Titration of supplemental *Bacillus subtilis* subsp. *subtilis* American Type Culture Collection PTA-125135 to broiler chickens fed diets of 2 different metabolizable energy concentrations

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ABSTRACT *Bacillus subtilis* subsp. *subtilis* American Type Culture Collection deposit number PTA-125135 has recently been studied by our laboratory as a potential probiotic strain for avian species. The objective of the present study was to evaluate growth performance and feed efficiency in broiler chickens in response to a dose titration of the *Bacillus* strain in feed. In addition to a nonsupplemented control, *Bacillus* spores were supplemented into broiler chicken diets at 4 levels, which were 8.1×10^4, 1.6×10^5, 2.4×10^5, and 3.2×10^5 CFU per g of feed. The titration was applied to two different dietary regimes of standard or low metabolizable energy (ME), which differed in ME by 22, 56, and 110 kcal/kg in starter, grower, and finisher dietary phases, respectively. All diets contained 249 g per metric ton of a previously patented synbiotic feed additive. Performance data were collected at day 14, 26, and 40 of age, and the effects of *Bacillus* and ME treatments were evaluated by factorial ANOVA. Treatment group means were further examined for significant ($P < 0.05$) pairwise differences among treatments and for significant ($P < 0.05$) linear and quadratic effects. At day 14 of age, significant linear effects for decreased feed conversion ratio (FCR) with higher CFU of *Bacillus* supplementation were observed within the standard ME diet. At day 26, a linear trend was observed for increased mortality with increased dose within the standard ME diet only. *Bacillus* supplementation at day 26 also significantly affected FCR and mortality-adjusted FCR, where supplementation with 3.2×10^5 CFU per g feed produced lower FCR and mortality-adjusted FCR than supplementation with 1.6×10^5 CFU per g feed. We conclude from linear effects related to feed efficiency observed at day 14 and from the significant separation of *Bacillus* treatment means within the titrated range of supplementation at day 26 that further evaluation for effects on performance should be made of doses at 2.4×10^5, 3.2×10^5, and greater CFU per g in feed.

Key words: broiler, probiotic, *Bacillus*

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INTRODUCTION

Avi-Lution (Agri-King, Inc., Fulton, IL) is a patented, synbiotic, feed-additive product containing live strains of *Saccharomyces cerevisiae*, *Enterococcus faecium*, *Bacillus subtilis*, and *Bacillus licheniformis*, as well as β-glucans, mannan-oligosaccharides, and fructo-oligosaccharides. A patent on the combination of *S. cerevisiae* and *E. faecium* as a probiotic product was first filed in 1998 and issued in 2003 as US patent 6,524,574, effective for the reduction of contaminating enteric bacteria in humans and monogastric animals (Spangler et al., 2003). A second patent (US patent 6,841,149) was issued on the combination of the same strains with prebiotic nutrients in 2005 (Spangler et al., 2005). Avi-Lution continues to be marketed and sold as a feed additive at the present time.

Although only a single study of Avi-Lution as a feed additive to poultry has been reported in the literature (Krueger et al., 2017), the additive is known to decrease the shedding of enteric pathogenic bacteria, including pathogenic strains of *Enterococcus coli*, *Salmonella*, and *Campylobacter* (Spangler et al., 2003, 2005). By decreasing pathogen exposure, Avi-Lution has been hypothesized to improve the body weight gain and feed efficiency of broiler chickens, and this hypothesis has been supported and further developed by numerous unpublished works, including comparisons with antibiotic growth promoters such as bacitracin, virginiamycin, and

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avilamycin (D. A. Spangler, unpublished data; L. A. Krueger, unpublished data). Importantly, modes of action for many of the ingredients in Avi-Lution have been reported in literature. The cell wall of *S. cerevisiae* has been shown to induce trophic effects on the intestinal mucosa of broilers, increase body weight gain, and improve feed efficiency (Santin et al., 2001). Similarly, a recent review (Elghandour et al., 2019) has discussed the application of live *S. cerevisiae* in poultry diets, whereby the organism improves feed efficiency and increases growth performance by causing competitive exclusion of pathogenic bacteria and stimulating the host immune system. In US patent 6,524,574, Spangler et al. (2003) demonstrated that a commensal strain of *E. faecium* (strain NCIMB 10415) improved the competitive exclusion of pathogens by *S. cerevisiae*. *E. faecium* demonstrates species diversity with regard to virulence risk, pathogenicity, and antibiotic resistance, but strain NCIMB 10415 has been proven to be safe and effective as a probiotic strain in humans and animal species (Holzapfel et al., 2018). Mannan-oligosaccharide and fructo-oligosaccharide, which are formulated into Avi-Lution as prebiotics, are known to serve as substrates for *E. faecium* (Spangler et al., 2005). Mannan-oligosaccharide has also been shown to promote intestinal tissue development and improve mucosal enzyme activities in broiler chickens (Iji et al., 2001; Hutsko et al., 2016). This collection of ingredients has therefore been shown to support the competitive exclusion of enteric pathogens, improve intestinal tissue health, and thereby improve growth performance and feed efficiency of broiler chickens.

Continued research for developing Avi-Lution as an improved combination feed additive must demonstrate that any ingredient increases body weight gain and feed efficiency in birds where the base combination product is also applied. *B. subtilis* subsp. *subtilis* American Type Culture Collection PTA-125135 (PTA-125135) has recently been studied by our laboratory as a production source for β-glucanase and protease enzymes, as described in US patent 10,138,444 (Ayangbile et al., 2017). Such enzymes have been studied extensively in poultry diets for effects on growth performance, feed efficiency, and intestinal health (Cowieson and Kluenter, 2019; Raza et al., 2019; Yadav and Jha, 2019). Indeed, previously known strains of *Bacillus* are known to produce carbohydrase and protease enzymes (de Boer et al., 1994; Guan et al., 2017), and strains of *B. subtilis and B. licheniformis* are included as ingredients in Avi-Lution.

Although qualitative carbohydrase and protease activities of PTA-125135 are mostly redundant to previously studied activities of other strains, our laboratory also has identified that PTA-125135 produces one or more lipophilic compounds into the extracellular biofilm during *in vitro* culture, and the biofilm has been found to be enriched for unsaturated fatty acids when compared with other *B. subtilis* strains (L. A. Krueger, unpublished data). Lipophilic compounds, upon fractionation from the biofilm, have been found to have surface tension–reducing or emulsifying properties, and a recent characterization by mass spectrometry of the fractionated residues identified peptide sequences with similarity to 2 putative lipoproteins that have previously been predicted or observed in *B. subtilis* strain 168 (L. A. Krueger, unpublished data). This common strain for laboratory study (Kunst et al., 1997) shares approximately 88% genetic similarity with PTA-125135 (L. A. Krueger, unpublished data). The recovery of emulsifying bioactivity related to putative lipoproteins is a primary distinguishing difference between strain PTA-125135 and any of the *Bacillus* strains that are presently formulated into Avi-Lution and could be beneficial to growing broilers by improving lipid digestibility (Roy et al., 2010) or by participating in *de novo* fatty acid synthesis to affect dietary fatty acids that are ultimately available for absorption (Grau and de Mendoza, 1993). Such bioactivities readily relate to the health of the gastrointestinal mucosal epithelium (Marion-Letellier et al., 2013).

In this regard, we have considered that the characteristics of PTA-125135 could be suitable for evaluation as an ingredient with Avi-Lution. Feed additives comprised of single *Bacillus* strains such as *B. subtilis* LS 1-2 and *B. subtilis* C-3102 which have been found to affect broiler growth performance or feed efficiency at applied doses of 1.0×10^5 to 3.3×10^5 CFU per g in feed (Fritts et al., 2000; Sen et al., 2012), but little is known about any lipid-active modes of action that might govern minimum or maximum effective doses of PTA-125135, especially in the presence of *S. cerevisiae*, *E. faecium*, and prebiotic ingredients. We hypothesized that by testing numerous doses of PTA-125135, a dose titration curve should be developed wherein increased body weight gain or improved feed efficiency (decreased feed conversion ratio [FCR]) should be explained by a linear effect of PTA-125135 treatment. Our hypothesis extended that the linear effects of PTA-125135 should become saturated or revert in quadratic fashion as the dosing level exceeded an optimal effective dose. Therefore, the null hypothesis projected that upon application of titrated dose levels of PTA-125135, no linear or quadratic effect on body weight gain or feed efficiency should be identified.

This hypothesis was tested in two dietary regimes comprised of starter, grower, and finisher phases, which are described in subsequent sections as standard or low metabolizable energy (ME) diets, where the low ME diets were formulated by decreasing the inclusions of soybean meal and soy oil. These feedstuffs were considered to be especially rich, collectively, in crude protein that is compatible with *Bacillus* proteases, nonstarch polysaccharides that are compatible with *Bacillus* carbohydrase enzymes, and triglycerides that could be compatible with the putative lipoproteins of PTA-125135. Therefore, the objective of applying "low-ME" diets was to decrease putative stimuli for expression of PTA-125135 bioactivities, rather than to strictly objectify a diet that was low in ME. The previously described hypothesis was therefore able to be tested in two different dietary scenarios, where the "standard ME" diets were projected to be more stimulatory for PTA-125135 bioactivities.

MATERIALS AND METHODS

Ethics Statement

All experimental procedures and conditions were designed and carried out in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching. The trial was performed at a commercial research facility (Virginia Diversified Research Corp, Harrisonburg, VA). All procedures were supervised by an attending veterinarian.

Animal Use and Handling

Straight-run, newly hatched Ross 708 broiler chickens (n = 1,650) were obtained on the day of hatch and randomly placed into 50 pens (33 birds per pen). Chicks received a coccidiosis vaccine (Advent, Cocci-Vac) at the hatchery before receipt and placement. At the time of placement, 200 randomly selected chicks were weighed to establish mean and standard deviation statistics for a representative subpopulation. The weight of all birds that were placed was measured within 2 standard deviations of the subpopulation mean.

Pen dimensions were approximately 1.52-m wide and 1.22-m long to provide initial stocking density of 0.056 m^2 per bird. Each pen contained a single Plasson water fountain (Plasson, Ma'agan Michael, Israel) and a single feed tube with 20.4-kg capacity. Birds were started on new wood shavings, and on day 4, birds were exposed to used litter sourced from healthy chickens not previously exposed to dietary enzymes or direct-fed microbials. Birds were exposed to continuous lighting for the first 3 Days, and then were exposed to 18 h of light each day thereafter.

Diet Formulation

Pens were arranged as 5 replicate blocks of 10 pens each. Each pen within a block was randomly assigned to 1 of 10 treatments, thus generating a randomized complete block design. Two levels of dietary ME (standard and low) and five levels (nonsupplemented control plus four levels of supplemental treatment) of *B. subtilis* supplementation (described in the following paragraphs) were established in a factorial arrangement.

Diets at each level of dietary energy (standard or low) consisted of a starter diet fed from day 0 to 14, a grower diet fed from day 15 to 26, and a finisher diet fed from day 27 to 40. All diets were in mash form. A brief description of nutrient specifications for complete feed formulations is described in Table 1. As-analyzed values in Table 1 were produced by a commercial feed analysis laboratory (Analab, Agri-King, Inc., Fulton, IL) using AOAC methods 990.03, 920.39, and 985.01 for crude protein, crude fat, and minerals, respectively (Latimer, 2019). Feed samples for analysis were not retained from the original batch mixes, so diets were remixed

Table 1. Diet composition as-formulated and as-analyzed, as-fed basis.[1]

	Starter		Grower		Finisher	
Nutrient	Formulated	Analyzed	Formulated	Analyzed	Formulated	Analyzed
Low-ME Diet						
ME, kcal per kg	3,042		3,086		3,142	
Crude protein, %	19.5	20.3 ± 0.1	18.4	19.1 ± 0.1	17.8	18.6 ± 0.2
Crude fat, %	3.56	3.37 ± 0.21	4.34	3.76 ± 0.07	5.54	4.63 ± 0.08
Crude fiber, %	2.89		2.96		3.04	
Arg, %	1.248		1.132		1.060	
Lys, %	1.234		1.130		1.031	
Met, %	0.596		0.534		0.464	
Cys, %	0.316		0.301		0.294	
Trp, %	0.213		0.190		0.176	
Leu, %	1.731		1.686		1.678	
Ile, %	0.820		0.752		0.713	
Ca, %	0.85	0.65 ± 0.03	0.80	0.56 ± 0.01	0.75	0.51 ± 0.02
P, %	0.66	0.58 ± 0.01	0.63	0.53 ± 0.01	0.60	0.52 ± 0.01
Na, %	0.23		0.23		0.23	
Standard ME Diet						
ME, kcal per kg	3,064		3,142		3,252	
Crude protein, %	21.1	21.0 ± 0.3	18.5	18.8 ± 0.2	17.7	17.6 ± 0.1
Crude fat, %	4.43	4.02 ± 0.11	4.90	5.18 ± 0.13	6.45	5.72 ± 0.12
Crude fiber, %	2.65		2.71		2.74	
Arg, %	1.36		1.14		1.06	
Lys, %	1.37		1.16		1.07	
Met, %	0.57		0.52		0.46	
Cys, %	0.37		0.33		0.32	
Trp, %	0.23		0.19		0.18	
Leu, %	1.83		1.69		1.65	
Ile, %	0.87		0.74		0.70	
Ca, %	1.00	1.05 ± 0.02	0.90	0.86 ± 0.03	0.80	0.81 ± 0.02
P, %	0.76	0.80 ± 0.01	0.69	0.72 ± 0.02	0.64	0.64 ± 0.01
Na, %	0.20		0.20		0.20	

Abbreviation: ME, metabolizable energy.

[1]Feed samples for analysis were not retained from the original batch mixes, so diets were re-mixed after the conclusion of the experiment for the purpose of providing analyzed nutrient values in feed.

after the conclusion of the experiment for the purpose of providing analyzed nutrient values in feed. Each treatment diet was remixed and sampled, so the reported nutrient values are the average of 5 treatment diets per phase and energy level. The standard ME diet was adopted from an integrated commercial producer, and the authors are obligated to not disclose ingredient formulations. Primary ingredients were corn, soybean meal, dried distillers grains, and meat and bone meal. ME was lowered (in low-ME diets) mostly by the subtraction of soy oil and by substitution of soybean meal for additional corn and meat and bone meal. Table 2 describes feed ingredient substitutions in low-ME diets for soy oil, soybean meal, corn, meat and bone meal, and dried distillers grains on a g per kg as-fed basis in complete feed.

All diets were supplemented with Avi-Lution at 249 g per metric ton in complete feed to provide approximately 3.1×10^4 CFU *S. cerevisiae* and 9.3×10^4 CFU *E. faecium* per g of complete feed. For the purposes of the present study, the Avi-Lution product was formulated with no basal inclusion of *B. subtilis* or *B. licheniformis*. The levels of *Bacillus* treatment were the nonsupplemented control and supplementation with spores of PTA-125135 at 8.1×10^4, 1.6×10^5, 2.4×10^5, and 3.2×10^5 CFU per g feed. *E. faecium* and *B. subtilis* were enumerated in feed samples that were mixed after the conclusion of the experiment and are reported in Table 3.

Performance Data Collection

Pen and feed weights were collected on day 14, 26, and 40. Cumulative mortality by period (starter, grower, and finisher) was recorded as percentage and calculated by treatment from daily mortality records. Bird body weight gain was recorded in g, whereas FCR was calculated as total feed consumed per total live weight produced, and mortality-adjusted feed conversion ratio (MAFCR) was calculated as total feed consumed per total gain, including weight of dead birds, for the pen.

Statistical Analysis

Pen was the experimental unit for all analyses. Data were analyzed by factorial ANOVA using Statistix 10 software (Analytical Software, Tallahassee, FL) according to the model statement, $Y_{ijk} = \mu + T_i + p_j + b_k + T_i \times p_j + e_{ijk}$, where T_i ($i = 2$) is dietary energy, p_j is probiotic treatment ($j = 1$ to 5), b_k is block ($k = 1$ to 5), and e_{ijk} is residual error. Where the P value associated with the F statistic for a main effect was significant ($P < 0.05$), treatment means were separated by pairwise comparisons with Tukey's honestly significant difference test with α of 0.05.

The model provided 36 degrees of freedom to e_{ijk}, which was the error term used to construct all statistical contrasts. Contrasts included an orthogonal contrast between control treatments "Low-control" and "Standard-control," which were not supplemented with *B. subtilis*, as well as linear and quadratic (polynomial) contrasts by *Bacillus* level within each level of dietary ME. It should be noted that the incremental change among treatment levels was equally spaced (8.1×10^4 CFU PTA-125135 per g complete feed). Results are presented as statistically significant where $P < 0.05$ or as a statistical tendency at $P < 0.10$. Data are presented in tables as least squares mean ± SEM for each treatment.

Table 2. Feed ingredient substitutions in low-ME diet compared with standard ME diet on a g/kg wet basis in complete feed.

Ingredient	Starter	Grower	Finisher
Corn	+82.5	+40.5	+29.0
Soybean meal	−85.5	−48.0	−52.5
Dried distillers grains	0.0	0.0	+20.0
Meat and bone meal	+40.0	+40.0	+40.0
Soy oil	−16.1	−12.2	−17.4

Abbreviation: ME, metabolizable energy.

RESULTS

Body Weight Gain

Body weight gain was affected neither linearly nor quadratically by *Bacillus* treatment within the standard or low-ME diets at day 14, 26, or 40 (Table 4). Within respective ME diets, body weight gain was similar for all PTA-125135 treatments and not different from the control at all time points. Body weight gain differed significantly ($P \leq 0.001$) for aggregate treatment groups of standard and low-ME diets during the starter and grower phases, where body weight was greater for birds fed the standard ME diet.

Feed Conversion Ratio

Bacillus treatment induced a significant ($P = 0.009$) linear effect on FCR within the standard ME diet at day 14 (Table 5), where FCR was lower with higher levels of PTA-125135 supplementation. However, *Bacillus* treatment was not a significant model term, and no significant separation of treatment means was identified at day 14. Dietary energy did not affect FCR during the starter phase.

In the grower phase, neither linear nor quadratic effects of *Bacillus* were observed within the standard ME diet, but both linear and quadratic effects tended to be significant ($P = 0.082$ and $P = 0.055$, respectively) within the low-ME diet, where FCR was lower with higher levels of PTA-125135 supplementation. Similarly, both the level of probiotic supplementation and dietary ME were significant model terms ($P = 0.039$ and $P = 0.009$, respectively). Separation of *Bacillus* treatment means for aggregated ME diets identified that supplementation with 3.2×10^5 CFU induced lower FCR than supplementation with 1.6×10^5 CFU. FCR was significantly lower during the grower phase for aggregate

Table 3. Enumeration of *Bacillus subtilis* and *Enterococcus faecium* in mixed feed, CFU/g.[1]

Probiotic	Control	Level 1	Level 2	Level 3	Level 4
B. subtilis					
Formulated	0.0×10^0	8.1×10^4	1.6×10^5	2.4×10^5	3.2×10^5
Recovered	$<1.0 \times 10^3$	7.7×10^4	9.6×10^4	2.4×10^5	3.3×10^5
E. faecium					
Formulated	9.3×10^4	9.3×10^4	9.3×10^4	9.3×10^4	9.3×10^4
Recovered	1.7×10^5	1.8×10^5	1.1×10^5	1.7×10^5	1.3×10^5

[1]Data are the mean recovered values from 6 replicate batches per Bacillus level.

treatments fed the low-ME diet than for those fed the high-ME diet.

Neither *Bacillus* nor dietary ME significantly affected FCR at day 40. Linear and quadratic effects of *Bacillus* were not significant for both standard and low-ME diets.

Mortality-Adjusted Feed Conversion Ratio

MAFCR (Table 6) at day 14 was linearly affected ($P = 0.009$) by *Bacillus* treatment within the standard ME diet, and a trend for a quadratic effect ($P = 0.079$) was observed in the low-ME diet. For both linear and quadratic trends, MAFCR was lower with higher CFU inclusions, but neither *Bacillus* treatment nor dietary ME were significant model terms, so no treatment means were found to differ from controls.

At the conclusion of the grower phase, both linear and quadratic effects of *Bacillus* tended to affect MAFCR ($P = 0.072$ and $P = 0.076$, respectively), where lower MAFCR was identified with higher PTA-125135 supplementation. Both *Bacillus* treatment and dietary ME significantly affected MAFCR, where supplementation at 3.2×10^5 CFU decreased MAFCR compared with 1.6×10^5 CFU. MAFCR was significantly lower for low-ME treatments than for standard ME treatments.

Upon completion of the finisher phase, no statistical differences were identified for *Bacillus* treatment or

Table 4. Body weight gain, g, of broilers at day 14, 26, and 40.[1]

Treatment	Day 14	Day 26	Day 40
Interaction of probiotic and energy, mean ± SEM[2]			
Low, control	310 ± 7	996 ± 16	1,765 ± 39
Low, 8.1×10^4 CFU/g	303 ± 5	1,009 ± 30	1,723 ± 120
Low, 1.6×10^5 CFU/g	312 ± 7	994 ± 14	1,803 ± 24
Low, 2.4×10^5 CFU/g	298 ± 9	1,001 ± 26	1,839 ± 24
Low, 3.2×10^5 CFU/g	297 ± 4	1,002 ± 12	1,775 ± 13
Standard, Control	332 ± 5	1,044 ± 20	1,831 ± 35
Standard, 8.1×10^4 CFU/g	317 ± 6	1,027 ± 17	1,824 ± 16
Standard, 1.6×10^5 CFU/g	331 ± 8	1,029 ± 24	1,829 ± 56
Standard, 2.4×10^5 CFU/g	339 ± 5	1,064 ± 19	1,888 ± 52
Standard, 3.2×10^5 CFU/g	327 ± 6	1,044 ± 7	1,816 ± 65
Aggregate within level of probiotic, mean ± SEM[3]			
Control	321 ± 5	1,020 ± 14	1,798 ± 27
8.1×10^4 CFU/g	310 ± 4	1,017 ± 17	1,774 ± 59
1.6×10^5 CFU/g	321 ± 6	1,011 ± 14	1,816 ± 29
2.4×10^5 CFU/g	319 ± 8	1,033 ± 19	1,863 ± 28
3.2×10^5 CFU/g	312 ± 6	1,023 ± 10	1,796 ± 32
Aggregate within level of dietary energy, mean ± SEM[4]			
Low ME	304 ± 3^y	1,000 ± 9^y	1,781 ± 25
Standard ME	329 ± 3^x	1,042 ± 8^x	1,838 ± 20
Significance of model terms, *P* value			
Probiotic	0.209	0.832	0.513
Dietary energy	<0.001	0.001	0.097
Interaction probiotic*energy	0.222	0.810	0.962
Significance of contrast statements, *P* value			
Low ME linear	0.108	0.937	0.415
Low ME quadratic	0.613	0.989	0.652
Standard ME linear	0.507	0.512	0.839
Standard ME quadratic	0.964	0.684	0.699
Low-control vs. standard-control	0.015	0.073	0.378

Abbreviations: HSD, honestly significant difference; ME, metabolizable energy.

[1]Data are reported as mean ± SEM.

[2]No significant interaction of main effects was identified ($P < 0.10$), so no analysis of means separation was carried out for the interaction term.

[3]No significant effect of probiotic treatment was detected ($P < 0.10$), so no analysis of means separation was carried out.

[4]Where dietary energy was a significant model term ($P < 0.10$), means were separated by Tukey HSD test. Means within a column with different superscripts are different, $P < 0.05$.

Table 5. Feed conversion ratio of broilers at day 14, 26, and 40.[1]

Treatment	Day 14	Day 26	Day 40
Interaction of probiotic and energy, mean ± SEM[2]			
Low, control	1.20 ± 0.02	1.25 ± 0.01	1.75 ± 0.03
Low, 8.1×10^4 CFU/g	1.23 ± 0.02	1.25 ± 0.03	1.86 ± 0.14
Low, 1.6×10^5 CFU/g	1.24 ± 0.05	1.26 ± 0.02	1.82 ± 0.05
Low, 2.4×10^5 CFU/g	1.25 ± 0.03	1.25 ± 0.02	1.74 ± 0.01
Low, 3.2×10^5 CFU/g	1.20 ± 0.02	1.20 ± 0.01	1.73 ± 0.02
Standard, control	1.24 ± 0.02	1.27 ± 0.01	1.78 ± 0.02
Standard, 8.1×10^4 CFU/g	1.22 ± 0.04	1.28 ± 0.03	1.87 ± 0.04
Standard, 1.6×10^5 CFU/g	1.24 ± 0.02	1.31 ± 0.01	1.82 ± 0.04
Standard, 2.4×10^5 CFU/g	1.15 ± 0.02	1.24 ± 0.01	1.81 ± 0.06
Standard, 3.2×10^5 CFU/g	1.16 ± 0.02	1.26 ± 0.02	1.83 ± 0.06
Aggregate within level of probiotic, mean ± SEM[3]			
Control	1.22 ± 0.02	$1.26 \pm 0.01^{x,y}$	1.77 ± 0.02
8.1×10^4 CFU/g	1.22 ± 0.02	$1.27 \pm 0.02^{x,y}$	1.86 ± 0.07
1.6×10^5 CFU/g	1.24 ± 0.03	1.29 ± 0.01^{x}	1.82 ± 0.03
2.4×10^5 CFU/g	1.20 ± 0.02	$1.24 \pm 0.01^{x,y}$	1.77 ± 0.03
3.2×10^5 CFU/g	1.18 ± 0.01	1.23 ± 0.01^{y}	1.78 ± 0.03
Aggregate within level of dietary energy, mean ± SEM[4]			
Low ME	1.22 ± 0.01	1.24 ± 0.01^{y}	1.78 ± 0.03
Standard ME	1.20 ± 0.01	1.27 ± 0.01^{x}	1.82 ± 0.02
Significance of model terms, *P*-value			
Probiotic	0.149	0.039	0.455
Dietary energy	0.254	0.009	0.240
Interaction probiotic*energy	0.140	0.406	0.892
Significance of contrast statements, *P* value			
Low ME linear	0.933	0.082	0.417
Low ME quadratic	0.108	0.055	0.652
Standard ME linear	0.009	0.262	0.841
Standard ME quadratic	0.526	0.283	0.701
Low-control vs. standard-control	0.318	0.295	0.657

Abbreviations: HSD, honestly significant difference; ME, metabolizable energy.

[1]Data are reported as mean ± SEM.

[2]No significant interaction of main effects was identified ($P < 0.10$), so no analysis of means separation was carried out for the interaction term.

[3]Where probiotic was a significant model term ($P < 0.10$), means were separated by Tukey HSD test. Means within a column with different superscripts are different, $P < 0.05$.

[4]Where dietary energy was a significant model term ($P < 0.10$), means were separated by Tukey HSD test. Means within a column with different superscripts are different, $P < 0.05$.

dietary ME, and no statistically significant linear or quadratic effects were identified.

Mortality

Mortality percentage (Table 7) was not significantly affected by *Bacillus* treatment or dietary ME treatment at day 14. A quadratic trend ($P = 0.065$) was identified at day 14 within the standard ME diet where mortality was lowest for the control and for the highest level of CFU supplementation. At day 26, *Bacillus* supplementation linearly increased mortality ($P = 0.033$), within the standard ME diet, whereas an opposite linear trend was observed within the low-ME diet ($P = 0.096$). A linear effect of *Bacillus* on mortality was observed at day 40 as a statistical trend ($P = 0.053$).

Although the interaction of probiotic and ME treatment tended to be significant ($P < 0.10$) at day 26 and at day 40, means separation failed to detect any significant differences among treatment means at either day. Mortality was significantly greater in aggregate treatments of standard ME diets than in those of low-ME diets at day 40 ($P = 0.042$).

DISCUSSION

The hypothesis tested in the present study was that a titration of supplemental PTA-125135 should induce linear or quadratic effects for improved broiler body weight gain or feed efficiency. Body weight gain data presented in Table 4 failed to demonstrate any significant response to PTA-125135 supplementation, and so we were unable to reject the null hypothesis. FCR, however, was dose responsive within the standard ME diet during the starter phase and dose responsive to aggregate ME diets through the end of the grower phase. These results suggest that PTA-125135 dose should be evaluated at higher CFU inclusions until a quadratic effect of dose is observed for body weight gain or feed efficiency.

The present data have demonstrated that doses of 2.4×10^5 or 3.2×10^5 CFU per g in feed improve broiler feed efficiency compared with lower CFU doses, especially in the starter and grower phases. Applied doses (per g feed) of other *Bacillus* strains that have been reported as efficacious in the literature span more than a 2-log range from 1.0×10^5 CFU for strain LS 1-2 (Sen et al., 2012) to 4.0×10^7 CFU (Bai et al., 2017). Other

Table 6. Mortality-adjusted feed conversion ratio of broilers at day 14, 26, and 40.[1]

Treatment	Day 14	Day 26	Day 40
Interaction of probiotic and energy, mean ± SEM[2]			
Low, control	1.19 ± 0.02	1.24 ± 0.01	1.67 ± 0.02
Low, 8.1×10^4 CFU/g	1.22 ± 0.02	1.25 ± 0.03	1.78 ± 0.13
Low, 1.6×10^5 CFU/g	1.24 ± 0.05	1.25 ± 0.02	1.67 ± 0.02
Low, 2.4×10^5 CFU/g	1.24 ± 0.03	1.24 ± 0.02	1.67 ± 0.01
Low, 3.2×10^5 CFU/g	1.19 ± 0.02	1.20 ± 0.01	1.66 ± 0.01
Standard, control	1.24 ± 0.02	1.27 ± 0.01	1.69 ± 0.02
Standard, 8.1×10^4 CFU/g	1.21 ± 0.05	1.27 ± 0.03	1.73 ± 0.02
Standard, 1.6×10^5 CFU/g	1.24 ± 0.02	1.29 ± 0.01	1.69 ± 0.02
Standard, 2.4×10^5 CFU/g	1.15 ± 0.02	1.23 ± 0.01	1.65 ± 0.02
Standard, 3.2×10^5 CFU/g	1.16 ± 0.02	1.24 ± 0.02	1.69 ± 0.04
Aggregate within level of probiotic, mean ± SEM[3]			
Control	1.22 ± 0.02	$1.26 \pm 0.01^{x,y}$	1.68 ± 0.01
8.1×10^4 CFU/g	1.22 ± 0.02	$1.26 \pm 0.02^{x,y}$	1.76 ± 0.06
1.6×10^5 CFU/g	1.24 ± 0.02	1.27 ± 0.01^{x}	1.68 ± 0.01
2.4×10^5 CFU/g	1.19 ± 0.02	$1.24 \pm 0.01^{x,y}$	1.66 ± 0.01
3.2×10^5 CFU/g	1.18 ± 0.01	1.22 ± 0.01^{y}	1.67 ± 0.02
Aggregate within level of dietary energy, mean ± SEM[4]			
Low ME	1.22 ± 0.01	1.24 ± 0.01^{y}	1.69 ± 0.03
Standard ME	1.20 ± 0.01	1.26 ± 0.01^{x}	1.69 ± 0.01
Significance of model terms, *P* value			
Probiotic	0.142	0.047	0.301
Dietary energy	0.295	0.030	0.886
Interaction probiotic*energy	0.158	0.444	0.883
Significance of contrast statements, *P* value			
Low ME linear	0.967	0.072	0.395
Low ME quadratic	0.079	0.076	0.411
Standard ME linear	0.009	0.148	0.596
Standard ME quadratic	0.689	0.338	0.974
Low-control vs. standard-control	0.229	0.351	0.701

Abbreviations: HSD, honestly significant difference; ME, metabolizable energy.

[1]Data are reported as mean ± SEM.

[2]No significant interaction of main effects was identified ($P < 0.10$), so no analysis of means separation was carried out for the interaction term.

[3]Where probiotic was a significant model term ($P < 0.10$), means were separated by Tukey HSD test. Means within a column with different superscripts are different, $P < 0.05$.

[4]Where dietary energy was a significant model term ($P < 0.10$), means were separated by Tukey HSD test. Means within a column with different superscripts are different, $P < 0.05$.

test doses have included 1.0×10^6 CFU per g for either a multistrain *Bacillus* probiotic (Hayashi et al., 2018) or for strain DSM 32315 (Sokale et al., 2019) or 10^7 CFU per g or greater for strains CGMCC 1.1086 (Li et al., 2016), American Type Culture Collection PTA-6737 (Abudobos et al., 2017), and CSL-2 (Oh et al., 2017). Therefore, although the present study effectively identified dose-responsive effects within the titrated range up to 3.2×10^5 CFU per g, it is possible that PTA-125135 doses well in excess of the titrated range could be tolerated by poults and could be favorable for improving feed efficiency. In the present study, we did not observe any incidence where a desirable outcome reverted in a significant quadratic effect, which indicates that observed linear effects were not saturated by the highest CFU dose tested. Enumeration results of supplemented bacteria in mixed feed support that the formulated doses were applied effectively in the present titration study.

One of the most striking observations of the present study was the highly significant linear effect of *Bacillus* supplementation on FCR in the standard ME starter diet, which was an effect that was not repeated in the low-ME starter diet. We have considered whether this result should be attributed to dietary substrates that support the vegetation of PTA-125135 for expression of beneficial bioactivities or whether this result should be attributed to a greater potential for improving digestibility of said substrates by application of PTA-125135. This distinction is important because it relates to identifying the most limiting factor for improved performance. While the latter mode of action is strictly nutritional by means of increasing feedstuff digestibility, the former proposed mode of action describes that dietary substrates support vegetation of PTA-125135 for expression of beneficial bioactivities, wherein the limiting factor for improving performance (especially feed efficiency) is the amount of dietary substrate that fuels the metabolism of the probiotic strain (Roels, 1980). Thus, as available substrate fuels cellular replication of the probiotic, the beneficial bioactivity might also increase (Marvasi et al., 2010). Where the beneficial bioactivity relates to improved feedstuff digestibility and nutrient uptake, a negative feedback loop is introduced into the digestive ecosystem, and the effect of incremental applied doses of the probiotic is saturated in the putative negative feedback loop. Data in the present study do not support that dose saturation of feed efficiency was accomplished

Table 7. Mortality percentage of broilers at day 14, 26, and 40.[1]

Treatment	Day 14	Day 26	Day 40
Interaction of probiotic and energy, mean ± SEM[2]			
Low, control	1.21 ± 0.74	3.75 ± 1.17^{x}	4.82 ± 1.38^{x}
Low, 8.1×10^4 CFU/g	0.61 ± 0.61	1.88 ± 0.77^{x}	2.07 ± 0.84^{x}
Low, 1.6×10^5 CFU/g	0.00 ± 0.00	1.88 ± 1.25^{x}	7.59 ± 3.84^{x}
Low, 2.4×10^5 CFU/g	1.21 ± 0.74	1.88 ± 1.25^{x}	4.14 ± 1.29^{x}
Low, 3.2×10^5 CFU/g	0.61 ± 0.61	0.63 ± 0.63^{x}	1.38 ± 0.84^{x}
Standard, control	0.00 ± 0.00	0.63 ± 0.63^{x}	2.76 ± 0.69^{x}
Standard, 8.1×10^4 CFU/g	1.21 ± 0.74	3.13 ± 1.98^{x}	7.59 ± 2.97^{x}
Standard, 1.6×10^5 CFU/g	1.21 ± 0.74	3.13 ± 1.40^{x}	6.21 ± 3.34^{x}
Standard, 2.4×10^5 CFU/g	0.61 ± 0.61	4.38 ± 1.25^{x}	8.28 ± 3.20^{x}
Standard, 3.2×10^5 CFU/g	0.00 ± 0.00	4.38 ± 1.25^{x}	8.97 ± 2.07^{x}
Aggregate within level of probiotic, mean ± SEM[3]			
Control	0.61 ± 0.40	2.19 ± 0.81	3.79 ± 0.80
8.1×10^4 CFU/g	0.91 ± 0.46	2.50 ± 1.02	4.83 ± 1.72
1.6×10^5 CFU/g	0.61 ± 0.40	2.50 ± 0.91	6.90 ± 2.41
2.4×10^5 CFU/g	0.91 ± 0.46	2.81 ± 0.87	6.21 ± 1.77
3.2×10^5 CFU/g	0.30 ± 0.30	2.50 ± 0.91	5.17 ± 1.65
Aggregate within level of dietary energy, mean ± SEM[4]			
Low ME	0.73 ± 0.26	2.00 ± 0.47	4.00 ± 0.93^{y}
Standard ME	0.61 ± 0.25	3.00 ± 0.61	6.76 ± 1.17^{x}
Significance of model terms, *P* value			
Probiotic	0.833	0.990	0.607
Dietary energy	0.749	0.180	0.042
Interaction probiotic*energy	0.252	0.069	0.096
Significance of contrast statements, *P* value			
Low ME linear	0.749	0.096	0.465
Low ME quadratic	0.419	0.774	0.254
Standard ME linear	0.749	0.033	0.053
Standard ME quadratic	0.065	0.474	0.536
Low-control vs. standard-control	0.158	0.064	0.484

Abbreviations: HSD, honestly significant difference; ME, metabolizable energy.

[1]Data are reported as mean ± SEM.

[2]Where the interaction of treatments was a significant model term ($P < 0.10$), means were separated by Tukey HSD test. Means within a column with different superscripts are different, $P < 0.05$. No significant differences or statistical trends ($P < 0.10$) among means were detected at day 26 or day 40.

[3]No significant effect was identified ($P < 0.10$), so no analysis of means separation was carried out for the Probiotic term.

[4]Where dietary energy was a significant model term ($P < 0.10$), means were separated by Tukey HSD test. Means within a column with different superscripts are different, $P < 0.05$.

in the starter phase (Table 5), else a significant quadratic effect should be identified. However, we propose that identifying dose saturation, rather than minimum effective dose, will be important for future modeling of probiotic applications in complex ecosystems (Kay et al., 1999). Admittedly, the modeling of ecosystem dynamics is outside the scope of the present probiotic strain titration, but this concept highlights the need for titrating single strains with other ingredients in defined combinations, such as with Avi-Lution, where each ingredient enacts a mode of action on the digestive ecosystem.

The propensity of resources within the digestive ecosystem to stimulate *Bacillus* vegetative growth and expression of bioactivities was alluded to previously in this article by the formulation of the "low-ME" diets. In the starter phase where feed efficiency results were exemplified, Table 1 documents approximately 22 kcal/kg difference in ME between low and standard ME starter diets, and Table 2 documents that approximately 85.5 g of soybean meal and 16.1 g of soy oil per kg feed were substituted out of the standard ME diet to achieve the low-ME diet. The difference between these diets of approximately 1.6% crude protein (as formulated) also should not be ignored, as numerous strains of *B. subtilis* have long been known to secrete proteases (Connelly et al., 2004). *B. subtilis* PTA-125135 is also known by our laboratory to secrete proteases, such as bacillopeptidase F (Sloma et al., 1990). Therefore, numerous nutritional hypotheses might explain the different results observed between the standard ME and low-ME diets, such as enzymatic digestion of protein and nonstarch polysaccharides in soybean meal or more efficient emulsification and digestion of dietary oil. Although we do not present evidence in this article for any mode of action in the gastrointestinal tract, all these modes of action have been demonstrated to improve broiler chick performance (Singh et al., 2017; Dabbou et al., 2019; Hosseindoust et al., 2019).

The significant linear trends for FCR and MAFCR at day 14 and the significant effect of PTA-125135 supplementation level at day 26 are the primary findings from which future evaluations should be developed. Observed linear effects indicate that higher doses that were tested in the present study could be evaluated in starter-phase diets to test dose saturation, although supplementation at 2.4×10^5 CFU per g feed produced the greatest

(but not significantly different from control) mean body weights and lowest mean MAFCR through 14 D and the duration of the study. A key finding from the present work is the distinction between low-ME and standard ME diets, in which soybean meal and soy oil were distinguishing ingredients, for the induction of a linear effect of *B. subtilis* supplementation on feed efficiency in the starter phase. Future evaluations will likely focus on identifying dose saturation and ecological effects of supplementing PTA-125135 at 2.4×10^5 CFU per g feed and greater CFU inclusions.

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REFERENCES

Abudabos, A. M., A. H. Alyemni, Y. M. Dafalla, and R. U. Khan. 2017. Effect of organic acid blend and *Bacillus subtilis* alone or in combination on growth traits, blood biochemical and antioxidant status in broilers exposed to *Salmonella typhimurium* challenge during the starter phase. J. Appl. Anim. Res. 45:538–542.

Ayangbile, G. M., J. F. Grzemski, Tobey, Jr, D. Spangler, and L. Krueger. 2017. U.S. Patent No. 10,138,444. U.S. Patent and Trademark Office, Washington, DC.

Bai, K., Q. Huang, J. Zhang, J. He, L. Zhang, and T. Wang. 2017. Supplemental effects of probiotic *Bacillus subtilis* fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. Poult. Sci. 96:74–82.

Connelly, M. B., G. M. Young, and A. Sloma. 2004. Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. J. Bacteriol. 186:4159–4167.

Cowieson, A. J., and A. M. Kluenter. 2019. Contribution of exogenous enzymes to potentiate the removal of antibiotic growth promoters in poultry production. Anim. Feed Sci. Tech. 250:81–92.

de Boer, A. S., F. Priest, and B. Diderichsen. 1994. On the industrial use of *Bacillus licheniformis*: a review. Appl. Microbiol. Biot. 40:595–598.

Dabbou, S., A. Schiavone, F. Gai, S. Martinez, J. Madrid, F. Hernandez, A. L. Martinez Marin, D. Soglia, S. Sartore, I. D. Kalmar, L. Gasco, and J. Nery. 2019. Effect of dietary globin, a natural emulsifier, on the growth performance and digestive efficiency of broiler chickens. Ital. J. Anim. Sci. 18:530–537.

Elghandour, M. M. Y., Z. L. Tan, S. H. Abu Hafsa, M. J. Adegbeye, R. Greiner, E. A. Ugbogu, J. Cedillo Monroy, and A. Z. M. Salem. 2019. *Saccharomyces cerevisiae* as a probiotic feed additive to non and pseudo-ruminant feeding: a review. J. Appl. Microbiol. 128:658–674.

Fritts, C. A., J. H. Kersey, M. A. Motl, E. C. Kroger, F. Yan, J. Si, Q. Jiang, M. M. Campos, A. L. Waldroup, and P. W. Waldroup. 2000. *Bacillus subtilis* C-3102 (Calsporin) improves live performance and microbiological status of broiler chickens. J. Appl. Poult. Res. 9:149–155.

Guan, X., P. Chen, Q. Xu, L. Qian, J. Huang, and B. Lin. 2017. Expression, purification and molecular characterization of a novel endoglucanase protein from *Bacillus subtilis* SB13. Protein Expres. Purif. 134:125–131.

Grau, R., and D. de Mendoza. 1993. Regulation of the synthesis of unsaturated fatty acids by growth temperatures in *Bacillus subtilis*. Mol. Microbiol. 8:535–542.

Hayashi, R. M., M. C. Lourenço, L. A. Kraieski, R. B. Araujo, R. Gonzalez-Esquerra, E. Leonardecz, A. Ferreira da Cunha, M. F. Carazzolle, P. S. Monzani, and E. Santin. 2018. Effect of feeding *Bacillus subtilis* spores to broilers challenged with *Salmonella enterica* serovar Heidelberg Brazilian strain UFPR1 on performance, immune response, and gut health. Front. Vet. Sci. 5:13.

Holzapfel, W., A. Arini, M. Aeschbacher, R. Coppolecchia, and B. Pot. 2018. *Enterococcus faecium* SF68 as a model for efficacy and safety evaluation of pharmaceutical probiotics. Benef. Microbes 9:375–388.

Hosseindoust, A., S. Lee, W. Gook Nho, Y. H. Song, J. S. Shin, S. Laxman Ingale, P. C. Rathi, J. Choi, B. Chae, and J. Kim. 2019. A dose–response study to evaluate the effects of pH-stable β-mannanase derived from *Trichoderma citrinoviride* on growth performance, nutrient retention, and intestine morphology in broiler chickens. Ital. J. Anim. Sci. 18:147–154.

Hutsko, S. L., K. Meizlisch, M. Wick, and M. Lilburn. 2016. Early intestinal development and mucin transcription in the young poult with probiotic and mannan oligosaccharide prebiotic supplementation. Poult. Sci. 95:1173–1178.

Iji, P. A., A. A. Saki, and D. R. Tivey. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. J. Sci. Food Agr. 81:1186–1192.

Kay, J. J., H. A. Regier, M. Boyle, and G. Francis. 1999. An ecosystem approach for sustainability addressing the challenge of complexity. Futures 31:721–742.

Krueger, L. A., D. A. Spangler, D. R. Vandermyde, M. D. Sims, and G. A. Ayangbile. 2017. Avi-Lution® supplemented at 1.0 or 2.0 g/kg in feed improves the growth performance of broiler chickens during challenge with bacitracin-resistant *Clostridium perfringens*. Poult. Sci. 96:2595–2600.

Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessières, A. Bolotin, and S. Borchert, et al. 1997. The complete genome sequence of the gram-positive bacterium B*acillus subtilis*. Nature 390:249–256.

Latimer, G. W. 2019. Official Methods of Analysis of AOAC International, 21st ed. AOAC International, Gaithersburg, MD.

Li, Y., Q. Xu, Z. Huang, L. Ly, X. Liu, C. Yin, H. Yan, and J. Yuan. 2016. Effect of *Bacillus subtilis* CGMCC 1.1086 on the growth performance and intestinal microbiota of broilers. J. Appl. Microbiol. 120:195–204.

Marion-Letellier, R., G. Savoye, P. L. Beck, R. Panaccione, and S. Ghosh. 2013. Polyunsaturated fatty acids in inflammatory bowel diseases: a reappraisal of effects and therapeutic approaches. Inflamm. Bowel Dis. 19:650–661.

Marvasi, M., P. T. Visscher, and L. Casillas Martinez. 2010. Exopolymeric substances (EPS) from *Bacillus subtilis*: polymers and genes encoding their synthesis. FEMS Microbiol. Lett. 313:1–9.

Oh, J. K., E. A. B. Pajarillo, J. P. Chae, I. H. Kim, D. S. Yang, and D. K. Kang. 2017. Effects of *Bacillus subtilis* CSL2 on the composition and functional diversity of the faecal microbiota of broiler chickens challenged with *Salmonella gallinarum*. J. Anim. Sci. Biotechnol. 8:1.

Roels, J. A. 1980. Application of macroscopic principles to microbial metabolism. Biotechnol. Bioeng. 22:2457–2514.

Santin, E., A. Maiorka, and M. Macari. 2001. Performance and intestinal mucosa development of broiler chickens fed diets containing *Saccharomyces cerevisiae* cell wall. J. Appl. Poult. Res. 10:236–244.

Singh, A. K., J. d. Berrocoso, Y. Dersiant-Li, A. Awati, and R. Jha. 2017. Effect of a combination of xylanase, amylase and protease on growth performance of broilers fed low and high fiber diets. Anim. Feed Sci. Tech. 232:16–20.

Sloma, A., G. A. Rufo, C. F. Rudolph, B. J. Sullivan, K. A. Theriault, and J. Pero. 1990. Bacillopeptidase F. of *Bacillus subtilis*: purification of the protein and cloning of the gene. J. Bacteriol. 172:1470–1477.

Sokale, A. O., A. Menconi, G. F. Mathis, B. Lumpkins, M. D. Sims, R. A. Whelan, and K. Doranalli. 2019. Effect of *Bacillus subtilis* DSM 32315 on the intestinal structural integrity and growth

performance of broiler chickens under necrotic enteritis challenge. Poult. Sci. 98:5392–5400.
Raza, A., S. Bashir, and R. Tabassum. 2019. An update on carbohydrases: growth performance and intestinal health of poultry. Heliyon 5:e01437.
Roy, A., S. Haldar, S. Mondal, and T. K. Ghosh. 2010. Effects of supplemental exogenous emulsifier on performance, nutrient metabolism, and serum lipid profile in broiler chickens. Vet. Med. Int. 262604.
Sen, S., S. L. Ingale, Y. W. Kim, J. S. Kim, K. H. Kim, J. D. Lohakare, E. K. Kim, H. S. Kim, M. H. Ryu, I. K. Kwon, and B. J. Chae. 2012. Effect of supplementation of *Bacillus subtilis* LS 1-2 to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. Res. Vet. Sci. 93:264–268.
Spangler, D. A., P. K. Brown, and T. E. Witzig. 2003. U.S. Patent No. 6,524,574. U.S. Patent and Trademark Office, Washington, DC.
Spangler, D. A., P. K. Brown, and T. E. Witzig. 2005. U.S. Patent No. 6,841,149. U.S. Patent and Trademark Office, Washington, DC.
Yadav, S., and R. Jha. 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. J. Anim. Sci. Biotechnol. 10:2.