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Evaluation of Selected Bacillus Direct-Fed Microbial Candidates in Reduced Energy Diets on Live Performance, Carcass Characteristics, and Foot Pad Dermatitis

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Evaluation of Selected *Bacillus* based Direct-Fed Microbial Candidates in Reduced Energy Diets on Live Performance, Carcass Characteristics, and Foot Pad Dermatitis

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Abstract

Bacillus spp. are ubiquitous, Gram-positive, spore forming bacteria that are commonly recovered from the environment and gastrointestinal tract (GIT) of poultry. These spores are capable of withstanding harsh condition such as feed pelletization, which facilitates inclusion in poultry feeds. Once ingested by the bird, spores germinate into metabolically active vegetative cells which can produce extracellular enzymes which can hydrolyze otherwise indigestible components of the feed. Soybean meal (SBM) is the gold standard vegetable protein source for non-ruminant animals worldwide and is included in practically all poultry diets in the United States at levels ranging from 10 to more than 30%. However, SBM also contains various indigestible carbohydrate fractions that can exert antinutritive effects, limiting some of the available energy and nutritional value of SBM for poultry. These anti-nutritive effects can primarily be attributed to soluble non-starch polysaccharides (**NSP**; e.g., pectins and β-mannans) and oligosaccharides (raffinose, stachyose, and verbascose). Soluble NSP increase digesta viscosity, leading to changes in intestinal physiology that not only inhibit digestibility of dietary energy and nutrients, but can also compromise gastrointestinal health. Thus, many nutritionists attempt to restrict SBM to inclusion levels lower than those that would be realized if relying solely on least-cost feed formulation. More than 50% of the U.S. commercial broiler industry has adopted antibiotic-free production methods, with as many as one-third of broilers fed all vegetable-based diets to align with growing consumer demand for these products. As such, demand for all-vegetable based diets presents a clear opportunity for increased SBM demand, but the aforementioned concerns related to high-SBM broiler diets are heightened under these production systems. The objective of the present dissertation was to evaluate the effect of *Bacillus* isolates ability to increase performance and welfare conditions of broilers fed diets with

elevated levels of SBM. In the first experiment, 6 trials were conducted to evaluate performance at 21 d post hatch in broilers fed a mash, corn-high SBM diet with either control, CTL (3,050 kcal/kg) or reduced energy, RED (2,925 kcal/kg) or RED supplemented with select *Bacillus* isolates. Consistent positive responses of increased body weight gain (BWG) and lower feed conversion ratios (FCR) were observed for birds fed RED + *Bacillus* isolate 46 (BI-46) compared to birds fed RED alone. Pooled data from trials 1-3 showed RED + BI-46 fed birds to be intermediate compared to CTL and RED for BWG and FCR. Data pooled for trials 4-6 showed birds fed RED + BI-46 had higher BWG and lower FCR compared to birds fed RED alone and were similar in performance to CTL group. This experiment confirmed not all isolates that performed well *in vitro* yielded performance benefits *in vivo*. In experiment 2, birds were fed similar diets as in experiment 1, in either mash or pelleted form, with a 125 kcal/kg difference in AME_n between the CTL and RED diets throughout the starter, grower, and finisher feeding phases. Birds fed pelleted diets had higher BWG and lower FCR in the starter and overall phases compared with birds fed mash diets. The birds fed RED + BI-46 had lower FCR in the starter phase than birds fed RED alone. Birds fed the CTL diet had the lowest cumulative FCR among all treatment group. The birds fed RED + BI-46 had greater hot and chilled carcass yield compared to birds fed the CTL. Birds fed RED + BI-46 had higher breast yield than birds fed RED and greater tender yield than birds fed CTL. Inclusion of BI-46 also lowered foot pad dermatitis lesion scores at 36 d post-hatch compared to the CTL group. Overall, this research demonstrates that BI-46 could potentially increase performance of birds fed higher levels of SBM and enhance welfare conditions.

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“Therefore, since we are surrounded by such a great cloud of witnesses, let us throw off everything that hinders and the sin that so easily entangles. And let us run with perseverance the race marked out for us, fixing our eyes on Jesus, the pioneer and perfecter of faith. For the joy set before him he endured the cross, scorning its shame, and sat down at the right hand of the throne of God.”

Hebrews 12:1-2

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Chapter 1: Introduction

Growing consumer demand for broilers fed all vegetable-based diets and raised without antibiotics has challenged the poultry industry to adopt novel production techniques to overcome production hurdles. An increased inclusion of soybean meal (SBM) in poultry diets has resulted from removal of animal by-products. Soybean meal is the gold standard vegetable protein source for non-ruminant animals worldwide and is included in practically all poultry diets in the United States at levels ranging from 10 to more than 30%.(Cromwell, 1999). Soybean meal is an excellent source of digestible amino acids in animal feeds, yet a considerable proportion of its energy is not available to poultry (Dozier et al., 2011). This untapped energy is largely contained within indigestible SBM carbohydrates that are not only poorly utilized themselves, but exert anti-nutritive effects (Slominski, 2011). However, SBM also contains various indigestible carbohydrate fractions that can exert anti-nutritive effects, limiting some of the available energy and nutritional value of SBM for poultry (Choct et al., 2010). These anti-nutritive effects can primarily be attributed to soluble non-starch polysaccharides (**NSP**; e.g., pectins and β-mannans) and oligosaccharides (raffinose, stachyose, and verbascose) (Coon et al., 1990; Choct et al., 2010; Hossain et al., 2012). Soluble NSP increase digesta viscosity, leading to changes in intestinal physiology that not only inhibit digestibility of dietary energy and nutrients, but can also compromise gastrointestinal health (Choct, 1997). Thus, many nutritionists attempt to restrict SBM to inclusion levels lower than those that would be realized if relying solely on least-cost feed formulation (Mukherjee et al., 2016).

There have been many attempts to alleviate these symptoms through SBM processing techniques (Coon et al., 1990) , genetic selection (Parsons et al., 2000), and enzyme supplementation (Bedford and Partridge, 2010). However, there are pitfalls to each of the aforementioned

approaches whether monetary or technical. The approach of this dissertation was to utilize a *Bacillus*-based DFM that was selected for its ability for *in situ* (i.e., within the gastrointestinal tract of broilers) production of digestive enzymes that target indigestible SBM carbohydrates. *Bacillus* spores application to poultry diets has steadily gained acceptance due to their long shelf life and ability to withstand pelleting temperatures in the feed milling process (Cartman et al., 2007). Previous studies have demonstrated the ability of *Bacillus* spores to be present, germinate, and survive in the gastrointestinal tract (GIT) of mice and poultry (Duc et al., 2003; Latorre et al., 2014). *Bacillus* have been shown to produce several extracellular enzymes that might increase nutrient availability for the animal, including phytase, lipase, protease, amylase (Latorre et al., 2016), cellulase, and xylanase (Latorre et al., 2015). The objectives of the present dissertation were to evaluate a selected *Bacillus*-DFM candidate in low energy, high SBM diets on live performance, carcass characteristics, and foot pad dermatitis.

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Chapter II. Literature Review

Indigestible Components of Poultry Feeds

The indigestible component in poultry feeds includes the fractions that are either totally undigested or incompletely digested (Ravindran, 2013). These include substrates for which poultry does not produce suitable enzymes, substrates that have anti-nutritional properties, or substrates that are inaccessible to enzymes because they are encapsulated or bound to other compounds. Each vegetable cell contains nutrients such as starch, lipids, and proteins that poultry can easily digest as they naturally produce enzymes like amylase, lipase, and protease (Meng et al., 2004). However, in cereal grains like corn, wheat, sorghum, barley, and rye or cereal by-products like distiller's dried grains with solubles, these nutrients are often protected from enzymatic digestion by cell walls comprised of non-starch polysaccharides (NSP) (Knudsen, 1997). Fiber is one of the most quantitatively important indigestible fractions of feed (Choct et al., 2010). Insoluble fiber passes through the upper part of the intestinal tract intact (Knudsen, 1997), locking in the nutrients present within the cell wall making them unavailable to the animal. Insoluble fibers can be broken down further into two categories: Neutral detergent fiber (NDF) and acid detergent fiber (ADF) (Raza et al., 2019). The NDF contains hemicellulose, cellulose, and lignin, while ADF consists of the more indigestible cellulose and lignin (Choct, 1997). The NSP are mainly concentrated in the cell walls of the endosperm, but are also in the bran (Knudsen, 1997). They can be divided into two categories, the water-soluble beta-glucans and pectins and water-insoluble cellulose fractions (Knudsen, 2014). The NSP content of cereal grains ranges from 7 to 19%. The outer cell wall tissues are made up mostly of cellulose and lignin, while the cell walls of the endosperm are predominately arabinoxylans and beta-glucans.

The complexity of arabinoxylans is variable among grains and highly influences the digestibility of feed (Knudsen, 1997).

Effects on Intestinal Health

Increased levels of NSP in poultry diets, particularly the soluble fraction, lead to decreased nutrient digestion and absorption (Choct and Annison, 1990). It is accepted the negative effects of NSP are linked to the increase in digesta viscosity, physiological and morphological changes to the digestive tract, and the interaction with the microflora of the gut (Choct, 1997). Elevated gut viscosity is credited to the water-soluble fraction as the arabinose branches provide soluble properties and the ability to bind water (Choct et al., 2010). This decreases the rate of diffusion of substrates and digestive enzymes and obstructs their interactions at the mucosal surface (Edwards et al., 1988). In addition to acting as a physical barrier to nutrient digestion and absorption, soluble NSP can also alter gut functions by changing endogenous secretions of electrolytes, water, lipids, and proteins (Angkanaporn et al., 1994). These changes within the GIT are accompanied by increased digestive secretions, enlargement of digestive organs, and decreased nutrient digestion (Choct, 1997).

In addition, it has been suggested that the anti-nutritive effects of NSP may be exacerbated by some anaerobic, opportunistic intestinal microflora as MacAuliffe and McGinnis (1971) reported dietary supplementation with antibiotics moderately improved the nutritive value of rye. Wagner and Thomas (1978) observed anaerobic counts in the ileum of birds fed rye or pectin-enriched diets was 2 to 3 logs higher compared to those fed a corn-soy diet and counts were reduced by 5 logs with the addition of penicillin to the diet. Moreover, soluble NSP results in a slower digesta passage rate (Van der Klis and Van Voorst, 1993), which could decrease oxygen tension to benefit the overgrowth of anaerobic bacteria. Choct et al. (1996) reported an

increase in fermentation in the small intestine of broilers by adding soluble NSP in the diet. This will increase volatile fatty acid production; however, the drastic modifications to the gut ecosystem resulted in decreased nutrient digestion accompanied by poor bird performance (Choct et al., 2010).

Effects on Litter Quality

Poultry diet composition and nutrient concentration has been shown to influence litter quality in turkeys and broiler chickens (Swiatkiewicz et al., 2017). Dietary protein is an important factor that affects litter quality. Ferguson et al. (1998) determined lowering the crude protein concentration significantly reduced litter moisture in broilers from 22 to 43 days of age. Dietary nitrogen not utilized by the bird is excreted as undigested protein in fecal waste or by the kidneys as uric acid. Approximately 50% of excreted N content is in the form of uric acid (Ritz et al., 2004). This N can be quickly converted to ammonia by hydrolysis, mineralization, and volatilization mostly by the primary uricolytic bacteria, *Bacillus pasteurii* (Oenema et al., 2008). As poultry houses become "tighter" with less frequent litter removal, an increase in house moisture, relative humidity, and nitrogen levels in the litter are observed (Ritz et al., 2004). This environment has the potential to markedly increase ammonia concentrations in poultry housing. The National Institute of Occupational Safety and Health has established an exposure time of 8 to 10 h at 25 ppm for humans, while similar recommendations have been made for poultry (Carlile, 1984). Ammonia in poultry houses has been shown to lower performance and possibly increase disease susceptibility (Beker et al., 2004). Broilers exposed to ammonia levels of 25 ppm or greater exhibited lower body weight gains (Miles et al., 2002) and decreased feed consumption and efficiency (Charles and Payne, 1966; Johnson et al., 1991). In addition to detrimental effects on performance, levels of 75 to 100 ppm resulted in loss of cilia and

increased the number of mucus-secreting cells in the respiratory epithelium (Oyetunde et al., 1978; Al-Mashhadani and Beck, 1985). Carr and Nicholson (1980) reported exposure of 46 to 102 ppm resulted in keratoconjunctivitis, which can result in blindness.

Soybean Meal Production and Nutritional Composition

United States SBM is included in 70% of the animal feed produced worldwide, making it the most frequently used source of protein for swine and poultry feeds (Dozier et al., 2011). Soybean meal is characterized as either from dehulled beans or beans having hulls (NRC, 1994). Dehulled soybean meal has a higher crude protein, amino acid, and metabolizable energy composition as opposed to soybeans having hulls (NRC, 1994). In the U.S., soybeans are generally processed using one of two methods, mechanical or chemical. While chemical processing is still the predominant method, recent increases in the production of biodiesel fuel have resulted in more plants switching to the extruder/expeller processing methods (Loeffler, 2012). In the chemical process, a hexane solvent is used to extract the oil, while in the mechanical method a two-step process of extrusion and expelling is used to remove the oil. The hexane solvent has been scrutinized, as it is highly flammable and considered a carcinogen (Balloun, 1980). The extrusion process is considered a more "natural" way to process the soybeans in comparison to hexane extraction. Additionally, capital cost for this process is much lower than a solvent extraction plant, so this is also popular in developing industries.

Solvent Extraction

Solvent extraction became the preferred method of oil extraction from soybeans around the 1930's because of its ability to remove all but about 0.5% of the soy oil. During solvent extraction, soybeans are dehulled and cracked using corrugated rollers. The hulls are removed because they contain very little oil and the cracked soybeans are conditioned at 70-75°C for 20-

30 minutes. This steam conditioning hydrates the cracked beans to condition them for further processing. Next, the beans are flaked under pressure and flaking rollers. It is vital that the flakes are thin enough for solvent penetration, but strong enough so they do not crumble. Flaking ruptures the beans cells, allowing the solvent to cover more surface area. The flaking process also exposes the oil cells, which allows the solvent to penetrate and increase oil extraction yield. The commercial solvent used is hexane at a ratio of 1:1 solvent-to-soybean. After leaving the extractor, the beans can contain up to 40% solvent and they must be air dried before use to remove the chemical. The extracted flakes are toasted and steam heated to inactivate anti-nutritional factors and remove any remaining hexane. In the final step, the flakes are dried and ground to an acceptable size for feed ingredients. The beans used to make SBM may be intact or they may be dehulled prior to flaking. If soybeans are not dehulled, the meal will contain more NDF and less protein, thus explaining the crude protein percentage spread below (NRC, 2012). The resulting SBM has a particle size ranging from 700-1,000 microns, contains around 44 to 48% crude protein, 3.5 to 7% crude fiber, and 1% crude fat. The beans are dried to approximately 10% moisture content and tempered to allow a moisture equilibrium to be reached. For poultry, the soybean meal contains approximately 2,230 – 2,440 kcal/kg of metabolizable energy (NRC, 1994).

Expeller-Extruded

The dry extrusion process was first introduced in 1969, as opposed to wet extrusion, the dry extruder does not require an external source of heat or steam (Said, 2000). The process uses friction generated from the extrusion process as the sole source of energy to cook and dehydrate the soybeans. These are single screw extruders put together around a shaft. In between the screw a restriction plate of different diameters can be placed to increase the cook and shear. When

material moves in the barrel and comes across these restrictions, it is unable to pass through, and consequently, pressure builds up and a back flow is created. Usually these restriction plates are arranged in such a way that they increase in diameter toward the die end of the screw creating more pressure and shear as they reach the die. This buildup of pressure and temperature, together with shear stresses developed, tends to plasticize (gelatinize) the raw materials into viscous paste or puffed shape, depending upon the raw material. In dry extrusion, pressure and temperature should be at a maximum just before leaving the die. The optimum extrusion temperature is around 150-160°C. This temperature and pressure is enough to denature anti-nutritional factors and rupture the oil cells. As soon as the material leaves the extruder dies, pressure is instantaneously released from the products, which cause internal moisture to vaporize into steam, making the product expand (Extruding, 2007). The final product contains 38% crude protein, 5-10% crude fat, and a metabolizable energy value for poultry of 3,200 kcal/kg. (NRC, 1994; NRC, 2012). This method produces higher metabolizable energy than solvent extraction and has a more predictable quality of fat, so there is no need to add fat to the diet (Loeffler, 2012).

Overheating of Soybean Meal

Although soybeans must be heat-treated through one of these processes to remove anti-nutritional factors, they can be overcooked which reduces the nutritional value of the meal for poultry (Renner et al., 1953; Warnick and Anderson, 1968; Araba and Dale, 1990). The overcooking of soybean meal decreases the digestibility of amino acids likely due to Maillard reaction (Lee and Garlich, 1992; Parsons et al., 1992). Parsons et al. (1992) autoclaved dehulled, solvent extracted SBM at 121°C and 105 kPa for 0, 20, 40, and 60 minutes to examine the effects of over processing. Increasing autoclave time reduced total concentration of lysine, arginine, and cysteine, but did not influence other amino acids. A growth assay using broiler chicks found

autoclaving at 121°C for 40 minutes reduced lysine availability by 15% compared to birds fed non-autoclaved soybean meal. The damage of lysine and arginine due to autoclaving and the browning of the meal indicates the presence of the Maillard reaction. During the Maillard reaction, the reactive carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid. This produces a Schiff base, which cyclizes to form a glycosylamine. The glycosylamine is then transformed into either an Amadori product (glucose) or a Heyns product (fructose). This reaction predominately affects the ε-amino group of lysine. The decreased absorption of lysine is due to the glycosylated lysine derivatives competing with lysine for absorption carriers. These derivatives are poorly utilized with greater than 75% of the absorbed amounts being excreted (Dozier et al., 2011).

Anti-Nutritional Factors

Soybeans contain a number of anti-nutritional factors such as, oligosaccharides, non-starch polysaccharides, phytate, lectins, and trypsin inhibitors, which can result in a reduction in nutrient utilization. These factors cause negative effects by different mechanisms, including binding to digestive enzymes and nutrients or increasing gut viscosity (Ravindran, 2013). The anti-nutritional factors can be split into two groups: 1) heat labile and 2) heat stable. The soybeans are heated to denature the native protein structure and inactivate the trypsin inhibitors and lectins. However, oligosaccharides are heat stable and the concentration of raffinose and stachyose cannot be reduced by heating (Loeffler, 2012).

Trypsin Inhibitors

Trypsin inhibitor is the primary anti-nutritional factor in soybean meal (Araba and Dale, 1990; Anderson-Hafermann et al., 1992; Mian and Garlich, 1995). It inhibits the conversion of zymogens to active proteases of trypsin and chymotrypsin. Trypsin inhibitor binds trypsinogen,

forming an irreversible compound, which stops the development of being able to produce an active protease. Trypsin inhibitors effects on chymotrypsin are less severe, as it forms a reversible dissociated compound that could potentially still become active (Loeffler, 2012).

Trypsin inhibitor also has an impact on pancreas size and amount of trypsinogen produced. Chernick et al. (1948) fed chicks diets containing raw soybean meal or heat-treated soybean meal and observed a 43% increase in trypsinogen content per gram of pancreas nitrogen content and a 56% increase in pancreas weight as a percent of body weight in chicks fed the raw soybean diet. This overstimulation of trypsinogen production in an attempt to compensate for the trypsin inhibitor leads to pancreatic hypertrophy. An enlarged pancreas not only results in increased enzyme production, but also in the increased secretion of nitrogenous products into the intestine, which could explain some of the growth-limiting effects of raw soybeans (Saini, 1989). Broiler growth has been reported to increase 140 to 150% by feeding heat treated raw, hexane extracted soybeans or soybean meal compared to non-heat treated raw, hexane extracted soybeans or soybean meal (Araba and Dale, 1990; Anderson-Hafermann et al., 1992).

There have been different proposed mechanisms as to how the trypsin inhibitor negatively affects growth rate. One mechanism is that the imbalance in amino acids, caused by the biosynthesis of enzymes in the pancreas increasing essential amino acid requirements, resulting in decreased protein digestion and lower concentration of essential amino acids necessary for optimal growth (Rackis, 1974). Another mechanism that has been proposed is that the trypsin and chymotrypsin in the intestine suppresses and controls pancreatic enzyme secretion by feedback inhibition and the dietary trypsin inhibitors counteract the suppression and initiate increased enzyme secretion (Green and Lyman, 1972; Niess et al., 1972).

Lectins

Another anti-nutritional factor in soybeans is lectins. Lectins are glycoproteins that can bind to cell surfaces on specific oligosaccharides or glycopeptides. They also have a high binding affinity to the intestinal enterocytes, which causes impairment of brush border continuity and ulceration of villi (Pusztai, 1991). Douglas et al. (1999) found that almost 15% of total growth reduction in chicks was associated with lectin presence in raw soybeans. Lectins, like trypsin inhibitors are heat labile and can be reduced with proper heating.

Oligosaccharides

The primary sugars present in SBM are sucrose and the oligosaccharides stachyose, raffinose, and verbascose. These galactooligosaccharides (GOS) contain a terminal sucrose that is linked to a chain of α-1,6 galactoses via an α-1,3 bond (Mul and Perry, 1994). These sugars can make up 7-8% of the DM within SBM as they are not removed during processing (Van Kempen et al., 2006). The GOS in SBM can only be enzymatically hydrolyzed by α-galactosidases, which are not produced in the intestinal tract of non-ruminants like poultry (Middelbos and Fahey Jr, 2008). They are considered anti-nutritional factors because they are poorly digestible and can cause reduced transit time, which leads to lower fiber digestion and TME value (Coon et al., 1990). Leske et al. (1993) observed the addition of raffinose and stachyose to a boiler diet significantly reduced TME values compared to controls, but ethanol extraction removal of the oligosaccharides resulted in an increased TME for roosters and broiler chicks (Coon et al., 1990; LESKE et al., 1991; Leske and Coon, 1999). The oligosaccharides can also cause wet feces, which can impact litter quality in poultry (Graham et al., 2002). This problem can be alleviated with low-oligosaccharide variety of soybeans or the addition of α-galactosidase enzyme. This also reduces the quantity of soybean meal needed due to greater

nutritional value and the increased concentration of digestible amino acids (Perryman and Dozier, 2012). Graham et al. (2002) found that a lower concentration of raffinose and stachyose will reduce the viscosity of the gut, leading to a faster passage rate, greater access of digestive enzymes to substrates, and diffusion of absorbable nutrients to the intestinal mucosa.

Non-Starch Polysaccharides

Soybean meal contains between 20-30% of NSP, including 8 and 17% of insoluble and soluble, respectively, on a dry matter basis (Smits and Annison, 1996). Non-starch polysaccharides are classified into three main groups specifically cellulose, non-cellulosic polymers, and pectic polysaccharides. The soy NSP are predominately a mixture of pectic polysaccharides with rhamnogalacturonans as the most abundant carbohydrate and cellulose as the second most abundant in SBM (Choct et al., 2010). The solubility of NSP is the main factor affecting their digestibility with the soluble fraction being more digestible than the insoluble. Digestibility of cockerels fed a diet containing 6.9% of NSP from defatted, de-hulled SBM resulted in an NSP digestibility value of 13% (Carré et al., 1990). Carre et al. (1995) also reported higher NSP digestibility in adult cockerels compared to broilers fed a corn-soy diet. They hypothesize the mature gut microflora adapts to increased glycanase production to more efficiently digest dietary NSP.

Litter Quality and Footpad Dermatitis

Currently, one of the main factors believed to be contributed to poor litter quality and also the main concern in the poultry industry in regards to litter quality is the skin condition known as footpad dermatitis (FPD). This condition was first described in broilers in the 1980s (McFerran et al., 1983; Greene et al., 1985). During this time period, the broiler paw market was beginning to develop along with greater attention being given to paw quality. Due to the market value of

this product along with increasing welfare concerns, the industry began to focus on how to reduce paw downgrades and condemnations (Shepherd and Fairchild, 2010). Footpad dermatitis is characterized by inflammation and necrotic lesions on the surface of the footpads and toes (Greene et al., 1985). Increased inclusion of SBM in all vegetable-based diets has been suggested as a possible cause of an economic, welfare, and food safety issue by possibly contributing to the incidence and severity of footpad dermatitis (FPD). One factor is the high potassium content in SBM. It has been reported that dietary electrolyte balance is a key factor influencing litter moisture and high dietary inclusion of Na and K as well as high dietary electrolyte balance increases water intake and litter moisture (Borges et al., 2003; Ravindran et al., 2008; Koreleski et al., 2010). Cengiz et al. (2012) reported high dietary Na concentration enhanced water consumption and litter moisture. Fuhrmann et al. (2016) observed similar findings when broilers were fed a diet with high levels of K. Another factor is the indigestible NSP can increase gut viscosity resulting in sticky droppings that can adhere to the foot and over time deteriorate the epidermis and keratin layers (Hess et al., 2004).

Strategies to Improve SBM Utilization

Reduced Oligosaccharide Soybean Varieties

There have been many attempts to reduce the negative effects of NSP and oligosaccharides in soybean meal. It has been demonstrated that removal of raffinose and stachyose yielded higher ME_n values for poultry by genetic selection (Parsons et al., 2000). Soybean varieties have also been genetically selected to have reduced raffinose and stachyose concentrations (Parsons et al., 2000; Baker and Stein, 2009; Baker et al., 2011). A 7 to 9% increase in TME_n was observed in roosters fed low oligosaccharide SBM compared to those fed conventional SBM (Parsons et al., 2000). In contrast, Baker et al. (2011) detected no difference in TME_n between low

oligosaccharide SBM and conventional SBM. New varieties of soybeans have been developed to ultra-low oligosaccharide content with over a 90% reduction in GOS compared to conventional SBM. Perryman and Dozier (2012) evaluated the use of a low oligosaccharide SBM and an ultra-low oligosaccharide SBM and reported increases in AME_n of 168 kcal/kg and 5.8% higher apparent ileal amino acid digestibility (AIAAD) for low oligosaccharide SBM compared to conventional SBM. They also observed an 8 and 17% increase in AIAAD for the first 5 limiting amino acids in broilers for low oligosaccharide and ultra-low oligosaccharide SBM, respectively, compared to conventional SBM. Perryman et al. (2013) also observed similar performance and carcass characteristics from 1 to 40 days of age with birds fed low oligosaccharide SBM compared to conventional SBM. Similar results were observed from 1 to 42 days of age birds fed low oligosaccharide SBM, while those fed ultra-low oligosaccharide SBM also showed no differences in bodyweight gain and carcass characteristics, but there was a 4 point reduction in FCR compared to those fed conventional SBM. The diets for the low and ultra-low oligosaccharide SBM were formulated with 28 to 71% less supplemental oil compared to diets formulated with conventional SBM translating to a major reduction in dietary costs.

Specialized Processing

There have also been reports of beneficial processing techniques to remove the oligosaccharide content of SBM. One of those methods is ethanol extraction (Coon et al., 1990; Leske and Coon, 1999). Ethanol extraction removes approximately 90% of water-soluble galactooligosaccharides and increases the TME_n of SBM for poultry (Coon et al., 1990). It has also shown to increase fiber digestibility, lengthen transit time, and increase cecal pH (Coon et al., 1990). Veldman et al. (1993) reported the addition of the ethanol extract back into the diet had a detrimental effect on ileal digestibility and resulted in fluid retention with increased microbial fermentation in the

gut of piglets. However, there has been contradicting research that concluded there was little or no anti-nutritional effect of the SBM oligosaccharides (Irish et al., 1995).

Another specialized processing method is the fermentation of SBM by fungal and bacterial strains. The predominant organism used for SBM fermentaion is *Aspergillus* due to its ability to produce enzymes such as hemicellulases, hydrolases, pectinases, protease, amylase, and lipases (Mathivanan et al., 2006). Bacterial fermentation is also accomplished with various *Lactobacillus* and *Bacillus* species being used (Yang et al., 2007). When compared to conventional SBM, fermented SBM was reported to have higher protein content and available amino acid compositions, higher protein digestibility, lower anti-nutritional factors and allergenic compounds, and an overall improved nutritional value (Feng et al., 2007a; b; Frias et al., 2008; Song et al., 2008). Feng et al. (2007a) reported an increase in average daily gain and feed intake of broilers for 6 weeks with a significantly lower FCR from weeks 1 to 3. This is in agreement with Mathivanan et al. (2006) who reported increased bodyweight and lower FCR at 6 weeks for broilers fed fermented SBM at 3 different levels and Hirabayashi et al. (1998) who also showed increased body weight gain from weeks 1 to 5 in broilers fed fermented SBM compared to those fed conventional SBM. In addition to performance parameters, improvements in phosphorous digestibility (Hirabayashi et al., 1998; Feng et al., 2007a), blood parameters (Feng et al., 2007a), and histological characteristics (Mathivanan et al., 2006) were also reported.

***Bacillus* as Direct-Fed Microbials**

Sporulation and Resistance

Endospore formation by *Bacillus* and other Gram-positive bacteria, like Clostridia, is a strategy used by these organisms to survive environmental stress and inhabit harsh environments. When these rod-shaped bacteria are starved for carbon, nitrogen, or sometimes a phosphorous source,

they produce an oval, dormant cell called a spore (Driks, 2002). This sporulation process takes approximately 7 hours at 37°C (Piggot and Hilbert, 2004). Sporulating *B. subtilis* cells are cannibalistic and feed on their siblings in order to delay committing to spore formation (González-Pastor et al., 2003). During initiation, the master transcription regulator Spo0A is activated by phosphorylation. The first morphological stage of sporulation from a vegetative cell (stage 0) is the formation of an axial filament of chromatin. During this stage, two copies of the chromosome condense and elongate to form a filament that reaches across the long axis of the cell (stage I). The cell then asymmetrically divides into two daughter cells (stage II). At the time of septation, only one-third of a chromosome is existent in the prespore. However, DNA translocase quickly transfers the remaining two-thirds yielding two cells with identical genomes, but unequal volumes. Subsequently, the prespore is engulfed by the mother cell and wrapped within the septal membrane resulting in a free-floating protoplast encircled by two membranes (stage III). Proceeding engulfment, two peptidoglycan layers, the primordial germ cell wall and the cortex are deposited between the membranes surrounding the prespore (stage IV) and are essential to spore dormancy (Driks, 2002). In stage V, the coat, a thick, complex structure of proteins on the outside surface of the prespore is assembled. The spore then matures, gaining resistance to high temperatures and UV radiation (stage VI). In the final stage (VII), the mother cell lyses, releasing the mature spore (Hilbert and Piggot, 2004). The spore coat is important in spore resistance to some chemicals, exogenous lytic enzymes that can degrade the spore cortex, and to predation by protozoa. However, the coat does not play a role in protection from chemicals, heat, or radiation (Setlow, 2006). The peptidoglycan-comprised cortex is essential for the formation of the dormant spore and in the reduction of water content in the core. While the germ cell wall under the cortex probably does not affect resistance, it does become the cell wall

of the outgrowing spore during germination (Setlow, 2006). The inner membrane is a tough permeability barrier that aids in spore resistance to chemicals (Nicholson et al., 2000). The core also contains three small molecules whose concentrations are important for resistance. Water is the first molecule and it makes up only 27-50% of the core wet weight compared to 75-80% of a growing cell. This low amount of free water in the spore core restricts macromolecular movement, aids in enzymatic dormancy, and is the most important factor determining resistance to wet heat (Gerhardt and Marquis, 1989). The second molecule is dipicolinic acid (DPA) which makes up 5-15% of the dry weight of spores and is usually chelated to Ca^{2+} (Gerhardt and Marquis, 1989). The large amounts of DPA assist in reducing the core water content and UV photochemistry of the spore DNA (Setlow, 2006). The mature spore exhibits little to no metabolic activity and is considered dormant.

Germination

Once spores are exposed to a suitable stimulus (germinant) they promptly lose their dormancy and resistance properties. There are several germinant agents such as nutrients, calcium dipicolinic acid (CaDPA), and high hydrostatic pressure (HP) that can elicit spore germination. In nature, it is likely that the presence of specific nutrients is what activates spore germination. Nutrient germinants bind to germinant receptors (GRs) in the inner membrane. For *B. subtilis*, L-alanine, L-valine, and L-asparagine have been shown to cause germination, while the D-amino acids are inactive (Atluri et al., 2006). When the nutrient germinants bind to receptors located in the spore's inner membrane a series of reactions is triggered which will ultimately result in a metabolically active vegetative cell. In *B. subtilis*, the nutritional germinant will bind to the GerA, GerB, or GerK receptors encoded by gerA, gerB, and gerK operons, which initiates "commitment" where germination will continue even if the germinant is removed (Paidhungat

and Setlow, 2000). Around the same time as commitment, the release of monovalent cations, Na^+, K^+, and H^+ from the spore core result in an increase in spore core pH to approximately 8. It is unknown whether this cation release is causally related to the previous commitment step (Setlow, 2013). Shortly after these two steps, the spore core's huge CaDPA depot is completely released in about 2 minutes and replaced with water, thus increasing spore water content (Kong et al., 2010). With this, Stage I of germination is complete (Setlow et al., 2001). The time it takes for spores to complete stage I is widely variable with some spores taking less than 10 minutes, while others may take an hour or even days. The main reason for differences seems to be in the time between germinant addition and initiation of rapid CaDPA release, termed "Tlag" (Yi and Setlow, 2010). This is predominately caused by variations in the spore's level of germinant receptors (Kong et al., 2010).

The final stage, stage II, in spore germination is triggered by events in stage I, particularly the release of CaDPA. In stage II, there is hydrolysis of the peptidoglycan (PG) cortex by cortex-lytic enzymes (CLEs). The CLEs specifically recognize cortical PG, via the muramic acid-δ-lactam (MAL) in the polysaccharide backbone that is not present in growing cell or germ cell wall PG (Setlow, 2003). Bacillus spores have been shown to have two enzymes, CwlJ and SleB, which are involved in the degradation of the cortex PG (Chirakkal et al., 2002). The hydrolysis of the cortical PG allow for the expansion of the spore core and the inner membrane which increases 1.5 to 2-fold without new membrane synthesis. (Cowan et al., 2004). Stage II of germination results in the spore core now containing approximately 80% wet weight as water and active enzymes within the core (Paidhungat and Setlow, 2001). Enzyme activity leads to the degradation of novel, acid-soluble proteins in the core and instigation of metabolism and macromolecular synthesis in the core (Setlow, 2013). Achievement of stage II of

germination also leads to breakdown of the spore coat and escape of the outgrowing spore (Plomp et al., 2007).

Potential for *Bacillus* DFM to Improve Poultry Health and Performance

The increasing concern of multi-drug resistant bacteria over the past decades has resulted in the Food and Drug Administration calling for companies to discontinue labelling antibiotics as growth promoters in agricultural animals (GFI #213). One promising antibiotic replacement is the integration of probiotic bacteria into feed as DFMs. In this regard, *Bacillus* spp. have a distinct advantage over other microbes like *Lactobacillus,* which require more careful handling, storage, and administration by bird caretaker on site via drinking water. *Bacillus* spp. spores possess the ability resist harsh environmental conditions such as, extreme pH, high pressures, dehydration, and long storage periods all of which make them suitable for commercial use in the poultry industry (Cartman et al., 2007). These characteristics allow spores to survive pelletization during the feed milling process, which makes administration of *Bacillus* spp. spores as DFMs convenient to producers and helped gain traction for their commercial use (Hong et al., 2005). Latorre et al. (2014) reported spores can persist and possibly complete a full life cycle development within the GIT, indicating these bacteria could be considered part of the metabolically active host microbiota. It is important to mention that not all *Bacillus* are created equal. Each isolate possesses distinctly different characteristics such as, heat resistance, rate of growth and sporulation, enzyme production, and anti-microbial production (Larsen et al., 2014). Thus, they have been shown to have many possible modes of action for improving gut health and nutrient utilization. One possible mode is the activation of intestinal function. Samanya and Yamauchi (2002) reported decreased blood ammonia concentration after supplementation with *B. subtilis natto*, which they hypothesize*,* resulted in increased cell mitosis in the intestines and

greater villus heights as ammonia has been reported to be toxic to villus histology, reduce cell proliferation, and gastric mucosal DNA synthesis. Similar results were observed by Yurong et al. (2005) with the addition of enhanced intestinal mucosal immunity following *Bacillus* supplementation. A second mode of action could be competitive exclusion in limiting the colonization of pathogenic bacteria. Jin et al. (1996) reported decreased population counts of intestinal *E. coli* in broilers fed feed supplemented with *B. subtilis.* La Ragione and Woodward (2003) found that an oral inoculation of *B. subtilis* spores 24 hours prior to *C. perfringens* challenge suppressed colonization of the pathogen in the distal GIT of chickens. Similarly, Latorre et al. (2015b) showed antimicrobial activity against *C. perfringens* for several different isolates of *Bacillus.* Shivaramaiah et al. (2011) administered spores of different *Bacillus spp*. strains to *Salmonella* Typhimurium challenged chicks and poults and observed a reduction in pathogen recovery in the crop and ceca of birds fed *Bacillus* supplemented diets. Wolfenden et al. (2011) saw similar results with a decreased level of colonization of *Salmonella* in commercial turkeys supplemented with a *Bacillus*-based DFM and similar body weight at 23 days of age as turkeys consuming a diet medicated with Nitarsone. A third potential mode of action could be enzyme production. Latorre et al. (2015b) determined cellulase and xylanase production of several *Bacillus* isolates, as well as amylase, protease, lipase, and phytase (Latorre et al., 2016). These possible modes of action suggest that *Bacillus* isolates could be an effective tool in replacing antibiotic growth promotors and combatting anti-nutritional factors in feed components.

Extensive work has been done with *Bacillus*-based DFMs and alternative ingredients as complementary approaches to lowering poultry diet costs. By-products of biofuel production (distiller's dried grains with solubles) and cereals like wheat and barley are being included in

poultry rations, effectively increasing the amount of less digestible NSP in the feed and raising concerns of digesta viscosity (Tellez et al., 2014, 2015). Latorre et al. (2015b) demonstrated the inclusion of a selected *Bacillus*-based DFM to a high NSP rye-based diet significantly reduced digesta viscosity, increased body weight, and lowered FCR at 28 d post hatch compared to untreated control fed birds (Latorre et al., 2015a). Salim et al. (2013) reported birds fed a standard corn-soy diet supplemented with a *Bacillus*-based DFM increased body weight gain of broilers from 0 to 21 d post hatch and reduced FCR from 0 to 7 days post hatch when compared to untreated controls. These performance increases were similar to birds fed diets supplemented with an antibiotic growth promotor (AGP), virginiamycin. Additionally, Harrington et al. (2015) found that birds fed low energy diets supplemented with *B. subtilis* were able to achieve higher 42 d post-hatch body weight gain and lower FCR than birds fed corresponding diets without *Bacillus* supplementation. In the same experiment, birds fed diets with a 2% reduction in metabolizable energy (ME) supplemented with *Bacillus* attained performance similar to that of birds fed non-supplemented feed formulated without a reduction in ME. A regression analysis determined that supplementation of the *Bacillus*-based DFM had an overall ME contribution of +62 kcal/kg feed. Knap et al. (2011) had previously demonstrated birds fed diets with a 4% reduction in ME and *Bacillus* supplementation had improved FCR, but not body weight, compared to birds fed an untreated control energy diet.

DFM Treatment and Welfare

As previously mentioned, increased SBM inclusion results in higher NSP concentrations in the diet, which can have negative effects on the birds GIT health and litter quality. The addition of NSP-degrading enzymes and selected *Bacillus* DFMs have been shown to significantly reduce digesta viscosity in diets containing high NSP (Choct et al., 1995; Latorre et

al., 2015b). Furthermore, research has been done to evaluate the synergistic effect of the combination of enzymes and DFMs. Dersjant-Li et al. (2015) evaluated the combination of a xylanase, amylase, and proteinase (XAP) with three strains of *Bacillus amyloliquefaciens* on welfare parameters in broilers reared under commercial conditions. The study reported improved litter quality and reduced FPD lesion scores in treated birds compared to untreated controls. Similar results were observed by Flores et al. (2016) where a reduction in FPD lesion scores was seen in birds fed the combination of XAP and *Bacillu*s-based DFM compared to the negative controls. In this dissertation, a *Bacillus*-based DFM that was selected for its ability to produce XAP enzymes was administered alone to a low energy, high soybean meal diet in the absence of pathogen challenge and reduced FPD lesion scores compared to un-supplemented controls. This indicates *Bacillus* isolates could not only have probiotic effects in the presence of pathogenic exposure, but also produce enzymes that could reduce the need for additional supplementation.

Conclusions

With increasing demand and outside pressure from consumers, producers are looking for alternatives to antibiotics that will allow for similar gains in performance and health seen with AGPs. *Bacillus* DFMs have been proven to have the ability to increase health, immune status, and performance parameters of broilers, while providing for a convenient and easy in-feed application method. However, little work has been done to determine potential for *Bacillus*-based DFM alone to improve litter quality and FPD scores for broiler when fed in all vegetable diets that may have a higher NSP content. Further research is needed to determine specific mechanisms for health and performance improvements seen when using *Bacillus* DFM, but there is potential for their use to possibly reduce negative effects of increased SBM in poultry diets.

Thus, allowing nutritionists to reduce diet costs, while maintaining or increasing the performance and welfare of the bird.

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Chapter 3: *In Vivo* Evaluation of *Bacillus* Isolates as Direct-Fed Microbials Based on Performance of Broiler Chickens Fed High Soybean Meal Diets

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Abstract

Bacillus isolates (BI) were selected as direct fed microbial (DFM) candidates in previous experiments based on their ability for *in vitro* degradation of carbohydrates found in soybean meal (SBM) that are poorly digested by poultry. In the current experiment, 6 *in vivo* trials were conducted to evaluate 5 different BI in broiler chickens reared to 21 d. In 3 floor pen trials, feed treatments were administered to 6 replicate pens of 12 birds ($0.09m^2$/bird; trials 1 & 2) or 8 replicate pens of 20 birds ($0.11m^2$/bird; trial 3) housed on new pine shavings. In 3 battery (trials 4-6), feed treatments were administered to 12 replicate cages of 8 birds ($0.04m^2$/bird). In all trials, birds were fed high soybean meal (40%) diets formulated to contain 3,050 [control, (CTL)] or 2,925 [reduced energy (RED)] kcal/kg of AME_n or the RED containing BI. Birds had *ad libitum* access to mash feed and water, and body weight gain (BWG) and feed conversion ratio (FCR) were recorded weekly. In all trials, except trial 6, birds fed the RED diet had lower BWG and higher FCR at 21 d than birds fed the CTL. In trial 1, birds fed the RED increased BWG by 17% compared to the birds fed CTL, while birds fed BI-46 had similar BWG to birds fed CTL. In trial 3, birds fed BI-40 had similar ($P>0.05$) BWG compared to birds fed the CTL. In trial 4 and 5, birds fed BI-40 and BI-46 had similar ($P>0.05$) BWG and FCR compared to birds fed CTL. In trial 6, BI supplementation tended ($P>0.05$) to increase BWG and birds fed BI-46 lowered ($P<0.05$) FCR compared to CTL. Data were pooled to further evaluate the responses across all trials in which BI-46 was used due to the consistent positive response. Thus, these data reveal a novel DFM candidate for broiler diets containing SBM and additionally indicate that not all BI with promising *in vitro* enzyme activity yield benefits *in vivo*.

Keywords: *Bacillus*, DFM, Soybean meal, oligosaccharide, non-starch polysaccharide

Introduction

Bacillus spp. are ubiquitous, Gram-positive, spore forming bacteria that are commonly recovered from the environment and gastrointestinal tract (GIT) of poultry (Latorre et al., 2015a). *Bacillus* species exist in an endosymbiotic relationship with their host and are able to temporarily survive and multiply within the GIT (Hong et al., 2005). In response to nutritional limitations, *Bacillus* produce a robust, resting cell called an endospore (Hong et al., 2005). These spores can remain dormant for years and are resistant to toxic chemicals, radiation, desiccation, and heat (González-Pastor et al., 2003; Setlow, 2006). This facilitates inclusion of spores in poultry feeds as they can withstand high temperature feed milling processes such as pelleting. Once ingested by the bird, the presence of nutrients promotes spores to germinate into metabolically active vegetative cells that can produce beneficial compounds for the host and intestinal microflora (Jadamus et al., 2001; Leser et al., 2008). Indeed, *Bacillus* have been shown to produce several extracellular enzymes that might increase nutrient availability for the animal, including phytase, lipase, protease, amylase (Latorre et al., 2016), cellulase, and xylanase (Latorre et al., 2015b). However, not all *Bacillus* strains produce the same enzymes, which necessitates isolate screening based on target substrates.

United States SBM is included in 70% of the animal feed produced worldwide, making it the most frequently used source of protein for swine and poultry feeds (USDA, 2015). While the demand for SBM is primarily driven by its desirable amino acid profile, it also contains approximately 35% carbohydrate and several anti-nutritional factors. Some anti-nutritional factors within SBM, such as trypsin inhibitors and lectins, can be inactivated by heat and are reduced significantly by normal soybean meal processing (Palacios et al., 2002). However, the non-starch polysaccharides (NSP) and oligosaccharides are not degraded by heating (Loeffler,

2012). Non-starch polysaccharide concentrations vary from 20-30%, of which approximately 8% are insoluble cellulose and 17% are partially soluble pectic polysaccharides (Choct et al., 2010). The water-soluble polysaccharides have been shown to be extensively degraded in the digestive tract of birds with digestibility values around 80 to 90%, while water insoluble NSP which remain almost completely undegraded (Carré et al., 1990). Oligosaccharides comprise 5-7% of SBM and consist of stachyose (4%), raffinose (≈1%), and verbascose (≤ 1%) and are also indigestible in the small intestine of poultry due to insufficient endogenous α-1,6 galactosidase enzyme production for their hydrolysis (Coon et al., 1990). As such, SBM oligosaccharides decrease the TME_n and fiber digestion of SBM (Coon et al., 1990; Cromwell, 2000; Choct et al., 2010) and impact water reabsorption, which can negatively affect litter quality in poultry (Bedford, 1995). Several approaches including selection of low-oligosaccharide soybean varieties (Parsons et al., 2000), fermentation (Mathivanan et al., 2006), alcohol extraction (Coon et al., 1990), enzymatic treatment of SBM (Jiang et al., 2006) prior to feeding, and dietary enzyme supplementation (Kocher et al., 2002) have been attempted to reduce the effects of these carbohydrates, but opportunities remain for novel approaches to help the birds better utilize these components in vivo.

The objective of the present study was to test five previously screened *in vitro Bacillus* isolates as direct fed microbial (DFM) candidates for their ability to degrade SBM carbohydrates and their potential to improve the growth performance of broiler chickens fed high soybean meal diets with reduced energy. Six trials were conducted to evaluate different isolates, concentrations, and combinations to identify potential DFM candidates. One isolate was included in all six trials, and data for this isolate were pooled across three floor pen trials and three battery cage trials to provide a more robust evaluation of its efficacy.

Materials and Methods

The University of Arkansas Institutional Animal Care and Use Committee approved all experimental procedures involving live birds #18125.

Isolation and characterization of Bacillus spp.

The *Bacillus* strains used in these trials were isolated from poultry sources and selected as superior producers of α-galactosidase, cellulase, mannanase, and xylanase based on a qualitative enzyme activity evaluation performed using a different selective media for each evaluated enzyme (unpublished data). Candidates were screened by placing on media containing only specific carbon sources to be utilized for growth. Four selective media were used for testing and included raffinose, arabinoxylan, galactomannan, and cellulose. Selection was based on assumed enzyme activity by their ability to grow on the provided substrates and also color changes which indicated hydrolysis of dyed carbohydrates. Sporulation of each selected *Bacillus* isolate was confirmed during the DFM-candidate selection process. Identification and characterization of the different isolates were conducted using a bioMerieux API 50 CHB test kit (catalog no. 50430, bioMerieux, Marcy l'Etoile, France), and each strain was subjected to 16S rRNA sequence analysis (Midi labs, Newark, DE, USA). Two of the five *Bacillus* strains (31 and 86) were identified as *B. subtilis*, two isolates (40 and 46) were identified as *B. amyloliquefaciens,* and one (65) was identified as *B. licheniformis*. Five *Bacillus* isolates were selected for *in vivo* evaluation and are referenced herein with the following numerical identifiers 31, 40, 46, 65, and 86.

DFM preparation

A solid state fermentation media (SS) developed by Zhao et al., (2008) was selected and modified to produce candidate *Bacillus* spores used in these experiments. Briefly, ammonia broth was added to a mixture of 70% rice hulls and 30% wheat bran at an inclusion of 58% by weight

and mixed. Then, the SS fermentation media was added to 250 mL Erlenmeyer flasks and sterilized by autoclaving for 30 min at 121°C. *Bacillus* isolates were cultured individually in 10 mL of tryptic soy broth (TSB, catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated statically at 37°C overnight for 18h. Following broth incubation for each isolate, 2 mL of turbid culture was added separately to the previously prepared SS fermentation media flasks. Inoculated flasks were incubated for 24 h at 37°C to promote vegetative cell growth of the *Bacillus spp.*, and then incubated for another 72 h at 30°C to induce sporulation. Next, the inoculated SS fermentation media was transferred from the Erlenmeyer flasks into sterile petri dishes and dried at 60°C. The dried SS fermentation media was aseptically ground into a fine powder using a Bunn G3 HD bulk coffee grinder on the Turkish setting (Bunn, Springfield, Illinois, USA). Fresh rice hulls were ground through the machine in between isolates to prevent contamination. The final dried and ground material contained approximately 10^{10} stable *Bacillus* spores per gram, with spore counts confirmed following a 1:10 dilution of product in 0.9% sterile saline in a 15mL conical tube (VWR, catalog no. 89039-668, Radnor, PA, USA). Subsequent 1:10 dilutions of the solubilized material from the 15mL conical tubes were plated on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated at 37°C for 18 h to allow germination of spores into vegetative cells before counts were conducted to calculate the number of viable spores per gram of product.

Bacillus spores from each of the isolated strains were included into experimental feeds at a calculated 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 number of spores per gram of feed after mixing steps. Feed samples were first pasteurized at 90°C for 10 min to eliminate vegetative cells present in the feed from final spore enumeration. Following

heat-treatment, feed samples were subjected to a 1:10 dilution with 0.9% sterile saline in 15mL conical tubes, and diluted samples were plated on TSA plates and incubated at 37°C for 18 h to promote germination of spores into vegetative cells. Vegetative cells were counted and used to calculate the number of viable spores present per gram of feed.

General Animal Husbandry and Diets

A total of 6 independent trials were conducted. For all trials, day-of-hatch male by-product breeder chicks from a Cobb 500 female line were obtained from Cobb-Vantress (Siloam Springs, AR, USA) and group weighed upon arrival. In 3 floor pen trials, experimental feeds were administered to 6 replicate pens of 12 birds (0.09 m^2/bird) (trials 1 and 2) or 8 replicate pens of 20 birds (0.11 m^2/bird) (trial 3). In all floor pen trials, pens contained a hanging feeder, a nipple drinker line, and fresh pine shavings. In 3 battery trials (trials 4-6), experimental feeds were administered to 12 replicate cages of 8 birds (0.04 m^2/bird). Battery cages (Alternative Design, Siloam Springs, AR) were equipped with one trough feeder and two drinker nipples. In all trials, temperature was maintained at 33°C for the first 5 d and was then gradually reduced according to recommended management practices until a temperature of 23°C was achieved at 21 d of age. Birds received 24 h of light on D0 and 23L:1D d1-d4. On d 5, a 20L:4D light schedule was implemented until d 15 when 18L:6D was used until the end of the trial. Target light intensities were verified at floor or battery cage level via light meter.

In all trials, birds were fed corn-soybean meal-based diets formulated to contain 3,050 (control, **CTL**) or 2,925 (reduced energy, **RED**) kcal/kg of apparent ME_n or the RED with the addition of select *Bacillus* isolates. A total of five *Bacillus* isolates with unique numerical identifiers BI-31, BI-40, BI-46, BI-65, and BI-86 were evaluated in these trials. Due to the primary objective of screening Isolates based on potential ability to improve utilization of

carbohydrates found within SBM, diets were formulated to contain higher SBM concentrations (40%) to increase target substrate availability. In all trials, a starter diet was fed throughout the experimental period (0 to 21 d). No antibiotics or coccidiostats were added to the feed. Mortality were collected and weighed twice daily. Feed Intake (FI) was calculated based on bird days and FCR was corrected to include the weight of any dead birds. Body weights and feed consumption were taken by pen at 0, 7, 14, and 21 d post-hatch to calculate FCR and body weight gain (BWG).

Experimental Designs

Floor pen trials 1, 2, and 3. Three separate 21 d floor pen trials were conducted. In trial 1, a total of 504 broiler chicks were randomly distributed to 42 floor pens on day-of-hatch. Broilers were fed 1 of 7 experimental diets: CTL, RED, RED + BI-46, RED + BI-65, RED + BI-86, RED + BI-65 + BI-86, or RED + BI-46 + BI-65 + BI-86. In trial 2, a total of 576 chicks were randomly distributed to 48 floor pens on day-of-hatch. Broilers were fed 1 of 8 experimental diets: CTL, RED, RED + BI-46L, RED + BI-46, RED + BI-46H, RED + BI-65L, RED + BI-65, or RED + BI-65H. The "L" denotes a lower dose of spores of 6.6×10^5 spores/g of feed and "H" denotes a higher dose of spores with a concentration of 3.3×10^6 spores/g of feed. In trial 3, a total of 960 broiler chicks were randomly distributed to 48 floor pens and fed 1 of 6 experimental diets: CTL, RED, RED + BI-40, RED + BI-46L, RED + BI-46, or RED + BI-46H. In all trials, all birds and feed were weighed on d 0, 7, 14, and 21 to determine BW, BWG, and FCR. Feed intake was calculated based on bird days for trials 1, 2, and 3.

Battery cage trials 4, 5, and 6. Three 21 d battery cage trials were conducted. In trial 4, a total of 576 broiler chicks were randomly allocated to 72 battery cages on day-of-hatch. Broilers were fed 1 of 5 experimental diets: CTL, RED, RED + BI-31, RED + BI-40, or RED + BI-46. In trials,

5 and 6, a total of 480 chicks were randomly distributed to 60 battery cages on day-of-hatch. Broilers were fed diets: CTL, RED, RED + BI-40, RED + BI-46L, or RED + BI-46H (trial 5) and CTL, RED, RED + BI-40, RED + BI-46, or RED + BI-40 + BI-46 (trial 6). In trial 5, "L" denotes a low dose of 1 x 10^5 spores/g of feed and "H" denotes a high dose of 1 x 10^7 spores/gram of feed. These doses were selected to determine if any detrimental effects were observed if spores were added at a lower or higher dose than recommended. All birds and feed were weighed on D0, D7, D14, and D21 to determine BW, BWG, and FCR. Feed intake was calculated based on bird days for trials 4, 5, and 6.

Statistics

In all trials, pen or cage was the experimental unit and data were subject to one-way ANOVA using JMP pro 13 (JMP®). Where appropriate, means were separated using Tukey's honestly significant difference (HSD) test with significance reported at $P \leq 0.05$. Because the control, RED, and isolate 46 treatment groups were included in all 6 trials, data from these treatment groups in trials 1-3 and 4-6 were pooled by experiment type (floor pen or battery cage) for additional analysis. Pooled datasets were subjected to a two-way ANOVA to evaluate the fixed effects of dietary treatment, trial, and their interactions. Following no treatment × trial interactions ($P > 0.05$), the main effects of dietary treatment were presented.

Results

Trials 1-3

Bird performance data from floor pen trials 1-3 are presented in Tables 2-4, respectively. In trial 1, chicks were fed the CTL diet or RED without or with *Bacillus*-based DFM candidates BI-46, BI-86, BI-65, BI-46 + BI-86 (combo 1), or BI-46 + BI-86 + BI-65 (combo 1). Dietary treatment

did not influence FI ($P > 0.05$) in trial 1, but tended to influence both BWG ($P = 0.07$) and FCR ($P = 0.09$).

In trial 2, chicks were fed the CTL diet or RED without or with *Bacillus*-based DFM candidates 46 and 65 at 3 different doses. Dietary treatment influenced BWG ($P < 0.01$), but not FI ($P > 0.05$) or FCR ($P > 0.05$). Body weight gain was lower ($P < 0.05$) than the CTL group and similar to the RED for birds in all DFM groups, except for BI-46, which was similar ($P > 0.05$) to both the CTL and RED groups.

In trial 3, chicks were fed the CTL diet or RED without or with *Bacillus*-based DFM candidates 40 and three different doses of 46. Dietary treatment did not influence FI ($P > 0.05$) or FCR ($P > 0.05$), but affected BWG ($P < 0.05$). The RED group had an 8% lower BWG ($P < 0.05$) compared with the control group. The addition of DFM candidate 40 and 46L increased BWG of birds to be intermediate to that of the CTL and RED groups, whereas BWG of birds fed RED + BI-46 and BI-46H were lower than those fed CTL and similar to those fed the RED.

Trials 4-6

Bird performance data from battery trials 4-6 are presented in Tables 5-7, respectively. In trial 4, chicks were fed the CTL diet or RED without or with *Bacillus*-based DFM candidates 31, 40, and 46. There was an 8% reduction in BWG ($P < 0.01$) and 14 point increase ($P < 0.01$) in FCR for birds in the RED group compared with those in the CTL group, with no difference in FI ($P > 0.05$) between these groups. Body weight gain of birds fed RED + BI-40 was intermediate to that of the birds in the CTL and RED groups, whereas BWG of birds fed RED + BI-46 was higher than those in the RED group and similar to those in the CTL group. Feed conversion ratio of birds fed RED + BI-40 was similar to birds in the RED and RED + BI-46 groups, whereas FCR

of birds fed RED + BI-46 was lower than those in the RED group and similar to those in the CTL.

In trial 5, chicks were fed the CTL diet or RED without or with *Bacillus*-based DFM candidates 40 and two different doses of 46. There was an 18% reduction in BWG ($P < 0.01$) and 40-point increase ($P < 0.01$) in FCR for birds in the RED group compared with the CTL group. There was a treatment effect on FI ($P < 0.01$) as birds fed RED + BI-46L decreased FI compared to the RED group and was similar to the CTL group. While birds fed RED + BI-46H was intermediate to that of the birds in the CTL and RED groups. Bodyweight gain of birds fed RED + BI-40 was higher than that of birds in the RED group and similar to the CTL group, whereas birds in the RED + BI-46H was intermediate to that of the birds in the RED and CTL groups. Birds fed RED + BI-46L had BWG similar to all groups, except for the birds fed CTL. Feed conversion ratio for birds in the RED + BI-46L and BI-46H were lower than the birds in the RED group and similar to those in the CTL group, while the FCR of the RED + BI-40 group was intermediate to the RED and CTL groups.

In trial 6, chicks were fed the CTL diet or RED without or with *Bacillus*-based DFM candidates 40, 46, and combo (40 + 46). Dietary treatment did not influence FI ($P > 0.05$), but tended to influence BWG ($P = 0.10$) and significantly affected FCR ($P < 0.05$). Feed conversion ratio was lower for birds fed RED + BI-40, highest for birds fed CTL, and intermediate for all other groups.

Pooled Trials

Data for the CTL, RED, and RED + BI-46 groups were combined into 2 pooled floor pen (trials 1-3) and battery trial (trials 4-6) datasets presented in Table 8. For the 3 floor pen trials, it was observed that the RED reduced ($P < 0.01$) 0 to 21 d BWG by 11% and increased ($P \leq 0.05$) FCR

of birds by 13 points compared with those fed the CTL diet. Supplementation of DFM isolate 46 to the RED increased ($P < 0.01$) BWG and decreased ($P \leq 0.05$) FCR of birds by 5% and 8 points, respectively, to values that were intermediate to the CTL and RED group. There were no trial by treatment interactions observed in the pooled floor pen trial dataset.

Across the 3 battery trials, there was no trial by treatment interaction for BWG, however interactions were observed for FI ($P < 0.01$) and FCR ($P < 0.01$). These interactions in FI and FCR resulted from unexplained high FI in trials 4 and 5 coupled with increased FCR of the CTL group in trial 6. For BWG, RED reduced 0 to 21 d BWG ($P < 0.01$) by 10% and increased FCR ($P < 0.01$) by 13 points compared with the CTL diet. Supplementation of DFM isolate 46 to the RED increased ($P < 0.01$) BWG and lowered ($P < 0.01$) FCR of birds by 8% and 13 points, respectively, compared to the RED group to values that were similar to the CTL group.

Discussion

In the present study, a reduced ME, high SBM diet was used to evaluate the energy sparing effects of *in vitro* selected *Bacillus* DFM candidates. Candidate DFMs were selected on their ability to utilize otherwise indigestible carbohydrate fractions of SBM, like the oligosaccharides and NSP. Hence, a high SBM inclusion was incorporated in the diets to ensure enough substrate was available for the DFMs to compensate for the reduction in ME. The CTL diet was formulated to represent a standard energy commercial diet, and the energy in the RED was reduced by 4% or 125 kcal/kg. This diet was supplemented with five different *Bacillus* DFM candidates to evaluate whether the performance of broiler chickens is higher or comparable to those fed a standard energy diet, which would allow an opportunity for lower cost feed formulation.

In the first three pen trials, observed differences were minimal due to low number of replications as sufficient pens were not available for testing preliminary groups. In trials 1 and 2, post-hoc power analyses indicated 9 replicate pens per group were required for differences of this magnitude and only 6 were used. We recognize another limitation to these data as they are only 21 d post-hatch BWG and FCR of broilers fed a mash starter diet throughout. This is the reason BWG increases and decreases were reported as percentages and not actual numbers, due to the fact that birds were not market age. It is unknown if improvements seen will carry over to market age birds fed pelleted, conventional phase diets. However, young broilers are generally less responsive to energy changes than older birds, so the separation observed between reduced energy and control diets of approximately 10% for body weight gain at 21d, indicates further separation could occur in grower and finisher diets leading up to market age. Consistent differences observed also demonstrates a reliable testing model for *Bacillus*-based DFM candidates specifically selected for SBM substrates. It is also undetermined if *Bacillus* candidates will have an effect when added to standard energy diets, like the CTL. Further investigation is also needed to determine the specific mode of action of the *Bacillus* isolates within the bird. Many possible modes of action for DFM have been hypothesized and include: (1) activation of intestinal function (Samanya and Yamauchi, 2002; Yurong et al., 2005); (2) oxygen consumption – creating a more favorable environment for beneficial anaerobic species (Molnár et al., 2011); (3) enhanced immune responses (Huang et al., 2008); (4) competitive exclusion of pathogenic bacteria, like, *Clostridium perfringens*, *E. Coli*, *Salmonella enterica* (Jin et al., 1996); (La Ragione and Woodward, 2003) and/or (5) enzyme production (Latorre et al., 2015b, 2016)

The current study demonstrated birds fed a corn-high SBM diet supplemented with select *Bacillus* strains in reduced energy diets, had improvements in weight gain and FCR comparable to those of birds fed standard energy diets. These trends for the first 3 trials were driven by the 15% reduction and 16-point increase in BWG and FCR for RED group compared with the control group, respectively. The greatest improvement with addition of any of the DFM candidates was a 14% increase in BWG and 14-point decrease for isolate 46 compared with the RED group, though these changes were not statistically different ($P > 0.05$). In trial 2, despite a 9% reduction in BWG and 11-point increase in FCR in RED group compared with the CTL group, there were no statistical differences in the measurements between these groups. Of the five isolates tested, supplementation of isolate 46 to RED was the most consistent with positive responses in BWG and FCR observed in all six trials. Addition of isolate 40 to RED provided positive responses in trials 3, 4, 5, and 6. In trials 5 and 6, birds fed RED + BI-40 tended to increase feed intake, but did not have the increase in BWG seen in the RED + BI-46 group, resulting in higher FCR. The increase in feed intake is most likely due to the reduced ME of the diet. Harrington et al. (2016) reported birds fed low energy diets had higher FI during d 0 to 21 than birds fed higher energy diets to compensate for lower energy intake per kilogram of feed. Poultry lack the endogenous enzymes to break down galactooligosaccharides and the complex cell wall of NSP that encapsulates other nutrients (Bedford et al., 1991; Bedford and Classen, 1993; Bedford and and Schulze, 1998). The lack of increased FI seen in birds fed RED + BI-46 could be due to the *Bacillus* isolate contributing ME, via breakdown of poultry indigestible carbohydrates, to make up for the energy reduced diet. The combination of isolate 40 and 46 showed improvements over the RED fed birds in BWG and FCR, but the birds did not perform as well as those fed isolate 46 alone. Birds fed isolates 31, 65, 86, or combinations of these

isolates showed no improvements or negative effects from birds fed RED. Harrington et al., (2016) observed similar results as the supplementation of *B. subtilis* to reduced energy diets in broilers recovered growth performance to birds fed a control standard diet. Although, to our knowledge there are no other studies directly targeting substrates in poultry diets with *Bacillus*-based DFM, it has been suggested that DFMs are more effective when birds are fed lower nutrient level feeds (Upadhaya et al., 2019). However, Knap et al. (2011) previously demonstrated that *B. subtilis* supplementation did not result in equal performance of birds fed the reduced energy diets compared to birds fed the control diets, most likely because a reduction of 4% (100 kcal/kg) ME was too high. In the current studies, a reduction of 4.3% (125 kcal/kg) was used with birds fed reduced energy diets supplemented with *Bacillus* isolate 46 showing similar BWG and FCR to birds fed control diets. Taken together, this indicates not all *Bacillus* isolates are created equal and even those within the same species can have significant phenotypic differences.

Bacillus spores have been suggested to be optimal DFM candidates (Latorre et al., 2014a) because of their capacity to resist tough environmental conditions, long storage life, survival during high temperatures of feed pelletization, as well as tolerance to pH, dehydration, high pressure, and chemicals (Hong et al., 2005). Spores are generally added during mixing of the feed, which is ideal for a more consistent mix that does not require equipment and post-pelleting processes, usually used for enzyme supplementation (Bedford and Partridge, 2010). In addition to ease of application, exogenous enzyme supplementation results, to moderate anti-nutritional effects of oligosaccharides and NSP, have been inconclusive and there is little indication of successful enzyme preparations on the growth performance of broiler chickens and turkeys fed corn-SBM diets (Slominski, 2011). Only small improvements in weight gain and FCR were

observed following enzyme supplementation with select combinations of protease, amylase, and xylanase (Troche et al., 2007; West et al., 2007; Yu et al., 2007; Cowieson and Ravindran, 2008; Cowieson et al., 2010). In contrast, previous studies reported similar results of increased performance with enzyme-producing DFM candidates for broilers fed grains with high NSP content (Latorre et al., 2014b, 2017). DFM performance corresponded with increased BWG and lower FCR observed with supplementation of isolate 46 in the current trial.

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Tables

Table 1. Ingredient and calculated nutrient composition (% as-fed) of control or reduced energy (**RED**) basal diets fed from 0 to 21 d post-hatch in all experiments.

Item	Control	RED
Ingredients (%)		
Corn	53.34	55.71
Soybean meal	40.00	40.00
Soy oil	2.88	0.53
Dicalcium phosphate	1.67	1.66
Limestone	0.99	1.00
DL-methionine	0.27	0.27
L-lysine·HCL	0.07	0.06
L-threonine	0.05	0.04
Salt	0.69	0.69
Vitamin premix	0.10	0.10
Mineral premix	0.10	0.10
Choline chloride (60%)	0.05	0.05
Se premix (0.06%)	0.02	0.02
Santoquin	0.02	0.02
Builder's sand[1]	0.02	0.02
Calculated composition		
AME_n, kcal/kg[2]	3,050	2,925
CP (%)	23.62	23.79
dLys (%)	1.20	1.22
dTSAA	0.88	0.88
dThr	0.80	0.80
Total Ca	0.93	0.93
AvP	0.47	0.47

[1]Spores were added at the expense of sand

[2] Abbreviations: AME_n = Nitrogen corrected apparent metabolizable energy; dLys = Digestible lysine; dTSAA; Digestible total sulfur amino acids; dThr = Digestible threonine; AvP = Available phosphorous

Table 2. Live performance (0 to 21 d) of broilers fed mash high soybean meal control (CTL) or reduced energy diets (RED) supplemented without or with *Bacillus*-based DFM candidates in floor pen trial 1.[1]

Item[2]	FI, g	BWG, g	FCR
CTL	903	594	1.53
RED	828	506	1.69
RED + BI-46	893	579	1.55
RED + BI-65	874	559	1.59
RED + BI-86	790	478	1.69
RED + combo 1	861	535	1.59
RED + combo 2	826	512	1.72
SEM	34.0	30.0	0.058
***P*-values[3]**	0.192	0.073	0.093

[1]Values are LSMeans of 6 replicate pens.

[2] Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125 kcal/kg reduction in apparent ME_n ; RED + BI-46 = RED with *Bacillus* isolate 46; RED + BI-65 = RED diet with *Bacillus* isolate 65; RED + BI-86 = RED diet with *Bacillus* isolate 86; RED + combo 1 = RED diet with *Bacillus* isolates BI-46 + BI-86; RED + combo 2 = RED diet with *Bacillus* isolates BI-46 + BI-65 + BI-86.

[3]Overall ANOVA *P*-values

Table 3. Live performance (0 to 21 d) of broilers fed mash high soybean meal control (CTL) or reduced energy diets (RED) supplemented without or with *Bacillus*-based DFM candidates in floor pen trial 2.[1]

Item[2]	FI, g	BWG, g	FCR
CTL	940	641[a]	1.49
RED	913	585[ab]	1.59
RED + BI-46L	855	563[b]	1.70
RED + BI-46	954	590[ab]	1.50
RED + BI-46H	872	532[b]	1.82
RED + BI-65L	913	558[b]	1.65
RED + BI-65	882	556[b]	1.81
RED + BI-65H	959	558[b]	1.66
SEM	32.0	15.0	0.101
***P*-values[3]**	0.190	0.001	0.140

[a-c]Means within a column that do not share a common superscript are different ($P < 0.05$).
[1]Values are LSMeans of 6 replicate pens with 12 individually tagged birds/pen
[2] Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125 kcal/kg reduction in apparent ME_n ; RED + BI-46L = RED with *Bacillus* isolate 46 @ 6.6 X 10^5; RED + BI-46 = RED diet with *Bacillus* isolate 46 @ 10^6; RED + BI-46H = RED diet with *Bacillus* isolate 46 @ 3.3 X 10^6; RED + BI-65L = RED diet with *Bacillus* isolate 65 @ 6.6 X 10^5; RED + BI-65 = RED diet with *Bacillus* isolate 65 @ 10^6; RED + BI-65H = RED diet with *Bacillus* isolate 65 @ 3.3 X 10^6.
[3]Overall ANOVA *P*-values.

Table 4. Live performance (0 to 21 d) of broilers fed mash high soybean meal control (CTL) or reduced energy diets (RED) supplemented without or with *Bacillus*-based DFM candidates in floor pen trial 3.[1]

Item[2]	FI, g	BWG, g	FCR
CTL	965	679[a]	1.44
RED	956	629[bc]	1.54
RED + BI-40	1,012	667[ab]	1.53
RED + BI-46L	1,017	645[abc]	1.56
RED + BI-46	992	625[bc]	1.59
RED + BI-46H	1,001	605[c]	1.68
SEM	26.0	18.0	0.062
***P*-values[3]**	0.518	0.028	0.161

[a-c]Means within a column that do not share a common superscript are different ($P < 0.05$).
[1]Values are LSMeans of 8 replicate pens.
[2] Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125 kcal/kg reduction in apparent ME_n ; RED + BI-40 = RED with *Bacillus* isolate 40 @ 10^6; RED + BI-46L = RED diet with *Bacillus* isolate 46 @ 6.6 X 10^5; RED + BI-46 = RED diet with *Bacillus* isolate 46 @ 10^6; RED + BI-46H = RED diet with *Bacillus* isolate 46 @ 3.3 X 10^6.
[3]Overall ANOVA *P*-values.

Table 5. Live performance (0 to 21 d) of broilers fed mash high soybean meal control (CTL) or reduced energy diets (RED) supplemented without or with *Bacillus*-based DFM candidates in battery trial 4.[1]

Item[2]	FI, g	BWG, g	FCR
CTL	1,049	695[a]	1.51[d]
RED	1,055	641[b]	1.65[ab]
RED + BI-31	1,063	609[b]	1.76[a]
RED + BI-40	1,056	658[ab]	1.62[bc]
RED + BI-46	1,115	709[a]	1.58[cd]
SEM	19.9	19.3	0.033
***P*-values[3]**	0.111	0.001	0.0001

[a-d]Means within a column that do not share a common superscript are different ($P < 0.05$).
[1]Values are LSMeans of 16 replicate pens for RED and 14 for the other treatments.
[2] Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125 kcal/kg reduction in apparent ME_n ; RED + BI-31 = RED with *Bacillus* isolate 31; RED + BI-40 = RED diet with *Bacillus* isolate 40; RED + BI-46 = RED diet with *Bacillus* isolate 46.
[3]Overall ANOVA *P*-values.

Table 6. Live performance (0 to 21 d) of broilers fed mash high soybean meal control (CTL) or reduced energy diets (RED) supplemented without or with *Bacillus*-based DFM candidates in battery trial 5.[1]

Item[2]	FI, g	BWG, g	FCR
CTL	1,002[abc]	687[a]	1.49[b]
RED	1,063[ab]	580[c]	1.89[a]
RED + BI-40	1,100[a]	666[ab]	1.72[ab]
RED + BI-46L	939[c]	615[bc]	1.56[b]
RED + BI-46H	951[bc]	628[abc]	1.57[b]
SEM	32.3	19.5	0.074
***P*-values[3]**	0.0006	0.0003	0.0002

[a-c]Means within a column that do not share a common superscript are different ($P < 0.05$).
[1]Values are LSMeans of 12 replicate pens.
[2] Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125 kcal/kg reduction in apparent ME_n ; RED + BI-40 = RED with *Bacillus* isolate 40; RED + BI-46L = RED diet with *Bacillus* isolate 46 @ 1 X 10^5; RED + BI-46H = RED diet with *Bacillus* isolate 46 @ 1 X 10^7.
[3]Overall ANOVA *P*-values.

Table 7. Live performance (0 to 21 d) of broilers fed mash high soybean meal control (CTL) or reduced energy diets (RED) supplemented without or with *Bacillus*-based DFM candidates in battery trial 6.[1]

Item[2]	FI, g	BWG, g	FCR
CTL	1,031	726	1.55[a]
RED	963	703	1.40[ab]
RED + BI-40	1,025	742	1.46[ab]
RED + BI-46	976	772	1.34[b]
RED + combo	1,023	765	1.38[ab]
SEM	26.8	20.32	0.048
P-values[3]	0.248	0.105	0.022

[a-b]Means within a column that do not share a common superscript are different ($P < 0.05$).
[1]Values are LSMeans of 12 replicate pens.
[2] Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125 kcal/kg reduction in apparent ME_n ; RED + BI-40 = RED with *Bacillus* isolate 40; RED + BI-46 = RED diet with *Bacillus* isolate 46; RED + combo = RED diet with *Bacillus* isolate BI-40 + BI-46.
[3]Overall ANOVA *P*-values

Table 8. Pooled data from six *in vivo* trials where broilers were fed mash high soybean meal control (CTL) or reduced energy diets (RED) supplemented without or with *Bacillus*-based

		Pen Trials (1-3)			Battery Trials (4-6)		
Item[2]		BWG, g	FI, g	FCR	BWG, g	FI, g	FCR
Main effect of Trial							
1	4	560[c]	875[b]	1.592	682[b]	1,073[a]	1.585[a]
2	5	605[b]	946[a]	1.531	632[c]	1,001[b]	1.654[a]
3	6	651[a]	979[a]	1.518	728[a]	990[b]	1.452[b]
SEM		14.8	19.9	0.0372	13.5	16.7	0.0372
Main effect of Trt							
CTL		638[a]	936[ab]	1.487[b]	703[a]	1,027	1.521[b]
RED		574[b]	899[b]	1.615[a]	641[b]	1,027	1.652[a]
RED + BI-46		604[ab]	966[a]	1.538[ab]	697[a]	1,009	1.518[b]
SEM		14.8	19.9	0.0372	13.5	16.7	0.0372
P-Values[3]							
Trial		<0.0001	0.0003	0.326	<0.0001	<0.0001	<0.0001
Trt		0.0007	0.032	0.058	0.0002	0.607	0.002
Trial x Trt		0.472	0.778	0.749	0.150	0.001	<0.0001

DFM candidates BI-46 for 21d post-hatch.[1]

[abc]Means within a column that do not share a common superscript are different ($P < 0.05$)

[1]Values are LSMeans of 18 replicate pens for Trials 1 + 2 and 24 replicate pens for Trial 3; 20 replicate pens for Trt; 44 replicate pens for Trial 4 and 36 replicate pens for Trial 5 + 6; 38 replicate pens for CTL and RED + BI-46, and 40 replicate pens for RED.

[2]Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125 kcal/kg reduction in apparent ME_n; RED + BI-46 = RED with *Bacillus* isolate 46; Trt = Treatment

[3]Overall ANOVA *P*-values

Chapter 4: Effect of Dietary Inclusion of Selected *Bacillus* DFM Candidates in Reduced Energy Diets on Live Performance and Carcass Characteristics of Broiler Chickens

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Abstract

Bacillus DFMs have the potential to provide *in situ* enzyme production within poultry following feed administration. These enzymes could hydrolyze otherwise indigestible feed components which could also be considered anti-nutritional factors for the bird. This may be especially helpful in all vegetable diets in which inclusion of soybean meal (SBM) is increased. The increasing concentration of SBM in poultry diets results in higher levels of anti-nutritional factors like NSP and oligosaccharides. Since poultry lack the endogenous enzymes capable of hydrolyzing these compounds, often decreased performance and welfare issues arise. In the current study, previously characterized *Bacillus* isolates (46 or 40+46) were evaluated based on their effect on live performance and carcass characteristics of birds fed a corn-high soybean meal diet with reduced energy. A 35 d trial was conducted in floor pens (0.08 m^2/bird) with birds being fed a mash or pelleted control diet (CTL), a reduced energy diet (RED), or the RED with isolate 46 or 40 + 46. The RED was formulated to maintain a 125 kcal/kg reduction of AME_n throughout the starter, grower, and finisher phase's realtive to the CTL diet. Treatments were administered to 12 replicate pens. Body weight gain and FCR were recorded at the end of each feeding phase (14, 28, and 35 d) and birds fed pelleted diets were processed at 36 d post-hatch for evaluation of paw scores and carcass characteristics. Birds fed the pelleted diets had higher ($P < 0.01$) BWG and lower ($P < 0.01$) FCR in the starter and overall phases compared with birds fed mash diets. There was no difference ($P > 0.05$) in BWG or FI during the starter and grower phases for birds fed all treatments. Addition of isolate 46 to the RED diet reduced ($P < 0.01$) FCR by 8 points during the starter phase. Though it was not significant, birds fed RED increased FCR by 5 points in the starter period compared to birds fed CTL. Birds fed the CTL diet had the lowest ($P < 0.01$) cumulative FCR in the overall phase among all treatments groups. The birds

fed RED-46 had higher ($P < 0.05$) hot and chilled carcass yields than birds fed the CTL. Breast yield was increased ($P < 0.05$) for birds fed RED-46 increased compared with birds fed RED, whereas increased tender yield ($P < 0.05$) of birds fed RED-46 was higher than the CTL fed birds.

Introduction

Changing production methods have correlated with an increase in use of direct-fed microbials (DFM) as substitutes to antibiotic growth promotors (AGP).In particular, the application of *Bacillus* spores in poultry diets has steadily gained acceptance due to their long shelf life and ability to withstand pelleting temperatures during feed manufacture (Cartman et al., 2007). Previous studies have demonstrated the ability of *Bacillus* spores to germinate and survive in the gastrointestinal tract of mice and poultry (Duc et al., 2003; Latorre et al., 2014). Furthermore, *Bacillus* isolates have been shown to produce a multitude of relevant enzymes to the poultry industry (Latorre et al., 2015, 2016). Recent research from our laboratory reported increased BWG and lower FCR of broilers at 21d post hatch when fed a reduced energy, corn and soybean meal-based diets in mash form and supplemented with different selected *Bacillus*-based DFM candidates.

Broiler diets in the United States are mainly composed of corn and soybean meal (SBM). Although SBM is an excellent source of digestible amino acids in animal feeds, a considerable portion of its energy is not available to poultry (Dozier et al., 2011). The unused energy is mainly contained within indigestible SBM carbohydrates that can exert anti-nutritive effects, limiting some of the available energy and nutritional value of SBM for poultry (Choct et al., 2010). The anti-nutritive effects can largely be credited to pectins, soluble non-starch polysaccharides (NSP) and oligosaccharides (raffinose, stachyose, and verbascose) (Middelbos, 2020). These

carbohydrates have been shown to increase digesta viscosity (Bedford, 1995), intestinal inflammation (Teirlynck et al., 2009), and diarrhea (Saini, 1989), ultimately compromising gastrointestinal health. This can lead nutritionists to restrict SBM inclusion levels to lower than those recommended by least cost feed formulation (Mukherjee et al., 2016). Yet, with growing consumer demand requesting antibiotic free production methods and broilers fed all-vegetable diets there is a clear opportunity for increased SBM use. Increasing the utilization of NSP and oligosaccharides inSBM would lower diet costs by decreasing added lipid inclusions

High dietary SBM linclusions may also contribute to the incidence and severity of footpad dermatitis (FPD), leading to economic, welfare, and food safety issues. Footpad dermatitis is a skin condition characterized by inflammation and necrotic lesions on the surface of the footpads and toes (Greene et al., 1985). The indigestible NSP can increase gut viscosity resulting in sticky droppings that can adhere to the foot and over time deteriorate the epidermis and keratin layers (Shepherd and Fairchild, 2010). The addition of NSP-degrading enzymes and selected *bacillus* DFMs have been shown to significantly reduce digesta viscosity in diets containing high NSP from cereal grains (Choct et al., 1995; Latorre et al., 2015). Therefore, in addition to improving performance and carcass characteristics, *Bacillus* DFMs selected for their capacity to degrade SBM carbohydrates may improve litter quality and reduce the incidence of FPD. Thus, the objective of the present study was to evaluate the role of *in situ* enzyme producing *Bacillus*-based DFMs in broilers reared to market age on growth performance, carcass characteristics, and incidence of FPD.

Materials and Methods

The University of Arkansas Institutional Animal Care and Use Committee approved all experimental procedures involving live birds #18125.

Isolation and characterization of Bacillus spp.

The *Bacillus* strains used in these trials were isolated from poultry sources (unpublished data) and selected as superior producers of α-galactosidase, cellulase, mannanase, and xylanase based on a qualitative enzyme activity evaluation performed using a different selective media for each evaluated enzyme (unpublished data). Candidates were screened by placing on media containing only specific carbon sources that could be utilized for growth. Four selective media were used for testing and included raffinose, arabinoxylan, galactomannan, and cellulose. Selection was based on assumed enzyme activity by their ability to grow on the provided substrates. Sporulation of each selected *Bacillus* isolate was confirmed during the DFM-candidate selection process. Identification and characterization of the different isolates were conducted using a bioMerieux API 50 CHB test kit (catalog no. 50430, bioMerieux, Marcy l'Etoile, France), and each strain was subjected to 16S rRNA sequence analysis (Midi labs, Newark, DE, USA). The two *Bacillus* strains isolates (40 and 46) were identified as *B. amyloliquefaciens.* Isolate 46 is referenced herein with the numerical identifier BI-46 and the combination of isolate BI-40 and BI-46 is referred to as the combo.

DFM preparation

A solid state fermentation media (SS) developed by Zhao et al., (2008) was selected and modified to produce candidate *Bacillus* spores used in these experiments. Briefly, ammonia broth was added to a mixture of 70% rice hulls and 30% wheat bran at an inclusion of 58% by weight. Then, the SS fermentation media was added to 250 mL Erlenmeyer flasks and sterilized by autoclaving for 30 min at 121°C. *Bacillus* isolates were cultured individually in 10 mL of tryptic soy broth (TSB, catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated statically at 37°C overnight for 18h. Following broth incubation for each isolate, 2 mL of turbid culture was

added separately to the previously prepared SS fermentation media flasks. 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 induce sporulation. Next, the inoculated SS fermentation media was transferred from the Erlenmeyer flasks into sterile petri dishes and dried at 60°C. The dried SS fermentation media was aseptically ground into a fine powder using a Bunn G3 HD bulk coffee grinder on the Turkish setting (Bunn, Springfield, Illinois, USA). Fresh rice hulls were ground through the machine in between isolates to prevent contamination. The final dried and ground material contained approximately 10^{10} stable *Bacillus* spores per gram, with spore counts confirmed following a 1:10 dilution of product in 0.9% sterile saline in a 15mL conical tube (VWR, catalog no. 89039-668, Radnor, PA, USA). Subsequent 1:10 dilutions of the solubilized material from the 15mL conical tubes were plated on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated at 37°C for 18 h to allow germination of spores into vegetative cells before counts were conducted to calculate the number of viable spores per gram of product.

Bacillus spores from each of the isolated strains were included into experimental feeds at a calculated 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 number of spores per gram of feed after mixing steps. Feed samples were first pasteurized at 90°C for 10 min to eliminate vegetative cells present in the feed from final spore enumeration. Following heat-treatment, feed samples were subjected to a 1:10 dilution with 0.9% sterile saline in 15mL conical tubes, and diluted samples were plated on TSA plates and incubated at 37°C for 18 h to promote germination of spores into vegetative cells. Vegetative cells were counted and used to calculate the number of viable spores present per gram of feed.

Animal Husbandry

A total of 1,296 day of hatch by-product breeder chicks were obtained from Cobb-Vantress (Siloam Springs, AR, USA) and randomly distributed to 96 floor pens. Chicks were subcutaneously vaccinated at the hatchery for Marek's disease. All broilers were reared in floor pens in a solid-walled, climate-controlled facility at the University of Arkansas poultry research farm. Upon arrival, broiler chicks were group weighed and placed in 0.9 x 1.2 m floor pens at 12 chicks per pen (0.08 m^2 per bird). Each pen contained a hanging feeder, a nipple drinker line, and fresh pine shavings. Feed and water were provided on an ad libitum basis throughout the trial. Initial temperature was set to 32.8°C at placement and decreased gradually to 16.7°C by the end of the trial. A lighting schedule of 24L:0D from d0 to 1, 23L:1D from d 2 to 7, and 16L:8D from d 8 to 35 was used, and target light intensities were verified at floor level via light meter (Photometric sensor; model LT300, Extech Intruments, Waltham, MA.). Starter diets were provided as crumbles or mash from 0 to 14 d of age, whereas the grower and finisher diets were fed as pellets or mash from 15 to 28 and 29 to 35 d of age, respectfully. Mortality were collected and weighed twice daily. Feed intake was calculated based on bird days, and FCR was corrected to include the weight of any dead birds. Body weights and feed consumption were taken by pen at 0, 14, 28, and 35 d post-hatch to calculate FCR and body weight gain (BWG). . Broilers were fed corn-soybean meal diets that contained 38 (starter), 31 (grower), and 28% (finisher) SBM. The consistently higher levels of SBM in the diets was to provide ample substrate for the selected *Bacillus* isolates to be able to utilize. The control diets were formulated to contain 3,025, 3,095, and 3,165 kcal/kg of apparent ME_n in the starter, grower, and finisher phases, respectively. In addition to the control diet, reduced energy diets (RED) at each phase had a 125 kcal/kg reduction in AME_n compared to the control diet resulting in 2,900, 2,970, and 3,040 kcal/kg

(Table 1). Birds were either CTL, RED, or RED with the addition of *Bacillus* isolates 46 or combo (40 and 46) at a concentration of 10^6 spores/gram of feed as a mash or pelleted ration. This resulted in a 2 (feed form) x 4 (treatment) factorial arrangement of 8 treatments (Feed Form x Treatment).

Processing

After final bird weights were taken at 35 d, all birds were taken from 8 randomly selected blocks per dietary treatment. Only birds fed pelleted diets were selected for processing and blocks were chosen instead of birds to eliminate selection bias and pen effects. On day 36, tagged birds were transported to the University of Arkansas Pilot Processing Plant following an overnight (10 h) feed withdrawl. Birds were individually weighed at the plant, electrically stunned, and exsanguinated via a jugular vein cut. Birds were then scalded and defeathered, and the neck, head, and feet were removed at the hock from each bird. Hot carcass and fat pad weights were taken immediately following manual evisceration before carcasses were place in ice water for a 4-h chill. Chilled carcasses were weighed and deboned to collect weights of the pectoralis major, pectoralis minor, wings, and leg quarters. Part weights were divided by individual back dock live weights for each bird to determine percentage yields for each part. The sum of pectoralis major and pectoralis minor weights are reported as total white meat and yield.

Footpad Dermatitis lesions

Footpad dermatitis lesion scores were assigned to each bird after scalding at the processing plant, just prior to feet removal. Footpads were scored on a scale from 0 to 2, with 0 meaning no lesions, a score of 1 from mild lesions and discoloration of the footpad, and a score of 2 for severe lesions with ulcers or scabs and swollen footpads.

Statistics

Pen was considered the experimental unit, and treatments were assigned to pens in a randomized complete block design with pen location serving as the blocking factor. The experiment was comprised of a 2 x 4 factorial arrangement, with each treatment represented by 12 replicate pens of 12 birds. All performance data were subject to a two-way ANOVA using JMP Pro 13 (JMP®) to evaluate the fixed effects of treatment, feed form, and their interactions. Following no feed form x treatment interactions, the main effects of treatments were presented. Where appropriate, means were further separated using Tukey's honest significant difference (HSD) test. Carcass characteristics were subjected to a one-way ANOVA prior to means separation by Tukey's HSD test. Foot pad dermatitis lesions scores were analyzed as of the proportion of birds with scores of 0, 1, or 2. Percentage data were arcsine square root transformed prior to one-way ANOVA analysis.

Results and Discussion

In the current study, a reduced energy diet with high SBM inclusion was used to evaluate *Bacillus* DFM candidates and their combination on their ability to increase bird performance in reduced energy diets. Candidates were previously selected for their *in vitro* ability to enzymatically hydrolyze SBM carbohydrates that are indigestible to poultry and their *in vivo* propensity to increase broiler performance parameters when supplemented to reduced energy diets (unpublished data). Dietary SBM was elevated to increase substrate, like oligosaccharides and NSP, for the DFMs to overcome the energy reduction of the RED diet. The CTL diet was formulated to reflect commercially-relevant energy levels, and the AME_n in the RED was reduced 125 kcal/kg. This diet was supplemented with *Bacillus* isolate 46 or the combination of 40 and 46 to determine if performance, carcass characteristics, and FPD lesions of broiler

chickens fed a reduced energy diet was similar or improved compared to those fed a standard energy diet. Additionally, rations were fed in either pellet or mash form to verify isolates were not affected by pelleting.

Effect of Feed Form

It is well documented that pelleting diets increases performance of broilers by increasing BWG of birds fed pellets compared to birds fed mash diets (Reece et al., 1985; Deaton, 1992; Jahan et al., 2006). In the current experiment, feeding pelleted diets increased FI and BWG and reduced FCR of broilers in the starter period and overall (0 to 35 d) over those fed mash diets. The growth response to pelleting has been suggested to be due largely to the physical form, but also chemical changes from high pressure and steam used in pelleting can alter the ingredients (Allred et al., 1957). This process could alter the ingredients so that more energy and/or protein is available to the bird (Reimer and Beggs, 1993). The destruction of pathogens and other microorganism, as well as the inhibitors present in feed ingredient is another benefit of heat. In addition, birds fed feed in pellet form can consume feed more efficiently with less energy expenditure than birds fed mash diets. Therefore, the growth increase might be explained partly by an increase in feed consumption with pelleted diets (Salari et al., 2006). Though our primary objective was to confirm the efficacy of potential DFM candidates, previously tested in mash feeds, after pelleting rather than study the influence of pelleting alone our results do align with expected responses in broiler performance to pelleting.

Effect of Treatment

Bacillus spores have been suggested to be ideal DFM organisms because of their ability to withstand harsh environmental conditions experienced during feed processing and passage through the gastrointestinal tract (Hong et al., 2005). They have been shown to have many possible modes of action within the gastrointestinal tract such as: Activation of intestinal

function (Samanya and Yamauchi, 2002; Yurong et al., 2005), oxygen consumption – creating a more favorable environment for beneficial anaerobic species (Molnár et al., 2011), enhanced immune responses (Huang et al., 2008), competitive exclusion of pathogenic bacteria, like, *Clostridium perfringens*, *E. Coli*, *Salmonella enterica* (Jin et al., 1996); (La Ragione and Woodward, 2003), and/or enzyme production (Latorre et al., 2015, 2016). In the present study, B. *amyloliquefaciens* isolate 46 and the combination of BI-40 and BI-46 were added to a diet with high soybean meal inclusion and a reduced energy content to evaluate the DFMs effect on broiler performance parameters. Results from the starter and grower phase are shown in Table 2. In these phases, there was no difference ($P > 0.05$) in BWG or FI among birds fed all four treatments. In the starter phase, birds fed the RED + BI-46 had ($P < 0.01$) an 8 point lower FCR compared to the birds fed RED. The FCR of birds fed CTL and RED + Combo were intermediate ($P > 0.05$) to those of birds fed RED and RED + BI-46. Similarly, Harrington et al. (2016), reported birds supplemented with *Bacillus* had increased BWG and lower FCR compared to their respective controls. However, *Bacillus* supplemented groups were only able to compensate compared to the controls fed the same energy reduction. Controls fed higher energy content outperformed all lower energy diets irrespective of *Bacillus* inclusion. Whereas in the current trial, at 14 d post hatch the RED + BI-46 group, which comprised 96% ME of the CTL, had similar BWG to the CTL. Molnár et al. (2011) also reported early BWG improvements with *Bacillus* supplemented birds at 7 d post hatch compared to non-supplemented controls. The early growth response observed could be attributed to exogenous enzyme production facilitating the hydrolysis of NSP that are known to exert adverse effects on performance and nutrient digestibility by increasing digesta viscosity (Bedford and Classen, 1992).

On the other hand, Bai et al., (2013) reported enhanced growth performance in the starter phase when supplementing antibiotics, which was similar to improvements in the current trial. Positive effects of dietary antibiotics is correlated to a reduction of the microbial population in the gastrointestinal tract that competes with the host for nutrients, as well inhibition of production and excretion of catabolic mediators by intestinal inflammatory cells (Barton, 2000). The production of antimicrobials by *Bacillus* isolate 46 could be a principle mechanism by which it inhibits pathogenic microorganisms in the GIT as *Bacillus* species have been shown to produce a large number of antimicrobials (Hong et al., 2005). Antimicrobial activity was observed for BI-46 in previous assays performed in our lab (unpublished data), as well as for other work by Latorre et al. (2015) who reported antimicrobial activity against *Clostridium perfringens*. It has also been suggested that *Bacillus* fed chickens have reduced ammonia concentrations within luminal contents (Endo et al., 1999) which was proposed to activate intestinal function including villus height and enterocyte cell area (Hong et al., 2005). This could lead to increased total surface area of the gut lumen and improved nutrient absorption.

In the grower phase, there were no differences ($P > 0.05$)observed for BWG or FI between all groups, however the birds fed the CTL diet had a lower ($P < 0.01$) FCR than birds fed all other treatments (Table 2). Results from the overall period are shown in table 2. In the overall period, there were no differences ($P > 0.05$) in BWG or FI between all groups. However, birds fed the CTL diet lowered ($P < 0.01$) FCR compared to all other groups.

Carcass Characteristics

Carcass characteristics from are shown in Table 3. Although the CTL fed birds tended to have higher BWG from 0 to 35 d, there was no difference ($P > 0.05$) among treatments in hot carcass weights Birds fed RED + BI-46 ($P < 0.05$) had greater hot carcass yield compared to birds fed

the CTL, while hot carcass yields for birds fed RED and RED + Combo were intermediate.. There was no effect of treatments ($P > 0.05$) on fat pad weight or yield. Similar to hot carcass, birds fed RED + BI-46 increased ($P < 0.05$) had the greatest chilled carcass yield, burds fed the CTL had the lowest, while birds fed the RED and RED + Combo were intermediate.

Parts Weights and Yields

Individual white meat weights and yields as well as total white meat (TWM) weight and yields are reported in table 4. There was no difference ($P > 0.05$) in breast weight among birds fed all treatments, Birds fed the RED + BI-46 increased ($P < 0.05$) breast yield compared to birds fed the RED, while birds fed CTL and RED + combo were intermediate. Tender weights and yield followed a similar trend with no differences observed in weight, RED + BI-46 fed birds increased ($P < 0.05$) tender yield compared to birds fed CTL, with RED and RED + Combo fed birds being intermediate. There was no difference ($P > 0.05$) in total white meat weight, TWM yield tended ($P < 0.07$) to increase in the birds fed RED + BI-46 and RED + Combo compared to birds fed RED and CTL. In this study, DFM supplementation to a reduced energy diet increased carcass, breast, tender, and total white meat yield compared to the CTL and RED without DFM supplementation. This is comparable to results observed by Llamas-Moya et al. (2020), in which birds supplemented with a multicarbohydrase containing α-galactosidase (CAG) enzyme maintained processing parameters equivalent to the positive control formulated with a higher dietary ME. Similarly, the DFMs candidates used in this study were previously selected to have high activity for α-galactosidase for application in high SBM diets. Similar responses of increased breast weight and total breast meat weight have been reported for other exogenous enzymes like xylanase, mannanase, and cellulase (Coppedge et al., 2012; Williams et al., 2014), which our isolates have also demonstrated the ability to produce these NSPases (unpublished

data). The digestibility of NSP is influenced by the NSP concentration, as the chicken microflora is simply limited in the amount of NSP it can digest within the short transit time of the digesta. Increased concentration of the soluble fraction of NSP has shown decreased nutrient digestion and absorption for poultry. They have also been shown to form complexes with digestive enzymes and regulatory proteins in the gut resulting in increased endogenous losses of amino acids for poultry (Angkanaporn et. al., 1994). This could be one aspect influencing the differences observed in increased breast and tender meat yield for birds fed the DFM. No differences ($P > 0.05$) were observed for wing and leg quarter weights or yields (Table 5).

Footpad Dermatitis Scores

Direct fed microbials like B. *amyloliquefaciens* used in this experiment have been shown to have a positive impact on litter quality (Pesti and Fletcher, 1983; Santoso et al., 1999), such as ammonia and fecal moisture content (Ribeiro Jr et al., 2014). The two major influencing factors on FPD are nutrition and litter quality, and these are interconnected as litter quality is affected by moisture, consistency, and amount of excreta, all of which are affected by the diet (Youssef et al., 2011). Increased litter moisture, ammonia content, and chemical irritants in the litter have been shown to increase the incidence of FPD (Mayne, 2005). The use of diets with high levels of SBM, as used in the present experiment have also been associated with higher incidence of FPD (Abbott et al., 1969; Eichner et al., 2007). Litter quality was not directly measured in this experiment; however foot pad dermatitis lesion scores were reported on d 36. Birds fed the RED + BI-46 and RED + Combo increased ($P < 0.01$) scores of "0" and decreased ($P < 0.01$) scores of "1" compared to birds fed CTL and RED (Table 6). Flores et al. (2016) reported similar results in reduction of footpad lesion scores for birds fed a combination of xylanase, amylase, and protease plus *Bacillus* based DFM. Likewise, Nagaraj et al. (2007) reported lower lesion

incidence with the addition of a protease and carbohydrase enzyme product to the all-vegetable diet. In addition, Santoso et al. (1999) showed DFM supplementation reduced litter ammonia concentration. The enzymatic hydrolysis of the NSP and oligosaccharides in the diet by the supplemented BI-46 and the combination (BI-40 + BI-46) could have reduced the digesta viscosity, resulting in increased nutrient digestibility, decreased litter moisture, and lower litter ammonia concentrations. Taken together, these factors could explain the positive effect of the DFMs on footpad lesion scores compared to the non-supplemented controls.

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Tables

Table 1. Ingredient and calculated nutrient composition (% as-fed) of Starter, Grower, and Finisher Diets containing Control or Reduced Energy without or with *Bacillus* isolates from 0 to 35 d Post-hatch.

	Starter		**Grower**		**Finisher**	
Item	Control	RED	Control	RED	Control	RED
Ingredients (%)						
Corn	54.19	57.07	62.19	65.08	64.87	67.76
Soybean Meal	38.91	38.45	31.14	30.68	28.27	27.81
Soy Oil	3.19	0.77	2.94	0.51	3.53	1.11
Dicalcium-Phosphate	1.63	1.62	1.51	1.50	1.31	1.29
Limestone	0.91	0.92	0.90	0.91	0.84	0.86
DL-methionine	0.26	0.26	0.28	0.28	0.25	0.25
L-lysine HCL	0.03	0.04	0.15	0.16	0.12	0.12
L-threonine	0.07	0.07	0.08	0.08	0.06	0.06
NB 3000	0.25	0.25	0.25	0.25	0.25	0.25
Salt	0.40	0.40	0.40	0.40	0.40	0.40
Choline Chloride (60%)	0.06	0.06	0.04	0.04	0.04	0.03
BioCox 60	0.05	0.05	0.05	0.05	-	-
Inert Filler[1]	0.02	0.02	0.02	0.02	0.02	0.02
Calculated analysis						
AME_n, kcal/kg[2]	3,025	2,900	3,095	2,970	3,165	3,040
CP (%)	23.00	23.00	20.00	20.00	18.75	18.75
dLys (%)	1.22	1.22	1.12	1.12	1.02	1.02
dTSAA	0.90	0.90	0.85	0.85	0.80	0.80
dThr	0.82	0.82	0.73	0.73	0.66	0.66
Total Ca	0.90	0.90	0.84	0.84	0.76	0.76
AvP	0.45	0.45	0.42	0.42	0.38	0.38

[1]Spores were added at the expense of sand

[2]Abbreviations: AMEn = Nitrogen corrected apparent metabolizable energy; dLys = Digestible lysine; dTSAA = digestible total sulfur amino acids; dThr = digestible threonine; AvP = Available phosphorous

Table 2. Live performance of broilers fed high soybean meal (CTL) or reduced energy diets (RED) without or with Bacillus-based DFM candidates during starter (0-14 d) and Grower (0-28 d) and Overall (0-35 d) feeding phases.[1]

	Starter (0-14 d)			Grower (0-28 d)			Overall (0-35 d)		
Item[2]	BWG, kg	FI, kg	FCR	BWG, kg	FI, kg	FCR	BWG, kg	FI, kg	FCR
Main effect of Feed Form									
Mash	0.280[b]	0.411[b]	1.490[a]	1.010[b]	1.477[b]	1.494[a]	2.038[b]	4.321[b]	1.548[a]
Crumble/Pellet	0.404[a]	0.530[a]	1.320[b]	1.275[a]	1.876[a]	1.451[b]	2.454[a]	5.027[a]	1.528[b]
SEM	0.010	0.010	0.018	0.021	0.031	0.006	0.034	0.059	0.005
Main effect of Trt									
CTL	0.350	0.478	1.394[ab]	1.160	1.673	1.436[b]	2.302	4.583	1.501[b]
RED	0.334	0.471	1.444[a]	1.129	1.665	1.496[a]	2.234	4.719	1.554[a]
RED + BI-46	0.344	0.460	1.361[b]	1.139	1.698	1.474[a]	2.237	4.705	1.546[a]
RED + Combo	0.339	0.472	1.421[ab]	1.141	1.670	1.485[a]	2.211	4.689	1.552[a]
SEM	0.013	0.014	0.025	0.030	0.044	0.009	0.049	0.083	0.008
P-Values[3]									
Diet	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.005
Trt	0.233	0.373	0.016	0.364	0.611	<0.0001	0.104	0.104	<0.0001
Diet x Trt	0.459	0.727	0.766	0.673	0.959	0.123	0.986	0.986	0.843

[ab]Means within a column that do not share a common superscript are different ($P < 0.05$).

[1]Values are LSMeans of 12 replicate pens

[2]Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125kcal/kg reduction in apparent ME_n; RED + BI-46 = RED with Bacillus isolate 46; RED + Combo = RED with Bacillus isolates BI-40 + BI-46.

[3]Overall ANOVA P-values

Table 3. Carcass characteristics of broilers fed high soybean meal (CTL) or reduced energy diets (RED) without or with *Bacillus*-based DFM candidates from 0 to 35 d and processed at 36 d post-hatch.[1]

	Hot Carcass		**Hot Fat Pad**		**Chilled Carcass**	
Item[2]	Weight, kg	Yield, %	Weight, kg	Yield, %	Weight, kg	Yield, %
CTL	1.808	73.03[b]	0.027	1.15	1.833	74.07[b]
RED	1.797	73.28[ab]	0.028	1.19	1.820	74.24[ab]
RED + BI-46	1.825	73.86[a]	0.029	1.19	1.854	75.04[a]
RED + Combo	1.803	73.65[ab]	0.027	1.29	1.827	74.62[ab]
SEM	0.015	0.22	0.0008	0.040	0.016	0.252
***P*-value[3]**	0.630	0.033	0.517	0.116	0.540	0.030

[ab]Means within a column that do not share a common superscript are different ($P < 0.05$).
[1]Values are LSMeans of 8 replicate pens
[2]Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125kcal/kg reduction in apparent ME_n; RED + BI-46 = RED with *Bacillus* isolate 46; RED + Combo = RED with *Bacillus* isolates BI-40 + BI-46.
[3]Overall ANOVA *P*-values

Table 4. Parts weights and yields of broilers fed high soybean meal (CTL) or reduced energy diets (RED) without or with *Bacillus*-based DFM candidates from 0 to 35 d and processed at 36 d post-hatch.[1]

	Breast		**Tenders**		**Total White Meat**	
Item[2]	Weight, kg	Yield, %	Weight, kg	Yield, %	Weight, kg	Yield, %
CTL	0.473	19.11[ab]	0.093	3.74[b]	0.566	22.86[ab]
RED	0.464	18.90[b]	0.093	3.80[ab]	0.560	22.84[b]
RED + BI-46	0.484	19.56[a]	0.096	3.92[a]	0.581	23.48[a]
RED + Combo	0.473	19.28[ab]	0.094	3.84[ab]	0.567	23.13[ab]
SEM	0.006	0.16	0.001	0.041	0.006	0.19
***P*-value[3]**	0.132	0.020	0.162	0.014	0.125	0.007

[ab]Means within a column that do not share a common superscript are different ($P < 0.05$).
[1]Values are LSMeans of 8 replicate pens
[2]Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125kcal/kg reduction in apparent ME_n; RED + BI-46 = RED with *Bacillus* isolate 46; RED + Combo = RED with *Bacillus* isolates BI-40 + BI-46.
[3]Overall ANOVA *P*-values

Table 5. Parts weights and yields of broilers fed high soybean (CTL) or reduced energy diets (RED) without or with *Bacillus*-based DFM candidates from 0 to 35 d and processed at 36 d post-hatch.[1]

	Wing		**Leg Quarters**	
Item[2]	Weight, kg	Yield, %	Weight, kg	Yield, %
CTL	0.197	7.99	0.573	22.95
RED	0.196	8.00	0.563	22.98
RED + BI-46	0.196	7.96	0.566	23.10
RED + Combo	0.195	8.00	0.569	23.26
SEM	0.001	0.037	0.006	0.186
***P*-value**[3]	0.857	0.806	0.624	0.369

[1]Values are LSMeans of 8 replicate pens

[2]Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125kcal/kg reduction in apparent ME_n; RED + BI-46 = RED with *Bacillus* isolate 46; RED + Combo = RED with *Bacillus* isolates BI-40 + BI-46.

[3]Overall ANOVA *P*-values

Table 6. Percentage distribution of footpad dermatitis scores of broilers fed high soybean meal (CTL) or reduced energy diets (RED) without or with *Bacillus*-based DFM candidates from 0 to 35 d.[1]

Item[2]	**0**	**1**	**2**
CTL	11.65[b]	83.14[a]	5.21
RED	10.21[b]	78.24[a]	11.55
RED + BI-46	34.47[a]	59.00[b]	6.53
RED + Combo	33.52[a]	57.95[b]	8.52
SEM	5.88	5.44	3.21
***P*-value**[3]	0.0002	0.0002	0.625

[ab]Means within a column that do not share a common superscript from arc sine transformed data are different ($P < 0.05$).

[1]Values are LSMeans of 8 replicate pens

[2]Abbreviations: DFM = direct fed microbial; RED = reduced energy diet with 125kcal/kg reduction in apparent ME_n; RED + BI-46 = RED with *Bacillus* isolate 46; RED + Combo = RED with *Bacillus* isolates BI40 + BI-46.

[3]Overall ANOVA *P*-values from arc sine transformed data

Chapter 5: Conclusions

The overall focus of this dissertation was to evaluate the effect of a *Bacillus*-based DFM on low energy, high SBM diets on live performance, carcass characteristics, and footpad dermatitis, with the ultimate goal of identifying a potential DFM candidate.

Regarding live performance of broilers, two candidates (BI-40 and BI-46) of the five tested showed positive improvements in BWG and FCR. However, one isolate (BI-46) showed consistent positive responses across the first six trials. Supplementation of BI-46 increased BWG and decreased FCR compared to birds fed the RED alone to levels similar to that of birds fed the CTL diet. In experiment 2, birds supplemented BI-46 reduced FCR in the starter phase, but no differences were seen in the overall period. This revealed that potentially, *in situ* enzymes produced by BI-46 could alleviate negative effects observed from increased SBM supplementation and degrade the indigestible carbohydrate fraction to increase the metabolizable energy of the diet.

Regarding carcass characteristics and footpad dermatitis, supplementation of BI-46 to the low energy diet increased hot carcass yield compared to birds fed the CTL diet. Additionally, birds fed diets containing BI-46 increased breast meat yield, tender yield, and total white meat yield compared to birds fed the CTL diet. This could indicated a possible increased amino acid digestibility or increased availability of those amino acids to the bird by the *Bacillus* isolate. Birds supplemented with BI-46 also had lower foot pad dermatitis lesion scores compared to birds fed the RED and CTL diets. This possibly could be attributed to BI-46 producing enzymes to hydrolyze the elevated NSP levels in the diet, which results in increased digesta viscosity and sticky droppings.

The positive responses mentioned above could have a profound impact on the poultry industry with the ability to use a less expensive diet and maintain performance, while increasing meat yields and welfare conditions for the bird. Collectively, data from this dissertation confirm a *Bacillus* isolate previously selected for *in vitro* enzyme activity could be a possible alternative to AGP and multicarbohydrase enzyme cocktails in broilers fed all vegetable-based diets with elevated SBM levels.

Chapter 6: Appendix

To: Billy Hargis
Fr: Craig Coon
Date: June 5th, 2018
Subject: IACUC Approval
Expiration Date: May 31st, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18125:** *Evaluation of performance parameters in broilers fed a full nutrient diet without direct fed microbial (DFM) or fed a low nutrient diet with or without DFM at varying concentrations*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 31st, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Lucas Graham, Kyle Teague, Callie McCreery, and Danielle Graham. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp