel industry has been steadily expanding. Consequently, corn utilization has been changing from the feed to the ethanol industries, having as a result high availability of by-products such as DDGS that are increasingly being included as raw materials in livestock diets. The impact of the inclusion of the selected DFM-candidate was evaluated in grower diets containing 8% DDGS and showed a constant improvement of growth performance parameters, making broilers more efficient in the use of this alternative ingredient. *Bacillus*-DFM supplementation enhanced bone quality and increased the number of LAB in the foregut and hindgut intestinal sections. Additionally, it was observed a significant development of the intestinal epithelia with a higher villus area of absorption that could be related with more production of short chain fatty acids in the gut lumen.

The compilation of studies of this dissertation confirm that poultry diets supplemented with *Bacillus* endospores from isolates selected based on *in vitro* enzyme activity could contribute to enhance intestinal physiological traits, improve nutrient absorption, increase bone quality, and help to maintain a healthy microbial balance. At the end, resulting in more efficient performance parameters in poultry when consuming diets that contained considerable percentages of alternative and/or conventional feed ingredients.

X. APPENDIX I

February 23, 2016

MEMORANDUM

TO:	Billy Hargis
FROM:	Ines Pinto, Biosafety Committee Chair
RE:	New Protocol
PROTOCOL #:	16020
PROTOCOL TITLE:	Experimental studies to evaluate efficacy of probiotic culture alternatives
APPROVED PROJECT PERIOD:	Start Date 02/11/2016 Expiration Date 02/10/2019

The Institutional Biosafety Committee (IBC) has approved Protocol 16020, "Experimental studies to evaluate efficacy of probiotic culture alternative". You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.