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

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

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

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INTRODUCTION

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

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defined enteritis (Wolfenden et al., 2007). These studies have indicated that selection of therapeutically efficacious probiotic cultures with marked performance benefits in poultry is possible and that defined cultures can provide an efficient alternative for conventional antimicrobial therapy (Tellez et al., 2001, 2006, 2012; Higgins et al., 2011). On the other hand, studies have indicated that *Bacillus* spores are involved in rapid activation of host innate immune functions (Rhee et al., 2004). Furthermore, some *Bacillus* species have the capacity to produce different exogenous enzymes including protease, lipase, cellulase, xylanase, phytase, and keratinase (Hendricks et al., 1995; Monisha et al., 2009; Mazotto et al., 2011; Mittal et al., 2011; Shah and Bhatt, 2011; Jani et al., 2012). These enzymes may help to decompose complex feed molecules, improve absorption of nutrients, diminish intestinal viscosity in nonstarch polysaccharide diets, and decrease the amount of substrates available for growth of pathogenic bacteria. Additionally, it has been shown that the presence of *Bacillus* isolates, such as *Bacillus subtilis*, enhance growth of other beneficial microorganisms such as *Lactobacillus* by production of subtilisin and catalase and decreasing intestinal pH (Hosoi et al., 2000). These studies, in total, have opened an exciting possibility for identification of vastly superior and more potent probiotics. A probiotic is effective against enteropathogens in various ways, including enhancing immune exclusion, competing for essential nutrients, competing for attachment regions, or secreting antimicrobial compounds against various enteropathogens (Cartman et al., 2007, 2008). However, for most of these actions to be effective, there is an inherent requirement for a metabolically active cell, implicating that germination of spores within the gastrointestinal tract could be a major factor to be considered to employ *Bacillus* spore-based DFM. In this context, studies in our laboratory have confirmed that selected heat-resistant spore-forming *Bacillus* species can markedly sporulate in high numbers (Wolfenden et al., 2010, 2011; Shivaramaiah et al., 2011). There is a growing body of evidence supporting the idea that some *Bacillus* species produced spores can germinate in the GIT of chickens, mice, pigs, dogs, and humans, thus potentially being metabolically active and possibly eliciting a mechanism of action similar to other probiotic bacteria (Hoa et al., 2000; Duc et al., 2004; Tam et al., 2006). However, it is of prime importance to understand the factors that affect spore germination along with their distribution pattern throughout the GIT. The complete mode of action of bacterial endospores is not understood comprehensively, but is presumed to be affected by physiological conditions (temperature, pH, and humidity), anatomical distribution, and pattern of germination in these anatomical segments of the GIT. Earlier studies have shown that *Bacillus* spores germinate by the effects of nutritional and nonnutritional factors known as germinants, for instance: availability of L-alanine, asparagine, glucose, fructose, potassium chloride, and the effect of a nonlethal heat treatment under different pressures (Setlow, 2003). However, due to anatomical differences in the GIT of avian systems, it may not be acceptable to completely apply the results obtained in other animal and mammalian models. Therefore, the objectives of the present study were to evaluate the in vitro germination and growth rate of the *B. subtilis* spores, as well as the evaluation of in vivo distribution and germination of *B. subtilis* spores in different anatomical regions of GIT in a chicken model, as an extension to enrich comprehension of the mechanism of action involving *Bacillus*-based DFM in poultry.

MATERIALS AND METHODS

Bird Source and Diet

Day-of-hatch, off-sex broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR) and were placed in isolators, in a controlled age-appropriate environment. Chickens were provided ad libitum access to water and a balanced unmedicated corn-soybean diet meeting the nutritional requirements of poultry recommended by NRC (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc., 2013). The common starter diet was an antibiotic-free corn-soybean meal diet (Table 1). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas. For experiments 2 and 3, 6 chickens were humanely killed with carbon dioxide asphyxiation upon arrival, and confirmed negative for *Bacillus* spp. vegetative cells and spores. Briefly, tissue samples from crop, ileum, and cecae were aseptically removed from 12 chicks, collected in sterile bags, homogenized, weighed, and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten-fold dilutions of these samples were plated on tryptic soy agar plates (**TSA**, catalog no. 211822, Becton Dickinson, Sparks, MD), incubated at 37°C for 24 h to confirm the absence of any aerobic vegetative cells. Additionally, all samples from each region received heat treatment in a water bath at 75°C for 10 min to eliminate counting of vegetative cells. Ten-fold dilution of these samples were plated on TSA, incubated at 37°C for 24 h to confirm the absence of *Bacillus* spp. spores per gram of sample.

Direct-Fed Microbial

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

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

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

[1]Inclusion of 10^6 spores/g of feed mixed with calcium carbonate.

[2]Vitamin premix supplied the following per 1,000 kg: vitamin A, 20,000,000 IU; vitamin D_3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K_3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO).

[3]Mineral premix supplied the following per 1,000 kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10 to 15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC).

[4]Ethoxyquin.

original spore inoculum used in both in vitro and in vivo experiments were tested to be at 4.3×10^{10} spores per gram of solid media.

In Vitro Determination of Germination and Growth Rate of Spores

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

In Vivo Evaluation of Distribution, Persistence, and Germination of Spores (Experiment 2)

Experiment 2 involved a total of 60 chickens that were randomly divided in 2 groups of 30 chicks per treatment (constant feed vs. single gavage dose). Each group of 30 chickens were allocated in isolation chambers with a wire floor (90 cm × 80 cm) with space underneath for excreta to minimize coprophagia and offered feed and water ad libitum. For the first group of birds receiving spores in feed, *B. subtilis* PHL-NP122 spores were thoroughly mixed with previously autoclaved feed in a rotary mixer for 15 min to ensure thorough distribution. The final concentrations of spores were also determined retrospectively by serial dilution and further plating on TSA for enumeration of actual cfu/gram and ensured to be 10^6 spores per gram of feed. The second group of chicks was gavaged with a single dose of 10^6 spores suspended in 0.25 mL of PBS per chick using a sterile ball-ended gavage needle, just before placement. Chickens were randomly selected, humanely killed with carbon dioxide asphyxiation, and tissue samples from the crop, ileum (from Meckel's diverticulum to the ileocecal junction), and ceca were aseptically removed from 5 chicks at 24, 48, 72, 96, and 120 h after spore consumption by constant feed or single gavage, collected in sterile bags, homogenized, and weighed, and 1:4 wt/vol dilutions were made with sterile 0.9% saline. All samples from each treatment received heat treatment in a water bath at 75°C during 10 min to eliminate counting of vegetative cells. Ten-fold dilution of these samples were plated on TSA and incubated at 37°C for 24 h to enumerate total cfu of *B. subtilis* spores per gram of tissue. Feces samples were also collected during the same time points, and viable spore counts were determined employing a similar dilution plate method as described above.

In Vivo Evaluation of Persistence and Distribution of Vegetative Cells and Spores Throughout the GIT (Experiment 3)

Similarly to experiment 2, this experiment involved a total of 60 chickens that were randomly divided in 2 groups of 30 chicks per treatment (constant feed vs.

single gavage dose). Each group of 30 chickens were allocated in isolation chambers with a wire floor (90 cm × 80 cm) with space underneath for excreta to minimize coprophagia and offered feed and water ad libitum. In this experiment, along with determining just the final viable spore count, vegetative cells were also measured to evaluate the extent of germination of spores in each of section of GIT sampled, at each time point mentioned before in experiment 2. Briefly, ingesta samples collected at each time point and loaded on to 96 well sterile plates, both pre- and postheat treatment (only heat-treated samples in experiment 2). Ten-fold dilutions of all samples were then plated on TSA and incubated for 24 h at 37°C to determine the spore count pre- and postheat treatment. The difference in the cfu between heat-treated and non-heat-treated samples was counted to be the amount of spores that germinated at each time point.

Germination/Sporulation Rates of a Bacillus-DFM in Different Sections of the GIT in Broiler Chickens Consuming Bacillus Spores Constantly in the Feed (Experiments 2 and 3)

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

$$\text{germination rate} = \text{crop spores} - \text{ileum spores}.$$

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

$$\text{sporulation rate} = \text{cecal spores} - \text{ileum spores}.$$

Statistical Analysis

Colony-forming units in all experiments were converted to $\log_{10}$ values. Comparison between total aerobic vegetative cells versus *B. subtilis* spores ($\log_{10}$ cfu/g) after heat shock in the crop, ileum, and ceca of broiler chickens after a single gavage dose or constant administration in the feed were subjected to one-way ANOVA (ANOVA). Germination/sporulation rate of a *Bacillus*-DFM in different sections of the GIT in broiler chickens consuming *Bacillus* spores constantly in the feed were subjected to ANOVA. All data were compared using the GLM procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC). Significant differences among the means were determined by using Duncan's multiple-range test at $P \leq 0.05$. Five replicates were evaluated per time point, using a complete randomized design.

RESULTS AND DISCUSSION

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

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

To begin with, *B. subtilis* spores were recovered from sample tissues of crop, ileum, ceca, and collected feces from broiler chickens, either given as a single gavage dose or constant administration in the feed. Recovered spores count was between 10^4 and 10^5 per gram of ingesta at all times in crop, ileum, and ceca when spores were constantly administered in the feed. Further, recorded reduction of about 1-$\log_{10}$, from the original

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

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

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Data are expressed as mean ± SE of 5 replicates.

spore count in the feed to the recovered count from GIT, suggested that germination of 90% of spores occurred, which is consistent with the results obtained in the in vitro crop assay (Table 2; Figure 1). Recovered spores from chicks that received a single gavage dose also followed the same pattern of 1-$\log_{10}$ reduction, but decreased over time. Further, spores administered either continuously in the feed or by single gavage followed a consistent pattern of change in the number of spores recovered from the crop to the cecae. A numerical reduction tendency in the spore count was observed from crop to ileum followed by a numerical increment from the ileum to the cecae, at all sampling time points (Table 4). The results of experiment 2 were confirmed and extended in experiment 3 (Table 3). Overall, our results are in agreement with Jadamus et al. (2001), arriving at similar conclusions, that the amount of spores recovered tends to diminish from the crop to the ileum, and increase again from the ileum to the cecae (Tables 3 and 4). This change could be related to the varying physiological, nutritional, and microbiological conditions of the GIT. The reduction of spore cfu in the small intestine could be as a result of germination in the crop, duodenum, or jejunum due to abundance of nutrients and favorable conditions in these anatomical segments. On the other hand, the increment of spores in the cecae could be a response due to the competitive microbial population, contending for oxygen and nutrients. This, accompanied by elevated concentrations of bacterial metabolites, such as NH_3 (Preston and Douthit, 1984; Jadamus et al., 2001), could stimulate sporulation of vegetative cells entering the ceca, providing increased chances of survival, before being excreted

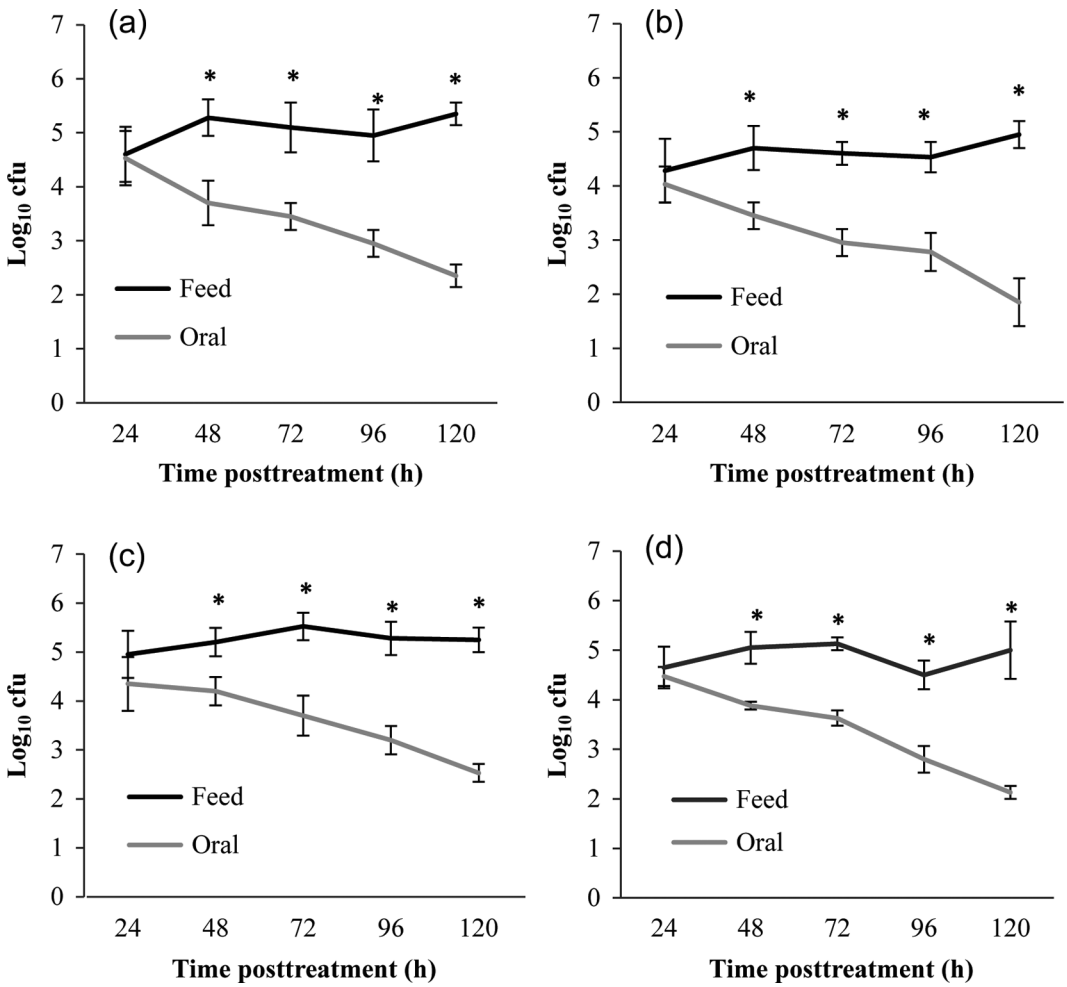

Figure 1. *Bacillus subtilis* ($\log_{10}$ cfu g^{-1}) in the crop (a), ileum (b), ceca (c), and feces (d) of broiler chickens given a single oral dose or constant administration of spores in the feed (experiment 2). *Data are expressed as means and SE of 5 replicates ($P < 0.05$).

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

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

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

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

[2]10^6 spores per 0.25 mL.

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

into the environment. The above observations, also suggest that spores transiting through the GIT, could potentially undergo a full life-cycle of germination and resporulation, also suggested by previous studies (Barbosa et al., 2005; Cartman et al., 2008). In the present study, when neonatal chickens received a single gavage dose of spores, a gradual decreased in the amount of spores recovered throughout different sampled sections of the GIT over time was observed (Figure 1; Tables 3 and 4); however, the long persistence of spores observed in feces in experiment 2 and as well as spores detected in each GIT organ evaluated in experiments 2 and 3, following one single gavage of spores, was longer than the estimated half-life, based on gut-passage time, which in chickens is around of 6.5 h (Shires et al., 1987), suggesting that some full life-cycle development may occurs within the GIT (Table 4). This is supported by the study showing the presence of a larger amount of spores excreted in the feces compared with the original inoculum administered to mice, suggested germination, growth and resporulation of the initial spore dose (Hoa et al., 2001).

To the contrary, Spinosa et al. (2000) have argued that the germination of *B. subtilis* spores and generation of beneficial effects by vegetative cells is highly improbable, due to the presence of low pH and secretory bile salts in certain regions of the GIT of mice, inhibiting spore germination or killing the new vegetative

Table 4. Germination/sporulation rates of *Bacillus* direct-fed microbials in different sections of the gastrointestinal tract in broiler chickens consuming *Bacillus* spores constantly in the feed (experiments 2 and 3)

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

[a–c]Means within a row with different superscripts differ ($P < 0.05$).

[1]Data are expressed as means ± SE of 5 replicates.

[2]Cp-Il = difference in spore count between the crop and ileum.

[3]Cc-Il = difference in spore count between the ceca and ileum.

cells. Nevertheless, other authors have shown that heat and low pH are conditions that instead of attenuating, could actually stimulate *Bacillus* spore germination, therefore providing evidence of survival within the GIT (Faille et al., 2002), partially supported by the results obtained in the present study.

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

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

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REFERENCES

Alvarez-Olmos, M. I., and R. A. Oberhelman. 2001. Probiotic agents and infectious diseases: A modern perspective on a traditional therapy. Clin. Infect. Dis. 32:1567–1576.

Barbosa, T. M., C. R. Serra, R. M. La Ragione, M. J. Woodward, and A. O. Henriques. 2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 71:968–978.

Boyle, E. C., J. L. Bishop, G. A. Grassl, and B. B. Finlay. 2007. *Salmonella*: From pathogenesis to therapeutics. J. Bacteriol. 189:1489–1495.

Cartman, S. T., R. M. La Ragione, and M. J. Woodward. 2007. Bacterial spore formers as probiotics for poultry. Food Sci. Technol. Bull. 4:21–30.

Cartman, S. T., R. M. La Ragione, and M. J. Woodward. 2008. *Bacillus subtilis* spores germinate in the chicken gastrointestinal tract. Appl. Environ. Microbiol. 74:5254–5258.

Cobb-Vantress Inc. 2013. Breeder Management Supplement. Accessed Feb. 2013. http://www.cobb-vantress.com//docs/default-source/cobb-500-guides/2013-january-15th-cobb-500ff-supplement-for-web.pdf.

Duc, H., H. A. Hong, T. M. Barbosa, A. O. Henriques, and S. M. Cutting. 2004. Characterization of *Bacillus* probiotics available for human use. Appl. Environ. Microbiol. 70:2161–2171.

Faille, C., J. Membre, M. Kubaczka, and F. Gavini. 2002. Altered ability of *Bacillus cereus* spores to grow under unfavorable conditions (presence of nisin, low temperature, acidic pH, presence of NaCl) following heat treatment during sporulation. J. Food Prot. 65:1930–1936.

Farnell, M., A. Donoghue, F. S. De Los Santos, P. Blore, B. Hargis, G. Tellez, and D. Donoghue. 2006. Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. Poult. Sci. 85:1900–1906.

Hendricks, C. W., J. D. Doyle, and B. Hugley. 1995. A new solid medium for enumerating cellulose-utilizing bacteria in soil. Appl. Environ. Microbiol. 61:2016–2019.

Higgins, J., S. Higgins, J. Vicente, A. Wolfenden, G. Tellez, and B. Hargis. 2007. Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. Poult. Sci. 86:1662–1666.

Higgins, J., S. Higgins, A. Wolfenden, S. Henderson, A. Torres-Rodriguez, J. Vicente, B. Hargis, and G. Tellez. 2010. Effect of lactic acid bacteria probiotic culture treatment timing on *Salmonella* Enteritidis in neonatal broilers. Poult. Sci. 89:243–247.

Higgins, J. P., R. L. Andreatti Filho, S. E. Higgins, A. D. Wolfenden, G. Téllez, and B. M. Hargis. 2008. Evaluation of *Salmonella*-lytic properties of bacteriophages isolated from commercial broiler houses. Avian Dis. 52:139–142.

Higgins, S., A. Torres-Rodriguez, J. Vicente, C. Sartor, C. Pixley, G. Nava, G. Tellez, J. Barton, and B. Hargis. 2005. Evaluation of intervention strategies for idiopathic diarrhea in commercial turkey brooding houses. J. Appl. Poult. Res. 14:345–348.

Higgins, S., A. Wolfenden, G. Tellez, B. Hargis, and T. Porter. 2011. Transcriptional profiling of cecal gene expression in probiotic-and *Salmonella*-challenged neonatal chicks. Poult. Sci. 90:901–913.

Hoa, N. T., L. Baccigalupi, A. Huxham, A. Smertenko, P. H. Van, S. Ammendola, E. Ricca, and S. M. Cutting. 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl. Environ. Microbiol. 66:5241–5247.

Hoa, T. T., L. H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P. H. Van, and S. M. Cutting. 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl. Environ. Microbiol. 67:3819–3823.

Hong, H., J. M. Huang, R. Khaneja, L. Hiep, M. Urdaci, and S. Cutting. 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. J. Appl. Microbiol. 105:510–520.

Hosoi, T., A. Ametani, K. Kiuchi, and S. Kaminogawa. 2000. Improved growth and viability of lactobacilli in the presence of *Ba-*

cillus subtilis (natto), catalase, or subtilisin. Can. J. Microbiol. 46:892–897.

Jadamus, A., W. Vahjen, and O. Simon. 2001. Growth behaviour of a spore forming probiotic strain in the gastrointestinal tract of broiler chicken and piglets. Arch. Anim. Nutr. 54:1–17.

Jani, S. A., C. J. Chudasama, D. B. Patel, P. S. Bhatt, and H. N. Patel. 2012. Optimization of extracellular protease production from alkali thermo tolerant *Actinomycetes*: *Saccharomonospora viridis* SJ-21. Bull. Environ. Pharmacol. Life Sci. 1:84–92.

La Ragione, R. M., and M. J. Woodward. 2003. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. Vet. Microbiol. 94:245–256.

Leser, T. D., A. Knarreborg, and J. Worm. 2008. Germination and outgrowth of *Bacillus subtilis* and *Bacillus licheniformis* spores in the gastrointestinal tract of pigs. J. Appl. Microbiol. 104:1025–1033.

Mazotto, A. M., R. R. Rodrigues-Coelho, S. M. Lage-Cedrola, M. F. Lima, S. Couri, E. Paraguai de Souza, and A. B. Vermelho. 2011. Keratinase production by three *Bacillus* spp. using feather meal and whole feathers as substrate in a submerged fermentation. Enzyme Res. 2011:1–7.

Mazza, P. 1994. The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. Boll. Chim. Farm. 133:3–18.

Menconi, A., A. Wolfenden, S. Shivaramaiah, J. Terraes, T. Urbano, J. Kuttel, C. Kremer, B. Hargis, and G. Tellez. 2011. Effect of lactic acid bacteria probiotic culture for the treatment of *Salmonella enterica* serovar Heidelberg in neonatal broiler chickens and turkey poults. Poult. Sci. 90:561–565.

Mittal, A., G. Singh, V. Goyal, A. Yadav, K. R. Aneja, S. K. Gautam, and N. K. Aggarwal. 2011. Isolation and biochemical characterization of acido-thermophilic extracellular phytase producting bacterial for potential application in poultry feed. Jundishapur J. Microbiol. 4:273–282.

Monisha, R., M. V. Uma, and V. Krishna Murthy. 2009. Partial purification and characterization of *Bacillus pumilus* xylanase from soil source. KATSU 5:137–148.

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Park, E., G. I. Jeon, J. S. Park, and H. D. Paik. 2007. A probiotic strain of *Bacillus polyfermenticus* reduces DMH induced precancerous lesions in F344 male rat. Biol. Pharm. Bull. 30:569–574.

Preston, R. A., and H. A. Douthit. 1984. Stimulation of germination of unactivated *Bacillus cereus* spores by ammonia. J. Gen. Microbiol. 130:1041–1050.

Rhee, K. J., P. Sethupathi, A. Driks, D. J. Lanning, and K. L. Knight. 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J. Immunol. 172:1118–1124.

Setlow, P. 2003. Spore germination. Curr. Opin. Microbiol. 6:550–556.

Shah, K. R., and S. A. Bhatt. 2011. Purification and characterization of lipase from *Bacillus subtilis* Pa2. J. Biochem. Tech. 3:292–295.

Shires, A., J. R. Thompson, B. V. Turner, P. M. Kennedy, and Y. K. Goh. 1987. Rate of passage of corn-canola meal and corn-soybean meal diets through the gastrointestinal tract of broiler and white Leghorn chickens. Poult. Sci. 66:289–298.

Shivaramaiah, S., J. Baila, S. Layton, C. Lester, Y. Kwon, L. Berghman, B. Hargis, and G. Tellez. 2010. Development and evaluation of an AaroA I AhtrA *Salmonella enteritidis* vector expressing *Eimeria maxima* TRAP family protein EmTFP250 with CD 154 (CD 40L) as candidate vaccines against coccidiosis in broilers. Int. J. Poult. Sci. 9:1031–1037.

Shivaramaiah, S., N. Pumford, M. Morgan, R. Wolfenden, A. Wolfenden, A. Torres-Rodriguez, B. Hargis, and G. Téllez. 2011. Evaluation of *Bacillus* species as potential candidates for direct-fed microbials in commercial poultry. Poult. Sci. 90:1574–1580.

Spinosa, M. R., T. Braccini, E. Ricca, M. De Felice, L. Morelli, G. Pozzi, and M. R. Oggioni. 2000. On the fate of ingested *Bacillus* spores. Res. Microbiol. 151:361–368.

Tam, N. K. M., N. Q. Uyen, H. A. Hong, L. H. Duc, T. T. Hoa, C. R. Serra, A. O. Henriques, and S. M. Cutting. 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. J. Bacteriol. 188:2692–2700.

Tellez, G., S. Higgins, A. Donoghue, and B. Hargis. 2006. Digestive physiology and the role of microorganisms. J. Appl. Poult. Res. 15:136–144.

Tellez, G., V. Petrone, M. Escorcia, T. Morishita, C. Cobb, and L. Villaseñor. 2001. Evaluation of avian-specific probiotic and *Salmonella* Enteritidis-, *Salmonella* Typhimurium, and *Salmonella* Heidelberg-specific antibodies on cecal colonization and organ invasion of *Salmonella* Enteritidis in broilers. J. Food Prot. 64:287–291.

Tellez, G., C. Pixley, R. Wolfenden, S. Layton, and B. Hargis. 2012. Probiotics/direct fed microbials for *Salmonella* control in poultry. Food Res. Int. 45:628–633.

Torres-Rodriguez, A., A. Donoghue, D. Donoghue, J. Barton, G. Tellez, and B. Hargis. 2007a. Performance and condemnation rate analysis of commercial turkey flocks treated with a *Lactobacillus* spp.-based probiotic. Poult. Sci. 86:444–446.

Torres-Rodriguez, A., S. Higgins, J. Vicente, A. Wolfenden, G. Gaona-Ramirez, J. Barton, G. Tellez, A. Donoghue, and B. Hargis. 2007b. Effect of lactose as a prebiotic on turkey body weight under commercial conditions. J. Appl. Poult. Res. 16:635–641.

Vicente, J. L., A. Torres-Rodriguez, S. E. Higgins, C. Pixley, G. Tellez, A. M. Donoghue, and B. M. Hargis. 2008. Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. Avian Dis. 52:143–146.

Vreeland, R. H., W. D. Rosenzweig, and D. W. Powers. 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature 407:897–900.

Williams, P. 2007. *Bacillus subtilis*: A shocking message from a probiotic. Cell Host Microbe 1:248–249.

Wolfenden, A., J. Vicente, J. Higgins, R. Andreatti Filho, S. Higgins, B. Hargis, and G. Tellez. 2007. Effect of organic acids and probiotics on *Salmonella enteritidis* infection in broiler chickens. Int. J. Poult. Sci. 6:403–405.

Wolfenden, R., N. Pumford, M. Morgan, S. Shivaramaiah, A. Wolfenden, C. Pixley, J. Green, G. Tellez, and B. Hargis. 2011. Evaluation of selected direct-fed microbial candidates on live performance and *Salmonella* reduction in commercial turkey brooding houses. Poult. Sci. 90:2627–2631.

Wolfenden, R., N. Pumford, M. Morgan, S. Shivaramaiah, A. Wolfenden, G. Tellez, and B. Hargis. 2010. Evaluation of a screening and selection method for *Bacillus* isolates for use as effective direct-fed microbials in commercial poultry. Int. J. Poult. Sci. 9:317–323.