Bacillus spp. as direct-fed microbial antibiotic alternatives to enhance growth, immunity, and gut health in poultry

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**ABSTRACT**

The increasing occurrence of antibiotic-resistant bacteria combined with regulatory pressure and consumer demands for foods produced without antibiotics has caused the agricultural industry to restrict its practice of using antibiotic growth promoters (AGP) in food animals. The poultry industry is not immune to this trend, and has been actively seeking natural alternatives to AGP that will improve the health and growth performance of commercial poultry flocks. *Bacillus* probiotics have been gaining in popularity in recent years as an AGP alternative because of their health-promoting benefits and ability to survive the harsh manufacturing conditions of chicken feed production. This review provides an overview of several modes of action of some *Bacillus* direct-fed microbials as probiotics. Among the benefits of these direct-fed microbials are their production of naturally synthesized antimicrobial peptides, gut flora modulation to promote beneficial microbiota along the gastrointestinal tract, and various immunological and gut morphological alterations. The modes of action for increased performance are not well defined, and growth promotion is not equal across all *Bacillus* species or within strains. Appropriate screening and characterization of *Bacillus* isolates prior to commercialization are necessary to maximize poultry growth to meet the ultimate goal of eliminating AGP usage in animal husbandry.

**Introduction**

Recovery of multi-drug resistant bacteria (“superbugs”) has been increasing over the past several decades, causing what some public health organizations consider a crisis with potentially “catastrophic consequences” as the world enters the post-antibiotic era (CDC, 2013a, b). The World Health Organization (WHO) has warned that superbugs are reaching dangerous levels worldwide, a problem that is exacerbated by the overuse and abuse of antibiotics. The WHO has specifically called on individuals, healthcare professionals, policy makers, and agricultural industries to make changes to prevent the spread of antibiotic resistance (WHO, 2017). In response, the United States Food and Drug Administration (FDA) has requested that drug companies voluntarily discontinue labelling antimicrobials for growth promotion in agricultural animals (Guidance #213), and antibiotics should only be prescribed for therapeutic uses through veterinary oversight. This voluntary approach would allow industry constituents to collaborate and cooperate with the FDA to quickly achieve a positive public health outcome rather than be mandated which would take more time and resources (US FDA, 2013, 2017).

In the United States, sub-therapeutic doses of antibiotics have been supplemented into the feed of animals intended for human consumption for over 60 years since the first discovery of their growth-promoting effect (Moore et al., 1946). Supplementation of streptomycin and sulfasuxidine along with folic acid in the feed of day-old chickens resulted in significantly increased growth. This growth-promoting result was confirmed by Jukes et al. (1950). Shortly thereafter, antibiotic-resistant bacteria were recovered from food animals given antibiotic growth promoters (AGP). Starr and Reynolds (1951) reported that coliform bacteria isolated from turkeys experimentally fed with streptomycin as a growth promoter were resistant to the antibiotic effect of streptomycin. Other studies reported the recovery of tetracycline-resistant bacteria from chickens fed sub-therapeutic levels of tetracycline (Elliot & Barnes, 1959; Dibner & Richards, 2005). Although AGP were classically used to promote the health of feed animals and have substantially improved their economic value by increasing growth and feed efficiency, the mounting concern of antibiotic resistance has forced the agricultural industry to seek alternatives to replace AGP in food bird production.

One promising antibiotic replacement is the incorporation of probiotics into feed to maintain bird health and promote growth. In the early 1900s, Nobel Prize winner Elie Metchnikoff established the groundwork...
for the modern-day theory of probiotics (Mackowiak, 2013). Since then, the scientific community has repeatedly verified the health- and growth-promoting benefits of probiotics (Vila et al., 2010; Mackowiak, 2013). In agriculture, popular probiotics, or direct-fed microbials (DFM), include Lactobacillus spp. and Bifidobacterium spp. Lactobacilli act by competitively excluding pathogenic bacteria from adhering to and invading the gut epithelium (Wine et al., 2009), and by improved feed digestibility (Zhao & Kim, 2015). Giang et al. (2010) found greater digestibility of crude protein within the first two weeks of weaning when the piglet weanlings’ feed was supplemented with L. acidophilus, L. plantarum, and L. plantarum at a rate of 4 × 10⁹, 2 × 10⁹, and 7 × 10⁹ CFU/kg, respectively. Bifidobacterium is associated with improved gut health by increasing intestinal immunostimulation and producing volatile fatty acids that are beneficial to its host (Williams et al., 1994; Haghhighi et al., 2005). Although promising, there are many challenges associated with industrial scale agricultural incorporation of these two bacteria. Lactobacilli and bifidobacteria are either microaerophilic or strict anaerobes, which makes their mass production and handling in an agricultural setting very difficult. The growth of both organisms is very slow, and they are sensitive to high temperatures that would be encountered during milling and pelleting of feed production (Silva et al., 2015; Quartieri et al., 2016). Further, both species are unable to survive the acidic conditions of the gastric environment, minimizing the number of cells able to colonize the small intestine. Santini et al. (2010) reported that only two of 11 different Lactobacillus and Bifidobacterium tested survived a simulated gastric environment with a pH of 2.5 after 1 h, and only one was recovered after 3 h, although its viability was greatly diminished.

In general, Bacillus spp. have a distinct advantage over Lactobacillus and Bifidobacterium as DFM. As a spore-forming facultative anaerobe, it can withstand temperatures up to 113°C for 8 min, which makes it easier to manipulate and increases its likelihood of surviving feed processing steps. In addition, Bacillus spores are resistant to low pH, bile salts, and other harsh conditions encountered in the gastric environment (Barbosa et al., 2005; Guo et al., 2006; Setlow, 2006; Chaiyawan et al., 2010; Shivaramaiah et al., 2011). Bacillus spores promote gut health not only by competitive exclusion, but by producing antimicrobial peptides (AMP) that are cytotoxic to bacterial pathogens and reduce signs associated with enteric infectious diseases, such as avian coccidiosis (La Ragione & Woodward, 2003; Lee et al., 2010b; Knap et al., 2011; Sumi et al., 2015). Bacillus DFM also improve gut health through the production of beneficial metabolites via alterations in the gut microflora. Bacillus stimulates the intestinal immune system by increasing the levels of cytokines and chemokines such as interleukin-1β (IL-1β) and interferon-γ (IFNγ) in the chicken gut (Lee et al., 2013), and some Bacillus probiotics have been used clinically to help alleviate gastric disorders in humans (Hun, 2009; Gareau et al., 2010). In fact, several studies showed that certain strains of B. subtilis promote the growth of chickens to a greater extent than AGP (Opalinski et al., 2007; Gadde et al., 2017b). Thus, Bacillus spp. have great potential to replace AGP and several commercial products are currently available (Table 1).

The objective of this review is to provide a collective overview of the direct and indirect mechanisms used by Bacillus spp. to improve gut health, immunity, and function as a probiotic growth promoter in poultry, more specifically broiler chickens. Although other non-poultry animals that incorporate Bacillus will be sparingly used as examples throughout this review, they will only serve to supplement and emphasize the various topics.

**Bacillus-produced AMP**

*Bacillus* has been a recognized producer of AMP for over 50 years, devoting up to 5% of its genome to AMP production, and produces at least 66 different AMP, of which several have been purified and commercialized (Stein, 2005; Sumi et al., 2015; Suva et al.,

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**Table 1.** Non-comprehensive list of commercially available *Bacillus* DFM marketed for agricultural poultry use.

| Brand name          | Bacillus strain(s)                  | Manufacturer                                      | Notes                                                                 |
|---------------------|-------------------------------------|---------------------------------------------------|                                                                     |
| **POULTRY-FEED**    | *Bacillus licheniformis*             | Biotechnix-International, [www.biotechnix-      | Also contains *S. cerevisiae* and *E. faecium*                     |
| **GALLIPRO® MS**    | *Bacillus subtilis* DS5750           | international.com](http://biotechnix.com)       |                                                                     |
| **B-Act™**          | *Bacillus subtilis*                 | Chr. Hansen Holdings, [www.chr-hansen.com](http://chr-hansen.com) | Also produce GALLIPRO Fit, GALLIPRO Max and GALLIPRO Tech         |
| **FloraFix-BIRDS**  | *Bacillus subtilis*                 | AgriHealth, [www.agrihealth.co.nz](http://agrihealth.co.nz) | Provided at a concentration of 3.2 × 10⁹ viable spores/gram     |
| **GUT START® – T** | *Bacillus subtilis*                 | BioGrow Australia, [biogrowcompany.com/            | Also contains *B. longum*, *E. faecium*, *L. acidophilus*, and *L. casei* |
| **Alterion®**       | *Bacillus subtilis*                 | Adisseo-Novozyme, [http://feedsolutions.        | Provided at a concentration of 1 × 10⁷ CFU/g                      |
| **Emviva®**         | *Bacillus amylobifaciens*            | dupont.com]                                         | Supplied at a concentration of 1 × 10⁸ CFU/g                      |
| **SPORULIN®**       | *Bacillus subtilis*                 | Novus International, [http://www.novusint.com](http://www.novusint.com) | Recommended dose is 7.5 × 10⁷ CFU/kg                               |
A number of these AMP have been well reviewed by Sumi et al. (2015). Bacteria can produce AMP both ribosomally, where AMP have a narrow antimicrobial range against closely related organisms, and non-ribosomally, where gene encoded precursor molecules are post-translationally assembled by enzymes to exert a broader antimicrobial range (Sumi et al., 2015). The activity of these small, positively-charged peptides is mediated through the disruption of bacterial membranes, making the development of resistance more difficult compared with traditional antibiotics that target enzymatic processes (Sang & Blecha, 2008). Andersson et al. (2016) thoroughly reviewed the potential for pathogens to become resistant to eukaryotic AMP in a clinical setting, and selection for phenotypes resistant to eukaryotic AMP could easily be acquired in vitro. Although not impossible, bacterial resistance to prokaryotic AMP in vivo could prove more difficult due to several factors, including the large variety of AMP produced and their primary mode of action of cell wall disruption. Maroti et al. (2011) described the interaction of AMP-sensitive and resistant bacteria within the gut microbiota as a game of “rock-paper-scissors”, where AMP-producing bacteria kill the AMP-sensitive strains, but are outcompeted in their growth by AMP-resistant strains. AMP-resistant bacteria, on the other hand, are then outcompeted by the growth of AMP-sensitive bacteria due to the lack of toxins in the environment, thus producing a balanced microbial microenvironment.

**Ribosomally produced AMP**

The major ribosomally produced AMP are bacteriocins which fall into three main classes (Zhao & Kuipers, 2016). Class I are the lantibiotics containing the modified amino acids lanthionine and methyl-lanthionine, class II are low molecular weight (<30 kDa) non-modified bacteriocins, and class III are non-heat tolerant high molecular weight proteins (>30 kDa). Bacteriocins form pores in the cell wall of bacteria, initially by attraction to the negatively charged cardiolipins, phosphatidylserine, or phosphatidylglycerol. After attaching to specific cell wall receptors, the spectrum of the antimicrobial activity is dependent on the peptide (Lee & Kim, 2011). The mechanisms and functions of the various bacteriocins produced by *Bacillus* are extensive (Sumi et al., 2015); the bacteriocins considered in this review will be limited to the amphiphilic *B. subtilis*-produced lantibiotic, subtilin (class I), subtilin-like enterin (class I), and the *B. thuringiensis*-produced thuricin and bacthuricin (class II).

*Bacillus* ribosomally produced AMP are mainly effective against Gram-positive bacteria that are closely related to it. For instance, Rea et al. (2010) identified a two-component thuricin which was effective against clinical strains of *Clostridium difficile*. These two-components, Trn-α and Trn-β, worked synergistically to reduce *C. difficile* from approximately 10^6 CFU/ml to below the limits of detection within 3 h. It is just as important to note that, at similar concentrations used to reduce *C. difficile*, this thuricin had no effect against *L. casei* and *B. lactis* which suggests that thuricin would have little effect against other commensal gut bacteria. It is important to note that *C. difficile* is a foodborne pathogen that is readily isolated in poultry, and studies have found genotypically identical strains of this organism between animals, food, and clinical strains isolated in the same geographical area (Harvey et al., 2011; Lund & Peck, 2015). Bacthuricin was also shown to be effective against food pathogens *Listeria monocytogenes* and *B. cereus*. The well-diffusion technique was used to demonstrate that Bacthuricin F4 greatly inhibited *B. cereus* after 24 h (Kamoun et al., 2005). Kamoun et al. (2011) showed similar results where Bacthuricin F103 was purified and characterized prior to applying 50 AU to 1 × 10^5 cells of *B. cereus* ATCC 14579, in vitro. They reported a reduction of 1.2 Log CFU/ml within the first 5 min, and this decline remained steady for 3 h resulting in a 4 Log reduction. This study then applied 500 AU of Bacthuricin F103 to beef that was artificially contaminated with 2 × 10^2 CFU/g of *L. monocytogenes*, and after 6 days the pathogenic load decreased by 1.61 Log CFU/g and was undetectable after 10 days showing its versatility in vitro as well as in a meat model. Another study reported that after purification, an unsuccinylated enterin, a subtilin-like lantibiotic, was effective against *Staphylococcus aureus* (MIC between 4 and 8 µg/ml), and *Enterococcus faecalis* (MIC between 8 and 16 µg/ml) (Fuchs et al., 2011). Both organisms are Gram-positive pathogens of interest commonly found in poultry (Persoons et al., 2009; Lee et al., 2010a), and enterin reduced them to levels similar to nisin A. This enterin was produced by *B. subtilis* DSM 15029, and it contained a 3-amino acid difference to the subtilin produced by *B. subtilis* ATCC 6633, and the ATCC 6633 strain did not produce enterin in discernable amounts (Fuchs et al., 2011). This suggests that different *Bacillus* strains can produce differently structured AMP, and it suggests *Bacillus* strains have the potential to evolve and produce different AMP that are like previously produced peptides but more effective.

A major class of ribosomally produced AMP that has been widely studied and has been used in agriculture production are the lantibiotic class of bacteriocins, more specifically subtilin (Lee & Kim, 2011). When mature, this *B. subtilis*-produced AMP contains 32 amino acids, a meso-lanthionine ring, and four methyl-lanthionine rings, a structure that closely resembles nisin, a *Lactococcus* antimicrobial that has been used in the dairy and cheese industry. Subtilin binds to

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bactoprenyl pyrophosphate in lipid II, and initiates a cascade of downstream reactions which results in disrupted cell barrier function and bacterial death (Chan et al., 1989; Parisot et al., 2008). The thick cell walls of Gram-positive organisms are most susceptible to subtilin. A suspension of B. cereus was significantly reduced after 4 h by 1 µg/ml of subtilin in a study conducted by Liu and Hansen (1992). Interestingly, this same study showed a site mutation at position 4 from GLU to ILE resulted in subtilin reducing B. cereus at lower concentrations, 0.3 µg/ml, compared to the wild-type which lends weight to the theory that slight mutations to the peptide’s structure might enhance the lethality of Bacillus-produced AMP. Like other bacteriocins, the production of subtilin is based on cell-density signalling, or quorum sensing, as an adaptive response to environmental stress including decreased availability of nutrients (Abriouel et al., 2011). Initiating subtilin production reduces the competition from the surrounding biota, and increases the available nutrients in the gastrointestinal (GI) environment, so more of these nutrients are readily available to the host and, in poultry production, this can equate to better feed efficiency. Subtilin does not cause complete bacterial interruption, and some beneficial organisms can be promoted in the presence of subtilin (Hosoi et al., 2000).

**Non-ribosomally produced AMP**

*Bacillus* also produces a series of non-ribosomally synthesized AMP through detailed mechanisms of assembly from over 300 different precursors mediated by a series of peptide synthases. These AMP have a broader range of microbial inhibitions and are effective against both Gram-positive and -negative bacteria, as well as viruses, fungi, and yeasts (Hancock & Chapple, 1999). Condensation of these molecules is catalysed by a thioesterase, and elongation typically occurs with three domains: an adenylation domain, a thiolation carrier domain, and a condensation domain (Stein, 2005). The most well-studied non-ribosomal AMP produced by *Bacillus* include bacitracin and gramicidin, which are both popular antimicrobials used in the medical field. Other non-ribosomal AMP produced by *Bacillus* include iturins and fengycins; these lipopeptides exhibit strong antifungal activity (Maget-Dana & Peypoux, 1994; Deleu et al., 2008).

A lipopeptidase produced by *B. subtilis*, surfactin, is one of the most powerful surfactants known, which interferes with biological membranes of bacteria, viruses, and mycoplasmas. Bio-surfactants have low toxicity and are biodegradable which makes them advantageous in agriculture, because pharmaceutical antibiotics may persist in the environment for long periods of time which increases the stability of resistance phenotypes in bacteria (Pérez-García et al., 2011). Fernandes et al. (2007) applied two surfactins, isolated from *B. subtilis* R14, to 29 multi-drug resistant bacteria and the surfactins were found to be effective against them all. One of the bacteria was a multi-drug resistant *E. coli* and, although *E. coli* is a commensal in the chicken gut, overgrowth of this organism can result in significant economic losses caused by colibacillosis and airsacculitis in poultry (Diarra et al., 2007). Surfactin disintegrated the cell membrane of several *Mycoplasma* spp. indicating its potential effectiveness against a similar species *Mycoplasma gallisepticum*, the causative agent of chronic respiratory disease in chickens (Vollenbroich et al., 1997). However, this theory requires further research.

The AMP discussed were applied to organisms *in vitro* or after the antimicrobial was purified. However, during large-scale poultry production, *Bacillus* spores would be supplemented in feed and/or water and the multitude of AMP produced by single or multiple strains would affect the gut community, *in vivo*, and in the presence of other microbiome interactions. In other words, the *in vitro* results of these studies may not translate directly with what occurs in the gut. Resistance to antimicrobials of human and veterinary importance is a risk when supplementing with whole bacterial organisms. In order to be considered suitable DFM, bacteria must show susceptibility to the following antibiotics: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, neomycin, erythromycin, clindamycin, quinupristin + dalfopristin, tetracycline, chloramphenicol, trimethoprime, and linezolid (Anadón et al., 2006). *Bacillus* spp. must also test negative for the toxigenic genes: haemolytic enterotoxin (*hne*), haemolysin BL (*hbl*), cytotoxin K (*cytK*), and cereulid (*ces*) (European Food Safety Authority, 2014).

**Bacillus-induced gut microflora modulation and resulting metabolite production**

Table 2 summarizes the results of some published studies that have examined the effects of *Bacillus* DFM on chicken growth performance. Although the exact mechanisms are unknown, it has been suggested that *Bacillus* supplementation as a DFM alters the gut microbiota, reduces the competition for nutrients between microbes and the host, and improves GI health. Microbial colonization of the chicken GI tract begins at hatch and rapidly increases. Within 24 h, the microbial load in the proximal and distal intestine can reach $10^8$ and $10^{10}$ cells/g, respectively (Apajalahti et al., 2004). The composition of the intestinal microflora is heavily influenced by the litter left from the previous flock (Lee et al., 2013). Early application of *Bacillus* spp. as a DFM is crucial if it is to maintain a lasting presence within the gut community. Studies have shown that supplementation of *Bacillus* spp. as a DFM improves overall intestinal health and growth.
Table 2. Effect of Bacillus DFM on chicken growth performance.

<table>
<thead>
<tr>
<th>Bacillus strain</th>
<th>Dosage</th>
<th>Length of study</th>
<th>Pathogen challenge</th>
<th>Growth performance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td>10^6 CFU/t</td>
<td>42 days</td>
<td>No</td>
<td>None</td>
<td>Teo and Tan (2007)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>10^5 CFU/t</td>
<td></td>
<td>No</td>
<td>None</td>
<td>Jeong and Kim (2014)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>10^5 CFU/g</td>
<td>35 days</td>
<td>No</td>
<td>Reported improved ADG but did not report final weight</td>
<td>Knap et al. (2011)</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>8 × 10^5 CFU/g</td>
<td>42 days</td>
<td>Yes, Salmonella</td>
<td>Weight gain was numerically significant but not</td>
<td>Knap et al. (2010)</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>8 × 10^5 CFU/g</td>
<td>28 days</td>
<td>Heidelberg</td>
<td>statistically significant</td>
<td></td>
</tr>
<tr>
<td>B. subtilis C-301</td>
<td>30 g/t feed</td>
<td>42 days</td>
<td>No</td>
<td>All these B. licheniformis concentrations caused a</td>
<td>Fritts et al. (2000)</td>
</tr>
<tr>
<td>B. subtilis 1781</td>
<td>1.5 × 10^5 CFU/g</td>
<td>14 days</td>
<td>No</td>
<td>weight gain significantly greater than the challenged</td>
<td>Gadde et al. (2017b)</td>
</tr>
<tr>
<td>B. subtilis (Avicorr™)</td>
<td>1.5 × 10^5 CFU/g</td>
<td>28 days</td>
<td>Yes, broilers were reared on Eimeria-positive used</td>
<td>Significantly greater body weights were reported in</td>
<td>Lee et al. (2015)</td>
</tr>
<tr>
<td>B. subtilis Calsporin (Calpis Co.)</td>
<td>1 × 10^19 CFU/g</td>
<td>42 days</td>
<td>No</td>
<td>the B. subtilis-treated group compared to the controls</td>
<td>Aliakbarpour et al. (2012)</td>
</tr>
<tr>
<td>B. subtilis (Bs27)</td>
<td>1.5 × 10^5 CFU/g</td>
<td>21 days</td>
<td>No</td>
<td>Significantly greater body weights were reported in</td>
<td>Lee et al. (2010a)</td>
</tr>
<tr>
<td>B. subtilis (3AP4)</td>
<td>1.5 × 10^5 CFU/g</td>
<td>21 days</td>
<td>No</td>
<td>the control and similar body weights as the LAB-treated</td>
<td></td>
</tr>
<tr>
<td>B. subtilis (BS18)</td>
<td>1.5 × 10^5 CFU/g</td>
<td>22 days</td>
<td>No</td>
<td>Increased body weights for five of the nine tested</td>
<td></td>
</tr>
<tr>
<td>B. subtilis (15AP4)</td>
<td>1.5 × 10^5 CFU/g</td>
<td>22 days</td>
<td>No</td>
<td>strains compared to control but none were significant:</td>
<td></td>
</tr>
<tr>
<td>B. subtilis (BS27)</td>
<td>1.5 × 10^5 CFU/g</td>
<td>22 days</td>
<td>No</td>
<td>Bs27, LSSAO1, 3AP4, 15AP4, Avicorr™</td>
<td></td>
</tr>
<tr>
<td>B. subtilis (Avicorr™)</td>
<td>1.5 × 10^5 CFU/g</td>
<td>22 days</td>
<td>Yes, Clostridium</td>
<td>Significantly increased body weights compared to</td>
<td>Lin et al. (2017)</td>
</tr>
<tr>
<td>B. licheniformis H2</td>
<td>1 × 10^6 CFU/g</td>
<td>22 days</td>
<td>perfringens</td>
<td>C. perfringens infected controls</td>
<td></td>
</tr>
</tbody>
</table>

in chickens but, again, the exact mechanisms are unknown. Bacillus spores are not reactive prior to ingestion. However, they quickly germinate once inside the chickens’ GI environment and vegetative cells can outnumber spores within 20 h of oral administration and could be detected along the small intestine, caeca, and colon (Cartman et al., 2008). Bacillus DFM could affect the distribution and colonization of the innate microflora along the GI tract and promote the growth and proliferation of other good symbiotic bacteria. One theory posits that the growth-promoting effects of DFM in poultry are linked to reduced number and diversity of the natural microbiota, thus allowing for increased nutrient utilization by host intestinal epithelial cells and reduced effects of detrimental microbial metabolites (Gadde et al., 2017a).

In general, lactobacilli and other bacteria of the phylum Firmicutes comprise 80–90% of the total small intestinal microflora, followed by members of Proteobacteria and Bacteroidetes phyla. The intestinal duodenum is predominated by Lactobacillus, Streptococcus, and coliforms, while the ileum houses mostly Lactobacillus, Streptococcus, coliforms, and members of the Enterobacteriaceae and Clostridiaceae families. Two bacteria known for their ability to improve chicken gut health are Lactobacillus spp. and Bifidobacterium, a genus of the phylum Actinobacteria. Both microorganisms ferment simple sugars, and both are relatively unaffected by Bacillus DFM’s (Kaplan & Hutkins, 2003; Moura et al., 2007; Rinttilä & Apajalahti, 2013; Choi et al., 2014). For instance, Teo and Tan (2007) recovered similar levels of Lactobacillus and Bifidobacterium spp. from the ileum of broilers given feed supplemented with 10^9 CFU/ml B. subtilis PB6 when compared to the negative control. In another study, B. subtilis (natto) enhanced the growth of beneficial bacteria L. reuteri and L. acidophilus (Hosoi et al., 2000), a result that was echoed by Jeong and Kim (2014) who reported a significant increase in Lactobacillus in the ilea and caeca after supplementing chicken feed with B. subtilis C-3102. The host can recover some of the energy lost through competition with microbes in this part of the gut by absorbing bacterially-produced nutrients and metabolites, such as lactic acid and volatile fatty acids, from fermenting bacteria. Some Bacillus DFM can facilitate the increase in beneficial gut biota in the upper GI tract to maintain or even increase the production of these nutrients.

Diets rich in carbohydrates, such as the corn- and soy-based diets typically given to production chickens, provide materials for fermenting biota in the upper gut, and those bacteria produce fermentation intermediates that include lactate, fumarate, and succinate. These metabolites are either absorbed by the host or used by other biota to produce other end products. After supplementing water with commercial organic acids, Chaveerach et al. (2004) reported greatly reduced amounts of lactate in the caecum compared with the crop, suggesting either lactate absorption by the host or utilization by other biota which could lead to the production of beneficial end
products, such as the short chain fatty acid (SCFA), butyrate. In an in vitro co-culture study, B. longum produced lactate in the presence of fructooligosaccharides and Eubacterium hallii fermented the lactate to produce significant amounts of butyrate. B. longum alone did not produce significant amounts of butyrate and E. hallii could not grow on fructooligosaccharides (Belenguer et al., 2006). Although this study was in vitro, it is likely that a similar microflora metabolite utilization and production occur along the gut, especially with increased levels of certain fermenting bacteria. Increased levels of fermenting bacteria in the families Bifidobacteriaceae, Bacteroidiaceae, and Lactobacillaceae strongly correlate with increased levels of β-glucosidase in the avian gut of grain-fed birds (Waite & Taylor, 2014). Deficiencies in β-glucosidase have been associated with increases of Bifidobacterium in the stool of healthy humans (Depeint et al., 2008); however, more research is needed to understand its exact impact on poultry.

Competition between host and gut biota occurs in the small intestine, mainly by the Lactobacillus spp. that is estimated to utilize approximately 3–6% of total dietary protein; however, this estimate can vary greatly depending on bacterial density and the host absorbs much of their needed amino acids in the proximal small intestine where bacterial densities are lower (Apajalahti & Vienola, 2016). Lactobacillus spp. and other protein-assimilating bacteria in the small intestine act as a filter to prevent the passage of amino acids to the caeca where they would be further fermented into toxic by-products, such as indoles and ammonia, by putrefying bacteria (Searle et al., 2009; Apajalahti & Vienola, 2016; Ghasemian & Jahanian, 2016). Indole toxicity uncouples the protein gradient across biological membranes and inhibits ATP production (Chimerel et al., 2013), and deamination production of ammonia adversely affects gut epithelial cell morphology, DNA synthesis, and metabolism, all of which result in decreased health and growth (Clausen & Mortensen, 1992; Apajalahti & Vienola, 2016). Greater nutrient absorption could lead to increased growth in poultry; however, this growth promotion is not guaranteed. Some studies, like Jeong and Kim (2014), Fritts et al. (2000), Gadde et al. (2017b), and Hosoi et al. (2000), reported improved growth performance that was either equal to or greater than the controls, while others report that their Bacillus DFM either had no difference in weight gain or caused a decrease in body weight, such as Teo and Tan (2007) (Table 2). Variation of DFM performance can even occur within the same study. Although Fritts et al. (2000) reported improved weight gain, this was only in one trial within the study. This same study also reported that B. subtilis C-301 did not improve growth in a separate independent trial under the same experimental conditions, which led the researchers to infer that heat stress may have reduced broiler performance, because the underperforming trial was conducted during a warm season.

The caeca have the highest bacterial density along the chicken gut, housing upwards of 10^{11} cells/g digesta, and the longest residence time of 12–20 h. The primary phyla in the caeca belong to Firmicutes, Bacteroides, and Proteobacteria with the order Clostridiales representing more than 50%. This organ is responsible for water regulation and carbohydrate fermentation and it is the largest SCFA producing organ in the chicken (Torok et al., 2011; Oakley et al., 2014). SCFA are necessary for epithelial cell maintenance and the inhibition of pathogenic organisms. Butyrate has been of interest to the poultry industry because it is the preferred energy source for enterocytes and colonocytes to maintain the integrity of the gut lining and effectively ward off Salmonella colonization (Van Immerseel et al., 2003). Supplementation with Bacillus has been reported to increase SCFA production either directly or indirectly by modulating SCFA producing bacteria. For instance, butyrate was significantly increased when chicken feed was co-supplemented with 5000 U/kg of serine protease and phytase along with 8 × 10^{6} CFU/g of B. licheniformis (Murugesan et al., 2014). It is worth mentioning that SCFA and nutrient production when supplemented with Bacillus is not equal and can differ from study to study. Novak et al. (2011) reported a decrease in butyrate and other SCFA produced when individually supplementing with B. subtilis and B. licheniformis while using a slow growing chicken breed in their model. Host breed, genomic and metabolic changes can influence gut microbial response to nutrient metabolism, as is evident in a study by Kim et al. (2015). Also, very few nutrients are absorbed in the caeca. When the caecal microflora produces SCFA and other nutrients such as vitamins B, possibly in response to Bacillus DFM supplementation and, to date, no study exists that correlates metabolomic production to gut flora modulation in the presence of Bacillus DFM. This novel area of study could lead researchers to possibly replace DFM with the beneficial metabolites they produce, which could decrease the variability that is often observed with DFM.
Pathogenic control by Bacillus DFM

Pathogen reduction and/or suppression by the GI commensal microflora is the result of multiple factors. Although the exact mechanisms are unknown, Bacillus as DFM could prevent pathogenicity by direct inhibition of pathogens through mechanisms of competitive exclusion such as the production of AMP or by enhancing the intestinal mucosal layer to prevent microbial diffusion across the membrane (Johansson et al., 2008). Pathogenic control is a major concern for the poultry industry from an economic and public health perspective. Salmonella is classically associated with poultry and causes approximately one million illnesses in the United States annually, and zoonotic pathogenic Clostridium spp. cost the American poultry industry roughly $6 billion annually (Wade & Keyburn, 2015; CDC, 2017). Studies show that Bacillus strains can reduce or inhibit poultry pathogens, both in vitro and as DFM. For instance, Teo and Tan (2007) showed that two strains of B. subtilis isolated from the gut of a chicken were antagonistic against C. perfringens ATCC 13124 after 24 h of incubation under anaerobic conditions, and Knap et al. (2010) reduced necrotic enteritis (NE) caused by C. perfringens in chickens with three concentrations of B. licheniformis (8 × 10⁵ CFU/g feed, 8 × 10⁶ CFU/g feed, and 8 × 10⁷ CFU/g feed). All three concentrations reduced clinical signs of NE to levels similar to growth-promoting doses of virginiamycin (50 g/ton) and they maintained similar body weight and feed conversion ratios (FCR) as the virginiamycin-treated group. Park and Kim (2014) reported that intestinal concentrations of Salmonella Typhimurium were significantly decreased in the presence of three concentrations of B. subtilis B2A (1 × 10⁴ CFU/g, 1 × 10⁵ CFU/g, and 1 × 10⁶ CFU/g). Although the weight gain of these birds was not significant, the B. subtilis B2A-fed group had less feed intake and better FCR, meaning they ate less to weigh the same.

In addition to controlling bacterial pathogens, Bacillus DFM has reduced the effects of coccidiosis, a disease caused by Eimeria spp. This parasite contributes to an estimated $3 billion annual loss, worldwide, and seven distinct species infect avian intestinal mucosa (Lillehoj & Trout, 1996; Shirley & Lillehoj, 2012). In an earlier study that tested eight individual strains of B. subtilis as a DFM against the clinical signs of coccidiosis, three Bacillus strains showed significant reduction in intestinal lesion scores, which is a post mortem observation of coccidiosis, and two of the three strains did not result in reduced body weight gain, also a clinical sign of coccidiosis (Lee et al., 2010b). Another study resonated these results and, after Eimeria infection, the treatment group that received B. subtilis maintained a body weight that was similar to the uninfected control and greater than the Eimeria infected group that was not alleviated with B. subtilis supplementation. This study also reported that, in addition to reducing the post mortem observations of coccidiosis, the Bacillus-treated group had enhanced immunological response towards the infection, marked by substantial up-regulation of proinflammatory cytokines and intestinal epithelial lymphocytes (Gadde et al., 2017c).

Rarely, if ever, is coccidiosis a unilateral parasite in poultry production, meaning Eimeria spp. typically promotes infections caused by the opportunistic pathogen C. perfringens to magnify the signs of NE (Park et al., 2008). Model development studies indicated that C. perfringens alone was not enough to cause NE, and intestinal lesion scores and body weight loss, the typical clinical signs in infected broilers, were mild or akin to the non-infected controls. However, after the addition of Eimeria spp., NE signs were dramatically worse resulting in broilers with noticeable intestinal lesion scores and markedly reduced body weights. After treating broilers with a cocktail of B. subtilis DFM for 28 days, there was a significant increase in body weight gain in the DFM-fed group compared to the salinomycin-fed group but not the controls following Eimeria and C. perfringens challenge infection. Bacillus DFM improve immunity by increasing antibodies against Eimeria spp. and serum nitric oxide (NO) levels; even if enhanced growth was not affected, these birds were more robust to fight infections and the symptoms associated with bacterial and parasitic pathogens, as is detailed in a study by Lee et al. (2014). A separate study had similar findings when using B. licheniformis to reduce NE infections in a dual C. perfringens-Eimeria model; however, this study took their analysis in another direction and used genomic sequencing to investigate the caecal microbial changes caused by DFM supplementation. In this case, B. licheniformis fed chickens had caecal profiles similar to the negative control group with Bacteroides being one of the most dominant taxa. However, Bacteroides was the lowest in the infection group that did not receive B. licheniformis alleviation. This study also noted a growth promotion response in the B. licheniformis-supplemented group (Lin et al., 2017).

Although performance variations depend on the supplemented DFM strain, the literature consistently reveals improved performance in the presence of a pathogen, either bacterial or parasitic (Ducatelle et al., 2014). Constant dosing with Bacillus as DFM is necessary for it to maintain its anti-pathogenic properties. Although Bacillus can survive in the gut, it is mainly a transient member and most of the cells are shed in faeces and must be continuously supplied in feed and water shortly after hatch through to harvest. In a study that compared the persistence of S. Enteritidis after a single dose of B. subtilis (1 × 10⁹ cells) followed by a single dose of S. Enteritidis (1 × 10⁵ cells),
there was mostly no significant reduction in the recovery of S. Enteritidis after 36 days in the liver, spleen, duodenum, jejunum, ileum, caeca, and colon. Inoculation with these organisms occurred within 48 h post-hatch, and the only observed reduction was very slight \( (P = 0.035) \) and was only on the first post-infection day.

**Gut morphological and immunological changes induced by Bacillus DFM**

Bacillus DFM interact directly with the host to produce a beneficial response to the chickens’ intestinal epithelium. This immunological and physiological response is critical to successful poultry production, because it directly correlates to improved growth and performance. Many factors related to disease and stress can cause an interruption to intestinal epithelial integrity which reduces nutrient absorption, increases pathogenic invasion, and increases inflammatory diseases, all leading to reduced growth performance (Yegani & Korver, 2008). Gut barrier function must be maintained if the body is to shield itself from unwanted biological and chemical invasions, and it does this with two major mechanisms. The first is the secretion of the mucous blanket by goblet cells that are dispersed throughout the small intestine luminal epithelium (Chichlowski et al., 2007). The mucus consists predominantly of mucin and is supported by other proteins, lipids, glycoproteins, and glycolipids. DFM, such as Bacillus, can up-regulate the mucin-producing gene, MUC2, to counteract the inflammation caused by pathogens. In a study by Gadde et al. (2017c), chickens were given intraperitoneal injections of E. coli LPS to induce an inflammatory response. The group that was given feed supplemented with B. subtilis strain 1781 showed significantly increased ileum levels of MUC2 and showed markedly lower signs of inflammation as noted by reduced α-1-acid glycoprotein, which is often associated with acute colitis (Hocheplied et al., 2002). This study also showed that up-regulation of MUC2 can happen in response to LPS circulating in the blood and not just as a reaction to direct contact with the intestinal epithelium. In other words, continual dosing with Bacillus DFM could combat residual infections or the pathogens that may have bypassed host defences and remain in circulation after the initial infection. An increase in MUC2 was observed in other studies that supplemented with Bacillus DFM in the absence of infection showing the ability of Bacillus to promote preventative gut infections (Lee et al., 2010a). Aliakbarpour et al. (2012) reported that Bacillus DFM-treated broilers produced significantly more mucin in the jejunum than the control and was comparable to the group that received a treatment of lactic acid bacteria cocktail containing L. casei, L. acidophilus, B. thermophilum, and E. faecium (Aliakbarpour et al., 2012). Both DFM-treated groups showed greater body weight than the control although all three groups consumed similar amounts of feed.

The second mechanism is the enhancement of the epithelial barrier integrity by increasing the regulation of tight junction proteins that bind to one another forming a continuous barrier that is impenetrable to pathogens and large molecules (Chichlowski et al., 2007). Shen et al. (2006) used electron microscopy to demonstrate some of the primary molecules involved in maintaining the integrity of this barrier which include junction adhesion molecule 2 (JAM2), occludin, and zona occludens 1 (ZO1) as well as the physiological response of increased intestinal villi crypt height. Gadde et al. (2017c) echoed similar findings and reported a distinct increase in tight junction genes JAM2, ZO1 and occludin in the ileum when chickens challenged with LPS were given B. subtilis as a DFM. Greater crypt depth increases the intestinal epithelial surface area making the organ better suited for nutrient absorption which could translate into growth promotion. When broilers were fed gradually increasing amounts of B. subtilis LS 1-2 over 35 days, not only were villus height and crypt depth significantly better in the duodenum and ileum, but the B. subtilis LS 1-2-treated group weighed significantly more and had decreased caecal Clostridium and coliforms compared to the control (Sen et al., 2012). In a C. perfringens challenge study, B. subtilis PB6 supplemented broilers had significant FCR and increased intestinal villi length of between 10.88% and 30.46% compared to infected controls demonstrating that Bacillus supplementation can improve host gut physiology and intestinal health in the presence of pathogens (Jayaraman et al., 2013). Avian physiological responses to Bacillus DFM cause an increase in gut barrier protein production, crypt height, and immune modulation via cytokine and defence molecule production.

Macrophages function as an important initiator and mediator in innate and adaptive immunity by recognizing pathogens and eliminating them via phagocytosis and cytotoxicity response which includes the production of proinflammatory cytokines: IL-1β, IL-6, IL-8, and TL1A (the chicken homologue of TNF-α (Takimoto et al., 2008)) and T-helper cytokines: IL-2 and IFNγ. Proinflammatory cytokines cause the production of defence molecules, NO, and inducible nitric oxide synthase (iNOS) that, at low concentrations, stimulate the maturation and activity of immune cells while, at higher concentrations, NO irreversibly binds to DNA, lipids, and proteins effectively killing the pathogen (Privett et al., 2012). Bacillus DFM have been marked by immunostimulatory production of proinflammatory cytokines and macrophage activation without causing cytotoxicity. In the presence of pathogens, Bacillus DFM cause a significantly noticeable increase in the up-regulation of cytokine production,
NO, and iNOS. One study reported a 1.5-fold increase in NO concentrations with *Bacillus* DFM in the presence of *E. coli* LPS, and an other study reported broilers raised on chicken litter that was positive for *Clostridium* caused gangrenous dermatitis; IL-1β was up-regulated along with IFNγ compared to the controls that did not receive *Bacillus* treatment (Lee et al., 2011, 2013). To understand the global gene expression that occurred in the presence of *B. subtilis* DFM and dual pathogens *Eimeria* spp./*C. perfringens*, one study utilized gene array techniques and identified 37 genes which were significantly related to the inflammatory response and were up- or down-regulated. This same study also reported an up-regulation of intestinal expression of IFNγ, IL-1β, and IL-12 (Lee et al., 2015). Augmentation of macrophage function is one way *Bacillus* DFM enhance immunity. It has also been reported to support the proliferation of lymphoid follicles along the intestinal mucosa and support the development of gut-associated lymphoid tissue thus increasing its immunoregulatory capacity (Rhee et al., 2004; Molnár et al., 2011). The up- or down-regulation of cytokines depends heavily on the strain of *Bacillus* DFM, especially when administered in the absence of a pathogen. Lee et al. (2010a) tested eight independent isolates of *Bacillus* and one commercially available cocktail of multiple strains in a non-pathogenic study and reported an up-regulation in expression of circulating IL-6 and IL-8, a result that was reiterated in a separate independent study where *B. subtilis* (natto) B4 spores induced several proinflammatory cytokines including IL-1β and IL-6, and IFNγ as well as an increase in macrophage NO and iNOS production. However, Lee et al. (2010a) also reported only one of the strains up-regulated IFNγ gene expression, whereas the remaining strains had decreased levels and two strains down-regulated it, two strains up-regulated IL-1β and one strain down-regulated it when compared to the control. These results further emphasize effective screening when selecting strains as DFM in poultry production because of the strain-to-strain variation, dose of the given DFM, and the persistence of that strain in the GI environment, a topic that is well reviewed by Huyghebaert et al. (2011).

Notably, *B. subtilis* also causes the production of the anti-inflammatory, or regulatory, cytokines IL-10, and IL-4 in response to increasing levels of proinflammatory cytokines as an autoregulatory negative feedback to control the acute inflammatory response (Platzer et al., 1995; Xu et al., 2012). Prolonged exposure to IL-1, TL1A, and IL-6 cytokines caused reductions in muscle cell translational efficiency which resulted in muscle proteolysis effectively destroying muscle mass (Fanzani et al., 2012). The chronic circulation of proinflammatory cytokines inhibits myogenic differentiation which could lead to diminished muscle growth. It could be argued that one of the multiple mechanisms by which *Bacillus* DFM could promote growth is by supporting muscle mass development through the reduction of prolonged proinflammatory cytokine production; however, this theory warrants greater study.

**Conclusion**

The use of *Bacillus* DFM in poultry is rapidly expanding as noted by the increase in research that studies its gut flora modulation and immune stimulation. This organism’s ability to survive feed processing and administer its benefits to the gut is an advantage that *Bacillus* has over some other commonly studied DFM, and several *Bacillus* DFM strains that are commercially available for use in poultry. There is evidence that *Bacillus* DFM reduce competition for nutrients by reducing the overgrowth of bacteria in the small intestine through the production of AMP, they promote the proliferation and production of beneficial bacteria and metabolites, and they alter the immune response towards the benefit of the host, all of which are modes of action that culminate to promote growth. Although the benefits of different *Bacillus* bacteria are not equal, the next steps of research could include the identification of global metabolites that are produced in the presence of well-performing *Bacillus* DFM to comprehend the interaction between bacteria and host.

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